GENETIC DIVERSITY ASSESSMENT OF LICORICE FOR *IN VITRO* PRODUCTION OF HIGH-YIELDING GLYCYRRHIZIN CONTAINING VARIETY

Thesis Submitted for the Award of the Degree of

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in

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By

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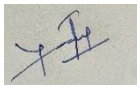
DECLARATION

I, hereby declared that the presented work in the thesis entitled "Genetic diversity assessment of licorice for *in vitro* production of high-yielding glycyrrhizin containing variety" in fulfilment of degree of **Doctor of Philosophy (Ph. D.)** is outcome of research work carried out by me under the supervision of Dr. Devendra Kumar Pandey, working as Professor, in the Department of Botany/ School of Bioengineering and Biosciences of Lovely Professional University, Punjab, India. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of other investigator. This work has not been submitted in part or full to any other University or Institute for the award of any degree.



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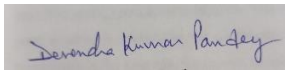
CERTIFICATE

This is to certify that the work reported in the Ph. D. thesis entitled "Genetic diversity assessment of licorice for *in vitro* production of high-yielding glycyrrhizin containing variety" submitted in fulfilment of the requirement for the reward of degree of **Doctor of Philosophy (Ph.D.)** in the Department of Botany/ School of Bioengineering and Biosciences is a research work carried out by N. W. Ayangla, Registration No. 11616823, is bonafide record of her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.



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ABSTRACT

Glycyrrhiza glabra Linn. (Fabaceae) or licorice or sweet root is a perennial herb/undershrub, which grows upto a height of 70 cm to 1.50 m. This particular herb is associated with Mediterranean basin areas and sevral portions of Asia and Europe. Nowadays, the plant is also cultivated in parts of Europe, USA and Asia, including India. The plant is well adapted in temperate, warm and sub-tropical climatic conditions. The plants thrive well in deep, loose, sandy and fertile soil, in full sun with sufficient supply of water. It can also grow well in dry regions having minimal annual rainfall. This plant is the oldest ones that the traditional medicinal community is aware of. The plant is prominent for its wide-ranging use in herbal medicines and formulations for innumerable diseases. The importance is attributed to the presence of glycyrrhizin, a non-sugar sweet-tasting agent, triterpene saponin with a characteristic sweet taste, its sweetness being 50-200 times more than sucrose. Glycyrrhizin which is mainly concentrated in the stolons and rhizomes, constitutes for about 2-15% dry weight which is dependent on species, geographical and environmental conditions. Besides, having tremendous pharmacological and biological properties, glycyrrhizin and its derivatives are widely used as food additives, in confectionery, cosmetics and flavouring agent as low-calorie sweetener. According to a recent report, the worldwide market for licorice stands at $ 848.9 million in the year 2022 and is speculated to increase. Glycyrrhizin originates in the stolons or rhizomes of the plant, resulting in extirpating the whole plant, which will endanger the sustainability of species populations, in the long run. Moreover, despite its recognition as an essential medicinal herb and commercial crop, yet it is diminutive of its management for mass cultivation. Little research has been done to examine the species' genetic foundation, especially, in India. It is time even in India to tap all the advantages of biotechnological advancements to move forward with breeding programmes by manipulating target genes and improving the crop yielding maximum glycyrrhizin. Nevertheless, considering the current status related to the plant, the research began to profile elite high-yielding population of *G. glabra*, followed by determination of the range of genetic variability in the species found in India and finally, establish a simple protocol that does not affect the biochemical production and confirm heritable variability of the regenerated plantlets.

Initially, optimization of various factors that affect the secondary metabolite extraction processes viz., interval, temperature, solvent mixture type, root particle measure, dilution ratio, pH, and sequence of steps involved in the recovery of glycyrrhizin were deliberated by RSM (response surface methodology), divided into a preliminary (Plackett-Burman model) followed by Central

composite model and compared with ANN (Artificial Neural Network). Estimation of the glycyrrhizin yield was done with the help of an optimized HPTLC technique. The key conditions that were observed for utmost recovery of glycyrrhizin through the aforementioned method stood at: 45 min. 55°C, 55% (v/v) ethanol, and 30 ml/g solvent-solute. The prototype was found to be suitable as it showed good consistency between the experimental value of 7.6 mg/g (0.76%) and the predicted value of 7.51 mg/g (0.751%) for glycyrrhizin amount. On comparison between RSM and ANN performance wise, ANN modeling was found to be far better in optimizing the glycyrrhizin yield.

In the study, 66 rhizome specimens of *G. glabra* were gathered from 12 distinct destinations across 6 states of India viz., Punjab, Haryana, Delhi, Himachal Pradesh, Uttar Pradesh and Maharashtra. The samples were approximately 2-3 years old and were collected during the fall and winter months. Extraction was done by green extraction method i.e., microwave-assisted extraction method and quantification of glycyrrhizin was done by HPTLC method. An average of 1. 38% of glycyrrhizin yield was observed from the experiment. The yield was slightly higher in Lucknow samples (2.18%) followed by Kurukshetra (2.12%) and the least amount was observed in the accessions from Pune (0.07%). Glycyrrhizin levels in Haryana samples were comparable to Lucknow samples which is perhaps due to analogous environmental settings in the two locations. Relationship studies was carried out to evaluate the association between glycyrrhizin yield and various environmental variables. The compound accumulation was observed to be highly related with longitude at $P<0.01$, $R^2=0.104$, and significantly with longitude at $P<0.05$, $R^2=0.086$.

Genetic diversity was evaluated in the cultivated, non-native *G. glabra* populations collected from different geographical locations, especially from Northern parts of India. For the study, 50 individuals from 7 populations, viz., Punjab (Phagwara, Ludhiana, Patiala), Haryana (Hisar, Kurukshetra), Uttar Pradesh (Lucknow), and New Delhi (South, West) were considered. DNA-based molecular markers viz., RAPD and ISSR were used to evaluate the genetic variation between and among the populations of the species. In total, 9 RAPD primers and 12 ISSR primers produced clear, distinct and discrete. The marker information obtained for RAPD (PIC= 0.14; MI=0.39; RP=1.32) and ISSR (PIC= 0.22; MI=3.59; RP=2.46) revealed that ISSR markers were much more effective in divulging information on polymorphism of the *G. glabra* populations. At the species level, genetic variation obtained was high while a moderate range of variability was recorded at the population level. On average, RAPD revealed (Ne=1.0617, H=0.0723, I=0.0463) while on the basis of ISSR it revealed (Ne=1.0246, H= 0.1782, I= 0.1122). The gene

disparity coefficient at the population strata with RAPD (G_{st} =0.2247) and with ISSR (G_{st}=0.2247) was lesser as compared to gene flow based on RAPD (N*m*=2.883) and ISSR (N*m*=1.7255). Low genetic variation is in line with AMOVA results that revealed more molecular variance within populations and less variation among the populations. The grouping investigation of collective RAPD and ISSR records bared the grouping of populations in one major cluster. Clustering was not formed in accordance with geographical distance as revealed by Mantel's test. In the dendrogram, LK, D, K, H, and L appeared in a major cluster, indicating a close genetic relationship. P and LP populations intended to move away from the major cluster exhibiting a lesser genetic relatedness. Results based on STRUCTURE were also in line with the dendrogram. The STRUCTURE showed assigning of the individuals into 3 different sets but exhibited admixture among individuals to some extent. In STRUCTURE analysis, P and LP populations were in one cluster, suggesting the possibility of having a similar origin for all the populations.

Based on the identification of high-yielding glycyrrhizin *G. glabra* species, nodal explants from the mother plant (Lucknow) were subjected to direct organogenesis via the *in vitro* tissue culture method. With growth media having MS and diverse doses of plant regulators, a prompt and easy micro propagation procedure was generated. Genetic integrity was assessed with the aid of molecular markers (ISSR). Furthermore, active compound (glycyrrhizin) consistency was evaluated with the help of HPTLC assay. Direct morphogenesis was accomplished using nodal explants. MS + BAP at a dose, 1.5mg/L showed the greatest recurrence of shoot development (96.45 %) while growth media with BAP (1.5mg/L) and IBA (0.50 mg/L) had the highest sprout multiplication (99.74%). Addition of IBA (1.0 mg/L) to 50% MS, and 5% sucrose stimulated in producing the highest regeneration frequency (92.36%). The plantlets so regenerated unveiled 92% survival rate. Additionally, ISSR-based studies supported genetic integrity and suggested authentic regenerants that are equivalent to the parent plant lacking soma clonal disparity. As evidenced from HPTLC, the proportion of glycyrrhizin in the regenerant root (0.076%) was fairly equal to that detected in parent roots (0.0752%), demonstrating its biochemical fidelity. As a means for continuous supply of glycyrrhizin-producing plants, the suggested *in vitro* method may be helpful.

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N. W. Ayangla

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ABBREVIATIONS

°C	Degree Celsius
μL	Microlitre
µM	Micrometre
2,4-D	2, 4-Dichlorophenoxyacetic Acid
AFLP	Amplified fragment length polymorphism
ANN-MLP	Artificial neural network – Multiplayer perceptron
B5	Gamborg B5
bp	Base pair
CAPS	Cleaved amplified polymorphic sequences
CBDP	CAAT box-derived polymorphism
cm	Centimetre
cpDNA	Chloroplast DNA
DAD	Diode-array detection
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EST-SSRs	Expressed sequence tag-SSR
GA_3	Giberellic acid
HCl	Hydrochloric acid
HSCCC	High speed counter current chromatography
ISSR	Inter-simple sequence repeat
LC-DAD	Liquid chromatography - Diode-array detection
LC-MS	Liquid chromatography-mass spectrometry
m	Metre
mg/L	Milligram per litre
mm	Millimetre
mM	Micromolar
NaCl	Sodium chloride
NaOH	Sodium hydroxide

ng	Nanogram
nL	Nanolitre
nrDNA	Nuclear DNA
ppm	Parts per million
RAPD	Random amplified polymorphic DNA
RFLP	Random Fragment Length Polymorphism
RH	Reverse phase
RT-qPCR	Qualitative reverse transcription
SCoT	Start codon targeted
SNP	Single nucleotide polymorphism
SRAP	Sequence-related amplified polymorphism
SSR	simple sequence repeats
TBE	Tris/Borate/EDTA
TDZ	Thiadizuron
TLC	Thin layer chromatography
UHPLC	Ultra high performance liquid chromatography
UV	Ultra violet

CHAPTER 1

INTRODUCTION

Plants and its products are long established to be inevitable for all communities across the world as source of food, timber, pharmaceuticals and fuel among other purposes. It is well known that the Sumerians used plants as their primary source of medicine at least 5000 years ago, and even ancient documents imply that people have been using plants for therapeutic purposes for a long time (Sharma *et al*., 2008). Historically, medicinal herbs were utilized in their crude form to alleviate abundant ailments, treat infections, injuries and other health related disorders as evidenced in Ayurveda, Unani and Chinese traditional medicine (CTM), Kampo etc (Itokawa *et al*., 2008). At the backdrop of traditional healing systems, modern world has come a long way in acknowledging the myriad of natural products. In this regard, natural products derived from plants act as templates for new drug discoveries and research. The Indian sub-continent harbours notable biological variation, making it amongst the seventeen mega hot-spots for biodiversity. A total of 45000 species out of 2, 50,000 higher order plants in the universe, have been reported from India, with different agricultural precincts, vegetations, biotic expanse and biomes (Sen *et al*., 2011). As the trend for trade of natural products bloom all over the world, it ultimately imposes a severe limitation on the resources that can lead to total loss of germplasm.

1.1 Prospective of therapeutic plants and biologically active substances

Medicinal plants as a potent library of bioactive compounds awaiting to be used as leads in drug discovery is well quoted (Adeola *et al*., 2021). Out of 250,000 higher plants on the planet (Farnsworth and Soejarto, 1985), only about 5% of them have been screened for medicinal values (Zakrzewski, 2002), suggesting a greater prospect of harnessing numerous active components as pharmaceutical and nutraceutical agents. Naturally occurring chemical compounds which are of paramount importance in supporting life processes in plants, also exhibit ecological functions. They are involved in adaptation to the environment by protecting from enemy attacks such as herbivores and microbes. Furthermore, they act as agents to charm insects/ animals for pollination and dispersal of seeds as well as allelopathic agents (Croteau *et al*., 2000).

Increasing interest in novel phytochemicals has enabled scientists and chemists to investigate and isolate several thousands of active principles from plants such as quinine from *Cinchona*, serpentine from *Rauwolfia serpentina*, cocaine from *Erythroxylum*

coca, codeine from *Papaver somniferum*, digitoxin from *Digitalis purpurea*, etc following scientific processes (Verma and Singh, 2008). It has been established that about 119 substances extracted naturally from about 90 plants are legally used in U.S (Cragg and Newman, 2001). Currently, a significant number (65%) of the 185 authorised therapeutic compounds for cancer therapy (from 1981 to 2019) are natural products in one form or another. (Newman and Cragg, 2020). Nowadays, a variety of drug molecules are introduced into market for different pharmacological targets including cancer, HIV/AIDS, Alzheimer's, auto immune diseases, senile dementia, malaria, and other infectious diseases. The estimation that 25%- 30% of prescribed medicines contain at least some plant components is now well established (Farnsworth and Soejarto, 1985; Shetty, 1997). Resurgence of the demand for plant-derived medicines and its products are driven by cost effectiveness, lesser side effects as they are "natural" rather than synthetic chemo-therapeutic compounds, beliefs and bioprospecting of new plant-derived drugs (Hoareau and DaSilva, 1999; Omogbadegun *et al*., 2011). Notwithstanding remarkable journey and progress in the synthetic industry, medicinal plants are exclusive in the pharmaceutical cultures in both developed and developing nations.

According to recent report, the global trade in medicinal plants stands at over USD 33 billion (Riaz *et al*., 2021). It is reported by the World Health Organization (WHO) that the annual growth rate of the economy of botanicals and their derived medicines is at 15%. It is projected by WHO, that the requirement of raw materials is predicted to proliferate beyond $ 5 trillion by 2050. Henceforth, the exploration of medicinal plants as source for active principles for therapeutics and also as starting materials for medicines, cosmetic, and food industries is anticipated.

1.2 Secondary metabolites

Organic substances known as secondary metabolites (SMs) or natural products are necessary for interacting with the environment but not for growth or life (Pagare *et al*., 2015). Unlike basic metabolites (sterols, lipids, nucleotides, amino acids etc) which are universally present in all plants, secondary metabolites (SMs) are constrained to certain taxa, with variation in the synthesis between and within plant groups and species, limited to specific tissues, development stages and seasons (Singh *et al*., 2010; Borges *et al*., 2017). SMs are broadly classified into terpenoids, phenolics and alkaloids (Hussain *et al*, 2012). Besides, flavonoids, coumarins, saponins, lignans, sulfides, curcumins, polyphenols, plant sterols and phathalides have been reported from plants (Craig, 1999).

Apparently, its utility and economy as therapeutic agents, food additives, flavours and other industrial substances has thrived the interest of many researchers to isolate thousands of SMs and their chemical structures elucidated (Rhodes, 1994). However, there is still a requisite for attaining maximum commercial applications (Vasconsuelo and Boland, 2007).

Plant extracts serve as the foundation for contemporary world of medicines. Thus, screening and isolation of the SMs from the medicinal plants is of paramount importance (Jain *et al*., 2019). Therefore, for separation and characterization of bioactive principles, sample preparation procedures and stability evaluation are highly crucial. The plant part and the solvent are two of the most significant factors influencing the efficiency of compound extract, with solvent type and strength acting as the limiting factors (Ngo *et al*., 2017). Besides, temperature, ratio, agitation speed etc., are also deliberated for optimal extraction (Azwanida, 2015). Nowadays, considerable advances have been made in the sample preparation procedures with continuous modifications, economically and in favour of green environment. Some modern techniques including microwave assisted extraction (MAE), supercritical fluid extraction (SFE), accelerated solvent extraction (ASE), pressurised liquid extraction (PLE) etc are stirred on decreasing or completely eliminating the use of organic solvents. These methods of extraction of SMs are optimized in favour of better recovery with lesser contaminants, cost and time and more refined products (Starmans and Nijhuis, 1996; Ong, 2004; Azwanida, 2015). Moreover, for further quantification and separation of target compounds, several innovative methods are applied including high performance thin layer chromatography (HPTLC), column chromatography (CC), flash chromatography (FC), High performance liquid chromatography (HPLC) etc.

1.3 Natural sweeteners

Sweeteners act as a stimulus for taste buds to induce a typical sweet response thus, improving the taste in which it is added. Sweeteners with varying degrees of sweetness ranging from several hundreds to over several thousand times than sucrose are used in food industries including the preparation of pastries, candies, beverages, chewing-gums and others. Furthermore, in pharmaceutical industries, sweeting agents are added as syrup base to retain stability, viscosity and to facade the unpleasantness of the medicine (Priya *et al*, 2011; Abraham and Mathew, 2014). Sweeteners are either natural, nutritive of saccharide origin (eg, honey, common sugar) or natural, non-nutritive of non-saccharide origin and artificial sweeteners that are synthetically

prepared (Polyák *et al*, 2010; Priya *et al*, 2011). It is well established that high intensity artificial sweeteners or chemically synthesized agents that includes aspartame, saccharin, cyclamate, neotame, alitame and acesulfame-k are in the market (Polyák *et al*, 2010).

However, over the last few decades, world-wide concerns for health implications such as chronic diseases have prompted an increased inclination for sugar substitutes, more specifically, low- calorie natural sweeteners which has provided a platform for research. It has been reported that natural sweeteners exist in about 150 plants belonging to more than 25 families including Leguminosae, Asteraceae, Verbenaceae, Marantaceae, Rutaceae etc and constituting a myriad of bioactive chemicals (Hussain *et al*, 1990; Kim and Kinghorn, 2002). Even though there are numerous naturally occurring plant products with sweetness (Table 1.1), very few of them are industrialized. Some examples such as **glycyrrhizin**, mogroside V, phyllodulcin, rebaudioside A, stevioside and thaumatin are all used as sugar substitutes elsewhere around the world (Kim and Kinghorn, 2002).

Table 1.1: Plants yielding natural sweeteners (Priya *et al*, 2011)

Plant species	Family	Sweetening principle	Chemical Class	No. of times sweeter than sucrose
Abrus precatorius	Leguminosae	Abrusoside and glycyrrhizin	Triterpene glycosides	30-100
Achras sapota	Sapotaceae	Glycyrrhizin	Triterpene glycosides	100
Baccharis gaudichaudiana		Gaudichaudioside-A	Diterpene glycosides	100
Cinnamomum osmophloeum	Lauraceae	trans-Cinnamaldehyde	Aromatic aldehyde	50
Citrus aurantium	Rutaceae	Neohesperidin dihydrochalcone	Dihydrochalcone	1000
Citrus limoni	Rutaceae	Hesperidin dihydrochalcone	Dihydrochalcone	300
Citrus sinensis	Rutaceae	Hesperidin dihydrochalcone	Dihydrochalcone	300
Citrus paradise	Rutaceae	Naringin dihydrochalcone	Dihydrochalcone	1000
Dioscoreophyllum Cuminsii	Menispermaceae	Monellin	Protein	2500

Glycyrrhiza glabra	Leguminosae	Glycyrrhizin	Triterpene glycosides	100
Hydrangea macrophylla	Saxifragaceae	Phyllodulcin	Dihydroisocoumarin	300-400
Lippia dulcis	Verbenaceae	Hernandulcin	Sesquiterpene	1000-1500
Perilla frutescens	Labiata e	Perillartine	Monoterpenoid	400-2000
Periandra dulcis		Periandrin V	Triterpene glycosides	100-200
Polypodium glycyrrhiza	Polypodiaceae	Polypodoside	Saponin glycosides	600
Polypodium vulgare	Polypodaceae	Osladin	Steroidal saponin glycosides	50-100
Pterocarya paliurus		Pterocaryoside A&B	Secodammaranoid saponin	50-100
Smilax glycyphylla	Liliaceae	Glycyphyllin	Dihydrochalcone glycosides	100-200
Stevia rebaudiana	Asteraceae	Steviosides	Tricyclicditerpenoid Glycosides	200-300
Siraltia grosvenorii		Mogroside V	Triterpene glycosides	250
Symplococos paniculata	Symplocaceae	Trilobatin	Dihydrochalcone Glycosides	400-1000
Thamatococcus	Marantaceae	Thaumatin	Protein	3000

1.3.1. Global status of natural low-calorie sweeteners

Health consciousness is a major driving force behind increasing inclination towards sugar free products. Today, a number of accepted low calorie natural sweeteners derived from plants are incorporated in food and beverages, herbal and traditional medicines. This has resulted in a surge for natural sweeteners, more specifically, of low-calorie sweeteners including **licorice** across the world. According to FMI report, (2022), the worldwide market for licorice, which was valued at $ 848.9 million in 2022, is expected to grow to $ 1.42 billion by 2032, showing a growth rate of 5.3%. China and India witnessed an increase in licorice consumption while major importers included countries such as Germany, France, US, China and Netherlands.

1.4. Plant genetic diversity: A basis for conservation

Biological diversity is the epitome all types of variation viz., genetic, species, and ecosystem diversity, in the living realms of the world (Begna, 2021). Genetic diversity refers to the genetic variability among populations and within individuals of the same species. Genetic diversity is denoted as differences in nucleotides, genes, chromosomes or whole genome of plants. Thus, genetic variety is a statistical term to denote variation among alleles of a gene or between populations or individual plants within the individual loci. It conveys the kind of allemorphs predominant in the gene pool that ultimately determines the type of genotype within populations. Genetic diversity is an inevitable hierarchy without which a population cannot evolve and acclimatize to environmental changes. Variation at the gene level acts as a raw material for adaptation in the changing environment. The more is the variation, the better is the chance of the individual survival which will further help in diversifying that particular population into subsequent generations. The understanding of genetic variation as the starting point of all forms of diversity thus, becomes crucial tool for taxonomic and evolutionary studies and further, for devising future conservation and management strategies of the genetic resources of a plant (Muhammed, 2012).

Identification, characterization and evaluation of genetic variation are the key activities to be kept in mind while designing for conservation of biodiversity. Plant characterization acts a discriminatory factor in establishing the uniqueness of each individual and its genetic relationship among various genotypes. For any selective breeding and for establishing conservation strategies, plant characterization is gauged on the basis of phenotypic, biochemical and genetic characterization and evaluation (Mondini *et al*, 2009). Morphological and biochemical characterization have its own sets of limitations to determine the variation between and within species. However, with the advent of molecular markers, it has become practical to portray gene diversification and also infer genetic relationships among organisms at DNA level. DNA-based molecular markers which are free from environmental interference are more promising over other kinds as they readily provide fine and consistent information on hereditary materials towards inferring genetic variations (Duran *et al*., 2009). A myriad of DNA-based markers such as RFLP, RAPD, AFLP, ISSR, SSR, CAPS, SNP etc. are being used today depending on the assay (hybridization or amplification- based) for ecological, evolutionary, taxonomical, phylogenetic and genetic studies of plants. Some existing

literatures that have used DNA- based molecular markers that have contributed to genetic diversity and conservation of medicinal plants are presented in table 1. 2.

Table 1.2: List of some DNA markers applied in medicinal plants

Medicinal plants	Type of molecular marker	Achievements	References
Acorus calamus	RAPD and Chloroplast microsatellite	Appraisal of genetic variation and population structure	Ginwal *et al*., 2011.
Mentha species	RAPD	Assessments of genetic relationships in 11 accessions from 6 taxa of Mentha developed by CIMAP	Khanuja *et al*., 2000.
Panax quinquefolius L.	RAPD	Evaluation and inter and intra specific variation	Schlag and McIntosh, 2012.
Stevia rebaudiana	RAPD	Estimation on accessions from India for gene variability	Chester *et al*., 2013.
Hypericum perforatum L.	ISSR	Gauging of genetic divereness and relationship of *Hypericum* L *spp.* From Iran.	Ma *et al*., 2021.
Solanum trilobatum L.	RAPD and ISSR	Estimation of gene variation in 14 accessions from five South Indian states	Shilpha *et al*., 2013.
Pimpinella anisum L.	RAPD and ISSR	Investigation genetic variation	Akçali Giachino, 2020
Tribulus terrestris	AFLP, SAMPL, ISSR and RAPD	Assessment of genetic polymorphism from populations of various precincts of India.	Sarwat *et al.*, 2008.
Moringa oleifera Lam.	AFLP	Analysis of genetic variation	Muluvi *et al*., 1999.
Origanum spp.	EST-SSR, SRAP	Genetic diversity assessment	Taşcıoğlu *et al*., 2018
Andrographis paniculata	RAPD-ISSR, SCoT and CBDP	Study of gene divergence and population structure	Tiwari *et al*., 2016.

1.5 Biotechnological manoeuvres to salvage therapeutic herbs

In India alone, out of approximately 45,000 plant species, nearly several thousand have claimed to possess medicinal properties (Kala *et al*., 2006). It has been reported that India has a rich medicinal plant resource of about 8000 species (Kadam and Pawar, 2000). However, due to the growing anthropogenic pressure to fulfil the ever-changing life styles the medicinal plants are diminishing rapidly from its natural habitats (Jain *et al.*, 2012). Hence, conservation of medicinal plants has grown into a global concern owing to a number of well-known pressures imposing a threat to plants' sustenance. According to a report, almost all the remedial raw resources for medicines are gathered from the natural flora (Verma *et al.*, 2012). The inconsiderate manipulation by locals and industries, destructive harvesting etc would jeopardize the medicinal plant population and its genetic resources (Shippmann *et al.*, 2003). The retention of medicinal plants and their genetic base is a subject of renewed attention worldwide due to a rise in interest in natural products. Conservation of medicinal plants is achieved either through on-site or off-site approaches. Numerous studies have found that both strategies for conserving plant genetic resources are effective (Barazani *et al*, 2008; Chen *et al*, 2016). While *in-situ* take measures to protect species and their habitats on site, *ex-situ* allows preservation of genetic resources via botanicals, seed storage and field gene banks. Nowadays, maximum efforts are channelled towards *ex-situ* conservation of plants with resources, as it is regarded as the most suitable way for protecting plants and their wild relatives away from their natural habitats (Pence *et al*., 2020). The National Medicinal Plant Board was established by the Indian Government in the year 2000 to coordinate and implement schemes and strategies for cultivation and safeguarding of medicinal plants. In coordination with the agency, the ministry of AYUSH has identified nearly 60 prioritized plants for cultivation, with ***Glycyrrhiza glabra*** being one of them.

Given the pressure for consistency in demand and supply side-by-side degradation of genetic resources, *in-vitro* techniques or biotechnological methods open up avenues for improved mass cultivation and thereby, conserving the resources. Revolutionary advancements in plant tissue culture have made it possible to quickly multiply plants, triumphing over barriers like seasonal constraints, disease manifestations and other physiological problems (Sharma *et al*, 2010). The multiplication of the cells, organs or tissues of plants in solid or liquid media under

controlled environmental conditions to improve upon strains or cell lines is well quoted (Gaurav *et al*., 2018). Breeding of botanicals, commercially, through meristematic and adventitious organs or callus cultures and somatic embryogenesis in cells or callus have been reported (Rout *et al*, 2005).

1.5.1. *In vitro* propagation.

There are primarily two approaches to plant *in vitro* propagation. The plants are either multiplied via axillary buds and apical shoots or via the formation of adventitious shoots or somatic embryos. Commonly, pre-existing axillary buds are frequently encouraged to develop into numerous shoots that increase logarithmically with succeeding subcultures (Sharma *et al*, 2010). Similar approaches to micropropagation include organogenesis and somatic embryogenesis. Adventitious shoot formation is synonymously regarded as direct organogenesis. In it, morphogenesis occurs without a callus or suspension phase. Indirect organogenesis involves the formation of tissues by way of callus initiation. Somatic embryogenesis can either siphon off straight away on explants intact or diverge from explant cells via callus or suspension phase. Generally, micropropagation consists of several steps like pre-propagation, initiation of explants, subculturing, shoot and root formation followed by acclimatization. Application of *in vitro* propagation techniques has a long history of its effectiveness in conserving important medicinal plants such as *Gymnema sylvestre* (Komalavalli, 2000); *Podophyllum hexandrum* (Nadeem *et al*., 2000); *Urginea altissima* (Baskaran *et al*., 2018). For mass cultivation, tissue culture techniques would produce aseptic, high-quality plants that would beneifit both the consumers and the farming community.

1.6. Aims and objectives

Glycyrrhiza glabra is one of the prioritized plants identified by the government for cultivation. The plant is recognized for its destructive harvest, which results in the exhaustion of high-yielding varieties or depletion of resources in the habitat. Environmental factors, seasonal variations and genes impact synthesis or build-up of active principles in medicinal plants. Hence, the study aims to determine any variation at the biochemical and gene levels and to devise future conservation strategies and plant breeding programmes through *in vitro* tissue culture techniques to ensure species ‘sustainability.

Objectives of the study:

1. Collection of licorice plants / germplasm (*G. glabra*) from different geographical locations of India.
2. Identification of high yielding glycyrrhizin containing lines by HPTLC fingerprinting of different populations of licorice
3. Assessment of genetic diversity of licorice by molecular markers.
4. Mass propagation of high yielding glycyrrhizin containing licorice through *in vitro* technique.

CHAPTER 2
REVIEW OF LITERATURE

2.1 Botanical description

Among the botanicals, *Glycyrrhiza glabra* Linn. is a prominently sought herb in several traditional systems. It is a perennial hard herb or under-shrub attaining a height of 70 cm to about l.50 m (Fenwick *et al.*, 1990; Henry *et al.*, 1991), the most important part being underground stems. The plants have a deep fusiform root system held by a subterranean stem or rhizome that extends more than 1m or up to 17m in length, especially in arid regions (Kushiev *et al.*, 2005). The compound leaf bears elongated ovalish leaflets numbering from 9 - 17, usually terminating in a single leaflet (Henry *et al*., 1991). The flowers are in an unbranched inflorescence, bunched with 20 to 30 flowers per spike that are lavender to violet in colour (Kaur *et al*, 2013). The pods are somewhat flattened and oblong, having few small rounded and greenish seeds (Henry *et al*, 1991; Yaneva *et al*., 2020).

Figure 2.1: *G. glabra* from herbal garden, LPU, Punjab.

Kingdom	Plantae
Division	Angiospermae
Class	Dicotyledonae
Order	Rosales
Family	Fabaceae
Genus	*Glycyrrhiza*
Species	*glabra* Linn.

Scientific classification (Sharma and Agrawal, 2013)

2.2 Distribution and Ecology

Glycyrrhizza spp. consists of about 30 species found wild or under cultivation. Of these, only few exhibits industrial potentials to be exploited including *G. glabra* L., *G. glabra* L. var. *glandulifera, G. uralensis* D, C and *G. echinata* L (Henry *et al,* 1991). *G. glabra* is indigenously distributed in Southwestern Asia and the Mediterranean countries (Hayashi and Sudo, 2009; Sharma and Agrawal, 2013). It is cultivated in Italy, Russia, France, UK, Germany, Spain, USA, China, Japan, Pakistan, Iran, Iraq, Afghanistan, Uzbekistan, Turkey etc. There are reports on cultivation of licorice in India such as Punjab, Haryana, Uttar Pradesh, Delhi, Dehradun, Jammu and Kashmir and in south India (Meena *et al*, 2010). The plant is well adapted in temperate, warm and sub-tropical climatic conditions (Anagha *et al*, 2012; Dastagir and Rizvi, 2016). The plants show optimal growth in deep, loose, sandy and fertile soil, in full sun near streams or rivers for sufficient supply of water. The plants are even well adapted in dry regions having annual rainfall not more than 50cm. Mohammad and Rehman, (1985) reported successful growth of *G. glabra* in irrigated as well as rainfed sand dune areas of Mastung area, Baluchistan. Reclamation of saline soils by growing *G. glabra* has been demonstrated in the Aral Sea basin (Kushiev *et al,* 2005). Similarly, reclamation of sodic lands by reducing soil pH and exchangeable sodium with the growth of licorice has been reported in Karnal, Haryana (Dagar *et al,* 2015).

2.3 Glycyrrhizin- the most active principle

Previous studies on the chemistry reveal that licorice is composed of a complex of compounds, of which glycyrrhizin is the most important constituent. Glycyrrhizin is a triterpene saponin with a characteristic sweet taste; its sweetness being 50 – 200 times more than that of sucrose. Glycyrrhizin which is mainly concentrated in the stolons and rhizomes, constitutes for about 2-15% dry weight which is dependent on species, geographical and environmental conditions. It occurs as calcium-potassium salts of glycyrrhizic acid/glycyrrhizic/glycyrrhizinic acid and a glycoside of glycyrrhetinic acid (Asl and Hosseinzadeh, 2008). Glycyrrhizin exhibits incredible pharmacological properties. Moreover, glycyrrhizin and its derivatives are widely used as food additives, in confectionery, cosmetics and as flavouring agent (Hayashi and Sudo, 2009).

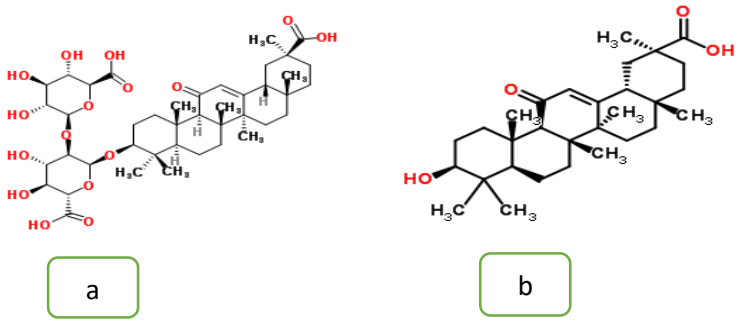

Figure 2.2: (a) Structure of glycyrrhizin, (b) Structure of its enoxolone, glycyrrhetenic acid.

2.4. Pharmacological perspective of *G. glabra*

G. glabra has a long antiquity of custom in traditional medicine systems around the world as a remedy for cold, cough, as a tonic and nutrition, to heal wounds, ulcerous sores etc (Davis and Moris, 1991). Licorice extract and derivatives of 18 glycyrrhetinic acid have been shown to have an antitussive effect similar to codeine (Anderson and Smith, 1961). Licorice has been long used to treat coughs, sore throats, and bronchial catarrh. It has demulcent and expectorant activities (Pastorino *et al*., 2018). Glycyrrhizin has been found to be a good agent of thrombin inhibitor, an important component of coagulation. Glycyrrhizin has been demonstrated to speed up the rates at which plasma recalcification, thrombin, and fibrinogen clot. Additionally, it prevented the platelet aggregation caused by thrombin (Mauricio *et al*, 1997). Mendes-Silva *et al.* (2003) also confirmed glycyrrhizin's ability to inhibit thrombin *in vivo*, implying that its anticoagulant effect is presumably due to its anti-inflammatory properties. *In vitro* studies have revealed glycyrrhizin as a potent antiviral agent against the Japanese encephalitis virus (Badam, 1997). Glycyrrhizic acid was also found to irreversibly inactivate the herpes simplex virus particles (Fiore *et al*, 2008). It has been demonstrated that glycyrrhizin obstructs the duplication of the human immunodeficiency virus (HIV) by managing the dosage (Ito *et al*, 1988) which is due to interference with virus-cell binding (De Clercq, 2000). Glycyrrhizin was demonstrated to inhibit the Hepatitis A (Crance *et al*, 1994), Hepatitis B (Takahara *et al*., 1994) and Hepatitis C virus in a dose-dependent fashion (Ashfaq *et al*., 2011). Glycyrrhizin was demonstrated to have antiviral effects on the SARS coronavirus *in vitro* (Cinatl *et al*., 2003). Due to its anti-inflammatory activity, licorice is reported to treat arthritis and allergies. Dhingra *et al*., (2004) reported that *G. glabra* can be used as a memory booster owing to its anti-inflammatory and antioxidant potential.

Although numerous health beneficial effects of licorice have been underlined, yet it is reported to be flanked by certain adverse effects. On administering graded doses of dried aqueous extracts of licorice root to healthy subjects, only the ones ingesting highest doses showed untoward side effects (Bernardi *et al*, 1994). Excessive or prolonged licorice consumption can result in hypermineralocorticoid–like effects. However, the effects of licorice are modifiable when consumption is stopped. A daily dose of only about 0.015-0.229 mg glycyrrhizin/ kg body weight is recommended (Isbrucker and Burdock, 2006).

Contemporary developments in pharmacological sciences have shown multiple healing benefits associated with *G. glabra*, its extracts, glycyrrhizin and herbal preparations, either by themselves or in synergy with other ingredients are enumerated (Table 2.1).

Table 2.1: Recent advances in pharmacological activities of glycyrrhizin

Bioacitivity/ adaptation disease	Model organism/cell	Result	Reference
Anticancer (liver carcinoma)	Human liver cancer cell line HepG-2	Inhibition of hepatocellular carcinoma cell migration, arrest of cell cycle at G2/M which in turn lead to induced apoptosis via the extrinsic pathway.	El-Senduny *et al*., 2019
Anticancer	Progesterone receptor membrane component 1 (PGRMC1) cancer cells	Binding of glucoglycyrrhizin (GlucoGL) to Progesterone Receptor Membrane Component 1 (PGRMC1) inhibited its function and suppression of PGRMC1-mediated cancer chemoresistance.	Kabe *et al*., 2021
Anticancer effects (colorectal cancer)	Human colorectal carcinoma cells SW48	Inhibition of cell growth in SW48. Inducted apoptosis with increase in Bax expression, Beclin-1, LC3B-I and LC3B-II. Supression of Bcl-2 expression.	Zhang *et al*., 2020

Antihyperglycemic effect (Diabetes mellitus)	Alloxan-induced diabetic mice.	Significant reduction in the blood glucose concentration comparable to reference drug.	Mustafa *et al*., 2019
Anti-Alzheimer's disease	Wounded normal cells (WI-38).	Glycyrrhizin and its derivative glycyrrhetinic acids showed remarkable healing activity to treated wounded cells.	Abdel Bar *et al*., 2019
Anti–diabetic (Alleviation of atherosclerotic lesion caused by diabetes)	Sprague Dawley rats induced with diabetic mellitus (DM)-antherosclerosis lesions (AS)	Decreased blood sugar level, inhibited biochemical parameters.	Zhao *et al*., 2021
Hepatoprotective activity	Carbon tetrachloride (CCl_4)-induced hepatic damaged rats	Reduction in liver wound by treatment with glycyrrhizic acids.	Huo *et al*., 2020
Anti-inflammatory and protective effects on acute lung injury	Lipopolysaccharide (LPS)-induced Acute lung injured (ALI) mouse	Reduction in protein amount, inflammatory cell counts and tumor necrosis.	Am Lee *et al*., 2019
Anti-viral activity (SARS-CoV-2)	SARS-CoV-2 isolate infected Vero E6 cells	Viral duplication was supressed with the treatment of glycyrrhizin blocking protease production.	Van de Sand *et al*., 2021
Antibacterial effect (Dental pathogens)	*Porphyromonas gingivalis, Treponema denticola, Tannerella forsythia, Streptococcus mutans,* and *Enterococcus faecalis*	Corneal surface was shown to inhibit production of new blood vesses. Phosphopyruvate hydratase protein present in all genera interacted with glycyrrhizin.	Balamithra *et al.*, 2020

Anti-Parkinson's disease	PD patients (YAHR staging ≤ 3)	Improved UPDRS, tremor with better motor and rigidity tests ($p > 0.05$). No abnormality in electrolyte, changes in blood pressure or blood glucose levels were observed.	Petramfar *et al*., 2020
Immunotherapeutic effect (cutaneous Leishmaniasis)	*Leishmania major*-infected BALB/c mice	Inhibition of the growth of *L. major* promastigotes and amastigotes, lessened the lesions and inhibited the parasites in the lymph regions.	Sheikhi *et al*., 2022

2.5 Extraction and analytical methods for determination of glycyrrhizin and related compounds.

The techniques for prototype preparation for further separation and categorization come first after isolation of active components from any pharmaceutically significant plants (Huie, 2002). Sample preparation requires the use of selective solvents to separate soluble plant metabolites from insoluble residues. The solvents used range from water to organic compounds of varying polarities to determine the solubility of the bioactive constituents. From the available literature, traditional techniques for sample preparation of *Glycyrrhiza spp*. include soxhlet extraction, heat reflux method, room temperature extraction, batch single pot extraction, bulk extraction method (Noori *et al*., 2018). Sample preparation technology has consistently grown from more exhaustive to less taxing and more reliable methods including ultrasonic-assisted extraction (Gupta *et al*., 2012; Charpe and Rathod, 2012; Jang *et al*., 2017; Lanjekar and Rathod, 2021); microwave-assisted extraction (Talebpour *et al*., 2009; Shams *et al*., 2015) and also coupled with ionic liquid (Bhan *et al*., 2017); pressurised hot water extraction (Mukhopadhyay and Panja, 2009); molecularly imprinted solid phase (Cirillo *et al*., 2011); super heated water (Shabkhiz *et al*., 2016); supercritical fluid (Hedayati and Ghoreishi, 2016); aqueous two phase (Zhang *et al*., 2019); enzyme-assisted extraction (Giahi *et al*., 2021). In almost all the methods, the most influential parameters are the solvent type and strength although, other factors including temperature, ratio, agitation speed, etc., are also considered for optimum extraction (Azwanida, 2015). Several separation techniques are available nowadays to determine the isolated secondary metabolites, which are depicted in Table 2.2.

Table 2.2: Summary of phytochemical recovery methods for estimation of glycyrrhizin and its related principles from *G. glabra.*

Analytical technique	Analyte	Instrument condition	Sample preparation	Reference
HPLC	Glycyrrhizin	RP-18 column. Mobile phase-methanol, water, acetic acid (60:34:6, v/v/v)	2g+30ml water+2mL NH_4OH (37%); pH 7.0 with H_3PO_4 + 1 ml 10% diastase; CH_3OH	Hurst *et al*., 1983
Capillary electrophoresis	Glycyrrhizin and glycyrrhetinic acid	Sample time 5 s hydrostatic; run time 10min; voltage 25kV; temp 25.0-25.5°C. Buffer:20% acetonitrile and 80% 0.02M sodium dihydrogenphosphate; pH 7.5 with 0.05m NaOH	70% methanol. Centrifuged for 10 min at 1500 g. 2.5mg cinnamic acid in 1ml of 70% methanol. Finally diluted to 25 ml with 70% methanol.	Chen and Sheu, 1993
NIR spectroscopy; RP- HPLC.	Glycyrrhizin	Mobile phase rate flow 1.0 ml/min. Column temp. 25±1°C. UV at 253 nm.	65% methanol	Chen and Sorensen, 2000
RP-HPLC	Glycyrrhizic acid	Retention time 4.35 min.	Microwave-assisted extraction (MAE), 50-60% ethanol(v/v)	Pan *et al*., 2000
HPLC	Glycyrrhizin	Reverse phase column C18. Mobile phase: methanol/water/acetic acid (67:26:7 by volume); 256 nm.	1.510.01% (w/v) NH_4OH, Ethanol/ phosphate aqueous two-phase system: alcohol (ethanol 60, v/v)/ salt (phosphate or sulphate) (15%, w/v).	Tianwei *et al*., 2002
HSCCC	Glycyrrhizin	Two phase solvent system: ethyl acetate–methanol–water (5:2:5 by volume).	Ultrasonic assisted extraction; Methanol: water (70:30, by volume).	Jiang *et al*., 2004
Isocratic HPLC	Glycyrrhizin	Water- acetonitrile (62:38, v/v). Mobile phase (pH 2.5, H_5PO_4). Flow rate 1.2ml/min	Supercritical fluid extraction: supply of CO_2 (15%) +modifiers (70% (v/v) methanol); 30MPa and 60°C.	Kim *et al*., 2004
HPLC	Glycyrrhizic acid	Mobile phase (CH_3 CN, H_2O), solvent modifier	Multi-stage counter current extraction	Wang *et al*., 2004

		(0.05% trifluoro acetic acid), flow rate 0.8 ml/min, retention time 9.9min.	(MCE; 5 stages, 60 min/ stage, 60°C, 6ml/g (solvent- water: licorice)	
HPLC+NIH imaging	Glycyrrhizin	Mobile phase: Acetic acid, acetonitrile(3:2v/v); flow rate 1.0ml/min; detection 254nm; retention time 10min.	Ultra sonic assisted extraction: 0.5ml methanol	Morinaga *et al*., 2005
ME-TLC	Glycyrrhizin and others	Mobile phase: ethyl acetate-formic acid-acetic acid (17:1:1, v/v/v)	Ultrasonic bath-40 ml methanol for 30 mins.	Cui *et al*., 2005
Isocratic HPLC	Glycyrrhizin and 18 β-Glycyrrhetenic acid	C_8 column-stationary phase. Mobile phase – acetonitrile, methanol, water, glacial acetic acid (35:35:30:1 by volume)	19ml of ethanol: water (1:1, v/v)	Sabbioni *et al*., 2006
HPLC	Glycyrrhizin	Mobile phase: (A) 0.1% formic acid in water, (B) 0.1% formic acid in acetonitrile. Detection at 254 nm. Temp 40°C and rate flow 1.0 ml/min.	Surfactant assisted pressurized liquid extraction (PLE), Sodium dodecyl sulfate (SDS) (0.1-0.4%, w/v) and triton X-100 (0.1-1.0%, v/v); sonication- methanol, water (70:30)	Eng *et al*., 2007
HPLC	18β-glycyrrhetinic acid	C-18 RP. Mobile phase: acetonitrile-phosphoric acid (3:1). Flow rate 0.6 ml/min (0.8 min), 0.4 ml/ min (8-20 min), UV detection 230 nm.	20 ml methanol. Shaking (30 min), centrigugation (5 min at 700 g). 20 ml methanol added to residue, decanted after 30 min. supernatants evaporated.	Esmaeli *et al*., 2010
On-line coupled capillary isotachophoresis-capillary zone electrophoresis	Glycyrrhizin	Leading, terminating and background electrolyte using acidified methanol of strength 30.	Ultra sonic- assisted extraction; 20% methanol in ultrasonic bath.	Kvasnicka and Voldrich, 2007

RP- HPLC	Glycyrrhizin and others	Reversed phase; mobile phase: 25mM phosphate buffer (pH 2.5), acetonitrile; peaks of detection 254 nm.	80% methanol (aqueous).	Wang and Yang, 2007
HPLC	Glycyrrhizin	Reverse phase gradient elution; Mobile phase (0.1%phosphoric acid, acetronile); flow rate (0.8ml/m); retention time (55.45 ± 0.31min); detection (254nm, 230nm, 280nm, 320nm).	Ultrasonic assisted extraction: 8ml methanol	Xie *et al*., 2010
RP-HPLC	Glycyrrhizic acid (GA) and glabridin	Mobile phase: methanol/water (70:30, v/v) +1% acetic acid. Flow rate 1.0ml/min; UV wavelength 252 nm.	Ultrasonic assisted extraction: Ethanol-Water (30:70, v/v); 60min dipping time under50° C.	Tian *et al*., 2008
UV spectrophotometer	Glycyrrhizic acid	Based on vanillin sulfuric acid method. Maximum recovery at 110° C, 5 atm with ratio of 0.01% (w/v) ammonia solution in 40 ml/g.	Pressurized hot water extraction (30-120° C). Dissolved in ammonia (0.01-4%, w/v) and pressurized with carbondioxide at varying pressure, water to feed and extraction time.	Mukhopadhyay and Panja, 2008
HPTLC	Glycyrrhizin	Mobile phase: ethyl acetate, glacial acetic acid, formic acid and water (15:2:1:1 by volume); detection 252nm.	Ultrasonic assisted extraction: 70% ethanol	Jadhav *et al*., 2009
HPLC	Glycyrrhizic acid	Mobile phase: Methanol/acetonitrile/water/glacialacetic acid mixture 35/35/30/1 by volume. Flow rate 0.5ml/ min.	Molecularly Imprinted Solid Phase Extraction (MISPE). Non covalent MIP-methacrylic acid (MAA), 2-(dimethylamino) ethyl dimethacrylate (EGDMA). Extraction	Cirillo *et al*., 2011

			20 ml ethanol/water (5/5, v/v)	
UV spectrophotometry	Glycyrrhizic acid	256nm; solvent- distilled water; distilled water as blank.	Ultrasonic assisted extraction: 16ml methanol	Raja *et al*., 2010
LC-DAD and LC-MS/MS; HPLC	Glycyrrhizic acid	Reverse phase C18 column. Mobile phase: phosphate buffer, acetonitrile (13:7); detection at 252 nm.	Hot water.	De *et al*., 2012
HPTLC	18β-glycyrrhetinic acid and β-sitosterol	Mobile phase: toluene-ethyl acetate-methanol (14.3:3.8:1.9, v/v/v). Post derivatization with 5% sulphuric acid. Retention 0.32 ± 0.01 and 0.71 ± 0.01.	Ultrasonic method: methanol, 1 hr (for β-sitosterol). Chloroform (for 18β-glycyrrhetinic acid).	Potawale *et al*., 2013
HPLC	Glycyrrhizic acid	C-18 column, isocratic elution with solvent A (0.5% acetic acid in H_2O) and solvent B (methanol) [A: B is 55:45]. Detection at 280nm.	Microwave assisted extraction (MAE); 60% ethanol, 1% ammonia.	Shams *et al*., 2015
HPTLC	Glycyrrhizin	Mobile phase ethyl acetate (EA): glacial acetic acid: methanol: water (6:2:2:1v/v/v)	Ultrasonic method, methanol solvent.	Siddiqui *et al*., 2015
HPLC	Glycyrrhizic acid	Mobile phase: potassium acetate buffer: acetonitrile (68.8:31.2, v/v). Detection by PDA at 254 nm.	Ultrasonic method. Aqueous	Abu-Shandi *et al*., 2015
HPLC	Glycyrrhizic acid	Mobile phase: Methanol 41%, water 59% (3% acetic acid); flow rate 1ml/min; detection at 248nm.	Supercritical method. 29.6 MPa, 68°C, CO_2 flow rate 2ml/min, time 108min, Methanol 46.5%	Hedayati and Ghoreishi, 2015
RP-HPLC	Glycyrrhizic acid	Mobile phase: A: water-acetic acid, B: methanol, isocratic mode at 42.6%	Superheated water extraction (SWE)-100ml ammonia (8g/l);	Shabkhiz *et al*., 2016

		and 57.4% with flow rate of 1.5mL/min.	100°C, 15 mL/min flow rate,120 min.	
RP-HPLC-DAD	Glycyrrhizin and others	Mobile phase: Glacial acetic acid, acetonitrile, water (6:30:64 V/V/V); Flow rate of 1.0 ml/min.	Ultrasonic method: 10%-90% glycerol; 360 W, frequency 35 Hz, temperature (20°C-70°C)	Ciganović *et al*., 2019
RP-HPTLC	Glycyrrhizin	Mobile phase: Methanol-water (7:3 v/v). Absorbance at 256 nm.	Percolation method-methanol (3x70 ml)	Alam *et al*., 2017
HPTLC	Glycyrrhizin	Mobile phase: Ethyl acetate, methanol, water, formic acid (15:2:1:1 volume); detection at 252nm.	Ethanolic extract	Salunke *et al*., 2020
HPLC	Glycyrrhizic acid and others	Mobile phase A: 0.2% glacial acetic acid; B: acetonitrile.	Ultrasonic method-70% ethanol, 30 min.	Li *et al*., 2021
HPLC	Glycyrrhizin	Mobile phase: Methanol, water and glacial acetic acid (7:3:1).	Ultrasonic method: NADES solvent-Chlorine chloride, lactic acid (1:1), 30 min, 30°C	Lanjekar and Rathod, 2021

2.6. Molecular markers for diversity assessment of Licorice (*Glycyrrhiza glabra*)

Molecular markers are now persistently utilized to control and conserve the genetic properties of medicinal floras. Genetic diversity can be gauged based on phenotypic and biochemical and molecular characterization and evaluation (Mondini *et al*, 2009). DNA-based molecular markers that are free from environmental interference are more promising than other kinds as they readily provide fine and consistent information on hereditary materials for inferring genetic variations (Subramanyam *et al*, 2009).With the foreseeable future in the importance of *Glycyrrhiza* spp., numerous efforts are underway to elucidate the genetic variety of *Gylcyrrhiza* species with the divergence of wild populations and genetic relations within the species as listed in Table 2.3. Recently, a phylogenetic study based on *cp*DNA variation in synergy with molecular markers on three *Glycyrrhiza* species, viz., *G. glabra*, *G. uralensis* and *G. korshinskyi* from North Eurasia established two main genetic lineages with considerable genetic

variation between the former first two. The study found evidence of past and present introgressive hybridization in all the species (Hantemirova *et al*., 2020). Genetic variation assessments at the nuclear DNA level and other related subjects in different *Glycyrrhiza* spp. have been accomplished utilizing RAPD (Gao *et al*, 2000; Khan *et al*, 2009), ISSR in *G. uralensis* (Yao *et al*, 2008), SSR/ microsatellites (Erayman *et al*, 2014; Liu *et al*, 2015; Um *et al*, 2016), RT-qPCR (Maroufi *et al*, 2016), SNPs (Dang *et al*., 2022). However, genetic assessment in *G. glabra* is limited and not well-founded. Certain DNA level indicating systems utilised in the evaluation of changes in hereditary materials in different *Glycyrrhiza spp*. are as below:

2.6.1 SSR markers

SSR markers or microsatellites are repetitons of short-sequences of DNA. They are co-dominant markers and possess large number of alleles (Souza *et al*, 2008). SSRs are highly polymorphic, specific and repeatable providing more detailed genetic insight although they are costly to develop (Zalapa *et al*, 2012). In order to study the genetic divergence of licorice, Erayman *et al.* (2014) endeavoured to transport SSR markers from three unrelated legumes to *Glycyrrhiza* species. They reported that *M. truncatula* markers show the maximum transferability rate with 33% and *P. vulgaris* markers exhibited the highest genetic diversity value. *Glycyrrhiza* species revealed higher genetic divergence than the unrelated legumes. Liu *et al.* (2015) in their study efficiently developed SSR markers in *G. uralensis* where sixty-four pairs of SSA primers could generate PCR amplification of genomic DNA. In the most recent work, Um *et al* (2016) established genetic variability of *G. uralensis* based on publicly available *Glycyrrhiza* EST databases. It generated 8 novel polymorphic microsatellites which when screened across *G. glabra* and *G. uralensis* revealed correlative genetic relationship between the species.

2.6.2 Random Amplified Polymorphic DNA (RAPD)

These group of primers are informative, easy to develop whereby; results are quickly and readily obtained but lack reproducibility (Virk *et al*, 2000). It does not require plant genetic expertise and only needs a small amount of genomic material. These powerful techniques are based on PCR amplification followed by agarose gel electrophoresis. These markers have been successfully used to study inter and intra specific gene relationships, genetic variability, and molecular characterization of licorice. In one study, it has been reported that Yamazaki *et al*. (1994) drew the interaction and relation between four species of licorice with the aid of RAPD and AFLP. Phylogenetic trees

construction based on the data, bared that glycyrrhizin-rich, *G. glabra* and *G. uralensis* are more connected to one another while remotely related to *G. inflata* and *G. pallidiflora*. Khan *et al.* (2009) demonstrated that 16 out of 52 primers produced discrete amplifications for the species annotating correct identification of *G. glabra*.

2.6.3. ISSR markers

ISSR markers are amplifies by PCR employing a single primer complementary to a target microsatellite. It is a dominant multilocus marker system which arbitrarily does not require genetic information of the plant (Bornet and Branchard, 2001). Using ISSR markers, the genetic variation of four wild populations of *G. uralensis* from various locales have been effectively evaluated (Yao *et al*., 2008). In the study, 92.2% polymorphic bands were generated with fourteen ISSR primers. Analysis of molecular variation (AMOVA) exhibited a maximum of genetic divergence within the species with 81% whilst among the populations was 19%.

Table 2.3: Genetic structure and variation studies using molecular markers in *Glycyrrhiza* spp. (Pandey & Ayangla, 2018)

Plant species	Tools used	Achievements	Reference
Glycyrrhiza species	RAPD & RFLP	Interspecific genetic association of *Glycyrrhiza glabra* and *Glycyrrhiza uralensis.*	Yamazaki *et al*., 1994
Glycyrrhiza uralensis	RAPD	Assessment on the effect of spaceflight on alterations in genomic DNA.	Gao *et al*., 2000
G. uralensis	ISSR	Estimation of genetic variation in wild populations.	Yao *et al*., 2008
G. glabra	RAPD	Validation and identification of *G. glabra* from *Arbus precatorius.*	Khan *et al.*, 2009
G. uralensis	AFLP	Evalualtion of genetic divergence.	Ge *et al*., 2009
Glycyrrhiza species	SSR	Estimation of marker transportability from remotely related legumes to *Glycyrrhiza* species.	Erayman *et al*., 2014
G. uralensis	SSR	Development of SSR molecular markers and transcriptome analysis.	Liu *et al*., 2015

G. uralensis	EST- based microsatellite markers	Estimation of the divergence of genes among the taxa.	Um *et al*., 2016
G. glabra, G. uralensis, G. inflata, G. pallidiflora	SSR	Comparison of genetic diversities and appraisal of genetic structures.	Liu *et al*., 2019
G. glabra	ISSR	Evaluation of structure and variation of hereditary materials.	Esmaeili *et al*., 2020
G. glabra	RAPD	Study of genetic distinction.	Mahboobeh *et al*., 2020
G. glabra	AFLP	Diversity estimation for selection of elite genotypes through association of markers.	Hosseini *et ali*., 2021

2.7. Biotechnological methods for large scale multiplication of *Glycyrrhiza glabra*

Commonly, vegetative propagation of the plants is achieved through cuttings of stolons, rhizomes with buds (Rao, 1993; Kohjyouma *et al*, 1995; Nezamabadi *et al*, 2007). However, growth of plants through such cuttings is slow and susceptible to viral infections (Kohjyouma *et al*, 1995). Commercial cultivation using seeds is also rarely practiced as it encounters delayed germination, consequently reducing multiplication rate (Gupta *et al*, 1997; Sawaengsak *et al*, 2011). Conventional methods of propagation thus obstruct industrial demands of the plant. Micropagation is increasingly envisaged as a propitious scheme for the mass propagation as well as for plant improvement and conservation. From the several available reports of *in vitro* multiplication of licorice, the researchers mostly highlight the success of micropropagation following shoot tip or nodal cultures.

2.7.1 Establishment of *in vitro* cultures: Direct oranogenesis

Selection of appropriate explants is crucial for any culture initiation. There have been reports of using a variety of explants in the micropropgation of *Glycyrrhiza glabra* namely, nodal segments, shoot tips, and leaves. Mousa *et al*. (2006) attempted to establish clonal plants cultures from an individual plant source grown in controlled condition for about three months. They reported that clipping of cultured plants enhanced shoots. Also, response was increased by more than 50% when explants from the clipped plants were compared to control. From the *in vitro* culture studies, it is evident that aseptic culture conditions are maintained by religiously treating the

explants with variety of chemicals. Mostly the chemical used includes mercuric chloride for surface sterilization with moderate change in concentration and treatment time. Such surface sterilization overcomes fungal and bacterial infections while maximizing explants survival rate (Arya *et al*., 2009).

There are few studies which uphold the fact that the time of explant harvest corresponds to regeneration rates. It is of the argument that explants gathered in late winter are the best receptive (Thengane *et al*., 1998), while on contrary, nodal explants collected in May through August show the highest regeneration rate (Yadav and Singh, 2012). In yet another work, nodal explants were induced to 90% rejuvenation (Arya *et al*., 2009). It was recently confirmed again that the nodal explants were the most effective method for establishing meristem culture, with a regeneration frequency of 66.67%. However, in recent times, it is documented that the most successful establishment of meristem culture was through nodal explants with a regeneration frequency of 66.67% which is in contrast to the previous studies (Badkhane *et al*., 2016). All pointing to the fact that nodes are enriched with meristematic cells.

For ideal results, careful selection and manipulations of elements such as culture media, the pH, plant growth regulators and their concentration are requisite. The most widely used culture medium for *G. glabra* micropropagation is MS (Murashige and Skoog, 1962), whether used as it is or in conjunction with cytokinins and auxins. Semi-solid basal medium was a preferred choice because of the observation of vitrification and faster rejuvenation (Arya *et al.*, 2009). Several growth hormones appended with basal media for efficient reawakening of *G. glabra* explants include BAP, NAA, IAA, Kn etc.

2.7.1.1. Shoot multiplication

Numerous shoot duplication is observed mostly by adding shoot and/or root enhancing hormones to the base as summerised in table 2.4 (Pandey and Ayangla, 2018). Media formulations, subculturing and other culture settings, for example, photoperiod, pH etc have significant impact (Mousa *et* al., 2006; Yadav and Singh, 2012). When MS medium alone was utilised for induction, callus development was observed at the proximal ends of explants (Kukreja, 1998). Addition of simply BAP at a concentration of 1mg/L was effective in encouraging the growth of numerous shoots (Kohjyouma *et al*., 1995). Contrarily, diverse number of shoot development was observed at higher concentration of BAP at 5 mg/L (Sarkar and Roy, 2015). Frequency of multiplication was speculated to enhance with BAP and NAA mixture rather than BAP only (Yadav

and Singh, 2012). Addition of adenine sulphate combined with low concentrations of BAP and Kinetin boosted multiplication rate by 8-9-fold (Arya *et al.*, 2009). It was recorded that lower BAP concentrations (2.22, 4.44 µM) showed efficacy towards hardiness and multiplication of shoots (Thengane *et al.*, 1998). Reduction of MS macrosalts to ¾ strength proved to have an added advantage of ten- fold on shoot multiplication but further reduction in MS macrosalts to ½ or ¼ strength proved to be lethal. However, full strength of MS macrosalts supplemented with low BAP was found to be the most effective combination for obtaining highest number of shoots per explants (1.5) and also for maximum shoot length (Sawaengsak *et al.*, 2011). This report is in line with the results of Mousa *et al.* (2006) who held the view that full strength MS basal medium enhanced with 2.0 mg/L BAP and 2.0 mg/L NAA significantly enhanced plantlet regeneration rate of secondary shoot cultures. They concluded that shoot cultures incubated in complete darkness for one week and then for three weeks in the light considerably lengthened stems and increased the number of micro-nodes to an average of nine per plantlet.

A more recent experiment extended the spectrum of *G. glabra* regeneration procedure using three combinations of cytokinin-auxin in varying concentrations (Badkhane *et al.*, 2016). The greatest growth for shoot was seen with BAP (2.0 mg/L) and NAA (0.50 mg/L), which was 86.67% while Kn and IBA was the least effective.

2.7.1.2. Root initiation

Root formation is an essential part of any micropropagation method that ultimately guarantees plantlet survival. For efficient root induction in the explants of *G. glabra*, auxin is observed to be indispensable. In the absence of auxin, the root either failed or took longer duration to react (Yadav and Singh, 2012; Badkhane *et al.*, 2016). Conversely, when explants were left in the culture vessel without any growth regulator, root development was witnessed (Kukreja, 1998; Sarkar and Roy, 2015). There have been studies utilising a variety of media doses with diverse auxin concentrations. The incidence of root rejuvenation was amplified when auxins viz., IAA, IBA were diluted to semi solid MS media (Arya *et al.*, 2009; Sawaengsak *et al.*, 2011; Badkhane *et al.*, 2016). Addition of IAA (Kukreja, 1998; Sawaengsak *et al*., 2011; Yadav and Singh, 2012; Sarkar and Roy, 2015) and with NAA (Kohjyouma *et al*., 1995; Gupta *et al.*, 2013); BAP (Mousa *et al*., 2006) enhanced root multiplication of licorice. Inclusion of 3% w/v of sucrose in a minute percentage of agar and IAA or IBA exaggerated root regeneration (Sawaengsak *et al*., 2011). They achieved high growth rate of root in any

of the auxins. While the addition of IBA was the most ideal for the increase of root length, IAA enabled maximum rooting ratio of explants. In contrary, Sarkar and Roy, (2015) observed that root induction on IBA yielded brittle roots and on addition of IAA root formation was the best. Yadav and Singh, (2012) studied the factors influencing *in vitro* rejuvenation of licorice where they observed a 100% root response frequency at 1.0 mg/L IAA. However, there was delayed response of root formation with increase in IAA with maximum callusing at the shoot base. Root initiation was found to be significantly related with sub culturing. Along with a reduction in the number of days needed for root development with succeeding subcultures, swelling and callusing at the base also gradually subsided. Furthermore, abundance of roots was observed at 2.0mg/L concentration of BAP, from 3rd to 6th node from the tip were selected as the inoculating material. Direct root initiation followed by stolon regeneration was also investigated (Gupta *et al.*, 2013). They observed prosperous root response with minutest amount of NAA along with 3% of sucrose in liquid MS medium.

2.7.1.3. Acclimatization

Consignment of micro propagated young plants to the field devoid of toughening and acclimation become incompatible to environmental conditions. Focus has to be channelled towards controlling of both physical and chemical conditions during this stage for substantial growth and reduced mortality in plantlets (Chandra *et al.*, 2010). To serve the purpose of hardening and acclimatization for *G. glabra* plantlets, a range of strategies have been described. Transfer of healthy plantlets to pots or poly bags with vermiculite (Kohjyouma *et al.*, 1995); different percentage of soil isto sand (Thengane *et al.*, 1998; Yadav and Singh, 2012; Gupta *et al.*, 2013); equal quantity of sand, farmyard manure, soil (Arya *et al.*, 2009; Badkhane *et al.*, 2016) were resourceful to accustom the regenerated plantlets in either greenhouse or glasshouse conditions. On transfer to field under natural conditions, varying degrees of survival rates (70%-100%) has been reported for *G. glabra.*

Table 2.4: *In vitro* clonal propagation through direct organogenesis in *Glycyrhizza glabra* (Pandey and Ayangla, 2018).

Explant	Shoot induction (PGR in mg/L)	Root induction (PGR in mg/L)	Reference
Stem segments with axillary buds	MS+1.00 BAP	MS+0.5-0.10 NAA	Kohjyouma *et al*., 1995

Apical tips and axillary buds.	MS+ 2.22-4.44 µM BA	50%MS+IAA (2.85µM) + IBA (4.90 µM)	Thengane *et al*., 1998
Stem segments with nodes, axillary buds, petiolar base	MS+2.0 BAP+1.0 IAA	MS / NB +1.0 IAA	Kukreja, 1998
Shoot culture	MS (FMS) + 2.0BA+ 2.0NAA or ½ MS+ 2.0 BA+2.0 NAA	MS(FMS)+2.0 BA	Mousa *et al*., 2006
Nodal segments with buds	MS+2.0BAP,0.5Kn,50 adenine sulphate	50%MS+0.1IAA	Arya *et al*., 2009
Shoot tip	MS(FMS)+0.5 BA	50% B5+5.0 IAA	Sawaengsak *et al*., 2011
Nodal explants	MS+ 2BAP+0.5 NAA	MS+1.0 IAA	Yadav and Singh, 2012
Leaf		MS+0.01 NAA+ sucrose (3%)	Gupta *et al*., 2013
Nodal segments with auxillary buds	MS+5.0 BAP	MS+3.0 IAA	Sarkar and Roy, 2015
Nodal segments	MS+2.0 BA+0.50 NAA	50% MS+1.0 IAA	Badkhane *et al*., 2016
Intermediate nodal segments	MS+2mg/L TDZ	MS+6.0 NAA	Shaheen *et al*., 2020

2.7.2. Indirect organogenesis and somatic embryogenesis

In the indirect organogenesis, morphogenesis occurs through an intermediate undifferentiated group of tissue, callus (George *et al*., 2008). While somatic embryogenesis incorporates the transformation into zygotic embryos passing through various developmental stages from a single or group of asexual donor cells (Hossein *et al*., 2006). Induction of callus and embryogenesis can be achieved for all plant species with the right choice of explants and by manipulations in culture media and environmental conditions (Von Arnold *et al.*, 2002). However, in case of *G. glabra*, exploration on rejuvenating the plants through callus phase and somatic embryogenesis are still emerging. It is indicative that in the process of indirect morphogenesis, types of plant parts and media composition with growth regulators and other elements are apparent. Explants such as leaf, stem, hypocotyl and root induced to callus and then cell suspension

is observed is documented (Hayashi *et al.*, 1988). Callus and suspension cultures were established with seeds in B5, sucrose (2%), 2, 4-D (1.0 mg/L), Kn (0.1 mg/L) (Arias-Castro *et al*., 1993). Bud segments were used for *in vitro* rejuvenation (Dimitrova *et al*., 1994). Seeds callused to the maximum using MS with 2, 4-D 0.5mg/L, and Kn 0.2 mg/L as well as with NAA 1 mg/L, 2, 4-D 0.5 mg/L, Kn 0.5 mg/L (Shams-Ardakani *et al*., 2007). When leaves were cultured in B5, BAP 1.0 mg/L, NAA 0.5 mg/L, callogenesis was best (Sharma *et al*., 2008). Shoot redevelopment from callus cultures of licorice was possible with with NAA and BA added to MS or TDZ of different doses (Wongwicha *et al*., 2008). Highest rate of callus was formed from nodes when cultured in B5, 2, 4-D (2.0 mg/L), sucrose (20g/L) and agar 7.5 g/L (Sharma *et al*., 2010). In recent times, leaf and stem segments were cultivated effectively in MS, BAP 2 mg/L, 2, 4-D 0.5 mg/L, ascorbic acid 50 mg/L (Jaiswal *et al*., 2021).

In the taxa of *Glycyrrhiza*, an investigation was conducted to rejuvenate the species by means of somatic embryogenesis and observed friable, white or yellow callus that subsequently developed into short, delicate adventitious roots (Kakutani *et al*., 1999). The effectiveness of suspension cultures and regenerating callus of *G. glabra* for the synthesis of glycyrrhizin was investigated by Mousa *et al* (2007). The modified Gamborg's B5 medium (Gamborg *et al.*, 1968) was the ideal way to start the basic embryogenic callus of all three clonal genotypes revealing nodular, compact hard yellow–green callus. They hold the view that secondary embryogenic callus formation and regeneration of the plants were affected by the age of cell suspension cultures. Germination response of both primary and secondary embryos showed shoot and root formation of 90% each in B5 medium devoid of any hormones. Somatic embryogenesis was initiated from hypocotyls resulting in callus with a maximum frequency of 94.3% clear and glandular callus (Chunhua *et al.*, 2010). In a recent report, Sarkar and Roy, (2015) attempted to establish optimal types and concentrations of carbon sources on somatic embryogenesis in *G. glabra*. When 2, 4-D was administered to the medium, they observed healthy calli. Induction of somatic embryogenesis was most efficiently promoted through suspension culture in a hormone - free medium with usual 3% sucrose, supplemented with 3% maltose. In about 10 days of germination of somatic embryos, its frequency was the highest at 47.0% with GA_3 combined with basal medium.

Table 2.5: Callus mediated, somatic embryogenesis & synthetic seed formation.

Explants	Results/Response.	Medium + Plant Growth Hormones (Pandey and Ayangla, 2018)				References
		Callus	Induction	Maturation	Germination	
Leaf	Callus	MS+NAA (1mg/L)				Kakutani *et al.*, 1999
Hypocotyl	SE	MS+ BAP (2.0 mg/L) + 2,4-D (0.5 mg/L)	MS+0.5mg/L BAP +0.5mg/L Kn+ IBA (0.1 mg/L)		MS+ME (malt extract) (500mg/L)	Chunhua *et al.*, 2010
Young leaflets	SE		B5, sucrose (2%) +2, 4-D (1.0 mg/L) + Kn (1.0mg/L).		B5 only	Mousa *et al.*, 2007
Leaf	SE	MS, 2,4-D (1.5mg/L)	MS+3% maltose		MS+ GA_3 (1.5mg/L), (With usual 3% Sucrose).	Sarkar and Roy, 2015
Leaf and stem	Callus	MS, 2 mg/L BAP, 0.5 mg/L 2,4-D and 50 mg/L				Jaiswal *et al.*, 2021

CHAPTER 3

Collection of *Glycyrrhiza glabra* species from different geographical locations of India (Punjab, Haryana, Delhi, Himachal Pradesh and Maharashtra) and identification of high-yielding glycyrrhizin containing population by HPTLC fingerprinting.

Optimization of extraction factors for improved recovery of glycyrrhizin

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3.1 Introduction

Glycyrrhiza glabra Linn. of the family: Fabaceae, is a classical therapeutic herb mainly distributed in South-western Asia and the Mediterranean countries (Hayashi and Sudo, 2009; Sharma and Agrawal, 2013). Nowadays, several countries including India has reportedly initiated its cultivation at different scales (Gupta, 2016). The importance of *G. glabra* is essentially because of the sweet- tasting component, glycyrrhizin, a triterpene-saponin which is 50 times sweeter than sucrose. Glycyrrhizin is mainly accumulated in rhizomes and stolons which accounts for about 2%-25% dry weight (Pastorino *et al*., 2018). The bioactive compound glycyrhhizin occurs only in three species out of 30 species namely, *G. glabra*, *G. uralensis* and *G. inflata*, with highest concentration (34.66 mg/g) being in *G. glabra* (Yang *et al*., 2018). Glycyrrhizin is known to have an extensive array of pharmaceutical and biological effects. Lately, there has been an escalation in willingness for items and medications made from plants (Omogbadegun *et al*., 2011). Currently, trade in licorice has also gained popularity because of its wide economic importance. However, over-exploitation and destructive harvesting nature cause problem to its sustainability which eventually may lead to genetic depletion (Karkanis *et al*., 2018). Taking into consideration the demand and supply sustainability of licorice, cultivation and thereof, conservation of medicinal plants is regarded as way for conforming the market needs and also for easing harvest stress on the plants (Schippmann *et al*., 2002, Esmaeili *et al*., 2019). Moreover, environmental elements including temperature, radiation, water, soil type and composition, salinity, geographical differences constantly interact with the plants throughout their ontogeny which certainly effect the total yield and quality of bioactive compounds. With that emphasis, it can be stated that even same plant species growing in dissimilar environments will show variation in a particular bioactive principle (Radušienė *et al*., 2012). Hence, it necessitates to screen for any variation in the occurrence of glycyrrhizin in different populations.

However, for analysis of any bioactive compound from medicinal plants, it is essential to follow sample preparation. Extraction and separation processes determine both the quality and quantity of the SMs from medicinal plants since they occur in very low amounts in the plants (Yang *et al*., 2018). Glycyrrhizin is extracted by conventional methods which are uneconomical and lengthy process. Today, extraction protocols are revolutionised and directed more towards green technology for greener environment such as microwave assisted extraction (Zhang *et al*., 2011). Extraction of SM is affected by factors viz., temperature, time, pressure, plant material (Azmir *et al*., 2013), sample matrix, target compound (Mustafa and Turner, 2011), solvent, particle size etc (Pandey and Tripathi, 2014). Hence, optimization of various extraction factors with the application of improved extraction techniques and modelling becomes important that aims at maximum recovery of the bioactive compound with the minutest variation in their functions (Giacometti *et al*., 2018). Nowadays, polished modeling and statistical techniques namely, Response Surface Methodology (RSM) and Artificial Neural Network (ANN) are broadly utilised in medicinal plants for processing and predicting wide range of complex information. RSM offers a dynamic platform to overcome the wastage of time, economy, chemicals, and plant materials and also allows to outperform multiple variable effect on the response in one test (Santelli *et al*., 2008; Aydar, 2018). ANN is yet another computational and tool to evaluate non linear and multifaceted data on the response function (Wesolowski and Suchacz, 2012; Simić *et al*., 2016). Further, fingerprinting of glycyrrhizin yield is evaluated by high performance thin layer chromatography (HPTLC), the most advanced technique of thin layer chromatography. HPTLC is a widely favoured technique for its efficiency, rapid, accuracy, requirement of less extract and solvent and comparatively less expensive method of measure (Bala *et al*., 2015, Misra *et al*., 2017).

3.2. Materials and methods for optimization

3.2.1. Standard chemical and other chemical substances

Ethanol, *n*-butanol, glacial acetic acid (S.D. Fine chemicals, Mumbai), glycyrrhizic acid (ammonium salt) of purity > 95% (Sigma- Aldrich Chemicals, Bangalore), all of molecular-grade were utilised.

3.2.2. Sample preparation and extraction

Gathering of rhizomes of *G. glabra* (of approximately 2 years old) were from herbal garden, Lovely Professional University, Phagwara, Punjab, India. The rhizomes of *G. glabra* were dried in hot air oven at 40˚C, cut into smaller pieces and pulverized in a grinder to pass

through mesh (30 and 16) for particle sizes (0.50 mm and 1 mm). 1 g of dry powdered rhizomes of *G. glabra* was extracted by heat reflux, using a hot water bath (30-70°C) and ethanol (30%-70%) in accordance to the experimental design. The suspensions were sieved using Whatman filter paper No. 1 to extrude the impurities and filtrates were finally reduced to 2 mL volume. The resultant prototypes were stored at 4°C for further examination.

3.2.3. HPTLC conditions for optimized glycyrrhizin yield

By using HPTLC, the entire amount obtained from the extracted samples of *G. glabra* was calculated. The sample applicator consisted of a Linomat 5 applicator with a small syringe, a scanner associated to winCATS application in the computer (CAMAG) and the TLC plates (20 x 10 cm; 0.2mm thick silica gel 60 F_{254}) were used. Loading of standard and extracts (2 μL) on TLC plates was executed at 6 mm band breadth, 8 mm from base, 15 mm from lower edge. The applicating frequency was maintained at 100nL/s for each solvent extract. The solvent was ethanol. The chromatographic panes were subsequently generated in a twin trough chamber (20.0 x 10.0 cm, Thermo Fischer Scientific) drenched with vapour of mobile phase beforehand, at relavtively 25°C and 52% moistness. Plate progress was performed using a linear ascending development. The solvent system used included a mixture of *n*-butanol, water and glacial acetic acid (7: 2: 1 by volume). After drying for 10 minutes, in hot air oven (60°C), the panes were densitometrically scrutinized at 254 nm (Fig. 3.1). The standard curve of the reference compound was used to determine the amount of glycyrrhizin present in the samples centred on peak regions.

3.2.4. Calibration curve of glycyrrhizin

The yield of glycyrrhizic acid was evaluated using a calibration curve obtained with a standard concentration range (200-1000 ng/spot). To achieve a final concentration of 200, 400, 600, 800 and 1000 ng per application, stock solution of glycyrrhizin was sprayed on the panes. The highest area for each concentration was plotted against the standard concentration and subjected to linear square regression analysis. Subsequently, recovery of glycyrrhizin was evaluated with the linear regression, equated with its corresponding peak area. By creating a final volume (10 mL)/ of concentration (1mg/mL) in volumetric flask, stock solution of glycyrrhizin (1mg/mL) was made in absolute ethanol.

3.2.5 Validation of HPTLC method

In accordance to the standards, the conditions of precision studies, limits of detection and quantification, specificity and recovery studies were taken into consideration using glycyrrhizin as reference, to authenticate the proposed HPTLC approach.

3.2.5.1.Precision studies

With replicated usage of reference for five times with 4 μL for every load, the instrument accuracy was unwavering. Likewise, for authenticating the repeatability (inter - assay), 3 varying reference doses were tested on the same day and repeated the same after three days to authenticate the reproducibility (inter- assay) and hence, Relative Standard Deviation (RSD) was computed.

3.2.5.2. Limits of detection and quantification

After loading varying quantity of reference solution and using ethanol as a blank, the standard deviation method was used to determine the limits of detection and quantification interpreted as ratio of spectrum signal and noise. S stands for the calibration curve while SD is the standard deviation of *y*-intercepts of regression line in the formulae for LOD (3x SD/S) and LOQ (10x SD/S).

3.2.5.3. Specificity

By relating and validating the R_f values of reference solution and spot spectrums of the samples, the accuracy of the suggested method to isolate glycyrrhizin from the sample matrix was assessed.

3.2.6. Response Surface Methodology

3.2.6.1. Plackett-Burman (PB) Design for screening the significant extraction parameters

In this study, initially, screening out for significant independent factors was tested by the Plackett-Burman design (Table 3.1). In this model, seven independent variables such as, extraction time (X_1), extraction temperature (X_2), solvent composition (X_3), particle size (X_4), solvent to solute ratio (X_5), pH (X_6) and steps/cycles (X_7) were selected to consider their possible influence on GA. The scheme operates on the first order equation:

$$Y= \beta 0+ \sum \beta i Xi \quad (1)$$

with Y as the response function, β_0 as the constant and β_i as the regression coefficients.

The model was performed at two level (+) indicating the maximum and (-) the minimum values consisting of 12 runs.

Table 3.1. Experimental domain of Plackett-Burman model showing extraction variables with responses

Independent variables	Variable code	Range and levels	
		High (+)	Low (-)
Extraction time (min)	X_1	60	30

Extraction temperature (°C)	X_2	70	35
Solvent composition (%)	X_3	70	30
Particle size (mm)	X_4	1.0	0.5
Sample to solvent ratio (g/mL)	X_5	1:40	1:20
pH	X_6	9	5
Steps/ cycles	X_7	3	1

3.2.6.2. Optimization of glycyrrhizin yield by Central Composite Design (CCD)

Response surface approach (RSM) improved the delineation of the ideal extraction conditions. Grounded on the preliminary PB design, those variables suggestively affecting the extraction efficacy were hence, subjected to a standard central composite design (CCD). The total compound yield as a function of the independent variable is predicted by the given quadratic model:

$$Y= \beta 0+ \beta 1X1+ \beta 2X2+ \beta 3X3+ \beta 5X5+ \beta 11X12+ \beta 22X22 +\beta 33X32 + \beta 55X52 + \beta 12X1X2+ \beta 13X1X3+ \beta 15X_1X_5 + \beta_{23}X_2X_3+ \beta_{25}X_2X_5+ \beta_{35}X_3X_5 \quad (2)$$

Where Y is the response variable, β_0 is the constant coefficients; $\beta_1\beta_2\beta_3\beta_5$; β_{11}, β_{22} , β_{33}, β_{55} and β_{12}, β_{13}, β_{15}, β_{23}, β_{25}, β_{35} are coefficients of linear, quadratic and interactive coefficients while X_1, X_2, X_3, X_5 are levels of extraction variables.

A full factorial Central composite design (CCD) with 31 runs having 16 factorial points, 7 centre points and 8 axial points was performed at five levels. The experimental range and levels of the selected factors are depicted (Table 3.2). Experimental runs along with the independent variables and yield of glycyrrhizin (GA) are shown.

Table 3.2 Experimental range of factors and corresponding levels (CCD)

Independent variables	**Symbol**		**Range and levels**				
	Uncoded	**Coded**	**-2**	**-1**	**0**	**1**	**2**
Time of extraction in minures	X_1	x_1	15	30	45	60	75

Temperature of extraction in celcius	X_2	x_2	17.5	35	52.5	70	87.5
Solvent composition (%)	X_3	x_3	10	30	50	70	90
Solute to solvent ratio in g/mL	X_4	x_4	10	20	30	40	50

3.2.7. Computation by ANN method

ANN was performed to modell the experimental data that resulted from the optimised extraction of glycyrrhizin. In the computation method of ANN, a multilayer perceptron (MLP) is utilised for evaluation of the non-linear relationships of the multidimensional variables. As a result, a three-layer ANN network design was created. The ANN complex included an output representing target function, an input with four nodes constituting the input parameters and one hidden stratum made up of a number of nodes. In the modelling, 70% of data was subjected for training, 15% for justification and 15% for complex testing. The number of neurons for the hidden layer ranged from 1–15 during the training stage of the data. Computations of ANN was performed with IBM SPSS Statistics 16 (IBM, Inc., New York, NY). The RSM and ANN (MLP) models were compared on the basis of validation and performance as per the following equations:

$$\text{RMSE} = \left(\frac{1}{n}\sum_{i=1}^{n}(Y_{predicted} - Y_{experimental})^2\right)^{1/2} \quad (3)$$

$$\text{AAD (\%)} = \left(\sum_{i=1}^{P}(\,|Y_{i,exp} - Y_{i,cal}\,|/Y_{i,exp})\,/P\right)\times 100 \quad (4)$$

Where RMSE is the root mean square error and ADD is the absolute average deviation.

3.2.8. Statistical analysis

The statistical software Minitab was utilised for the optimization experimental work. Analysis of variance (ANOVA) determined the extent to which the data fit statistically and the competence of the model was predicted by the co-efficient of determination R^2, *p* values showing the significance of the regression.

3.3. Results and discussion

3.3.1. Method validation

In the present densitometric study, the validation of the method and related features for estimation of glycyrrhizin are presented (Table 3.3). The linear regression obtained was Y= 8.9265X + 3750.2, which showed a good linear correlation between the applied concentrations of the standard versus observed peak areas (200 – 1000 ng per spot). The observed correlation coefficient (R^2) was found to be significant 0. 9957 ($P<0.05$) as in fig. 3.1. Without applying any post-derivatization techniques, separated components of the mixture of standard compound and plant extract peaks were visible at 254 nm. The spectrum of reference (Rf 0.36) was found to be similar in all of the extracts (Fig.3.2. A, B, C, D).

Table 3.3 Validation of analytical method (Pandey and Ayangla, 2018)

S. No	Limitations	Glycyrrhizin
1	Linearity range (ng/spot; n= 12[a])	200-1000
2	Correlation coefficient (r^2)	0.9957
3	Regression equation	Y= 8.9265X + 3750.2
4	Calculated SD value (CATS software)	2.82
5	[b] Limit of detection (LOD) (ng) [3 x SD/S]	30
6	[b] Limit of quantification (LOQ) (ng) [10 x SD/S]	90
7	R_f and λmax (nm)	0.36 and 254
	Precision and accuracy	
8	Intra-day RSD (%), n = 5	0.602
9	Inter-day RSD (%), n = 5 (day-1/day-2/day-3)	0.793
	Recovery	
10	Amount of standard in stolon samples (µg mg^{-1}) containing highest glycyrrhizic acid	7.60
11	Amount of standard added in stolon sample (µg mg^{-1})	4, 8, 12
12	Amount of standard found (µg)	11.7, 15.52, 19.68
13	Recovery (%)	100.862, 99.487, 100.408, 100.25

[a] Four concentration levels in triplicates

[b] SD is the standard deviation of the blank response and S is the slope of the calibration curve

3.3.1.1. Precision

Loading of 4 μL of the reference compound on the plate for five times, it was possible to assess the repeatability of the devised method expressed as a percentage of RSD (Relative

Standard Deviation). The devised approach is accurate, since RSD % was found to be less tha 2%.

3.3.1.2. Specificity

The purity of the glycyrrhizin compound in the extracts was assessed to detetrmine its specificity, which was validated by comparing the spectra of the sample solutions to retardation factor (R_f) of the reference solution. Glycyrrhizin was isolated from the excipients with R_f of 0.36. It was found that the excipients in the sample matrix did not obstruct with the spectrum of glycyrrhizin.

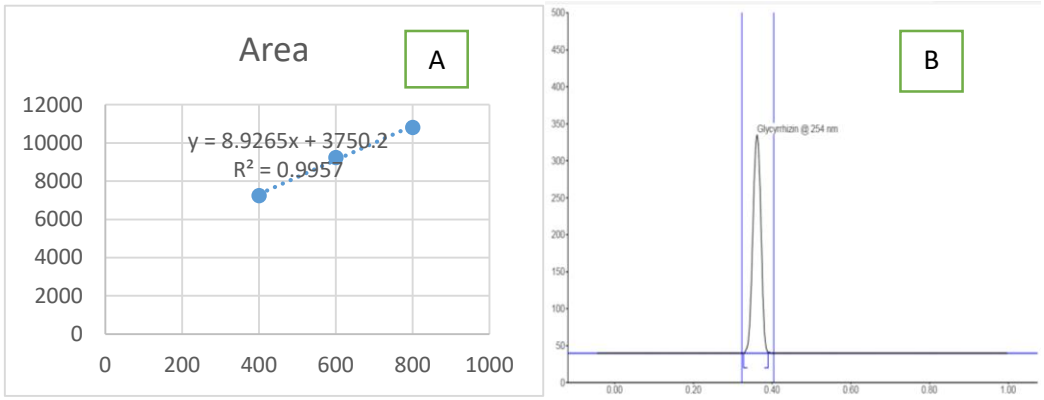


Fig 3.1: (A) Calibration curve and (B) HPTLC chromatogram of standard glycyrrhizin

3.3.2. Response Surface Methodology

3.3.2.1. Screening of the significant extraction parameters by Plackett and Burman Design.

In this study, efficacy of seven independent variables, namely, time of extraction (X_1: 30-70 min), temperature of extraction (X_2: 35-70 °C), solvent composition (X_3: 20-40 %), particle size (X_4: 0.5-1.0 mm), solvent to solute ratio (X_5: 10-50 mL/g) and pH (X_6: 5-9) and extraction steps (X_7:1-3) were examined. Significant correlations at ($P< 0.05$) were observed between the yields of the compound and four independent variables (Table 3.4) summarized from statistical analysis to further elucidate the interactive effects on the response (Table 3.5). Interestingly, when subjected to linear regression based on the preliminary PB design, four independent variables, namely, extraction time, extraction temperature, solvent composition and solute-to-solvent ratio, were shown to be significant. Further, CCD design was assigned to experiment the effect and relationship of four independent variables on the response function of the compound of interest within the range of experiment (Tables 3.2). More so, glycyrrhizin yield was quantified from *G. glabra* by densitometric HPTLC method.

Table 3.4: Estimated effects and coefficients for glycyrrhizin yield (Plackett-Burman Model)

Term	Effect	Coefficient	Standard Error coeficient	T-value	*P*-value
Constant		0.50833	0.005893	86.27	0.000
Time	0.08000	0.04000	0.005893	6.79	0.002
Temperature	-0.07667	-0.03833	0.005893	-6.51	0.003
Aqueous Ethanol	-0.08333	-0.04167	0.005893	-7.07	0.002
Particle size	-0.01667	-0.00833	0.005893	-1.41	0.230
Solid: Solvent	0.08333	0.04167	0.005893	7.07	0.002
pH	0.02667	0.01333	0.005893	2.26	0.086
extraction steps	0.01667	0.00833	0.005893	1.41	0.230
S = 0.0204124 PRESS = 0.015					
R-Sq = 98.02% R-Sq(pred) = 82.14% R-Sq(adj) = 94.54%					

Table 3.5: ANOVA for glycyrrhizin yield (Plackett-Burman)

Source	Degree of freedom	Sum of squares	Adjusted Sum of squares	Mean square	*F*	*P*
Main effects	7	0.082300	0.082300	0.0117571	28.22	0.003
Residual error	4	0.001667	0.001667	0.0004167		
Total	11	0.083967				

Table 3.6 Experimental runs of Plackett-Burman and yield of total glycyrrhizin (GA)

Run order	Time	Temp	Solvent compo	Particle size	Sample: solvent	pH	Steps/ Cycles	Yield %
1	+	-	+	-	-	-	+	0.53
2	+	+	-	+	-	-	-	0.48
3	-	+	+	-	+	-	-	0.42
4	+	-	+	+	-	+	-	0.49

5	+	+	-	+	+	-	+	0.58
6	+	+	+	-	+	+	-	0.52
7	-	+	+	+	-	+	+	0.36
8	-	-	+	+	+	-	+	0.48
9	+	-	-	+	+	+	-	0.61
10	+	-	-	-	+	+	+	0.69
11	-	+	-	-	-	+	+	0.46
12	-	-	-	-	-	-	-	0.48

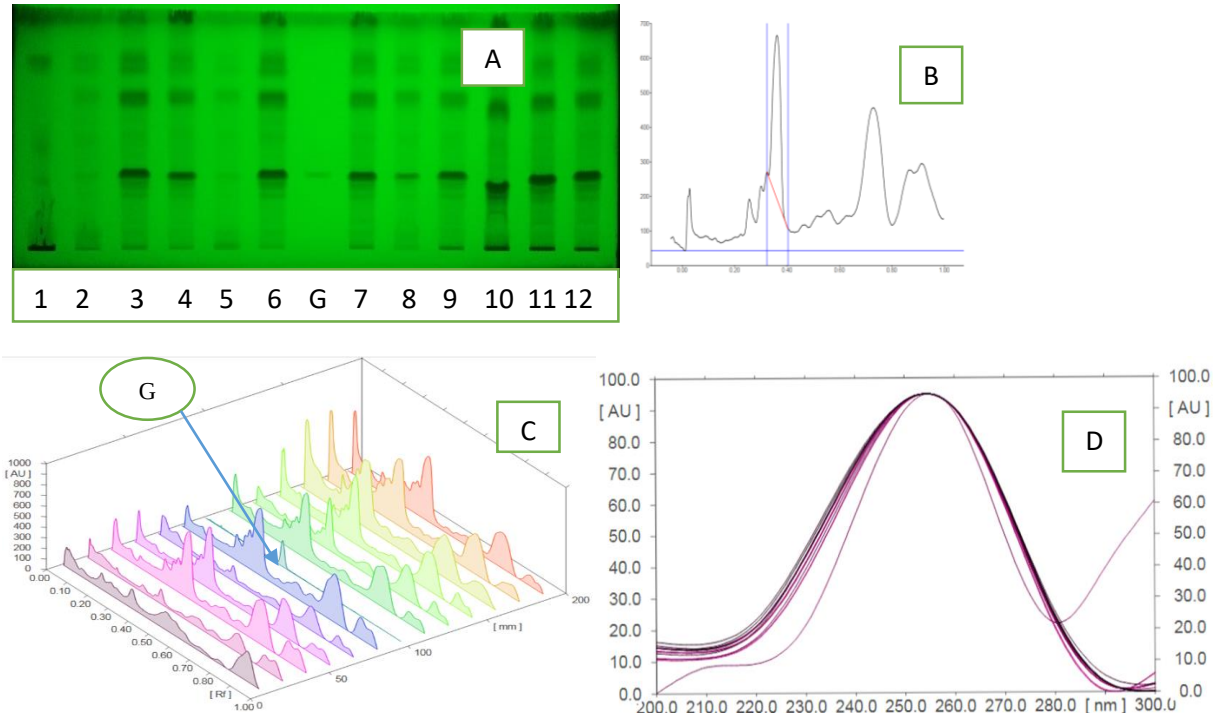


Fig 3.2. HPTLC fingerprint profiles obtained from Plackett-Burman (A), (B), (C) Densitometric chromatogram and (D) overlay spectra of all 12 *G. glabra* samples compared with standard.

3.3.2.2. Model fitting by Central Composite Design (CCD)

Tables 3.7 and 3.8 below provide an overview of the ANOVA results, the appropriateness, and suitability of the suggested model. The association between the response and input variable was demonstrated using the second-degree polynomial equation below:

$$Y=0.755857 + 0.007042X_1 + 0.011625X_2 + 0.021042X_3 + 0.007125X_5 - 0.057537X_1^2 - 0.034537X_2^2 - 0.042912X_3^2 - 0.048287X_5^2 - 0.007688X_1X_2 - 0.014063X_2X_5 - 0.070813X_3X_5 \quad (5)$$

The coefficient of determination (R^2) was assessed to evaluate the goodness of fit of the model (Table 3.7). The regression model was found to be fairly significant as the R^2 value obtained was 99.60% with only 0.4 % of total variations unexplained by the model. The value of R^2_{Adj} 0.9924 which is also near to 1 implies a good correlation between the observed R^2 value (0.9960) and predicted R^2 value (0.9798) (Jang *et al*., 2017). Since the statistical significance was based on the confidence level (95%), the following model conditions with $p<0.05$ were significant on the response variable (Table 3.8). Amongst the independent variables, solvent composition and time factor exhibited the maximum (0.021) and minimum (0.007) effects on the response function.

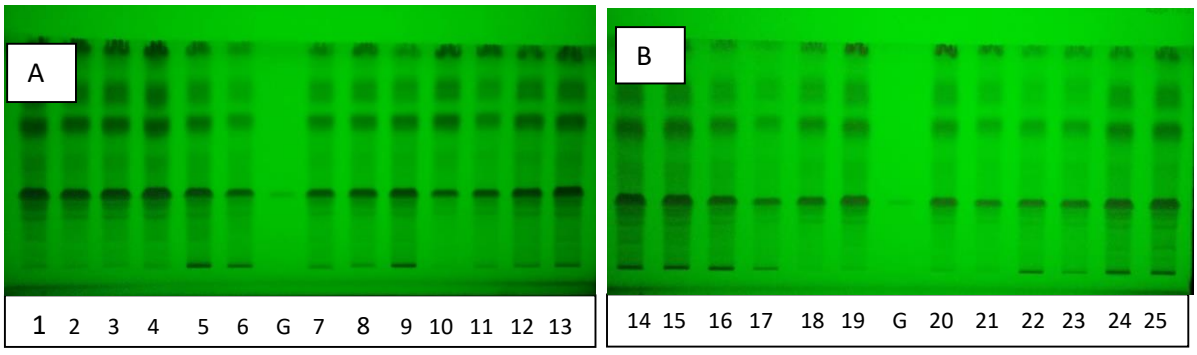
Table 3.7: ANOVA for extraction variables on linear, quadratic and interactions on response variable

Source	**Degree of freedom (D.F)**	**Sum of squares**	**Adj SS**	**Mean square**	**F**	**P**
Regression	14	0.291732	0.291732	0.020838	281.33	0.000
Linear	4	0.016278	0.016278	0.004069	54.94	0.000
Quadratic	4	0.190965	0.190965	0.047741	644.55	0.000
Interaction	6	0.084489	0.084489	0.014082	190.11	0.000
Residual	16	0.001185	0.001185	0.000074		
Lack-of-fit	10	0.000978	0.000978	0.000978	2.84	0.107
Pure error	6	0.000207	0.000207	0.000034		
Total	30	0.292917				

Table 3.8: Estimated regression coefficients of quadratic equations

Regression coefficient	Coefficient estimate	Standard error	T-Value	P-Value
constant	0.755857	0.003253	232.365	0.000
Linear				

Extraction time	0.007042	0.001757	4.008	0.001
Temperature	0.011625	0.001757	6.617	0.000
Solvent composition	0.021042	0.001757	11.978	0.000
Sample to solvent ratio	0.007125	0.001757	4.056	0.000
Quadratic				
Time x time	-0.057537	0.001609	-35.750	0.000
Temp x temp	-0.034537	0.001609	-21.459	0.000
Composition x compo	-0.042912	0.001609	-26.663	0.000
Sample: solvent x sample:solvent	-0.048287	0.001609	-30.003	0.000
Interaction				
Time x temp	-0.007688	0.002152	-3.573	0.003
Time x composition	-0.002688	0.002152	-1.249	0.230
Time x sample: solvent	-0.001437	0.002152	-0.668	0.514
Temp x composition	0.000187	0.002152	0.087	0.932
Temp x sample: solvent	-0.014063	0.002152	-6.536	0.000
Composition x sample: solvent	-0.070813	0.002152	-32.912	0.000



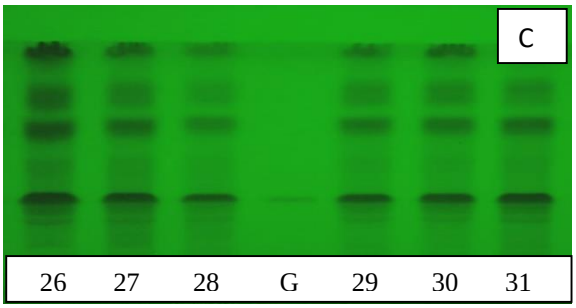


Fig 3.3. HPTLC fingerprint profiles of (A) tracks 1-13; (B) tracks 14-25; (C) tracks 26-31 *G. glabra* stolon along with glycyrrhizin standard (G) by CCD under visible light.

3.3.2.3. Interaction between the variables

As shown in Fig. 3.5, 2D-level plots were constructed to better understand the collaborations between the chosen independent factors and how they affected the response variable. In plot Fig. 3.5.A. solvent composition and solvent to solute ratio have an effect on yield of glycyrrhizin. As glycyrrhizin compound is a complex of calcium and magnesium salts, with 3 carboxyl and 5 hydroxyl groups, it is polar that readily gets solvated by ethanol-water solution. With increasing ethanol concentration (10 %- 50 %) and ratio volume (10-30 mL/g), yield of glycyrrhizin improved significantly attaining a maximum efficacy at 50% ethanol concentration and 30 mL/g. However, further increase in both solvent composition and volume ratio showed no difference in extraction which may be due to excessive distension of plant material exhibited by maximum absorption of solvent by the component. This outcome is in line with a recent investigation on triterpenoid saponin extraction from *Jatropha curcus* leaves (Wei *et al*., 2015). As observed in Fig. 3.5.B. at approximately 53°C and 30 mL/g ratio, percentage yield was found maximum but tends to decline beyond these range which may be due to degradation of glycyrrhizin. With increase in temperature, extraction of the compound surges because temperature facilitates faster desorption of the components into the solution and improves solubility of solutes. Furthermore, higher temperature increases diffusivity as it enhances the probability of particles collision (Li *et al*., 2010). Fig. 3.5.C. also showed that at approximately 55°C and 50 % ethanol composition, extraction is effective which is similar to the observations on triterpenoids by Pandey and Kaur, (2018). Meanwhile, in Fig. 3.5.D. yield was escalated from 0.28%- 0.73% (w/w) when time increased from 20-45 min. and solvent to solute ratio amplified from 10- 30 mL/g, but beyond this range, the response of the target compound diminished, indicating unfavourable effects with time extension resulting from conversion of analytes in the solution and over

swelling of the plant material at higher solvent volume. Likewise, higher yields were influenced by time and ethanol concentration as shown (Fig. 3.5.E.). However, as in Fig. 3.5. F. after climaxing the yield till 45 min. and 53°C, it gradually has no further effect or show negative effect as on the extraction. Similar results have been reported on how higher temperature leads to destruction of the characteristic property of the analyte and decreases the yield of saponins in bitter melons (Tan *et al*., 2015).

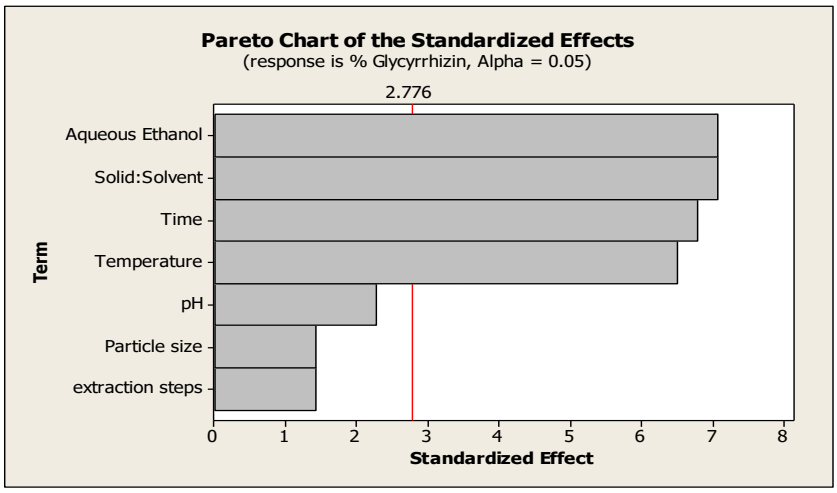
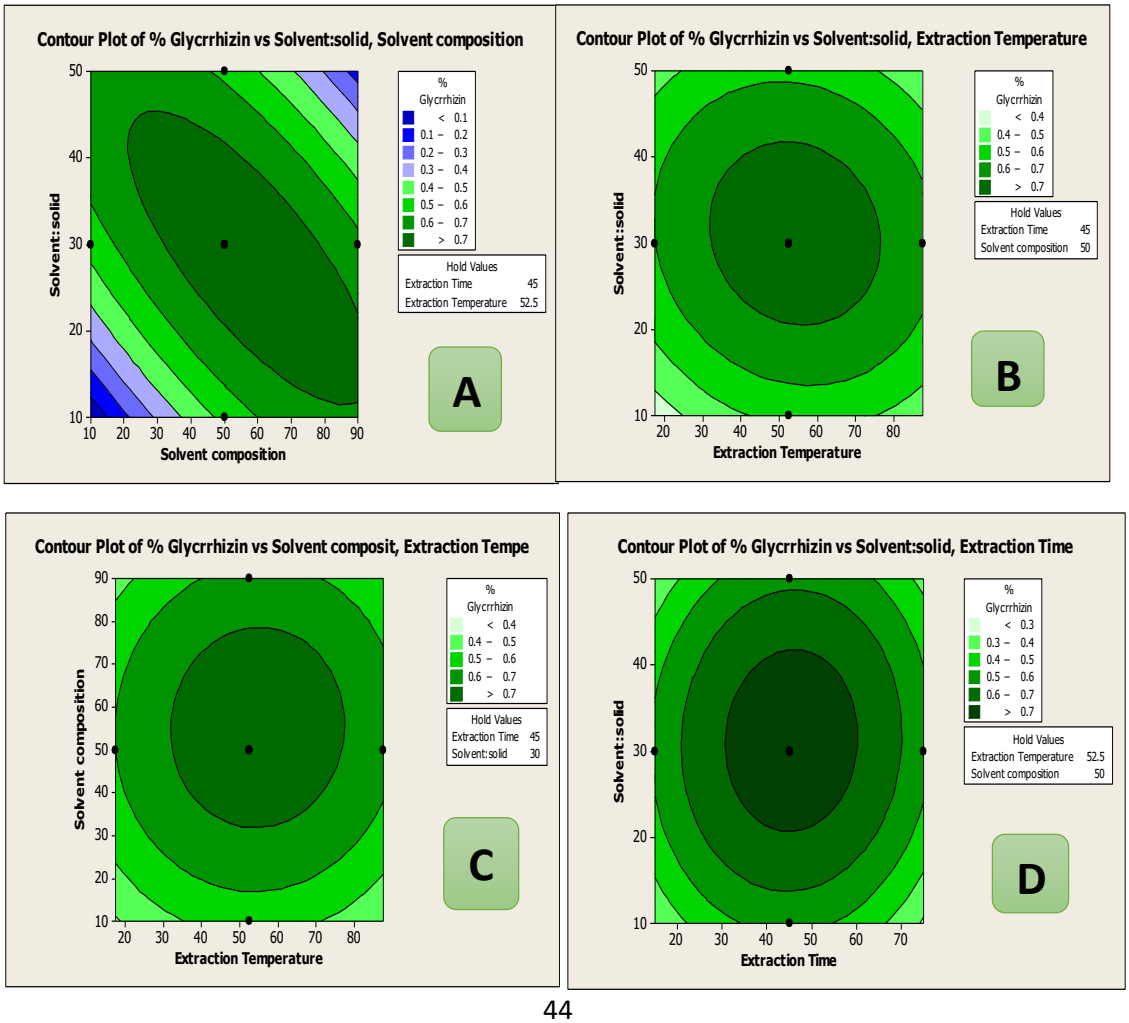


Fig 3.4. Relative effect of the selected parameters for optimum extraction of glycyrrhizin.



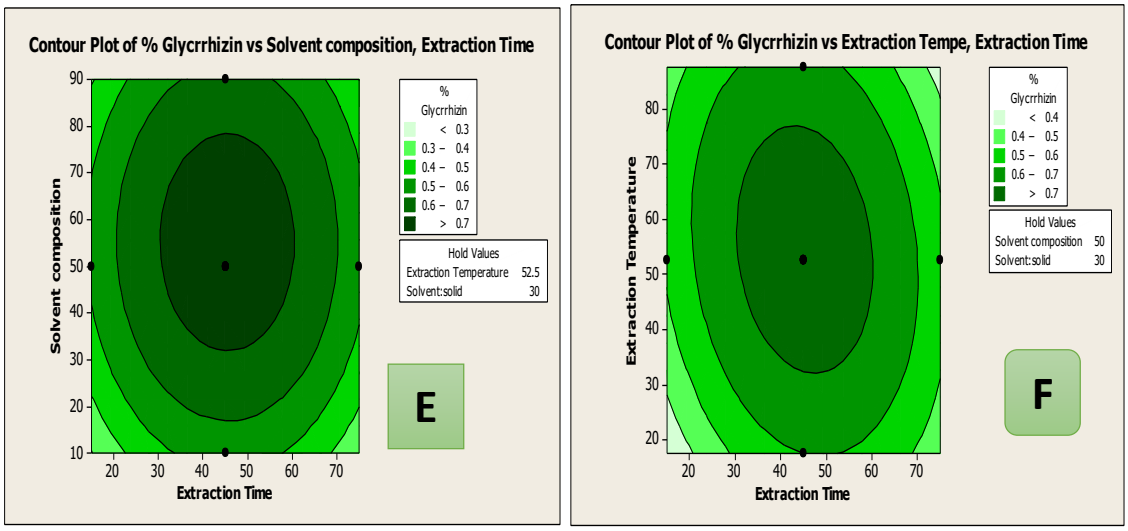


Fig 3.5. Contour plots showing effects of (A) solvent to solute ratio and solvent strength; (B) solvent to solute ratio and temperature; (C) solvent strength and temperature; (D) solvent to solute ratio and duration; (E) solvent strength and duration; (F) temperature and duration, on the total glycyrrhizin yield.

3.3.2. Artificial Neural Network (ANN)-(MLP) modelling

ANN consists of a network of mimicking neurons which is capable of providing accurate estimation of functions (Yadav *et al*., 2013). ANN-MLP, the most widely used model was utilised to protray the glycyrrhizin yield in *G. glabra* root. As depicted in Fig. 3.6., the constructed ANN complex contained a three-level network, an input with four nodes labelled as X1, X2, X3, X4; an output denoted as Y for the target function and a hidden layer. In The experimental data matrix applied in RSM was utilised for ANN computation (Table 3.9). In the ANN modelling, the total 31 runs of CCD experimental data were subdivided into 3, with 21, 5 and 5 points to train, validate and test. In the process of model development, input variables were limited by selecting the best combination that resulted in the activation performance. In addition, the input variables and targets were also normalised from -1 to 1. In the activation function which is the transfer function that decides the total input of neurons into the output amplitude, plotting of hyberbolic tangent to softmax in the output was done. The ANN for activation function was a feedforward architecture without any previous link from output to input neurons. Thus, by training a variety of feed-forward networks with a range of topologies, the ideal combination was selected with the highest R^2 and lowest MSE for deciding the number of neurons in the hidden set. The selection of the ideal feedforward arrangement of the network with its nodes and lines for the response function was carried

out as follows: MLP-Y: (4:6:1), which corresponded to the neuron's existent in the input, hidden, and output covers respectively (Fig 3.6).

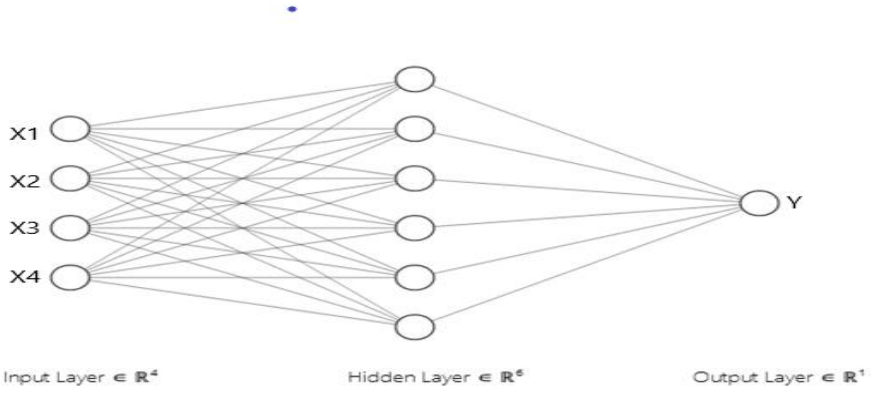


Fig 3.6 General architecture of the developed artificial neural network comprising of 3 layers.

Table 3.9. Total response on the yield of glycyrrhizin based on response surface methodology (CCD) and ANN modelling

Run order	**Time** x_1	**Temperature** x_2	**Solvent composition** x_3	**Sample: solvent ratio** x_4	**Glycyrrhizin yield (%) Y**	**RSM Y**	**ANN Y**
1	-1	-1	-1	-1	0.433	0.429	0.430
2	1	-1	-1	-1	0.461	0.466	0.460
3	-1	1	-1	-1	0.496	0.495	0.500
4	1	1	-1	-1	0.512	0.502	0.510
5	-1	-1	1	-1	0.623	0.618	0.625
6	1	-1	1	-1	0.645	0.645	0.640
7	-1	1	1	-1	0.685	0.685	0.682
8	1	1	1	-1	0.691	0.681	0.689
9	-1	-1	-1	1	0.614	0.616	0.606
10	1	-1	-1	1	0.646	0.648	0.626
11	-1	1	-1	1	0.624	0.626	0.627
12	1	1	-1	1	0.630	0.627	0.630
13	-1	-1	1	1	0.510	0.521	0.520
14	1	-1	1	1	0.550	0.542	0.540
15	-1	1	1	1	0.546	0.532	0.540

16	1	1	1	1	0.517	0.523	0.520
17	-2	0	0	0	0.511	0.511	0.510
18	2	0	0	0	0.535	0.539	0.533
19	0	-2	0	0	0.600	0.594	0.600
20	0	2	0	0	0.630	0.641	0.630
21	0	0	-2	0	0.543	0.542	0.543
22	0	0	2	0	0.620	0.626	0.620
23	0	0	0	-2	0.540	0.548	0.540
24	0	0	0	2	0.580	0.577	0.580
25	0	0	0	0	0.754	0.755	0.754
26	0	0	0	0	0.762	0.755	0.754
27	0	0	0	0	0.760	0.755	0.754
28	0	0	0	0	0.761	0.755	0.754
29	0	0	0	0	0.745	0.755	0.754
30	0	0	0	0	0.755	0.755	0.754
31	0	0	0	0	0.754	0.755	0.754

3.3.4. Comparison between RSM and ANN methods

Three statistical indices, R2, RMSE, and AAD, were used to assess the estimation abilities and predictive performance of the RSM and the ANN (MLP) models. The anticipated values of the target responses produced from the RSM and ANN models are shown in Table 3.9. A statistical comparison supported the conclusion even more (Table 3.10). The R2, RMSE, and AAD values showed that the ANN model outperformed the RSM in terms of prediction performance since it had higher R2 values and lower RMSE and AAD values (Fig 3.7). Table 3. 10 shows that prediction performance of ANN is better than RSM. According to Kamparia *et al*., (2020), the ability of ANN models to approximate nonlinear systems generally may account for their superior predictive power, whereas RSM remains relevant when subjected to linear regression of quadratic polynomial (Pandey and Kaur, 2018). As a result, it was determined that ANN was more effective than RSM and showed greater precision and dependability of performance estimate and also, apt target yield.

Table 3.10: Predictive ability of RSM and ANN, a comparative study

Parameters	**RSM**	**ANN**

Coefficient of determination (R^2)	0.996	0.997
Root mean square error (RMSE)	0.00639	0.00564
Absolute average deviation (AAD)	0.018653	0.01847

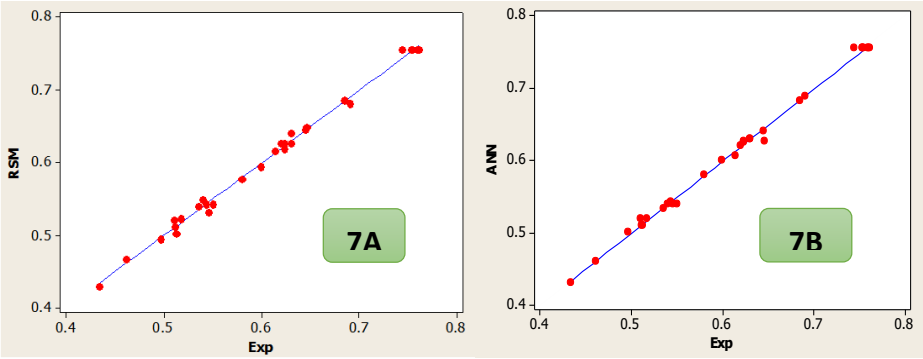


Fig 3.7. Graphical representation showing the comparison between RSM and ANN

3.4. Conclusion for optimization of glycyrrhizin extraction

The present work is the first to describe how to use RSM and ANN model systems to enhance the separation of glycyrrhizin from roots of *G. glabra*.

The generated model equation's efficacy for maximising the response of the bioactive substance was authenticated using the ideal conditions. Further, HPTLC analysis has been demonstrated as an efficient method for quantitative estimation of glycyrrhizin content. The established process has been proven to be simple, precise, and unambiguous. The effectiveness of RSM for optimizing glycyrrhizin extraction from *G. glabra* roots is elucidated. It was observed that the linear terms, such as extraction time, temperature, solvent composition, and solvent to solute ratio, and also relations between these variables and others, had a considerable impact on the compound's overall yield. The results of the analysis of variance confirmed concurrence between experimental and predicted data, illustrating accuracy and applicability of the created quadratic polynomial scheme. The findings of the performance estimation suggested that ANN was a much better modelling technique than RSM.

3.5 Materials and methods for screening of elite population

3.5.1 Chemicals

HPTLC grade solvents all gotten from S.D. Fine chemicals, Mumbai, India were used. Obtained standard marker compound, glycyrrhizic acid (purity > 95%) from Sigma- Aldrich Chemicals Bangalore.

3.5.2. Sample collection and preparation

Plant materials of *G. glabra* were collected from 12 location sites, across 6 states of India (Punjab, Haryana, Delhi, Himachal Pradesh, Uttar Pradesh and Maharashtra). Geographical data of collection sites along with some climatic parameters are as shown in Table 3.11 and 3.12. Two cultivars (Haryana Mulhati-1) from HAU, Hisar, (Mishree) from CIMAP, Lucknow were collected. All the collection of rhizomes were done on-site from herbal gardens of various institutions/ botanical gardens of universities/ public, except samples from Pune (procured from herbal vendor). Collection of the plant samples were done during the period of 2018-2020, at fall and winter months which were approximately of 2-3 years old and authenticated voucher specimens were deposited in the Department of Bioengineering and Biosciences, Lovely Professional University, Punjab, India for future reference. The rhizomes were then washed, dried in hot air oven at 40˚ C and powdered. Microwave-assisted extraction method (MAE), a green extraction method was used for the extraction. In 100 mL glass beaker, powdered sample (1/2 g), dissolved in ethanol of dose 70% and solute to solvent ratio of 1: 20 in g/mL was irradiated in a household microwave for 2 min for two cycles (Xu *et al*., 2012). The suspensions were avoided overheating with 10 s power on and off in each cycle (Pan *et al*., 2000). Following extraction, the filtrated residue of the material was desiccated using a rotary evaporator before concocting a final volume of 1 mL with the polar solvent. All the sample extracts were centrifuged at 10,000 rpm for 15 mins then, strained for additional analysis.

Table 3.11. Geographical data of different *G. glabra* collection sites

Acc. No.	Collection sites	Latitude (˚N)	Longitude (˚E)	Alt. (m)
L1	Phagwara, Punjab	31.2232	75.7670	240
L2	Phagwara, Punjab	31.2552	75.7050	241
L3	Phagwara, Punjab	31.2693	75.7811	245
L4	Phagwara, Punjab	31.2707	75.7275	240
L5	Phagwara, Punjab	31.2754	75.7764	242

L6	Phagwara, Punjab	31.2961	75.6982	245
L7	Phagwara, Punjab	31.2433	75.6855	213
L8	Phagwara, Punjab	31.2519	75.6901	215
P1	Patiala, Punjab	30.2132	76.2716	268
P2	Patiala, Punjab	30.2229	76.2779	269
P3	Patiala, Punjab	30.25681	76.2798	272
P4	Patiala, Punjab	30.29201	76.2843	280
P5	Patiala, Punjab	30.31729	76.2894	283
P6	Patiala, Punjab	30.3542	76.2987	291
P7	Patiala, Punjab	30.3645	76.3614	293
P8	Patiala, Punjab	30.3709	76.3691	297
P9	Patiala, Punjab	30.3996	76.4120	300
PA1	Moti Bagh, Patiala, Punjab	30.1852	76.2339	246
PA2	Moti Bagh, Patiala, Punjab	30.1890	76.2971	248
PA3	Moti Bagh, Patiala, Punjab	30.1914	76.3519	250
LA1	Ludhiana, Punjab	30.1966	76.3519	229
LA2	Ludhiana, Punjab	30.6731	75.5490	236
LA3	Ludhiana, Punjab	30.7311	75.6510	247
LA4	Ludhiana, Punjab	30.8098	75.8071	250
LA5	Ludhiana, Punjab	30.5440	75.4757	253
PO1	Pune, Maharashtra	18.5195	73.8469	550
PO2	Pune, Maharashtra	18.5210	73.8567	554
PO3	Pune, Maharashtra	18.5491	73.9102	562
PO4	Pune, Maharashtra	18.5694	73.9206	567

H1	Hisar, Haryana	29.1416	75.7112	211
H2	Hisar, Haryana	29.1484	75.7168	212
H3	Hisar, Haryana	29.1491	75.7192	212
H4	Hisar, Haryana	29.1523	75.7211	213
H5	Hisar, Haryana	29.1554	75.7280	213
H6	Hisar, Haryana	29.1571	75.7293	214
H7	Hisar, Haryana	29.1500	75.7322	215
H8	Hisar, Haryana	29.1672	75.7849	215
H9	Hisar, Haryana	29.1689	75.7879	223
H10	Hisar, Haryana	29.1723	75.7898	235
C1	CPB, Hisar, Haryana	29.1400	75.6893	217
C2	CPB, Hisar, Haryana	29.1418	75.6917	219
C3	CPB, Hisar, Haryana	29.1436	75.7036	221
C4	CPB, Hisar, Haryana	29.1466	75.7247	223
C5	CPB, Hisar, Haryana	29.1496	75.7182	229
K1	Kurukshetra, Haryana	29.5732	76.4846	257
K2	Kurukshetra, Haryana	29.6631	76.5894	258
K3	Kurukshetra, Haryana	29.7463	76.6129	256
K4	Kurukshetra, Haryana	29.9690	76.8798	259
LK1	Lucknow, U. P	26.8210	80.9691	120
LK2	Lucknow, U. P	26.8329	80.9720	121
LK3	Lucknow, U. P	26.8467	80.9462	122
N1	Nauni, Solan, H. P	30.5144	77.1080	1520
N2	Nauni, Solan, H. P	30.6216	77.1701	1523

N3	Nauni, Solan, H. P	30.7625	77.1649	1529
N4	Nauni, Solan, H. P	30.8192	77.1729	1530
N5	Nauni, Solan, H. P	30.8600	77.1730	1532
J1	South, New Delhi	28.3047	77.1447	238
J2	South, New Delhi	28.4512	77.1905	238
J3	South, New Delhi	28.4910	77.2101	240
J4	South, New Delhi	28.5496	77.1832	241
PB1	Punjabi Bagh, New Delhi	28.4090	77.3120	217
PB2	Punjabi Bagh, New Delhi	28.5409	77.4918	218
PB3	Punjabi Bagh, New Delhi	28.6620	77.5242	219
PB4	Punjabi Bagh, New Delhi	28.7091	77.6120	219
PB5	Punjabi Bagh, New Delhi	28.8632	77.8827	220

Note: Acc. No.- Accession numbers, La-latitude, Lo-longitude, Alt-altitude

Table 3.12. Climatic conditions of the collection sites in India

Acc. No	AMP. (mm)	AMT. (˚C)	AMx Temp (˚C)	AMn Temp (˚C)	Climate type
L	686	24.1	33.6	12.7	Local steppe
P	754	24.5	33.5	13.6	Local steppe
PA	754	24.5	33.5	13.6	Local steppe
LA	726	24	33.9	12.9	Sub-tropical
PO	763	25	31.8	23	Local steppe
H	459	25.1	34.4	13.8	Local steppe
C	459	25.1	34.4	13.8	Local steppe
K	763	24.4	33.4	13.6	Local steppe
LK	1001	25.7	33.8	16	Warm temperate

N	1413	17.4	24.7	8.6	Warm temperate
J	693	25.2	31.0	18.0	Local steppe
PB	693	25.2	31.0	18.0	Local steppe

Note: Acc. No: Accession number, L: Phagwara, Punjab, P: Patiala, Punjab, PA: Moti Bagh, Patiala, LA: Ludhiana, Punjab, PO: Pune, Maharashtra, H: Hisar, Haryana, C: Centre for Plant Biotechnology, Hisar, Haryana, K: Kurukshetra, Haryana, LK: Lucknow, Uttar Pradesh, N: Nauni Solan, Himachal Pradesh, J: South Delhi, New Delhi, PB: West Delhi, New Delhi, AMP: annual mean precipitation, AMT: annual mean temperature, AMxT: annual maximum temperature, AMnT: annual minimum temperature

3.5.3. HPTLC analysis for glycyrrhizin fingerprinting

Estimation of glycyrrhizin contents in the extracts was enabled using a HPTLC technique consisting of a Linomat 5 automatic sample applicator, a small syringe (100 µl), a twin-trough plate development chamber (20cmx10cm), TLC scanner with WinCATS software (all from CAMAG). With the aid of the spotter fixed with injector, 3 µl of each sample was loaded onto the HPTLC plates (20 x 10 cm, 60 F-254, E. Merck, Germany) at a speed of 150 nL/s, retaining a band width of 6 mm and 8 mm between bands. The loaded panes were subsequently run in a twin-through that had already been drenched with the eluent composed of *n*-butanol, water, and glacial acetic acid in an approximate percent of 70%, 20% and 10%. For each chromatography 10 mL of mobile phase used. The development of plates was carried out at normal room temperature (25 ± 2°C) and a relative humidity (64 ± 5) in a linear ascending fashion. The plates were then dehydrated, staged on to the scanner which was visible at 245 nm at a speed of 20 mm/s and split dimension of 4.00 x 0.30 mm. The information so derived was evaluated using WinCATS 4 software.

3.5.3.1 Preparation of stock solution

Glycyrrhizin stock solution was prepared by diluting 10 mg of reference in 10 mL of ethanol to achieve a final concentration of 1 mg/mL.

3.5.3.2. Calibration curve of glycyrrhizic acid

Using a calibration curve generated with a standard concentration range (200-1000 ng/spot), the yield of the bioactive principle was measured. For calibration of the standard curve, stock solution of the marker compound of glycyrrhizic acid (1 mg/mL) was prepared. The reference solution was loaded to the panes in 5 different doses and by linear square regression, the topmost area for each concentration which was plotted against the standard

concentration was computed. Subsequently, the quantity of the analyte was evaluated with the linear regression equated with the corresponding peak area.

3.6. Results and discussion

Quantitative densitometric HPTLC method was conducted to estimate the total yield of glycyrrhizin in all the *G. glabra* accessions. The linear regression obtained was $Y = 11.235X + 5106.1$ (Fig.3.8), showing a good linear coefficient differentiation between the applied concentrations of the standard versus observed peak areas (200 – 1000 ng/spot). The observed correlation coefficient (R^2) was established to be significant 0. 9931 ($P<0.05$). The ensuing chromatograms from the standard glycyrrhizin compound and extracted samples, the spikes were all visible at 254 nm (Fig. 3.9). The spots from all the test samples corresponded to R_f 0.31 of the standard compound and the proportions of glycyrrhizin content in all the accessions of *G. glabra* was calculated consistent to its peak area as presented in Fig. 3.10 (A-L). HPTLC spectra of all samples obtained from MAE method is shown (Fig. 3.10). The results indicated variation in the glycyrrhizin contents among the various populations (Table 3.13). In congruence to the present findings, variations in phytochemical contents have been reported in *G. glabra* populations collected from different geographical locations (Hosseini *et* al., 2014; Basar *et al*., 2015; Esmaeili *et al*., 2019; Eghlima *at al*., 2020). An average, 1. 38% of glycyrrhizin yield was observed from the experiment. As evidence from the result, concentration of glycyrrhizin was found to be slightly higher in Lucknow samples (2.18%) followed by Kurukshetra (2.12%) and the least amount was observed in the accessions from Pune (0.07%) as shown in Fig. 3.11. The present result is in good agreement with the literature which revealed variation of glycyrrhizin content from 0.177–0.688% of dry weight obtained from different geographical origins (Basar *et al*., 2014). Rezaei *et al.* (2017) reported that amount of the active principle varied due to various environmental aspects. Likewise, in our present study, Haryana samples show glycyrrhizin comparable to Lucknow populations which may be paralleled to similar climatic conditions. Moreover, on an attempt to evaluate relationship between glycyrrhizin yield and various environmental variables, it was found that the compound accumulation was positively correlated with latitude at $P<0.05$, $R^2=0.086$ and strongly positively correlated with longitude at $P<0.01$, $R^2=0.104$. In line to the findings, considerable differences in the glycyrrhizin yield were detected owing to environmental factors viz., latitude (Oloumi and Hassibi, 2011), longitude (Eghlima *et al*., 2020). There are reports that temperature plays an imperative effect in the compound accumulation (Hosseini *et* al., 2014, Rezaei *et al*., 2017).

However, in our case, temperature was not found to be significant. Variation of the phyto-active compound in *G. glabra* may be therefore, an interactive result of various other elements such as soil and morphological characteristics (Rezaei *et al*., 2017), morphological elements alone (Behdad *et al*., 2020), season of harvest (Cheel *et al*., 2012), genetic constrains (Kojoma *et al*., 2011, Hosseini *et al*., 2014), besides geographic factors.

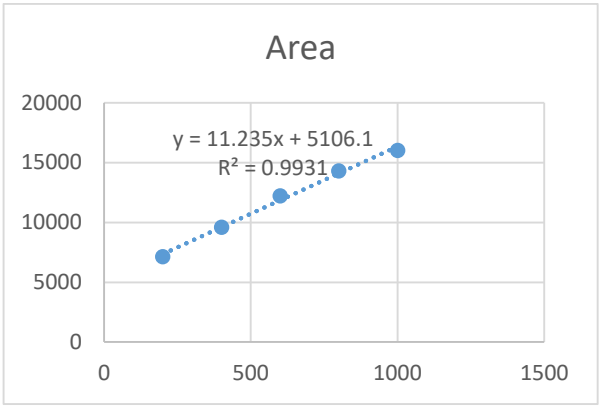


Fig. 3.8. Linearity study of glycyrrhizin standard

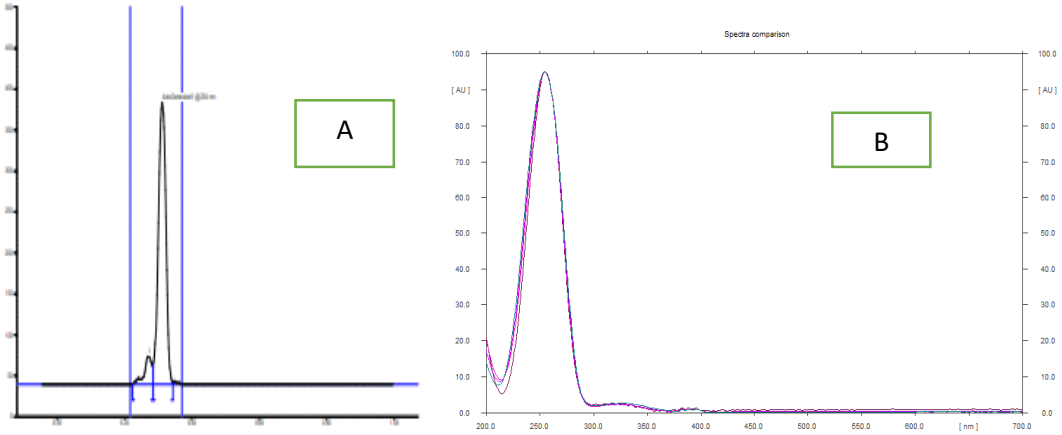
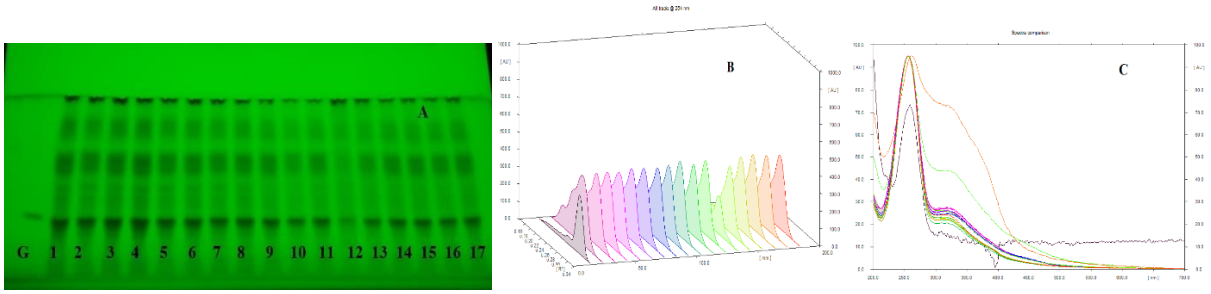


Fig 3.9. (A) HPTLC densitometric chromatogram and (B) Overlay absorption spectra of glycyrrhizin at wavelength 254 nm.



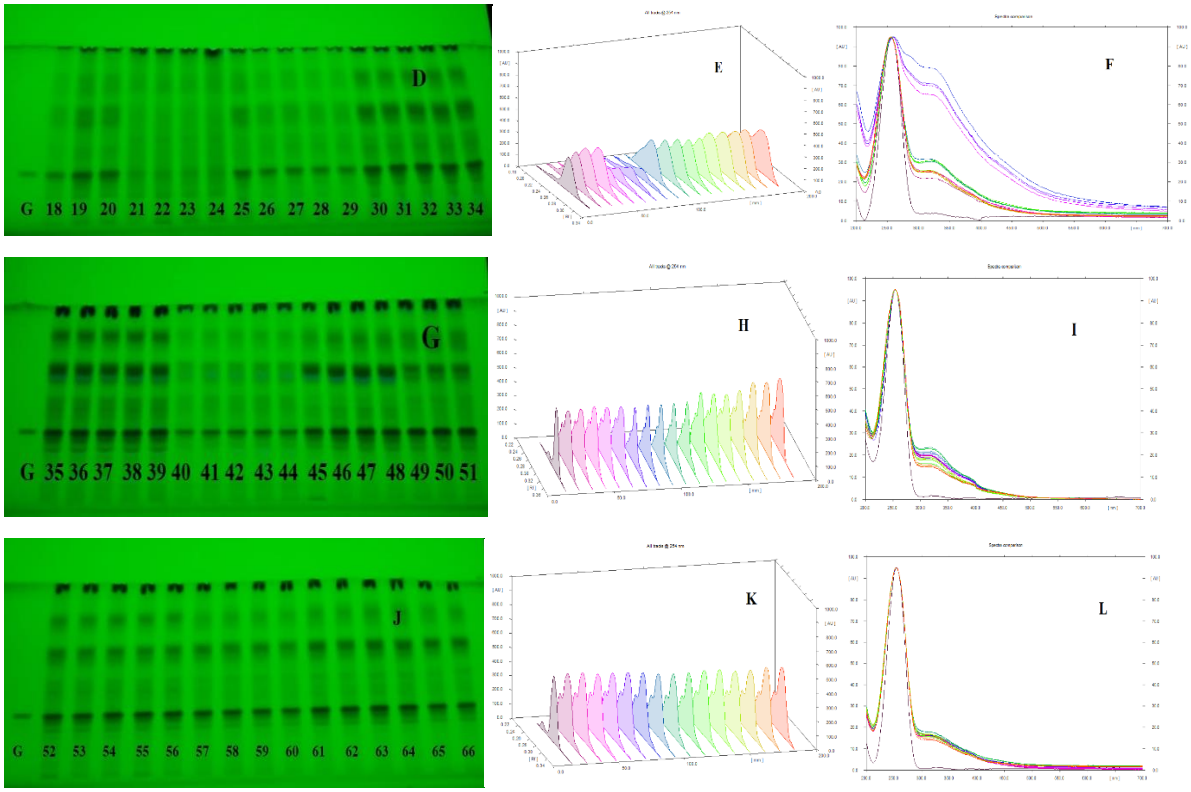


Fig. 3.10. HPTLC fingerprint profiles of glycyrrhizin: (A, G, J) Densitometric chromatograms; (B, H, K) 2-D chromatograms; and (C, I, L) Overlay spectra of *G. glabra* samples compared with standard compound (G).

Table 3.13. Total glycyrrhizin yields in different populations of *G. glabra*

Acc. No.	Collection sites	Glycyrrhizic acid (%)
L1	Phagwara, Punjab	0.35 ± 0.01
L2	Phagwara, Punjab	0.36 ± 0.02
L3	Phagwara, Punjab	0.39 ± 0.01
L4	Phagwara, Punjab	0.39 ± 0.01
L5	Phagwara, Punjab	0.42 ± 0.03
L6	Phagwara, Punjab	0.43 ± 0.01
L7	Phagwara, Punjab	0.42 ± 0.01
L8	Phagwara, Punjab	0.46 ± 0.01
P1	Patiala, Punjab	1.70 ± 0.01
P2	Patiala, Punjab	1.77 ± 0.00

P3	Patiala, Punjab	1.90 ± 0.05
P4	Patiala, Punjab	1.87 ± 0.02
P5	Patiala, Punjab	1.66 ± 0.01
P6	Patiala, Punjab	1.68 ± 0.01
P7	Patiala, Punjab	1.78 ± 0.01
P8	Patiala, Punjab	1.80 ± 0.02
P9	Patiala, Punjab	1.88 ± 0.00
PA1	Moti Bagh, Patiala, Punjab	1.39 ± 0.02
PA2	Moti Bagh, Patiala, Punjab	1.47 ± 0.01
PA3	Moti Bagh, Patiala, Punjab	1.50 ± 0.05
LA1	Ludhiana, Punjab	0.46 ± 0.01
LA2	Ludhiana, Punjab	0.42 ± 0.01
LA3	Ludhiana, Punjab	0.48 ± 0.01
LA4	Ludhiana, Punjab	0.36 ± 0.02
LA5	Ludhiana, Punjab	0.54 ± 0.03
PO1	Pune, Maharashtra	0.08 ± 0.01
PO2	Pune, Maharashtra	0.05 ± 0.01
PO3	Pune, Maharashtra	0.06 ± 0.01
PO4	Pune, Maharashtra	0.09 ± 0.01
H1	Hisar, Haryana	1.67 ± 0.01
H2	Hisar, Haryana	1.69 ± 0.02
H3	Hisar, Haryana	1.80 ± 0.01
H4	Hisar, Haryana	1.67 ± 0.02
H5	Hisar, Haryana	1.97 ± 0.01

H6	Hisar, Haryana	1.95 ± 0.01
H7	Hisar, Haryana	1.96 ± 0.02
H8	Hisar, Haryana	1.95 ± 0.01
H9	Hisar, Haryana	1.96 ± 0.01
H10	Hisar, Haryana	1.98 ± 0.01
C1	CPB, Hisar, Haryana	1.67 ± 0.02
C2	CPB, Hisar, Haryana	1.65 ± 0.04
C3	CPB, Hisar, Haryana	1.57 ± 0.01
C4	CPB, Hisar, Haryana	1.60 ± 0.01
C5	CPB, Hisar, Haryana	1.72 ± 0.01
K1	Kurukshetra, Haryana	2.07 ± 0.01
K2	Kurukshetra, Haryana	2.13 ± 0.01
K3	Kurukshetra, Haryana	2.15 ± 0.01
K4	Kurukshetra, Haryana	2.12 ± 0.01
LK1	Lucknow, U. P	2.18 ± 0.01
LK2	Lucknow, U. P	2.19 ± 0.01
LK3	Lucknow, U. P	2.18 ± 0.01
N1	Nauni, Solan, H. P	1.22 ± 0.01
N2	Nauni, Solan, H. P	1.25 ± 0.01
N3	Nauni, Solan, H. P	1.31 ± 0.01
N4	Nauni, Solan, H. P	1.30 ± 0.01
N5	Nauni, Solan, H. P	1.29 ± 0.01
J1	South, New Delhi	1.65 ± 0.02
J2	South, New Delhi	1.59 ± 0.02

J3	South, New Delhi	1.58 ± 0.06
J4	South, New Delhi	1.56 ± 0.04
PB1	Punjabi Bagh, New Delhi	1.83 ± 0.02
PB2	Punjabi Bagh, New Delhi	1.82 ± 0.02
PB3	Punjabi Bagh, New Delhi	1.86 ± 0.03
PB4	Punjabi Bagh, New Delhi	1.86 ± 0.03
PB5	Punjabi Bagh, New Delhi	1.81 ± 0.03
PB6	Punjabi Bagh, New Delhi	1.70 ± 0.04

SD= standard deviation; with SD (n=3).

Table 3.14 Correlational studies between various climatic parameters and yield of glycyrrhizin

Climatic parameters	Latitude	Longitude	Altitude	AMP. (mm)	AMT. (˚C)	AMxT (˚C)	AMnT (˚C)	Gly. yield
Latitude	1	.219	.004	.048	-265*	.015	-.811**	.293*
Longitude	.219	1	.119	.126	-.013	-.099	.033	.323**
Altitude	.004	.119	1	.818**	-.944**	-.909**	-.378**	-.156
AMP. (mm)	.048	.126	.818**	1	-.825**	-.836**	-.326**	-.107
AMT. (˚C)	-.265*	-.013	-.944**	-.825**	1	.825**	.624**	.173
AMx Temp (˚C)	.015	-.099	-.909**	-.836	.852**	1	.203	.087
AMn Temp (˚C)	-.811**	.033	-.378**	-.326**	.624**	.203	1	-.120
Gly. yield	.293*	.323**	-.156	-.107	.173	.087	-.120	1

Note: AMP: annual mean precipitation, AMT: annual mean temperature, AMxT: annual maximum temperature, AMnT: annual minimum temperature. *Correlation is significant at 0.05 level (2-tailed); **correlation is significant at 0.01 level (2-tailed).

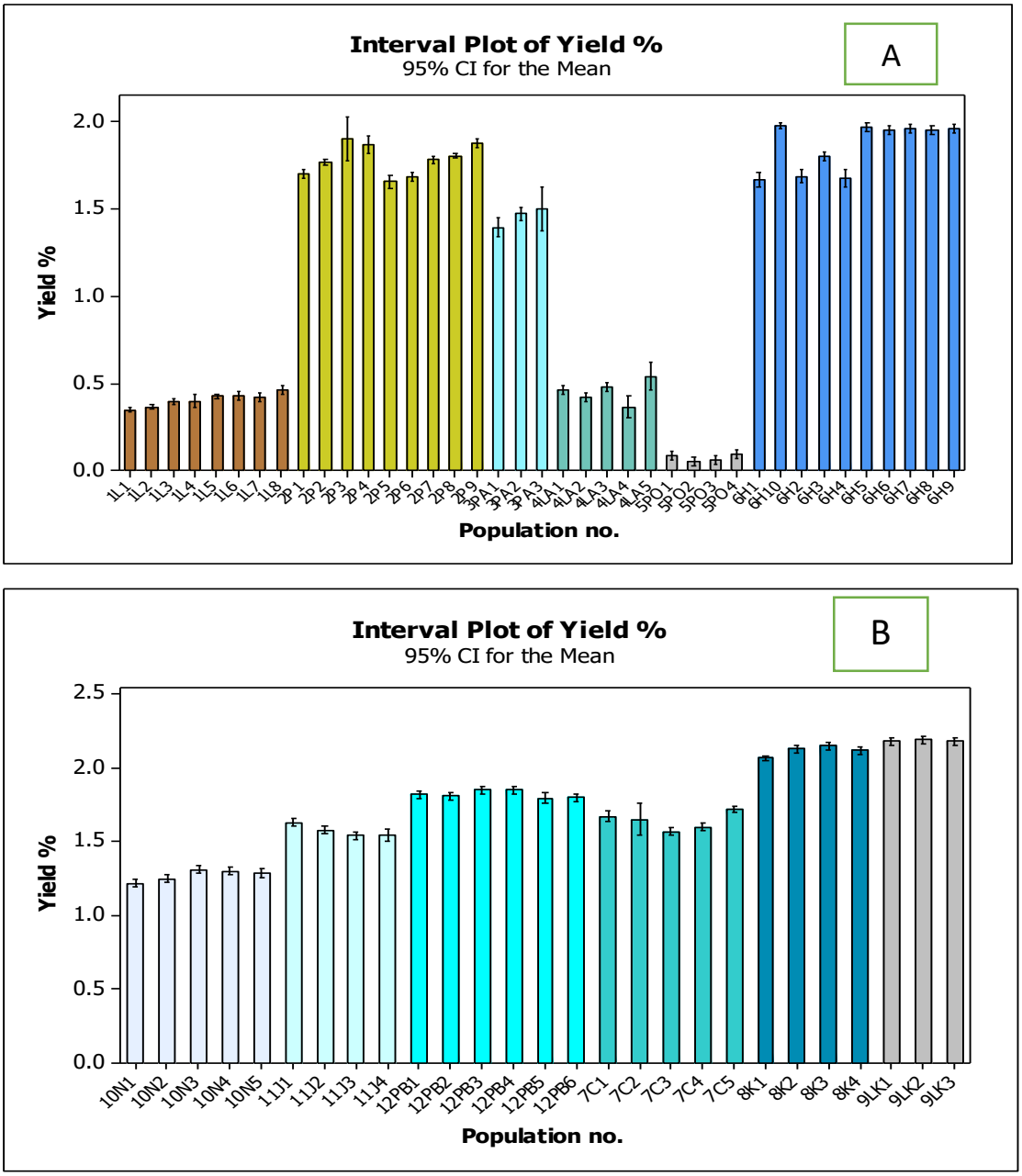


Fig. 3.11 (A and B). Percentile representation of glycyrrhizin yield in all 66 samples of *G. glabra*.

3.7. Conclusion

In the present study, twelve populations of *G. glabra* collected from different geographical locations across five states of India were subjected to HPTLC method to screen for elite population. The mentioned method was found to be simple, efficient and economical in quantifying the glycyrrhizin yield. From the results, it was found that yield in the glycyrrhizin ranged from 0.07-2.18% dry weight among the collected accessions. On the basis of result, content of the compound was found to be significantly correlated with latitude and longitude, indicating the influence of geographical factors. However, variation

in the glycyrrhizin content within and between populations of *G. glabra* was not conspicuous, hence, influence of genes also cannot be ruled out. The information ascertained from the present study could be useful for selection of the population with highest glycyrrhizin content for further plant breeding programmes and cultivation purposes for species' sustainability.

CHAPTER 4

Assessment of genetic diversity of licorice (*G. glabra*) populations by RAPD and ISSR markers.

4.1. Introduction

Glycyrrhiza glabra Linn., a perennial herb of the family Fabaceae, is one of the oldest herbs used in medicine, cosmetics, food and beverages. The presence of glycyrrhizin, a non-sugar sweet-tasting agent found in the stolons or rhizomes of the plant, is attributed to its significance. The plant is indigenous to northern parts of Asia, middle east countries and southern parts of Europe (Um *et al*., 2016) and also cultivated in parts of Europe, USA and Asia including, India (Parvaiz *et al*., 2014). The herb is broadly acknowledged for its antiviral (Luo *et al*., 2020), antibacterial (Varsha *et al*., 2013), antioxidative and inflammatory (Dhingra *et al*., 2004), antitumor (Huang *et al*., 2014), anti-diabetic (Rani *et al*., 2017), hepatoprotective (Chigurupati *et al*., 2016), and other activities. Natural populations have been rapidly declining over time as a result of over-exploitation of the species and high level of plant demad in the pharmaceutical industry. Nowadays, the plant is categorized as a protected species in Mongolia (Chunhua *et al*., 2010), prioritized as an endangered plant in Bulgaria (Kozhuharova *et al*., 2017) or as threatened species in Iran (Hosseini *et al*., 2021). To this end, prior knowledge of the genetic composition and variation at the allelic level is crucial, for any successful breeding programme as part of crop improvement and for the conservation and management of plant resources (Ismail *et al*., 2019). Despite the versatility and reputation of *G. glabra* as a popular medicinal plant, little research has been done on how to assess genetic variability in order to preserve the species.

For any selective breeding and for establishing conservation strategies, plant characterization is determined on the basis of phenotypic, biochemical and genetic characterization and evaluation (Mondini *et al*, 2009). However, unlike morphological and biochemical characterization with its own limitations, with the advent of DNA molecular markers, it is now possible to infer genetic variation and inter-relationships across organisms. It provides pieces of evidences on numerous matters, namely, allelic frequency distribution, the degree and spread of genetic variation both within and between individuals of populations, population construction, etc. DNA-based molecular markers that are free from environmental interference are more promising than other kinds as they readily provide fine and consistent information on hereditary materials toward inferring genetic variations (Duran *et al*., 2009). RAPD and ISSR alone or in combination are proved to be useful, extremely proficient in

evaluating interspecific and intraspecific genetic variation, and phylogenetic relations in plants with therapeutic significance (Tripathi *et al*., 2012; Rameshkumar *et al*., 2019). The RAPD markers provide several advantages in that they exhibit amplifications without prior knowledge of nucleotide sequence, require a limited amount of sample, detect variability among individuals, easy to apply, provide fast results and are cost-effective (Sevindik *et al*., 2019; Bi *et al*., 2021). ISSR markers are, reproducible, presence of abundant regions of microsatellite, reveal high level of allelic variation. The markers are also simple to use, economical and efficiently reliable (Palkar *et al*., 2019; Subositi *et al*., 2020). The previous reports have been documented on the genetic differentiation of *G. uralensis* (Yao *et al*., 2008; Um *et al*., 2016; Dashzeveg *et al*., 2018) and on taxa (Yamazaki *et al*., 1994; Atlay *et al*., 2016; Yang *et al*., 2018). The genetic variability studies of *G. glabra* have received limited attention up to this point, and the data that is available is scanty (Sorkheh *et al*., 2019; Hosseini *et al*., 2021). Hence, in order to appraise the interspecific and intraspecific genetic variation and also population organisation of *G. glabra* from various geographical sites of India, the current study is conducted by utilizing RAPD and ISSR markers.

4.2. Materials and methods

4.2.1. Plant resources and population sampling

Overall, 60 individual plants of *G. glabra*, representing 7 populations were gathered from several Indian sites spread through 5 states (Fig. 4.1). The altitude of populations under study ranged from 120-300 m. The proximity of the collected samples from the locations varied from 41.5 km (approx.) apart between LP and L to a maximum of 907.5 km (approx.) between L and K samples. Five to twelve individuals were picked at each location, spaced at least five metres in between, in accordance with the population size (Mutegi *et al*., 2015). All the samples were taken from plants that had been grown vegetatively in botanical gardens and fields. Fresh juvenile leaves weighing about 5 grams from each individual were collected in zip-lock poly bags containing silica gel. Then, once the samples were brought to the laboratory, they were immediately stored at -20 °C prior to DNA extraction.

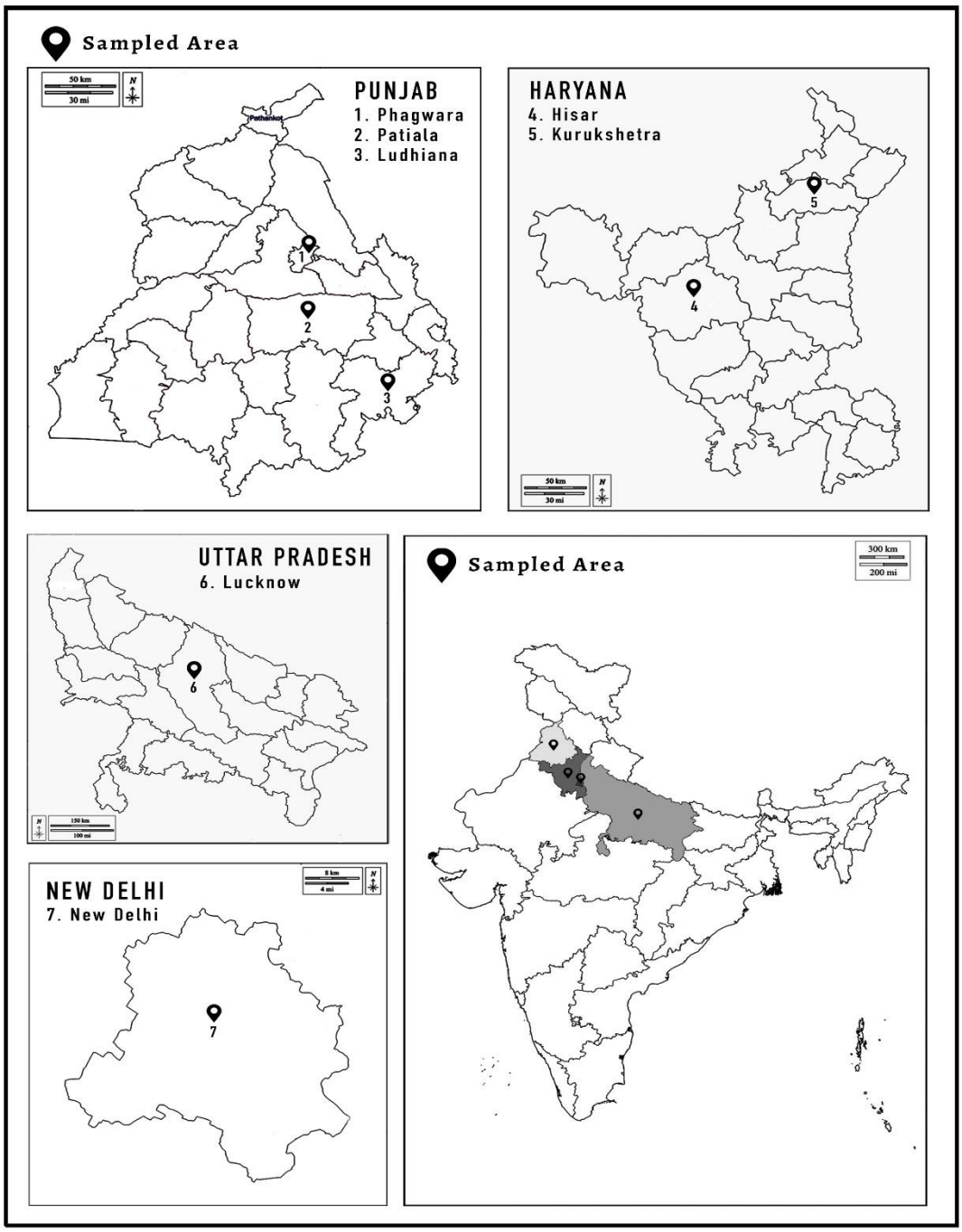


Fig. 4.1. Map identifying the locations of the overall fifty individual samples of seven populations of *G. glabra* across the Northern-parts of India.

4.2.2. DNA isolation

Genomic DNA from the juvenile leaves were extracted by CTAB methods with required modifications (Doyle and Doyle, 1990). About 200mg of dry young leaves was initially dipped in chilled absolute ethanol (15min) before crushing with 1 ml CTAB buffer including 2% PVP (Polyvinylpyrrolidone). Separation of the slurry (10,000 rpm) was done after incubating at 65°C for one hour (12 min). Subsequently, the afloating fluid was relocated to

new eppendorf tubes, appended with RNase (2 µl) was left to sit at normal ambient conditions (20 min). At this stage, phenol, chloroform, and isoamyl alcohol were poured in equal parts, shaked slowly and thoroughly for about five minutes, ensuing isolation. The salvaged separated mixture was treated with $1/10^{th}$ of a volume of 3.0 M sodium acetate and thoroughly shaked before filling the tube with chilled isopropanol. DNA precipitation was carried out by storing sample tubes at -20 °C overnight for proper DNA precipitation. Centrifugation at 11,000 rpm (15 min.) was done, followed by decantation of the supernatant. The DNA pellets so obtained were washed with 500 µl of chilled 70% ethanol, accompanied with centrifugation at 10,000 rpm (10 min). Finally, the DNA pellets were air dried (15 min) or until properly dried, then dissolved in 50 µl of TE buffer and stowed at -20 °C for all additional investigation. Spectrophotometry at 260 nm and 280 nm was engaged to estimate the DNA's consistency and amount. A total of 50 samples were chosen for further study from 60 samples that were screened for DNA isolation. The purity of the obtained DNA was estimated with 1.4% agarose gel electrophoresis where the resultant amplification was compared with 1kb and 100 bp DNA ladder and opted for 100 bp DNA ladder in the subsequent PCR analysis. DNA bands so obtained were finally detected in a gel documentation system (BIO-RAD) imaging system.

Table 4.1. Working concentration of CTAB-DNA extraction buffer.

Reagents/solvent	**Amount to add (for 10ml)**	**Final concentration**
Tris-Cl buffer (pH 8.0) 0.1 M	2 ml	2%
EDTA 0.025 M	0.5 ml	0.5%
NaCl 1.5 M	3 ml	3%
CTAB	3ml	3%
β-mercaptoethanol	0.1 ml	0.1%
PVP	0.2 g	0.2%
PCR water	1.4 ml	1.4%

Note: CTAB extraction buffer was freshly prepared every time for immediate use.

4.2.3. RAPD/ISSR- PCR analysis

A total of 21 PCR primers (Table 4.2), 9 out of 13 RAPD primers (Genei, India) and 12 out of 20 ISSR primers (Sigma, USA) that exhibited clear amplifications were screened for

further molecular characterization of *G. glabra* populations. The DNA amplification mixture for both RAPD and ISSR comprised of 20 µl volume (Table 4.3). A thermal cycler having 96 wells (AB, USA), was used to carry out throughout the PCR analyses. For ISSR settings, at the start of PCR, attenuation was at 94 °C for 5 min, trailed by 45 cycles of attenuation at 94 °C for 1 min, tempering at 50.5 °C for 1.30 min, extension step at 72 °C for 1.30 min, and finally amplifying at 72 °C for another 7 min. For RAPD amplifications, same conditions were followed except for tempering temperature which was ideal at 39°C. The amplified DNA was resolved on 1.4 % agarose gel in 1 X TBE buffer at 90–100 V and visualised under a gel documentation (BIO-RAD) imaging system. Every gel subjected to electrophoresis was estimated using a reference of a 100 bp DNA ladder (Hi-media).

Table 4.2. RAPD and ISSR markers applied in the study of genetic diversity assessment of *G. glabra* populations

Sl. No	RAPD	Primer sequence	Sl. No	ISSR	Primer sequence
1	OPJ01	CCCGGCATAA	1	UBC807	AGAGAGAGAGAGAGAGT
2	OPJ02	CCCGTTGGGA	2	UBC808	AGAGAGAGAGAGAGAGC
3	OPJ03	TCTCCGCTTG	3	UBC810	GAGAGAGAGAGAGAGAT
4	OPJ04	CCGAACACGG	4	UBC826	ACACACACACACACACC
5	OPJ05	CTCCATGGGG	5	UBC828	TGTGTGTGTGTGTGTGA
6	OPJ06	TCGTTCCGCA	6	UBC834	AGAGAGAGAGAGAGAGYT
7	OPJ07	CCTCTCGACA	7	UBC835	AGAGAGAGAGAGAGAGYC
8	OPJ08	CATACCGTGG	8	UBC841	GAGAGAGAGAGAGAGAYC
9	OPJ10	AAGCCCGAGG	9	UBC843	CTCTCTCTCTCTCTCTRA
			10	UBC845	CTCTCTCTCTCTCTCTRG
			11	UBC848	CACACACACACACACARG
			12	UBC855	ACACACACACACACACYT

Table 4.3. PCR reaction mixture for RAPD and ISSR analysis.

Biochemicals	Volume for 1 Reaction
10x Taq DNA polymerase Buffer (Genei)	2 µL
2.5 mM $MgCl_2$ (Thermo Scientific)	1.5 µL
10mM dNTPs (Thermo Scientific)	1 µL
RAPD/ ISSR Primers (Genei)	1.5 µL
1U/ µL Taq DNA polymerase (Sigma)	1 U
DNA template	2 µL
PCR grade water	11 µL

4.2.4. Statistical analysis

The RAPD and ISSR amplified bands were recorded in binary format for presence (1) or absence (0) of distinct, clear amplicons. The distinguishing characteristics for each primer (Table 4.4 and Table 4.5) such as Polymorphic Information Content (PIC), Marker Index (MI) and Resolving Power (RP) were computed in Microsoft excel. PIC was evaluated as: PIC=2f (1-f) where f means the frequency of amplified fragments (Lanteri *et al*., 2004). MI which provides information of polymorphism was calculated as: MI=PIC x EMR (effective multiplex ratio), where EMR= *n* β, *n* being the mean of amplicons and β being the fraction of polymorphic loci i.e., $\beta = \frac{PB}{PB+MB}$ (Powell *et al*., 1996, Milbourne *et al.*, 1997). The resolving power (RP) was computed with the formula, $R_P = \Sigma I_b$, where I_b (Informative fragments) =1-(2|0.5 - p|), with p representing the number of individuals having the amplified bands (Prevost and Wilkinson, 1999).

The binary data matrices of both ISSR and RAPD primers were determined by PopGene 1.32 software version (Yeh *et al*., 1999). All genetic diversity parameters including allele number and frequencies in each locus and genotypic allele frequencies were evaluated by PopGene 1.32 (Yeh *et al*., 1999). In order to define the level and relationships in the populations and genotypes, Shannon information index (*I*), Nei's genetic diversity (*H*) were determined by the same. Distance based analyses including AMOVA, PCoA and Mantel test were performed by GenALEx 6.5 version (Peakall and Smouse, 2006). A tree and neighbour joining dendrogram based on Nei's genetic distance matrix is constructed by using MEGA 6 (Tamura *et al*., 2013). Genetic stratification of *G. glabra* populations, dependent on the

Bayesian cluster analysis approach was assessed by STRUCTURE version 2.3.4 (Pritchard *et al*, 2000). In the algorithm, the span of burn-in for each run consisted of 10,000 iterations of Markov Chain Monte Carlo (MCMC), by ancestry-admixture conjecture and linked allele frequency model. Before replications, K was fixed to a number between 2 and 8, with 10 repeats for each value. Parsing and summarization of the STRUCTURE outfile was evaluated by STRUCTURE HARVESTER (Earl *et al*, 2012) to infer optimum *K* value that best fit the data by executing the simulation method (Evanno *et al*, 2005).

4.3 Results

4.3.1. RAPD and ISSR based polymorphism

Based on RAPD, all the marker informative parameters are listed in Table 4.4. For RAPD, the amplification ranged from 2 to 14 with OPO3 and OPO10. The least number of amplicons was obtained with OPO3, while the maximum 14 bands was obtained with OPO10, exhibiting a total of 81 bands. The amplified products varied from 100 bp to 1,200 bp in molecular size. The polymorphic information content (PIC) varied from 0.10-0.20 for RAPD. The minimum of PIC (0.10) was assessed for OPO1, OPO2, OPO5 and the maximum PIC (0.20) was revealed by OPO3 with an average of 0.14 for every primer. Marker index (MI) ranged from 0.16-0.65, whereby, the highest (0.65) was attained with OPO 10 and least was observed for OPO5 (0.16), with a mean of 0.39 for every primer. The resolving power (RP) fluctuated between 0.44-2.20 where the maximum RP value was observed for OPO 10 (2.20) and minimum for OPO3 (0.44), with an average 1.32.

Table 4.4 Description of RAPD marker information for *G. glabra* populations of India.

Primers	TB	PIC	MI	RP
OPO1	8	0.10	0.21	0.88
OPO2	8	0.10	0.21	0.88
OPO3	2	0.20	0.24	0.44
OPO4	9	0.16	0.54	1.56
OPO5	12	0.10	0.16	1.24
OPO6	10	0.16	0.65	1.80
OPO7	7	0.16	0.44	1.24

OPO8	11	0.14	0.43	1.64
OPO10	14	0.14	0.61	2.20
Total	81			
Average		0.14	0.39	1.32

Note: TB: Total bands, PIC: Plymorphic Information Content, MI: Marker index and RP: Resolving power of the marker.

Altogether 13 ISSR markers were chosen for further analysis. All the marker informativeness of the 13 ISSR primers used are listed in Table 4.5. The ISSR primers produced amplicons ranging from 5 bands (UBC 845) which was the lowest, to 11 bands (UBC 828) as the highest. The ISSR products showed molecular size varied between 100 bp to 1,400 bp, revealing a total of 88 amplified bands. The polymorphic information content (PIC) for ISSR was found between 0.09-0.36, the highest shown for UBC 826 (0.36) and lowest for UBC for UBC 808, UBC 843 (0.09), with a mean of 0.22 for each primer. MI value was observed between 0.05-10.71, demonstrating a maximum for UBC 826 (10.71) and a minimum for UBC 808 (0.05), indicating a norm of 3.59 for each primer. The resolving power (RP) revealed values from 0.40-4.84, with its highest value observed RP for UBC 834 (4.84) and lowest for UBC 808 (0.40), having an average of 2.46.

Table 4.5. Description of ISSR marker information for *G. glabra* populations of India.

Primers	TB	PIC	MI	RP
UBC 807	7	0.16	0.43	1.12
UBC 808	4	0.09	0.05	0.40
UBC 810	10	0.15	0.57	1.72
UBC 826	9	0.36	10.71	4.64
UBC 828	11	0.27	4.45	3.80
UBC 834	10	0.30	8.69	4.84
UBC 835	7	0.12	0.20	0.96
UBC 841	6	0.34	10.04	3.92
UBC 843	6	0.09	0.08	0.48

UBC 845	5	0.30	3.43	2.36
UBC 848	7	0.22	2.41	3.40
UBC 855	6	0.26	2.03	1.92
Total	88			
Average		0.22	3.59	2.46

4.3.2. Interspecific genetic variability as revealed by RAPD and ISSR markers.

Both RAPD and ISSR markers were used to access any variations within all seven populations as well as at the species level of *G. glabra* indicated in Table 4.6 and Table 4.7. From the findings, it was observed that genetic polymorphism (100%) was high at the species level, as revealed by ISSR while with RAPD it was recorded at 97.43%. At the species level, the observed number of alleles (N_a) for RAPD and ISSR were RAPD=1.9743, ISSR=2.0000; number of effective alleles (N_e) for RAP was 1.0788 and that of ISSR was 1.2111; Shannon information index (I) was 0.0710 with RAPD and 0.1555 and ISSR; Nei's gene diversity (H) for RAPD=0.1518 and ISSR=0.2747. However, the diversity at population level were low with only an average of 20.63% (RAPD) to moderate with an average of 41.49% (ISSR).

4.3.3. Level of genetic disparity and its interactions.

With RAPD, 0.0441 was obtained for the genetic diversity within population (H_s) and 0.0518 for total genetic diversity (H_t). With ISSR, H_s value stood at 0.1122 and H_t was 0.1447. The genetic differentiation among populations (G_{st}) was found to be 0.1479 (RAPD) which confirmed a moderately low genetic variation of 14.79% within populations. However, with ISSR, G_{st} value was found to be higher at 0.2247, showing genetic variation of 22.47% within the populations. The results indicated a substantially higher gene flow among the populations with N_m value at 2.883 (RAPD) than with ISSR with its value recorded at 1.7255. The outcomes of AMOVA (Fig. 4.2) disclosed that RAPD markers (85%) exhibited higher variance within the population than did ISSR markers (81%). Genetic variation among populations was low as exhibited by RAPD markers (15%) and ISSR markers (19%). At P= 0.003 and P=0.002, respectively, the differences for both RAPD and ISSR analysis was statistically apparent (Table 4.8). Furthermore, Mantel's test was computed to consider any link between genetic and geographic distance. However, it was found to be moderately negatively correlated between geographical and genetic distance as shown by RAPD (r= -

0.176 and *P*= 0.752) and ISSR markers (*r*= -0.247 and *P*= 0.799) (Fig. 4.3). The genetic distance based on ISSR markers varied from 0.006 between H and D populations to 1.08 between P and D populations. However, based on RAPD it was found to be nil between K and LK; K and D: and LK and D respectively, whereas, the maximum (0.026) was noted between LP and K; LP and LK and between LP and D (Table 4.9).

Table 4.6. A comphrensive gene diversification and limitations in all the populations of *G. glabra* by RAPD.

Populations	Observed No. of alleles ($N_{a)}$	Effective No. of alleles (N_e)	Shannon's Information Index (I)	Nei's gene diversity (H)	PPB %		H_t	H_s	G_{st}	N_m
LP	1.6914	1.2929	0.194	0.3080	69.14					
P	1.0988	1.0275	0.0359	0.0209	9.88					
L	1.0864	1.0201	0.0163	0.0291	8.64					
H	1.5556	1.0894	0.0758	0.1443	55.56					
K	1.0123	1.0023	0.0020	0.0037	1.23					
LK	1.000	1.000	0.000	0.000	0.00					
D	1.000	1.000	0.000	0.000	0.00					
Average	1.2064	1.0617	0.0463	0.0723	20.63					
Species level	1.9743	1.0788	0.0710	0.1518	97.43	Total	0.0518	0.0441	0.1479	2.883

Where, PPB is percentage of polymorphic bands; H_t is total genetic diversity; H_s is genetic diversity within populations; G_{st} is the relative degree of genetic differentiation among populations; N_m is the estimate of gene flow among populations.

Table 4.7. A comphrensive gene diversification and limitations in all the populations of *G. glabra* by ISSR

Populations	Observed No. of alleles (N_a)	Effective No. of alleles (N_e)	Shannon's Information Index (I)	Nei's gene diversity (H)	PPB %		H_t	H_s	G_{st}	N_m
LP	1.7234	1.4072	0.2479	0.3750	72.34					
P	1.4468	1.2348	0.1479	0.2270	44.68					
L	1.3298	1.1267	0.0852	0.1383	32.98					
H	1.7447	1.1526	0.1177	0.2128	74.47					
K	1.4255	1.1818	0.1171	0.1860	42.55					
LK	1.1915	1.0860	0.0546	0.0861	19.15					
D	1.0426	1.0246	0.0149	0.0224	4.26					
Average	1.4149	1.0246	0.1122	0.1782	41.49					
Species level	2.0000	1.2111	0.1555	0.2747	100	Total	0.1447	0.1122	0.2247	1.7255

Where, PPB is percentage of polymorphic bands; H_t is total genetic diversity; H_s is genetic diversity within populations; G_{st} is the relative magnitude of genetic differentiation among populations; N_m is the estimate of gene flow among populations.

Table 4.8. AMOVA showing the level of genetic disparity in *G. glabra* populations by RAPD and ISSR markers.

Marker	Source of variations	Degree of freedom	Sum of squares	Mean square	Variance components	% of total variance	*P* value
RAPD	Among populations	6	60.290	10.048	0.805	15	<0.003

	Within populations	43	189.950	4.417	4.417	85	<0.003
ISSR	Among populations	6	147.187	24.531	2.174	19	<0.002
	Within populations	43	401.233	9.331	9.331	81	<0.002

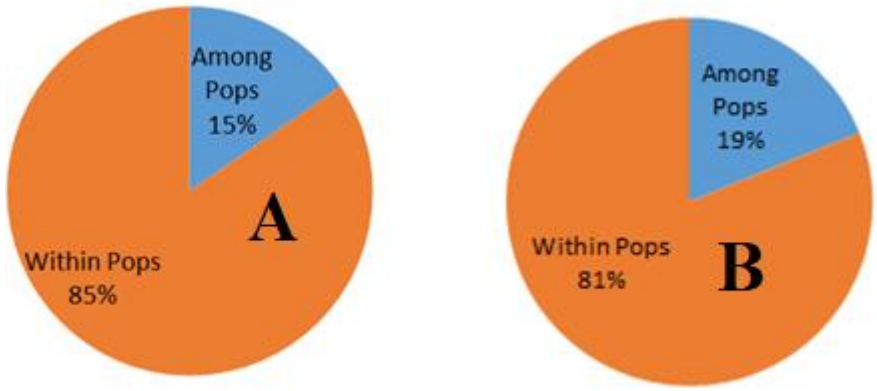


Fig. 4.2: Results of AMOVA exhibiting gene variation (A) RAPD showing 15% among and 85% within; (B) ISSR showing 19% among and 81% within the populations

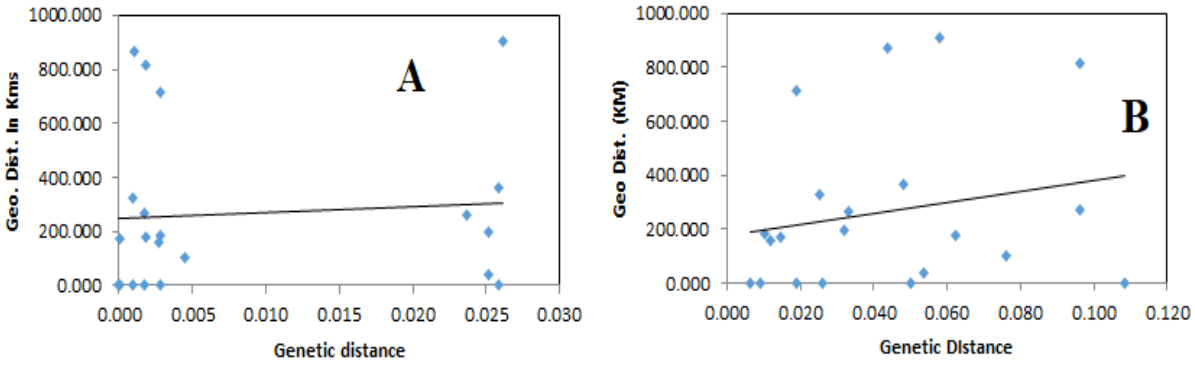


Fig. 4.3. Scatter plots of genetic and geographic distance among the populations (A) RAPD (B) ISSR

Table 4.9: Estimates of Nei's unbiased measures of genetic identity, read above diagonal and genetic distance, read below diagonal among the seven populations of *G. glabra* obtained based on RAPD and ISSR and pooled data analyses.

RAPD

LP	P	L	H	K	LK	D	Population
******	0.975	0.977	0.975	0.974	0.974	0.974	LP
0.025	******	0.998	0.996	0.998	0.998	0.998	P
0.024	0.002	******	0.997	0.999	0.999	0.999	L
0.025	0.004	0.003	******	0.997	0.997	0.997	H
0.026	0.002	0.001	0.003	******	1.000	1.000	K
0.026	0.002	0.001	0.003	0.000	******	1.000	LK
0.026	0.002	0.001	0.003	0.000	0.000	******	D

ISSR

LP	P	L	H	K	LK	D	Population
******	0.948	0.967	0.968	0.944	0.953	0.951	LP
0.053	******	0.940	0.927	0.908	0.908	0.891	P
0.033	0.062	******	0.988	0.957	0.975	0.981	L
0.032	0.076	0.012	******	0.981	0.990	0.994	H
0.058	0.096	0.044	0.019	******	0.985	0.974	K
0.048	0.096	0.025	0.010	0.015	******	0.991	LK
0.050	1.08	0.019	0.006	0.026	0.009	******	D

Combined

LP	P	L	H	K	LK	D	Population
******	0.961	0.972	0.972	0.958	0.963	0.962	LP
0.040	******	0.968	0.960	0.953	0.952	0.946	P

0.029	0.032	******	0.993	0.977	0.987	0.990	L
0.029	0.041	0.008	******	0.089	0.993	0.995	H
0.042	0.048	0.023	0.011	******	0.992	0.986	K
0.037	0.049	0.014	0.007	0.008	******	0.995	LK
0.039	0.056	0.010	0.005	0.014	0.005	******	D

4.3.4. Evaluation of clustering and principal coordinate analysis

The combined form of UPGMA cluster algorithm for both RAPD and ISSR based on Nei's unbiased genetic distances was evaluated to compare the genetic divergence between the species and populations (Fig. 4.4). While LK, D, K, H and L are present in one major cluster, LP and P populations are present alone. Population P and LP showed an inclination to deviate away from the major cluster, with LP closer to L population. Population LK and D populations were found in a sub-cluster within the major cluster which lie in close proximity to one another indicating its genetic relatedness, with D population lying closer to the rest through H population. P population was found closer to LK population. To further depict the relationship among all the fifty individuals of the seven populations, neighbour-joining cluster analysis based on Nei's genetic distance was performed. Comparable outcomes were portrayed from the neighbour-joining technique using both RAPD and ISSR data (Fig. 4.5). In both cases, most of the individuals of the same population remained in same clusters indicating close relations, genetically. However, some individuals were scattered and intermixed with individuals of other populations, representing genetic diversity.

Principal coordinate analysis (PCoA) was carried out to confirm the genetic differentiation as demonstrated by cluster analysis algorithms and to ordinate the genetic distances amongst the individuals in the form of two-dimensional scatter plots. PCoA scattergram generated for RAPD, ISSR and collective records (Fig. 4.6 A-C) exposed that the total genetic variation for the first and second coordinates accounted for 17.07% and 7.18 % based on RAPD. For ISSR, it accounted for 12.85% and 8.99% for first and second coordinates whiles in the combined scatter plot, the first coordinate represented 10.54% and the second coordinate portrayed 9.39% of the total genetic diversity. The representation of individual genotypes in the scatter plot and cluster analyses were found to be concordant to each other. The

individual genotypes of LP and P were observed to be dispersed independently while the individuals of other populations were nested as a mixture.

Population structure on the basis of Bayesian cluster analysis was obtained as in Fig. 4.6. At K = 3, it was discovered that the delta K value derived from STRUCTURE HARVESTER was ideal (Fig. 4.5). The population structure obtained by using ISSR dataset revealed similar pattern as that of UPGMA dendrogram combined tree. As depicted in the structure pattern, the individuals were assigned into 3 different sets (Fig. 4.6). The individuals of populations LP and L (Green colour) were classified in 1 group; the populations of P, H, and K (Blue colour) were in another cluster while populations D and LK (Red colour) were assigned into yet another cluster. In the UPGMA dendrogram P and LP were found to be deviated from the other populations however, in STRUCTURE analysis, they were in one cluster.

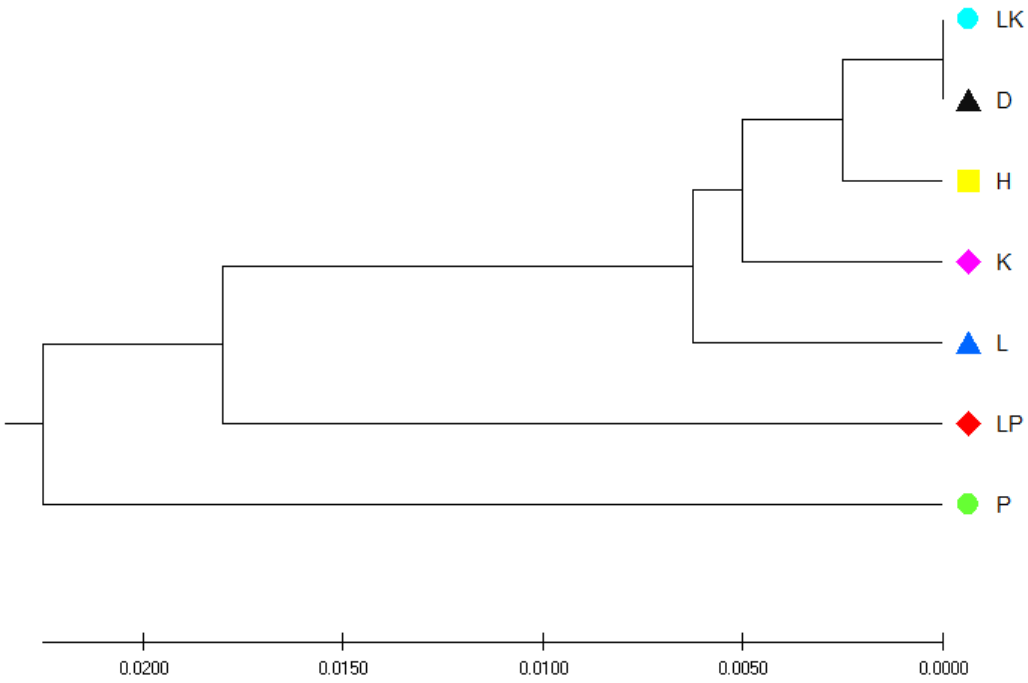


Fig. 4.4. Dendrogram construction for all seven populations of *G. glabra* on combined RAPD and ISSR data matrix following UPGMA.

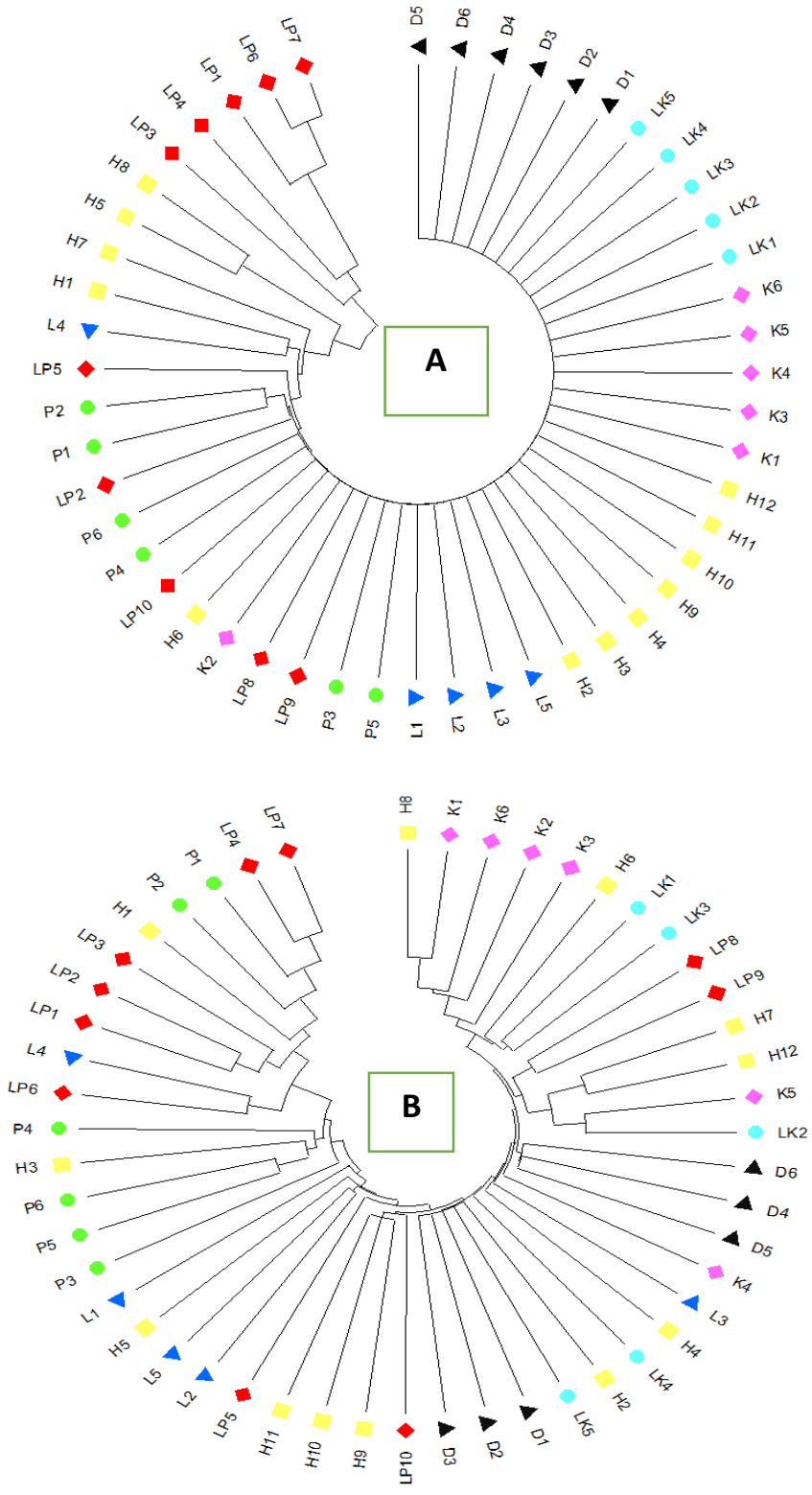


Fig. 4.5. Neighbour joining dendrogram circular clustering pattern for all individuals of *G. glabra* (A) RAPD, (B) ISSR

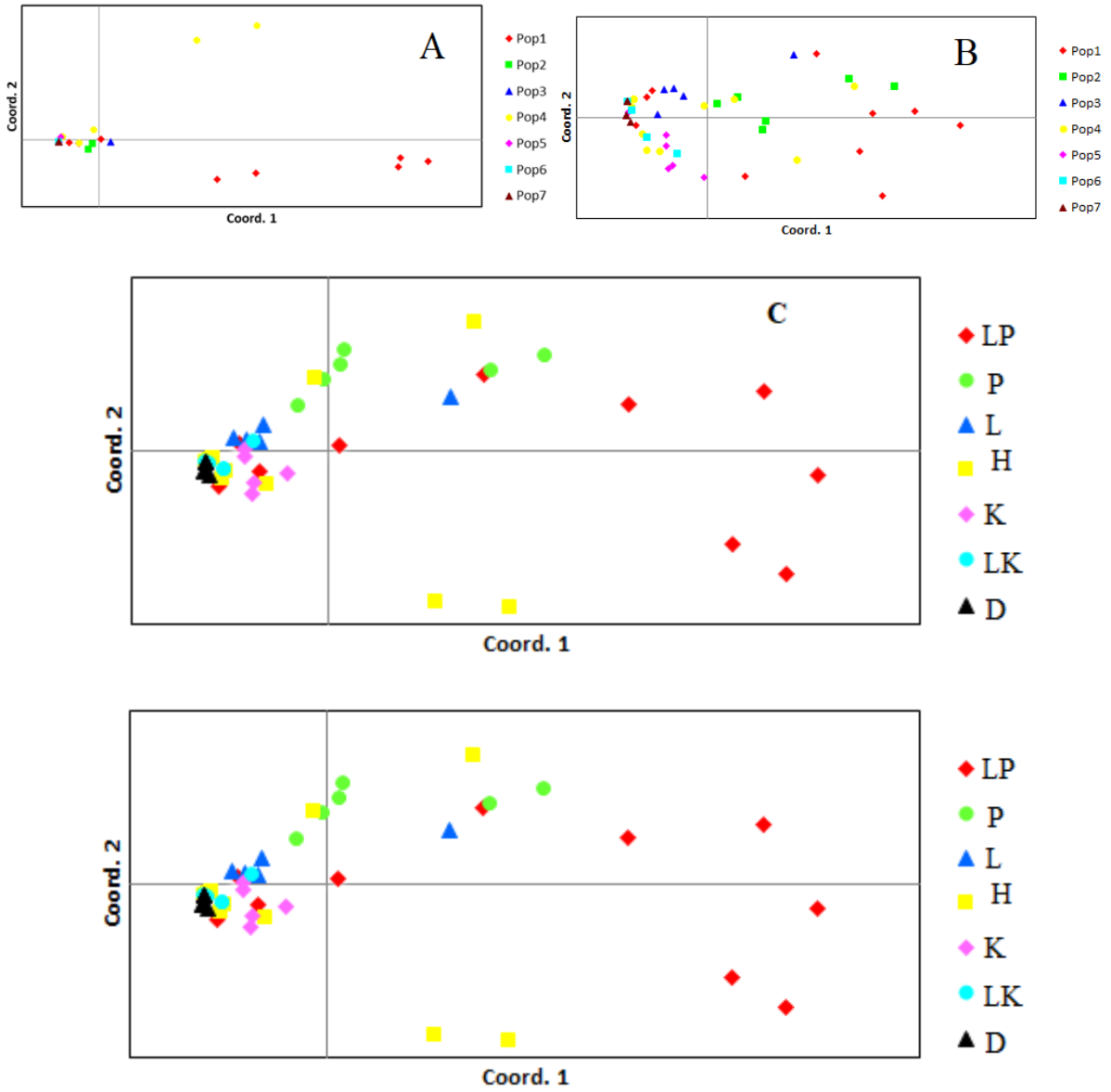


Fig. 4.6. Scatter-plots displaying cluster of all the individuals of *G. glabra* obtained from (A) RAPD (B) ISSR markers (C) collective data matrix.

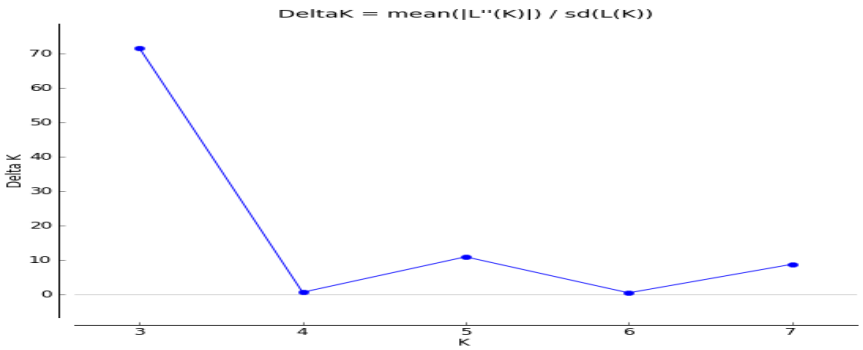


Fig. 4.7. Slope of log probability curve (ΔK) with maximum K value estimated at 3.

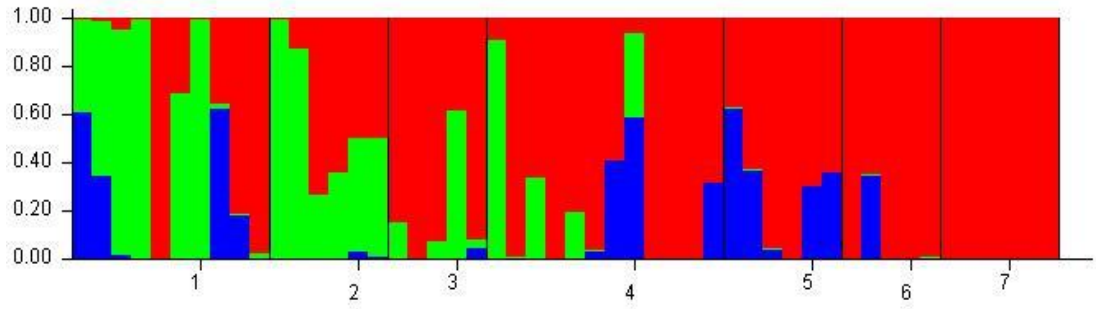


Fig. 4.8. Allocation of all populations of *G. glabra* into three clusters with STRUCTURE at *K*=3.

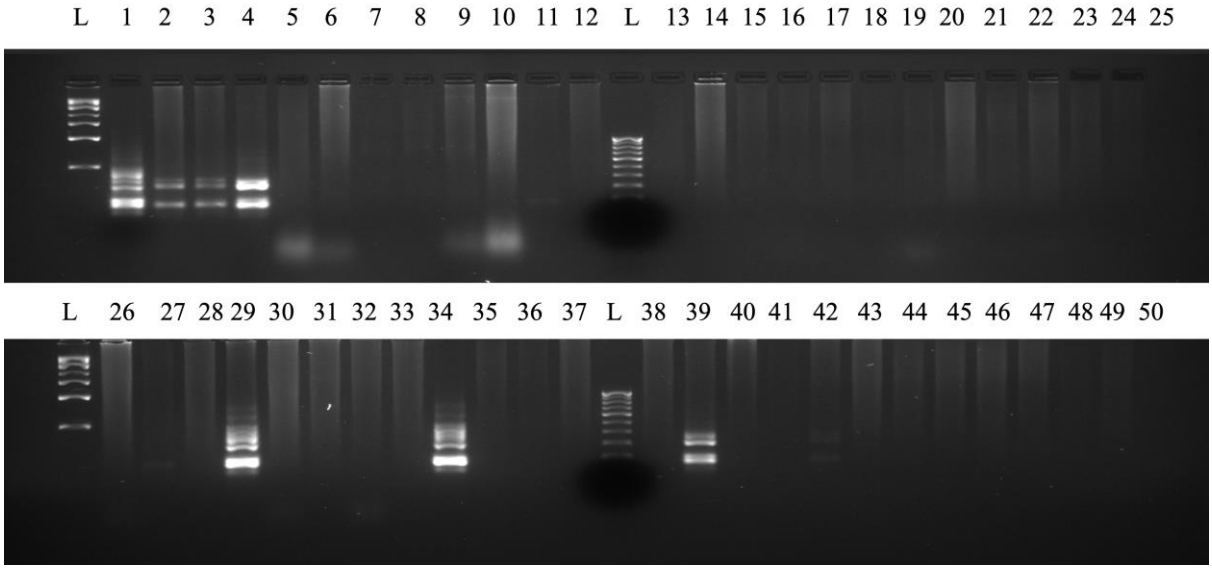


Fig. 4.9 A. Bands produced with UBC 807; L lane 1-10 are LP samples, lane 11-16 are P samples, lane 17-21 are L samples, lane 22-33 are H samples, 34-39 are K samples, lane 40-44 are LK samples and lane 45-50 are D samples.

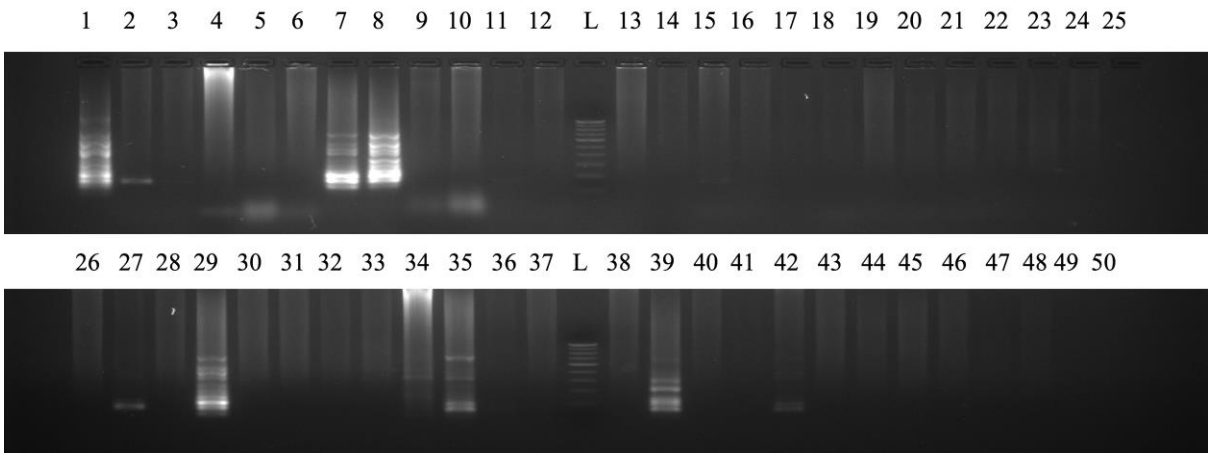


Fig. 4.9 B. Bands produced with UBC 810; L=DNA ladder, lane 1-10 are LP samples, lane 11-16 are P samples, lane 17-21 are L samples, lane 22-33 are H samples, 34-39 are K samples, lane 40-44 are LK samples and lane 45-50 are D samples.

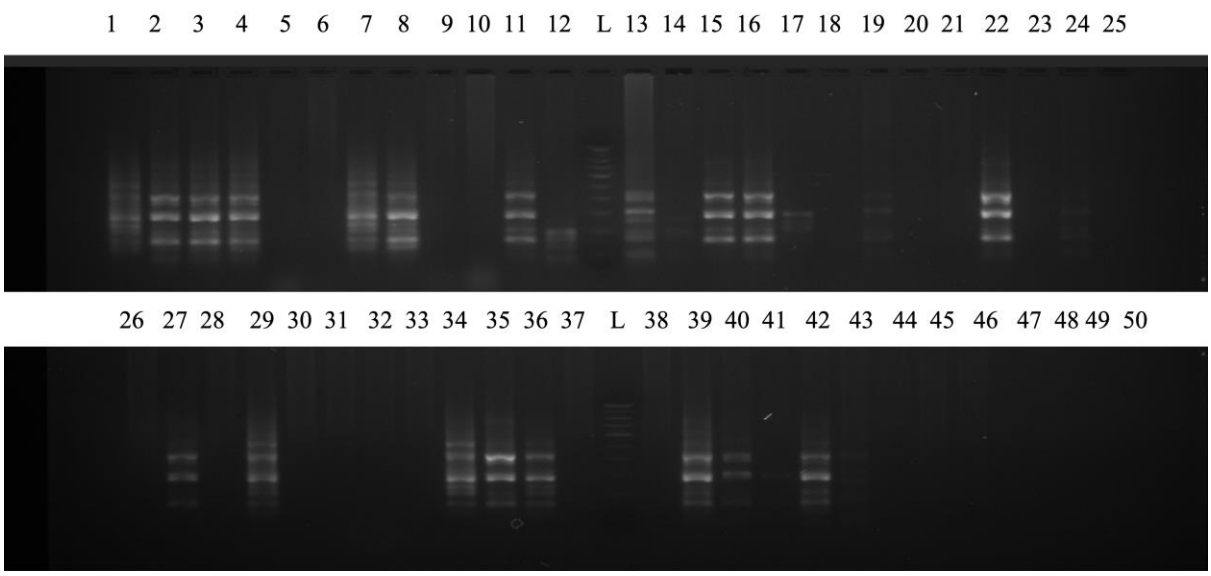


Fig. 4.9 C. Bands produced with UBC 826; L=DNA ladder, lane 1-10 are LP samples, lane 11-16 are P samples, lane 17-21 are L samples, lane 22-33 are H samples, 34-39 are K samples, lane 40-44 are LK samples and lane 45-50 are D samples.

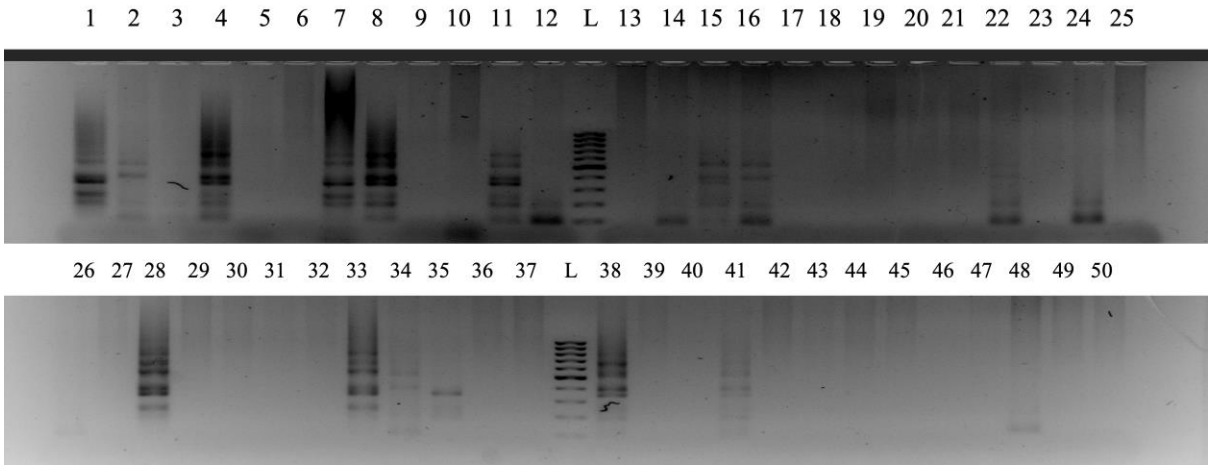


Fig. 4.9 D. Bands produced with UBC 828; L=DNA ladder, lane 1-10 are LP samples, lane 11-16 are P samples, lane 17-21 are L samples, lane 22-33 are H samples, 34-39 are K samples, lane 40-44 are LK samples and lane 45-50 are D samples.

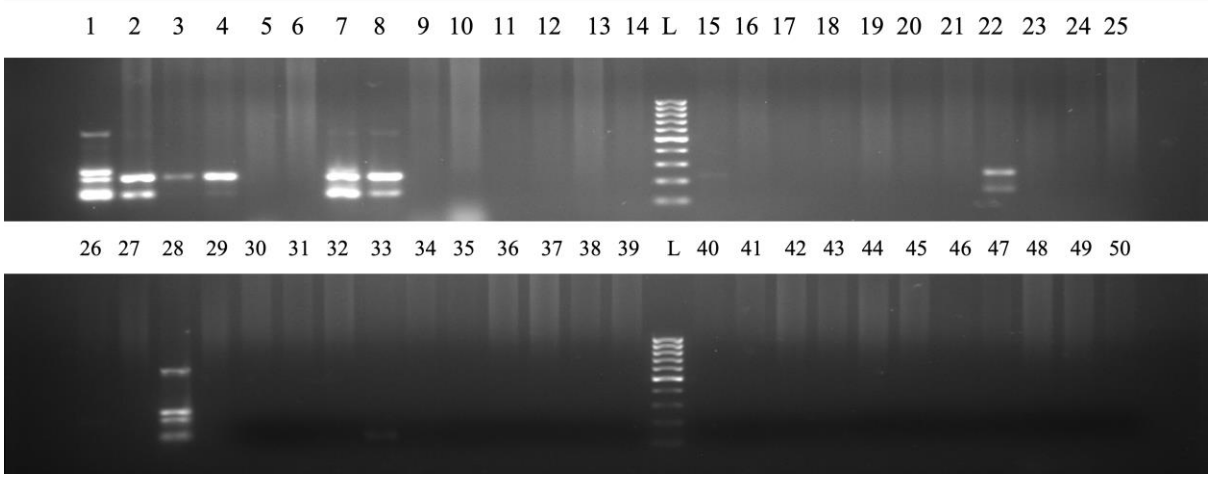


Fig. 4.9 E. Bands produced with UBC 834; L=DNA ladder, lane 1-10 are LP samples, lane 11-16 are P samples, lane 17-21 are L samples, lane 22-33 are H samples, 34-39 are K samples, lane 40-44 are LK samples and lane 45-50 are D samples.

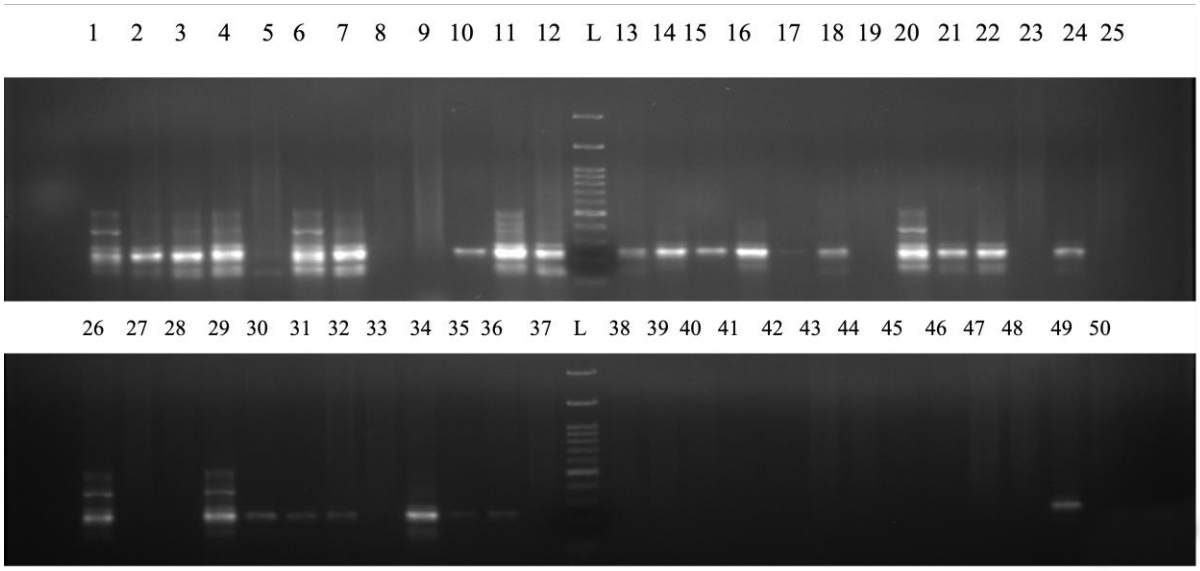


Fig. 4.9 F. Bands produced with UBC 841; L=DNA ladder, lane 1-10 are LP samples, lane 11-16 are P samples, lane 17-21 are L samples, lane 22-33 are H samples, 34-39 are K samples, lane 40-44 are LK samples and lane 45-50 are D samples.

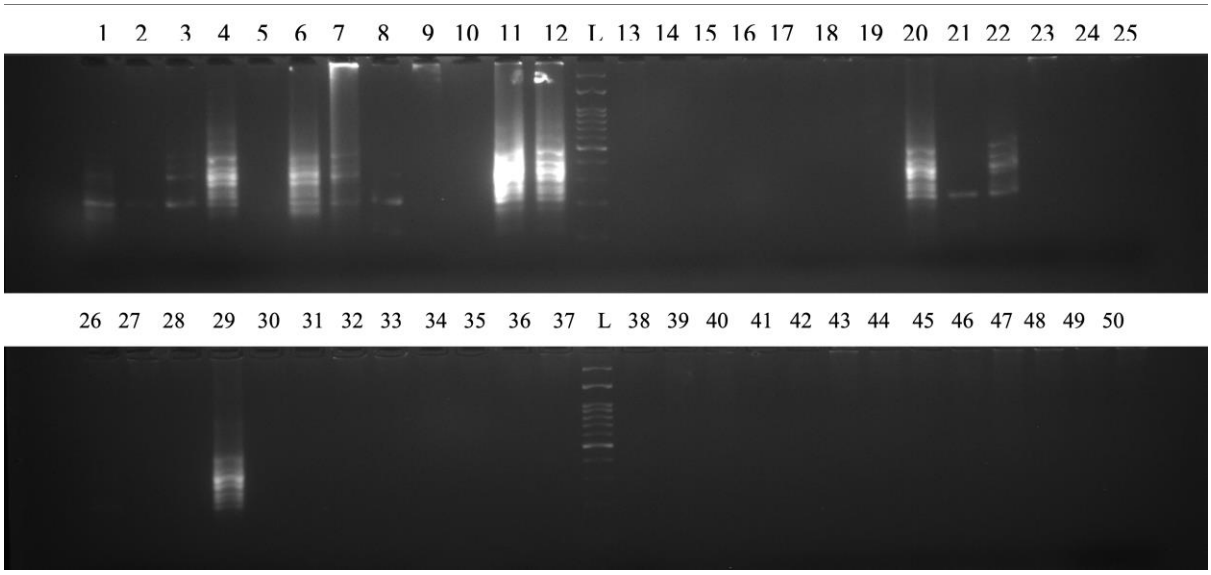


Fig. 4.9 G. Bands produced with UBC 848; L=DNA ladder, lane 1-10 are LP samples, lane 11-16 are P samples, lane 17-21 are L samples, lane 22-33 are H samples, 34-39 are K samples, lane 40-44 are LK samples and lane 45-50 are D samples.

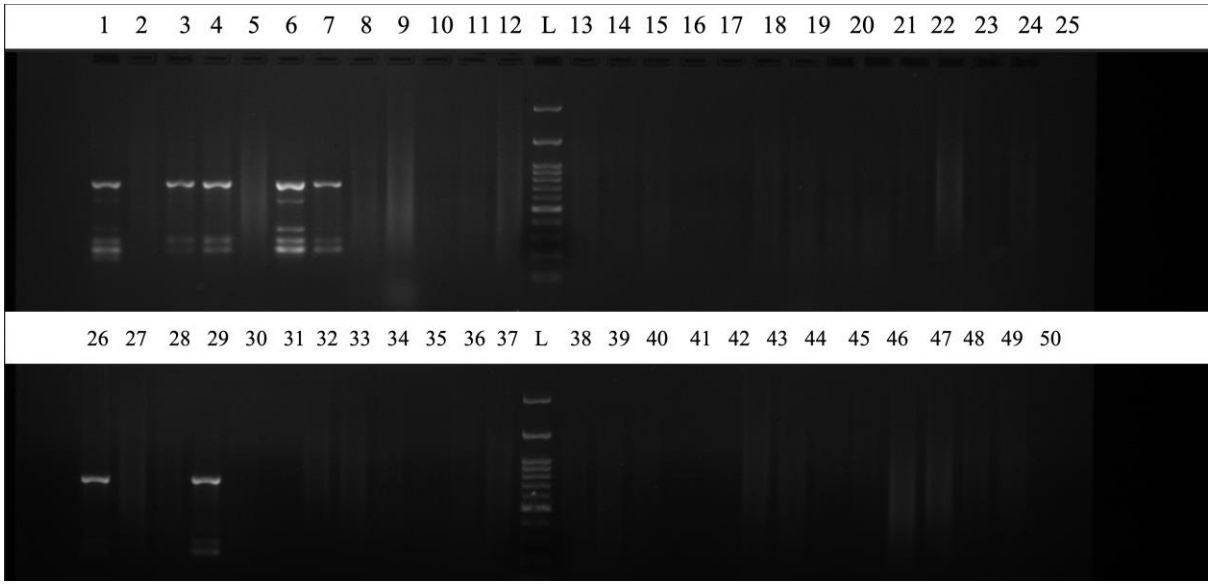


Fig. 4.9 H. Bands produced with OPJ01; L=DNA ladder, lane 1-10 are LP samples, lane 11-16 are P samples, lane 17-21 are L samples, lane 22-33 are H samples, 34-39 are K samples, lane 40-44 are LK samples and lane 45-50 are D samples.

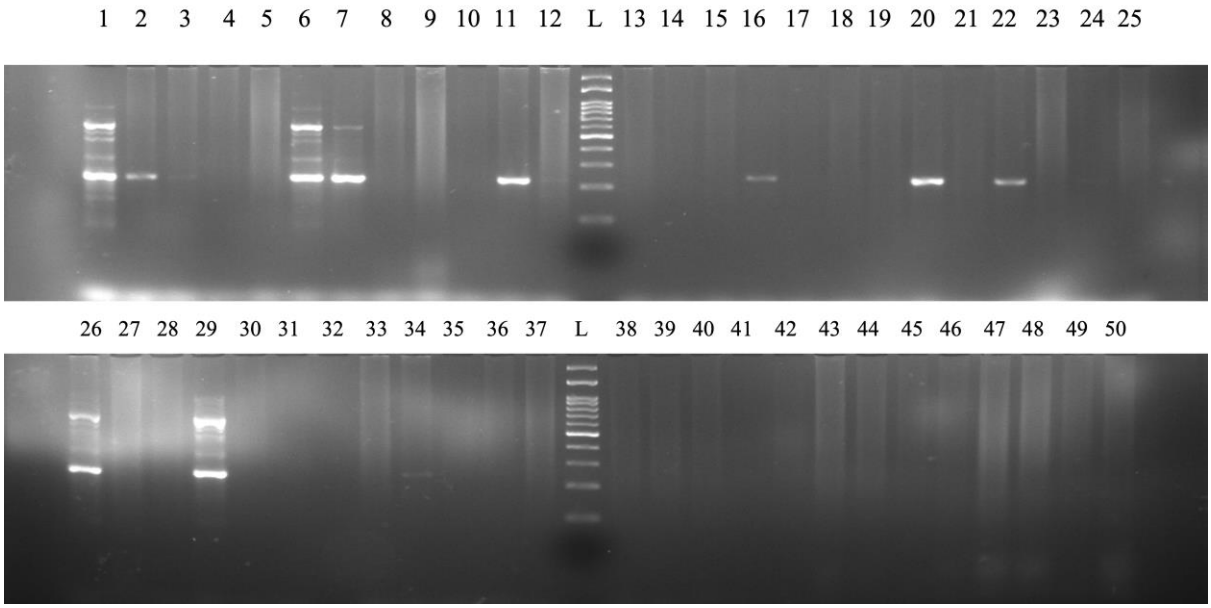


Fig. 4.9 I. Bands produced with OPJ04; L=DNA ladder, lane 1-10 are LP samples, lane 11-16 are P samples, lane 17-21 are L samples, lane 22-33 are H samples, 34-39 are K samples, lane 40-44 are LK samples and lane 45-50 are D samples.

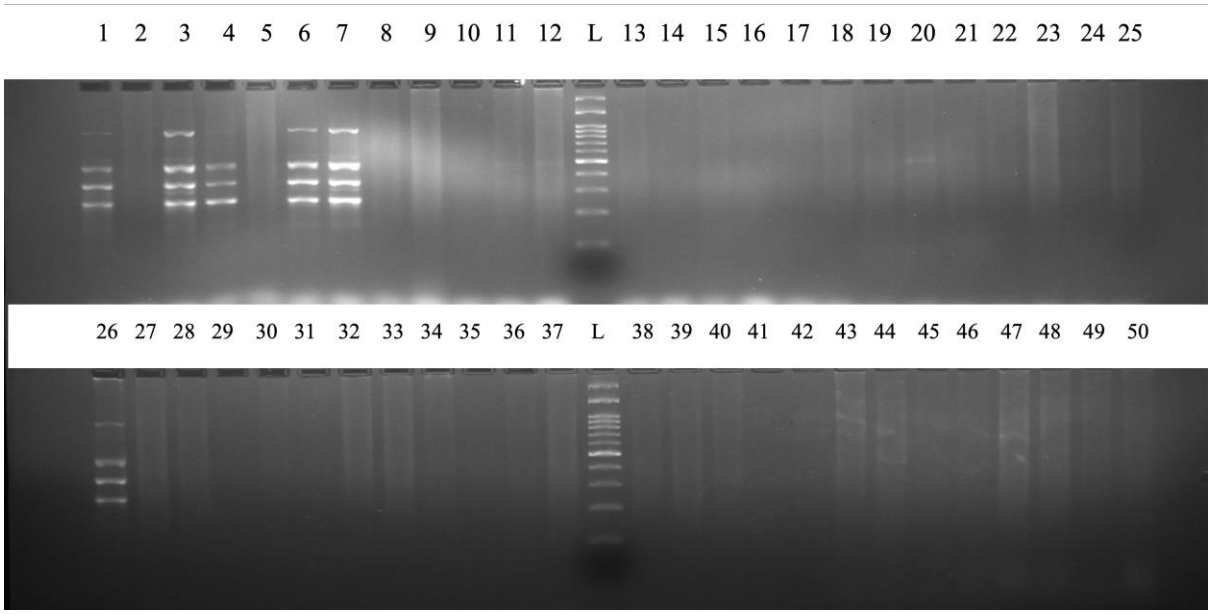


Fig. 4.9 J. Bands produced with OPJ05; L=DNA ladder, lane 1-10 are LP samples, lane 11-16 are P samples, lane 17-21 are L samples, lane 22-33 are H samples, 34-39 are K samples, lane 40-44 are LK samples and lane 45-50 are D samples.

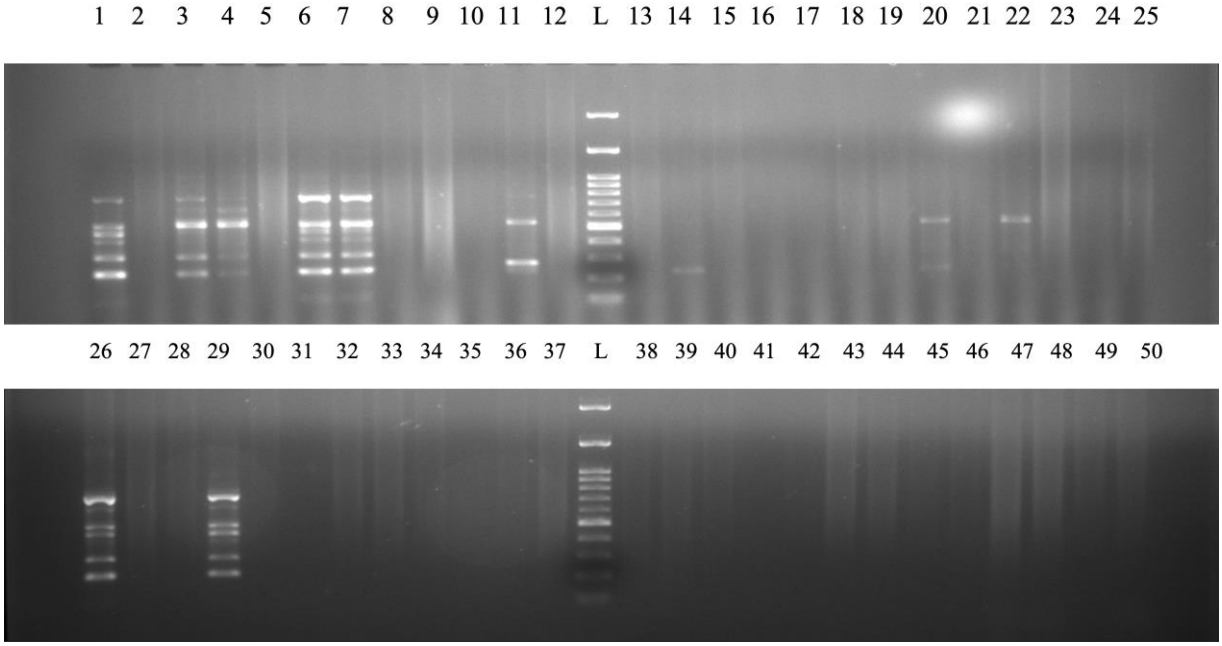


Fig. 4.9 K. Bands produced with OPJ10; L=DNA ladder, lane 1-10 are LP samples, lane 11-16 are P samples, lane 17-21 are L samples, lane 22-33 are H samples, 34-39 are K samples, lane 40-44 are LK samples and lane 45-50 are D samples.

4.4. Discussion

For any breeding programmes or species introduction and for large-scale cultivation, prior information at the gene level is an obligation (Tripathi *et al*., 2012). Hence, the genetic diversity and population structure of cultivated species of *G. glabra* plant from northern India were investigated using ISSR, RAPD and joint markers. Until now, both the markers have

been efficaciously applied by the scientific community to appraise the hereditary variability of medicinal flora from different regions (Shilpha *et al*., 2013; Rameshkumar *et al*, 2019; Suyal *et al*., 2022). The markers were used in combination because it allows wider details of the plant genomics as well as gives better knowledge and information on inferred data (Izzatullayeva *et al*. 2014; Selyutina *et al*., 2021).

At the instance of analysis, RAPD showed PIC=0.14, MI=0.39 and RP=1.32 while with ISSR, PIC=0.22, MI=3.59 and RP=2.46 were obtained. The results indicated that ISSR markers were far more capable of divulging better information when revealing the genetic variation in *G. glabra* populations. Similar outcomes have been reported in *Ocimum spp.* (Patel *et al*., 2015), *Hypericum* L *spp.* (Ma *et al*., 2021) etc. The genetic variation within the *G. glabra* populations was found to be low, as shown by Shannon's information index (*I*) and Nei's gene diversity (*H*) by RAPD that revealed 0.0463 and 0.07723 while ISSR markers revealed 0.1122 and 0.1782 respectively. The PPB values by both RAPD=97.43% and ISSR=100% demonstrated an elevated genetic variation at the species division, while a moderate range of genetic diversity was recorded at the population level with 41.49% of PPB (ISSR) and a low level of variation was detected by RAPD at 20.63%. These results were similar to previously reported literature on *G. uralensis* by using ISSR markers (Yao *et al*, 2008) and a little higher than perennial herb *Plumbogo spp* through RAPD and ISSR markers (Panda *et al*., 2015). Likewise, these outcomes coincide with previously reported literature on the genetic diversity of other species of the Fabaceae family, assumed from ISSR and RAPD (Bishoyi *et al*., 2014).

The population of long-lived herbaceous plant species is expected to have a significant genetic diversity (Nybom *et al*., 2000). However, self-pollinated plants tend to exhibit lower levels of gene flow inferring a lower genetic variability (Govindaraju *et al*., 1989). The degree of genetic differentiation (G_{st}) values obtained were relatively moderate to high, RAPD= 0.1479 ISSR=0.2247. These results revealed by RAPD were a little lower than the average G_{st} = 0.19 for long-lived plants and average G_{st} =0.032 for dicots while the results based on ISSR were higher than the average of perennial plants but a little lower than dicotyledons of the same (Nybom and Bartish, 2000; Nybom, 2004). Gene flow (N_m) was higher (RAPD=2.883, ISSR=1.7255), confirming low genetic variation in self-pollinated plants. The results are in agreement with that of various phytogeographical populations of *G. glabra* grown in Iran, as inferred by using AFLP markers (Hosseini *et al*., 2021) and also in the same taxa collected different provinces of China, using SSR markers (Liu *et al*., 2019). Low total

genetic variation (H_t) (RAPD=0.0518, ISSR=0.1447) and (H_s) (RAPD=0.0441, ISSR=0.1122) values as revealed by both markers may be due to the effect of sparse population of *G. glabra* in India. Similarly, low genetic variation was reported in *G. echinata* species as a result of less population in Turkey (Altay *et al*., 2016). Scarcely distributed plants generally exhibit lower levels of genetic variability when compared to their counterparts in areas with higher abundance due to genetic drift and restricted gene flow (Nybom *et al*., 2004). This is confirmed by the outcomes of intraspecific variation analysis as exhibited by AMOVA that displayed greater disparity in the hereditary elements in the individuals, as expected in perennial plants (Hamrick *et al*., 1992). Since the species is a non-native plant, gene flow may have happened as a result of the plants' essential medicinal value, leading to their transfer from one location to another and creating a restricted genetic base (Bishoyi *et al*., 2014). Lower genetic disparity at the interspecific level is further substantiated by overlapping and intermixing of genotypes as shown by PCoA and confirmed by cluster analysis, which is parallel to previous reports (Heikrujam *et al*., 2017, Selyutina *et al*., 2021). The cluster analysis of pooled data showed that the populations were not grouped by geographic distance, denoting that there is no association between the genetic and geographical distance as verified by Mantel's test. This result corroborates with another research work (Shilpha *et al*., 2013, Akçali Giachino, 2020). The dendrogram depicted that populations LK, D, K, H and L, appeared in a major cluster, indicating a close genetic relationship. Populations of LK and D were clustered next to each other which depicted a closer relationship between the two. However, P and LP populations intended to move away from the major cluster exhibiting a lesser genetic relatedness. The clustering pattern is comparable to that documented for *G. uralensis* (Yao *et al*., 2008). The STRUCTURE result also did not vary much with the cluster analysis. No distinct cluster was observed in accordance with their respective populations and geographical locations. However, one of the reasons for the existence of admixture among the individuals in all the populations can be attributed to the presence of high inter- and intra-cohort gene flow. While in cluster analysis P and LP seemed to be isolated, they appear grouped in the STRUCTURE, demonstrating that there is a possibility of having similar origins for all the populations. Extremely low seed formation, longer seed germination with selfing and occasional inbreeding could be involved in maintaining a narrow genetic base and slower genetic diversification (Gustafsson and Gustafsson, 1994; Bishoyi *et al*., 2014). Thus, based on the uncommon perennial life form and limited population density, this threatened species should be prioritized for intense protection and conservation, considering its high medicinal niche in Ayurveda.

4.5. Conclusion

This is a first comprehensive report on RAPD and ISSR markers-based population genetics and structure to appraise the degree of genetic variability of *G. glabra* from Northern parts of India. The investigation depicted a substantial disparity at intraspecific and a moderate variation in the hereditary materials among the population. Measures and strategies including, *ex-situ* conservation and cultivation need to be established to manage the existing genetic base, enhance the population and acclimatize to the fluctuating surrounding settings. The present results authenticate that RAPD and ISSR markers have the ability to measure the heritable information of *G. glabra*. The evidence obtained from the study could certainly contribute to the knowledge of genetic materials, which would be helpful in management of the species and in devising any breeding programs for the species to thrive well in India.

CHAPTER 5

Mass propagation of high yielding glycyrrhizin containing licorice (*G. glabra* L) through *in vitro* tissue culture technique.

5.1. Introduction

Glycyrrhiza glabra Linn. (Family Fabaceae), is a lucrative undershrub with a variety of pharmaceutical values. Diverse bioactivities, mostly from *G. glabra* root extracts are well reported. The herb has demonstrated anti-diabetic properties (Pratama and Gusdinar, 2019), anti-ulcer (Jalilzadeh-Amin *et al*., 2019), anti-oxidant (Martins *et al*., 2015), anti-microbial (Jafari-Sales *et al*., 2018), anti-cancer (Goel *et al*., 2021), anti-inflammatory (Vasanth *et al*., 2020) and anti-viral activities (Srivastava *et al*., 2020). The chemicals contained in licorice have gained attention recently due to their numerous uses, including those in pharmaceuticals, the food industry, and cosmetics. It is prevalent throughout the Mediterranean, as well as in a few areas of Asia and Europe. The plant is extensively grown in some European nations, although it is only sporadically grown in nations like India (Parvaiz *et al*., 2014). Even if the use of licorice in the Ayurvedic therapeutic community is well established and dates back millennia, little to no determined effort has been made to develop effective farming practises and conservation measures (Badkhane *et al*., 2016). According to a report, we still depend on other countries for the stolons and rhizomes (Sharma *et al*., 2010). A greater overexploitation of medicinal plants from their natural environment is perceived at a fast pace. According to an estimate, over 70% of therapeutic crops are harvested destructively (Sharma *et al*., 2010), *G. glabra* being one of them. Moreso, ruinous or unreasonable manipulation of collection of plants stresses out the persistence of the species. *G. glabra* is now an endangered species in Mongolia (Chunhua *et al*., 2010) and Bulgaria (Kozhuharova *et al*., 2017). Yet, because of its high demand and harsh harvesting methods, it has become a threatened species in Iran (Hosseini *et al*., 2021). Using diverse biotechnological tactics for the preservation and management of plant species should be regarded as part of conservation strategies and resource management (Chen *et al*., 2016). In order to restore the population of high-yielding medicinal plant species that have been depleted, it is essential to create effective large-scale multiplication techniques (Moraes *et al*., 2021).

Stolons or rhizome cuttings are frequently used to breed *G. glabra*, despite showing a detrimental effect due to the longer duration taken to mature and harvest the rhizomes plus persistence rate is only about 40–50% (Sharma *et al*., 2010). Additionally, the asexual cloning

of *G. glabra* is hindered by the few seeds per pod, low seed germination and growth, as well as cost (Wang *et al*., 2013). Thus, ordinary routes for propagation of the plant species are not effective. The plant tissue culture approach, on the other hand, is seen as a sustainable method for generating exceptional, immune plants for bulk multiplication for the welfare of both consumer and farmer communities. Consequently, it is essential to create a successful *in vitro* propagation technique for mass multiplication in order to balance off the damaging manipulation and, furthermore, to retain the genetic consistency of this important therapeutic plant (Ramak *et al*., 2011; Savitikadi *et al*., 2020). Molecular markers have widespread applications in medicinal herb community for establishing linkage maps and various breeding programs (Kordrostami and Rahimi, 2015). Numerous researchers have validated the analogy of *in vitro* plantlets to donor source in all respects, among various plant species using molecular markers. ISSR markers have been shown to be very useful in determining gene variability, gene tagging, genomic maps, fingerprinting and others (Pradeep Reddy *et al*., 2002; Bakshipour *et al*., 2019). They are easy to use, highly informative, economical and valuable (Pathak *et al*., 2012). ISSR markers have demonstrated efficacy in revealing homogeneity of hereditary components between cultured plantlets and donors, in many botanicals with therapeutic benefits including, *Stevia rebaudiana*, a natural sweetner (Lata *et al*., 2013); *Ochreinauclea missionis*, an endemic plant (Chandrika and Rai, 2009); *Corallocarpus epigaeus*, an endangered plant (Vemula *et al*., 2020); *Lilium davidii*, also an edible plant (Yang *et al*., 2021) to name a few. Several researchers have documented the successful *in vitro* multiplication of *G. glabra* however, only sparse reports are available on the genetic fidelity estimation of tissue-cultured *G. glabra* plantlets (Mehrotra *et al*., 2012). Based on the aforementioned viewpoint, the experiment was designed to create an effective plant regeneration strategy for *G. glabra* mass propagation. Somaclonal differences in the progenies were also ruled out by ISSR and phytochemical resoluteness was examined with HPTLC between cultured plantlets and donor plant.

5.2. Supplies and techniques

5.2.1 Plant resource and explant decontamination

Plants growing in CSIR- CIMAP, Lucknow were selected as the donor plants for the study. The nodes having axillary buds were preferred as the explants for the experiment. After washing the explants under running water to remove any surface debris or microorganisms, they were then submerged in tween-20 solution containing 0.5/100 mL/mL distilled water (DW) (10 min) trailed by medication with bavistin solution, probably containing 2 gm in 100

mL (DW) to protect them from fungus for an additional 10 minutes. Finally, all chemical residues were removed with distilled water. To further eliminate all the pollutants, the effects of two disinfectants, $HgCl_2$ 0.1g/100mL and Ca $(OCl)_2$ 5g/100mL, were then checked for a few times varied times under airflow hooded workstations. The explants were then rinsed with distilled water that had been autoclaved.

5.2.2. Growth broths, growth settings and bud formation

Growth broth was made up of MS (Murashige and Skoog's, 1962) that had been augmented with 3% (w/v) sucrose and 0.8% agar as well as many plant growth hormones and used in variable doses. The media was autoclaved (121˚C) for 15 minutes and maintained acidic (5.7). Then, the cultured jars were kept in a continuous lit environment using cool-white fluorescent light at a rate of 50 µ mol m -2 s -1 at a temperature of 25 ± 2 °C. The disinfected nodal portions of the plant were divided into lengths of 2 cm and immunised in growth base, previously treated with varying doses of cytokinins including BAP and kinetin with altered doses for encouraging bud rejuvenation. Through 1 month and half, the frequency of the effect of hormones on the buds were noted.

5.2.3 Shootlet enhancement and root formation

Transferring to new jars was carried out by inserting in new growth base containing 6 BA of concentration 1.5 mg/L and altered measures of IBA starting from 0.10 to 1.50 mg/L in order to induce shoot multiplication. The secure young plants were then immunized in MS base or 50% MS base broth, both of which were enhanced with IBA in different dosages (0.10-1.50 mg/L) and 05% sucrose, for root induction, and monitored the growth for around 2-3 months. In every instance, PGR-free media were used as controls. After six weeks, the root reaction was evaluated. Next, the plantlets with healthy roots were put to the test to acclimatize.

5.2.4 Adaptation to natural settings

The regenerants with good roots were washed to eradicate any remaining agar and transplanted in small pastic cups filled with vermiculite. All those plants that endured were transferred to small bags filled with two parts of topsoil and a part of medium grain sand. The plantlets were housed in environmental chambers by maintaining adequate settings for about 14 days. Slowly, the young plants that persisted were finally relocated to conservatory to expose them to native surroundings.

5.2.5 Evaluation of heritable steadfastness

Randomly chosen micropropagated plantlets were outlined for testing genetic conformity and gauged using an ISSR approach based on PCR. Separation of nucleic DNA from nascent leaves of the mother and *in vitro* acclimated plants were carried out by a slightly modified CTAB technique (Doyle and Doyle, 1990). Just 7 of the 15 ISSR primers were evaluated for scoring bands. An optimised 20 µL PCR mixture containing 0.5 U Tag DNA was utilised for each reaction in a thermocycler under optimised PCR settings according to objective two. The DNA molecules of all plant samples were separated in a gel electrophoresis by adding ethium bromide (EtBr) to 1 X TBE buffer for illumination of DNA bands. Electrophoresis (Bangalore Genei, India) was accomplished within 3 hours at about 90 volts and finally, the gel images were envisaged with UV source through gel documentation system (Bio- Rad, USA). A 100 bp DNA ladder (Bangalore Genei, India) was applied in one of the well to estimate the size of DNA molecules. Reproducible and clear DNA amplicons were documented for preparation of binary data matrix.

5.2.6 Phytochemical analysis

5.2.6.1. Sample preparation

The rhizomes of *G. glabra* plants were processed into a fine powder with the aid of a home-based crusher. The root powder was soaked in ethanol solution (1:1) for 1 day 24 h in normal settings. The separated filtrates were then dehydrated completely in an evaporator at 40 °C. The extract was diluted with ethanol, filtered through syringe mesh and then quantitative estimation of glycyrrhizin was done with the help of HPTLC (Mallavadhani *et al*., 2019).

5.2.6.2. HPTLC conditions and evaluation of glycyrrhizin

By using a modified version of the HPTLC method reported by Mallavadhani *et al*., (2019), the extent of glycyrrhizin in the extracted samples was assessed. Stock solution (1 mg/mL) strength was made with 10 mg of standard glycyrrhizin by combining with 10 ml of ethanol. A TLC scanner connected with winCATS software, Linomat 5 applier and a needle for spotting were inclusive of the HPTLC. At a dosage rate of 100 nL/s, every spotting (2.0 µL) of the sample was applied to the plates with a band width (6 mm). Afterwards, the panes were allowed to stand in a twin trough at normal ambience while being drenched with the mobile phase. The experiment employed ethanol as the solvent, *n*-butanol, water and glacial acetic acid (70%, 20% and 1%) by volume as the eluent and silica gelled TLC plates as the stationary phase. Skimming of bands was performed at wavelength of 254 nm and the bioactive compound was spotted at R_F (0.35).

5.2.7. Data prediction

Throughout each trial, 10 explants were used in triplicates for each treatment. Along the experiments, morphological changes were observed and noted regularly. The detailed records were then fed to SPSS for ANOVA and at 95% confidence interval, assessed with post hoc test.

5.3. Results and discussion

5.3.1 Explant decontamination and *de novo* generation.

External sterilisation of the node pieces with 0.1% $HgCl_2$ for 3 min. was observed to be the most suited for producing most robust shoots, with a survival rate of 88.78%, according to early testing using $HgCl_2$ (0.1 g/100 ml) and Ca $(OCl)_2$ (5g/100mL) (Table 5.1). Both an increase in the exposure time to $HgCl_2$ and decrease in the same exposure period, as well as all Ca $(OCl)_2$ treatments, resulted in infection or browning of the explants.

The greatest mortality rate (98.24%) was seen after a 1-minute treatment with 5% calcium hypochlorite. However, the survival rate improved with longer Ca $(OCl)_2$ therapy periods. (Bello *et al*., 2018). As demonstrated in Table 5.2, when the sterilised explants were transferred to cultures with various doses of BAP and Kn, they showed differential response frequencies. In PGR-free media, the explants failed to exhibit shoot initiation. Effective morphological changes, were visible in the presence of PGRs within a week of the inoculation. After three weeks, the shoot initiation on MS basal with 6-BA of 1.50 mg/L displayed the highest mean shootlets at a rate (96.45%). Hence, for further shoot proliferation treatments, this concentration was chosen. The frequency was significantly decreased when the explants were grown in media with lower or higher does of cytokinin-6-BA. Comparably, in *Holostemma ada-kodien* highest bud break in 6 BA at 1.50 mg/L was reported (Martin, 2002). There are also claims that *G. glabra* shoot development was more successfully induced by MS basal enchanced with weaker 6 BA. When treatment changed to kn (0.50-3.50 mg/L), all the samples countered some growth. Nonetheless, when decontaminated nodes were injected to fresh media containing Kn (0.5mg/L), the least frequency of shoot growth was observed (17.34 %) in our case which was in congruence with the observations that at more kinetin dosage, response frequency was also elevated (Arya *et al*., 2009). The diverse morphogenic changes of the botanical, *de novo* is portrayed in Fig. 5.1 (a-j).

Table 5.1. Treatment of nodal explants with different concentrations of chemicals and time in minutes to optimize surface sterilization.

0.1% $HgCl_2$/m	5% $CaOCl_2$/m	% contamination
0	-	100±0.00
1	-	89.02±0.01
2	-	57.19±0.03
3	-	11.22±0.02
-	0	100±0.00
-	1	98.24±0.02
-	2	76.68±0.01
-	3	39.05±0.02

Table 5.2. Impact of growth hormones on *G. glabra* nodes during shoot rejuvenation (Ayangla *et al*., 2022)

MS + PGRs in mg/L		Frequency of shoot induction (%)	Mean No. of shoots±S.E	Mean shoot length±S.E (cm)
BAP	Kn			
0	-	00.00±0.00	0.00±0.00	0.00±0.00
0.5	-	69.45±0.04	1.45±0.13	1.68±0.24
1.5	-	96.45±0.20	3.33±0.29	4.78±0.11
2.5	-	50.77±0.56	2.29±0.15	4.08±0.35
3.5	-	42.65±0.19	3.28±0.16	2.86±0.11
-	0	00.00±0.00	0.00±0.00	0.00±0.00
-	0.5	17.34±0.10	2.13±0.12	3.93±0.30
-	1.5	24.48±0.58	2.36±0.13	3.40±0.26
-	2.5	46.60±0.75	1.61±0.16	3.14±0.22
-	3.5	68.66±0.39	1.83±0.33	1.35±0.02

For each experiment, ten explants were cultured and it was carried out three times. Post dhoc test was conducted, with mean values significant at p=0.5.

Table 5.3. Impact of growth hormones on *G. glabra* nodes during shoot proliferation (Ayangla *et al*., 2022)

MS + PGRs BAP + IBA	Conc. in mg/L	Frequency of proliferation (%)	Mean shoot for each explant±S.E	Mean length ± S.E (cm)
BAP + IBA	0.0 + 0.00	00.00± 0.00	0.00±0.00	0.00±0.00
BAP + IBA	1.5 + 0.00	46.92±0.34	2.81±0.22	3.49±0.17
BAP+ IBA	1.5 + 0.10	53.87±0.36	1.68±0.21	3.96±0.32
BAP + IBA	1.5 + 0.50	99.74±0.26	6.26±0.05	7.47±0.12
BAP + IBA	1.5 + 1.00	62.26±0.03	3.93±0.33	5.16±0.05
BAP + IBA	1.5 + 1.50	31.66±0.02	2.81±0.43	3.50±0.26

For each experiment, ten explants were cultured and it was carried out three times. Post dhoc test was conducted, with mean mean values significant at p=0.5.

Combining cytokinin and auxin is said to increase the responsiveness of *G. glabra* shoot multiplication (Yadav and Singh, 2012). Consequently, after three weeks of culture, the healthy plants were transplanted in fresh base containing 6 BA of 1.5 ppm and altered doses of IBA, 30g/L sucrose and 7g/L agar for the purpose of promoting shoot multiplication. The best response rate (99.74%) was obtained with the BAP (1.5 ppm) and IBA (0.5 ppm), which also produced the highest mean shoots of 6.26 and mean length of 7.47. The lowest mean bud of 1.68 and length of 3.96 cm were obtained with IBA concentrations as low as 0.1 mg/L (Table 5.3). Relatedly, numerous studies have shown that minute doses of 6 BA and IBA have a beneficial collegial result on shoot multiplication (Martin, 2002; Ramak *et al*., 2011).

The well-developed regenerants were sub-clultured in two different MS basal types with variation in IBA dosage, augmented with sucrose 50g/L, for effective root induction. Root generation of sugarcane was found to benefit from the addition 5% sucrose to culture conditions (Gopitha *et al*., 2010). It was observed that root development in semi-solid MS culture conditions was preferable to full strength (Table 5.4), which concur the observations

in root regeneration documented (Thengane *et al*., 1997; Arya *et al*., 2009). Transferring the botanical in semi-solid base along with IBA of 1.0 ppm, highest root frequency of 92.36% was observed with 3.98 roots on each planted model and a length of 4.99 cm. Nevertheless, the culture media with with IBA (0.5 ppm), the rhizome measure was maximum with 8.23 cm. Numerous researchers have noted that lower IBA concentrations were observed to increase the root records and length for each plant in a variety of medicinal plants, including *Psoralea corylifolia*, *G. glabra*, *Stevia rebaudiana, Rauwolfia serpentina* (Anis *et al*., 2005; Sawaengsak *et al*., 2011; Laribi *et al*., 2012; Susila *et al*., 2013).

The plantlets were transferred to pots under controlled atmospheric conditions and finally transplanted to polybags containing sand and soil mix in order to acclimatise the newly sprouted plants to normal natural settings. After two weeks, the vigorous, acclimated plants were grown successfully in the open (92% survival rate). The acclimated saplings were revealed to be equivalent to their mother plants in all respects, without any dissimilarity in the morphology.

Table 5.4. Impact of growth hormones on *G. glabra* nodes during rhizome initiation (Ayangla *et al*., 2022)

Solid MS + IBA (mgL^{-1})	Semi solid MS+IBA (mgL^{-1})	Frequency of rhizome development (%)	Mean rhizome on each explant±S.E	Length ± S.E (cm)
0.0	0.0	00.00±0.00	00.00±0.00	00.00±0.00
0.1	-	18.00±0.36	0.91±0.26	1.54±0.21
0.5	-	28.63±0.13	3.64±0.53	2.61±0.39
1.0	-	30.16±0.06	2.65±0.20	1.74±0.01
1.5	-	44.46±0.22	1.61±0.31	1.43±0.23
0.0	0.0	00.00±0.00	00.00±0.00	00.00±0.00
-	0.1	33.11±0.51	1.37±0.15	1.86±0.41
-	0.5	70.13±0.02	2.61±0.27	8.23±0.12

-	1.0	92.36±0.12	3.98±0.37	4.99±0.28
-	1.5	50.73±0.27	2.97±0.48	3.39±0.06

For each experiment, ten explants were cultured and it was carried out three times. Post dhoc test was conducted, with mean values significant at p=0.5.

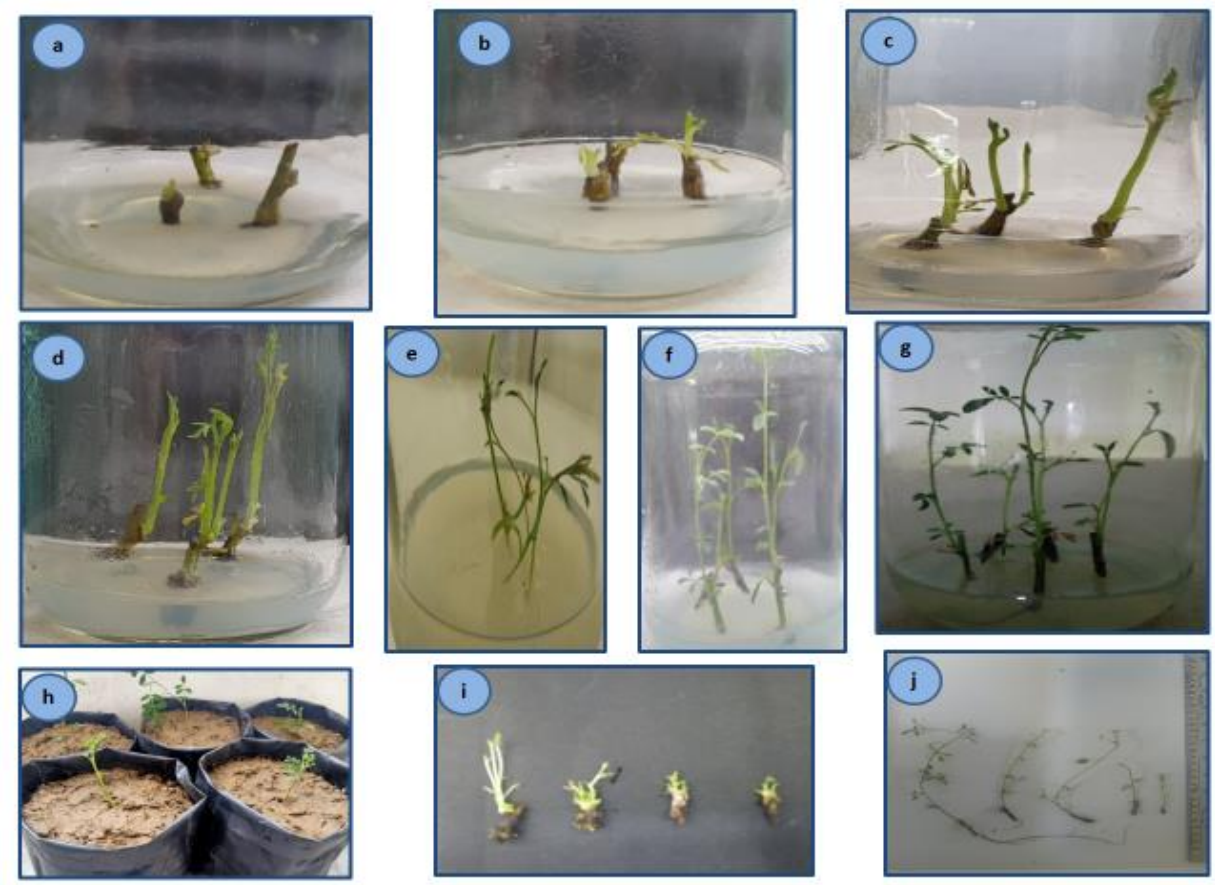


Fig. 5.1. Different phases of *in vitro* sprouting in *G. glabra*: (a, b and c) Shoot rejuvenation (d, e and f) Shoot proliferation and growth (g) Rhizome initiation (h) Hardening in poly bags (i and j) Shoot and rhizome development in one and a half month.

5.3.2 Genetic conformity of micro propagated plants

One of the main issues with tissue culture technique is the prevalence of somaclonal variation which occur due to minute alterations in the nucleotides or a locus (Biswas *et al.*, 2022). Maintaining the homology in hereditary elements in the cultured-grown plants is crucial and necessary for any plant regeneration operations (Bakhshipour *et al.*, 2019). Therefore, in the current investigation, the genetic consistency of ten randomly chosen plantlets that had been acclimated were compared to the parent plant using few ISSRs. Altogether, seven markers were utilized that characterised the DNA bands with 46 amplified

fragments, ranging from 5-8 bands with an average of 6.57 bands for each marker (Table 5.5). The lowest product was produced by ISSR 6 (five amplicons), while the highest bands were produced by ISSR 4 (eight amplicons) as shown in the table. In the study, monomorphic banding pattern was exhibited by all the ISSR primers in both *in vitro* produced plants and donor plant, indicating that micropropagated plants are genetically indistinguishable to their mother plants (Fig. 5.2). The findings confirm that *de novo* rejuvenation through meristematic propagation is more advantageous for attaining analogous true-to type duplications (D'Amato and Bayliss, 1985). We did not observe any somaclonal variability, monomorphic banding pattern was detected in the ISSR experiment. Parallel observations in ISSR – based inherent stability tests in micro-clones have been successfully demonstrated in numerous commercially important therapeutic plants, such as, *Ochreinauclea missionis* (Chandrika and Rai, 2009), *Stevia rebaudiana* (Lata *et al*., 2013), *Moringa peregrina* (Khateeb *et al*., 2013), *Abutilon indicum* (Seth and Panigrahi, 2019), *Solanum khasianum* (Chirumamilla *et al*., 2021), *Lilium davidii* (Yang *et al*., 2021).

Table 5.5. ISSR primers used in evaluating genetic stability in the regenerants of *G. glabra*

S. No	Primer code	5′- 3′ sequencing	No. of scorable bands per primer	Total scale of bands amplified (bp)
1	ISSR 4	ACACACACACACACACC	8	100 - 800
2	ISSR 5	TGTGTGTGTGTGTGTGA	7	100 - 650
3	ISSR 6	AGAGAGAGAGAGAGAGYT	5	200 - 800
4	ISSR 8	AGAGAGAGAGAGAGAGYC	6	150 - 500
5	ISSR 10	CTCTCTCTCTCTCTCTRG	6	100- 600
6	ISSR 11	CACACACACACACACARG	7	100 - 700
7	ISSR 13	ACACACACACACACACYT	7	180 - 600

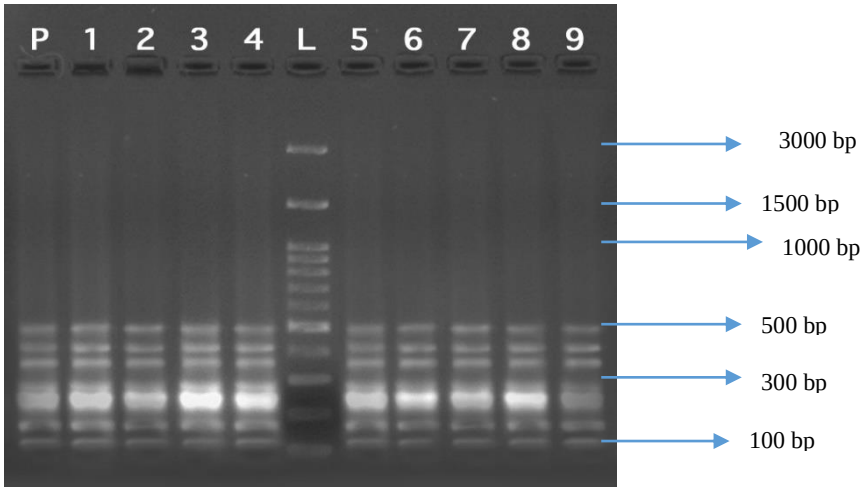


Fig. 5.2. A fingerprint profile by ISSR 8 of *G. glabra* with DNA marker (Lane L), donor plant (Lane P), arbitrarily picked micro-clones (Lane 1-9).

5.3.3. Biochemical fidelity

Evaluations of biochemical homogeneity in mother plants and clones is crucial for the hypothesised culture to be successful. The biological components contained in the botanicals are what give them their curative properties. In the present investigation, HPTLC was used to quantitatively analyse glycyrrhizin yield. On the basis of the spiked area, the glycyrrhizin concentration in the extracts of roots from the donor and the regenerated plantlets was determined. The HPTLC results displayed that the total of glycyrrhizin in the plantlets was 0.076%, which was proportional to the yield of the parent plant (0.0752%) (Fig. 5.3). According to the present research, plants grown using a particular technique do not have any detrimental impact on the production of glycyrrhizin. Equivalent investigations evaluating the biochemical reliability of other medicinal herbs using HPTLC have been reported in *Hedychium coronarium* (Behera *et al*., 2018).

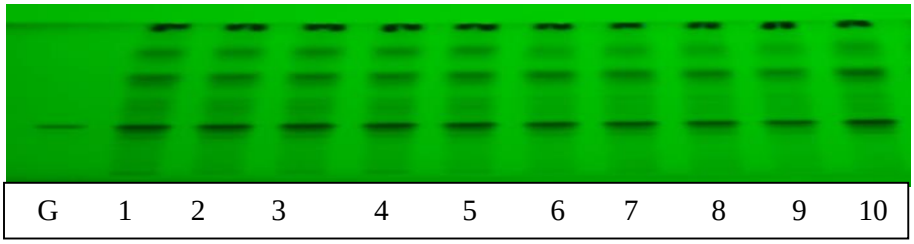


Fig. 5.3. HPTLC fingerprint profile of *in vivo* rhizome extracts: G=glycyrrhizin standard, 1= *in vivo* root, 2-10= *in vitro* roots.

5.4. Conclusion

The current study reveals a straightforward, dependable, and effective micro-clonal morphogenesis from axiliary nodes of the valuable commercial botanical, *G. glabra*. The revived microplants with the developed process were also assessed for genetic integrity because any breeding procedure must unquestionably preserve genetic consistency. Contrary to some reports that found modest polymorphism, the current investigation found no genetic variation. Correspondingly, the consistency of the most active principle i.e., glycyrrhizin was compared between the mature plant and plantlets by HPTLC. The suggested *in vitro* method can be used to genenerate in bulk, without any differences in the bioactive compound and hereditary materials to maintain and sustainably grow plant *G. glabra*.

SUMMARY AND CONCLUSION

G. glabra L. also known as licorice/ sweet root, is one of the most important and oldest medicinal herbs used in its crude form in various drugs and herbal formulations across the world, including, India. The most active bio-component of this plant, glycyrrhizin, is found in the rhizomes and is widely utilised as a seasoning and sweetening agent in the culinary industry and confectioneries. In recent times, demand for natural products, including licorice has been on the rise. However, as a requirement for medicine, glycyrrhizin content has to be over 2.5 % which makes it pertinent to search for the best raw material in terms of glycyrrhizin content and intensity of sweetness. Since, harvesting of the rhizome/stolons for obtaining glycyrrhizin, is rather destructive, it can deplete the populations of the plant which will eventually lead to extinction. Nowadays, many countries have assigned the plant for protection and conservation as the plants have become threatened or endangered. In India, the plant populations are quite limited as the species is non-native but yet the plants have a long history in Ayurveda. This made it more important to delve to the core of the genome of the species so that knowledge on the genetic variation can be tapped to develop successful breeding programmes and to improve the crop. Further, with the intervention of *in vitro* tissue culture technique, the demand, cost and supply of elite *G. glabra* plants can be resolved in a shorter duration. Mass propagating the elite plant species would enable incessant supply for cultivation or ex-situ conservation for a wider scope.

For the purpose, a comprehensive study was planned and implemented to optimize methods to estimate the maximum yield of glycyrrhizin, evaluated interspecific and intraspecific genetic variation of *G. glabra* species, aimed to contribute information on the genetic resources for efficient management of the crop and developed a simple and efficient revival of the species.

Secondary metabolites occur in very small amount, hence, to ensure high quality and quantity of bioactive compounds optimization is required. To this end, optimization of various factors that affect the extraction processes were considered by RSM (response surface methodology) and compared with ANN. Through preliminary Plackett-Burman design, significant variables viz., time, temperature, solvent composition, solid to solvent ratio were subjected to central composite design (CCD). Estimation of the glycyrrhizin yield was done with the help of optimized HPTLC technique. The ideal conditions for maximum yield of glycyrrhizin through RSM was found to be fifty-five-degree celcius, for forty-five minutes of

separation with 55 mL of ethanol solution and at 30 mL for a gram of solute in approximation. The proposed model was found to be appropriate since a good agreement was drawn between experimental (0.76%) and predicted (0.751%) percentage of glycyrrhizin. On comparison between RSM and ANN performance wise, ANN modelling was found to be far better in optimizing the glycyrrhizin yield.

For screening of the elite population producing the highest glycyrrhizin yield, *G. glabra* rhizomes were collected from 12 geographical locations across 6 states of India viz., Punjab, Haryana, Delhi, Himachal Pradesh, Uttar Pradesh and Maharashtra. A total of 66 samples were collected including, Haryana Mulhati-1 from HAU, Hisar and Mishree from CIMAP, Lucknow during 2018-2020. The samples were approximately 2-3 years old which were collected at fall and winter months. Extraction was done by green method i.e., microwave assisted extraction method and quantification of glycyrrhizin was done by HPTLC method. An average of 1. 38% of glycyrrhizin yield was observed from the experiment. As evidence from the result, concentration of glycyrrhizin was found to be slightly higher in Lucknow samples (2.18%) followed by Kurukshetra (2.12%) and the least amount was observed in the accessions from Pune (0.07%). In the study, Haryana samples showed glycyrrhizin comparable to Lucknow populations which may be attributed to similar climatic conditions in the two locations. Variations in the glycyrrhizin accumulation was observed which may be due to various environmental factors. This was validated by evaluating relationship between glycyrrhizin yield and various environmental variables, it was found that the compound accumulation was found to have positive connection with latitude at $P<0.05$, $R^2=0.086$ and strong positive association with longitude at $P<0.01$, $R^2=0.104$. However, contrary to some report, in this case, effect of temperature on compound accumulation was ruled out. Variation of the phyto-active compound in *G. glabra* may be therefore an interactive result of various other elements such as soil characteristics, season of harvest, genes along geographic factors.

Since time immemorial, *G. glabra* has a long history with Ayurveda. Despite the importance of the plant, less efforts are focussed towards cultivation and conservation of the non-native medicinal plant. Till date, only limited efforts are being shown towards assessment of genetic variation of *G. glabra* and if available are only scanty. Evaluation of genetic diversity will demonstrate the magnitude of genetic foundation, allowing breeders to apply the information for the crop improvement. Hence, in order to determine the genetic variation, DNA-based molecular markers which are more advantageous than phenotypic and biochemical markers were selected as they are free from any environmental interference. The

study used RAPD and ISSR markers because of their ease of use, degree of polymorphism, speed and cost effectiveness, and simplicity and efficiency. Altogether, 50 individuals representing 7 populations were nominated from Punjab (Phagwara, Ludhiana, Patiala), Haryana (Hisar, Kurukshetra), Uttar Pradesh (Lucknow) and New Delhi. DNA isolation of juvenile leaves was done by modified CTAB method. For PCR analysis, amplification conditions for both markers were optimized. In total, 9 RAPD primers and 12 ISSR primers were screened for discrete amplifications. The marker information obtained for RAPD (PIC= 0.14; MI=0.39; RP=1.32) and ISSR (PIC= 0.22; MI=3.59; RP=2.46) revealed that ISSR markers were much more effective in divulging information on polymorphism of the *G. glabra* populations. ISSR produced PPB=100%, while RAPD showed PPB=97.43%, depicting substantial blending of hereditary elements within the populations. A moderate range of genetic diversity was recorded at the population level based on RAPD (PPB=20.63%) and ISSR (PPB=41.49%). *G. glabra* is a perennial plant expected to have high genetic diversity. Also, the plant has selfing mode of breeding for which gene flow is expected to be lower. However, genetic differentiation (G_{st}) was found to be lower than gene flow (N_m) in both cases, implying the movement of genes among the individuals than in between the populations. Low genetic variation (H_t) and (H_s) values may be the effect rendered by limited population of *G. glabra* in India. These findings are also in congruence with AMOVA results that revealed more molecular variance within populations and less variation among the populations. Gene flow may have occurred due to the movement of plants from one region to another, attributed to medicinal value of the plant species. PCoA analysis and later UPGMA cluster analysis supported the presence of low genetic variation by revealing overlapping and intermixing of genotypes. The cluster analysis of pooled data of RAPD and ISSR revealed differentiation of populations in one major-cluster. Clustering was not formed in accordance with the geographical distance as revealed by Mantel's test. In the dendrogram, LK, D, K, H and L appeared in a major cluster, indicating close genetic relationship. LK and D populations were clustered next to each other which depicted a closer relationship between the two. However, P and LP populations intended to move away from the major cluster exhibiting a lesser genetic relatedness. Results based on STRUCTURE were also in line with the dendrogram. The STRUCTURE showed assigning of the individuals into three different sets but exhibited admixture among individuals to some extent. The individuals of populations LP and L (Green colour) were classified into one group; the populations of P, H, and K (Blue colour) were in another cluster while populations D and LK (Red colour) were assigned to yet another cluster. In the UPGMA dendrogram, P and LP were found to be deviated from the

other populations however, in STRUCTURE analysis, they were in one cluster, suggesting the possibility of having a similar origin for all the populations. Thus, considering its limited population density in India, the plant has to be assigned for conservation and breeding programmes have to be devised for improving the genetic variability of the species.

G. glabra is propagated asexually via rhizome cuttings and sexually via seed formation and germination. However, due to fewer seeds per pod, low seed viability, high cost and slow succession. Furthermore, practice of destructive harvesting to obtain glycyrrhizin from the plant may lead to population depletion. Hence, strategies have to be developed to alleviate the demand for and availability of superior plant stock at a faster pace. Towards this end, plant tissue culture was chosen as means to abridge the exploitation and demand by mass-producing the plants with genetic uniformity. The mother plant was obtained from Lucknow as it showed a higher content of glycyrrhizin. For direct organogenesis using the *in vitro* tissue culture approach, nodes with axillary buds were employed. For shoot initiation, effects of PGRs + MS viz., BAP and *Kn* were evaluated for response frequencies. With BAP (1.5 mg/L), maximum response rate (96.45%), shoots (3.33 cm) and length (4.78 cm) were yielded. For shoot multiplication, maximum growth (99.74), shoot/explant (6.26 cm) and length of shoot (7.47 cm) was observed with low doses of 6-BA and IBA. The best MS medium for root stimulation were half-strength versions with 5% sucrose. When subcultured in improved basal medium pooled with IBA (1.0 mg/L), the highest rate of rooting (92.36%) was perceived with 3.98 cm roots per explant and roots length measuring 4.99 cm. With IBA (0.50 ppm), we observed maximum rhizome length of 8.23 cm. Genetic fidelity was investigated on the acclimated in vitro regenerants with a 92% survival rate to rule out any somaclonal changes, which is an unfavourable phenomenon in regenerates. A total of seven ISSR markers produced distinct, clear and discrete amplifications ranging from 100 bp to 800 bp in size. The resolution of monomorphic banding patterns by all the markers demonstrated the consistency in heritable elements in the arbitrarily picked regenerates and donor. The genetic soundness was established since no genetic disparity was observed, nor polymorphic banding was seen. Furthermore, biochemical fidelity was also evaluated by means of HPTLC to check the consistency of glycyrrhizin content in the tissue cultured raised plantlets and donor plants. Glycyrrhizin yield in the regenerated plants exhibited (0.076%) which was akin to the concentration found in the parent plant (0.0752%), according to HPTLC data. The investigation showed that the proposed tissue culture methodology had no effect on the glycyrrhizin accumulation. In order to preserve improved plant quality and the long-term

viability of *G. glabra*, a producer of glycyrrhizin, the recommended method of micropropagation could be applied.

BIBLIOGRAPHY

Abdel Bar, F. M., Elimam, D. M., Mira, A. S., El-Senduny, F. F. and Badria, F. A., 2019. Derivatization, molecular docking and in vitro acetylcholinesterase inhibitory activity of glycyrrhizin as a selective anti-Alzheimer agent. *Natural Product research*, *33*(18), 2591-2599.

Abraham, J. and Mathew, F., 2014. Taste masking of paediatric formulation: a review on technologies, recent trends and regulatory aspects. *International Journal of Pharmacy and Pharmaceutical Sciences*, *6*(1), 12-19.

Abu-Shandi, K., Halawah, A. R., AlSayed Sallam, G. and Al-Edwan, A. R., 2015. Simultaneous analysis of glycyrrhizic acid and preservatives in licorice aqueous extract by HPLC/PDA detection. *Bulgarian Chemical Communication*, *47*(1), 135-143.

Adeola, H.A., Bano, A., Vats, R., Vashishtha, A., Verma, D., Kaushik, D., Mittal, V., Rahman, M.H., Najda, A., Albadrani, G.M. and Sayed, A.A., 2021. Bioactive compounds and their libraries: an insight into prospective phytotherapeutics approach for oral mucocutaneous cancers. *Biomedicine & Pharmacotherapy*, *141*, 111809.

Akçali Giachino, R. R., 2020. Investigation of the genetic variation of anise (*Pimpinella anisum* L.) using RAPD and ISSR markers. *Genetic Resources and Crop Evolution*, *67*(3), 763-780.

Alam, P., Foudah, A. I., Zaatout, H. H., Kamal, Y. T. and Abdel-Kader, M. S., 2017. Quantification of glycyrrhizin biomarker in *Glycyrrhiza glabra* rhizome and baby herbal formulations by validated RP-HPTLC methods. *African Journal of Traditional, Complementary and Alternative Medicines*, *14*(2), 198-205.

Altay, V., Karahan, F., Öztürk, M., Hakeem, K. R., Ilhan, E. and Erayman, M., 2016. Molecular and ecological investigations on the wild populations of *Glycyrrhiza* L. taxa distributed in the East Mediterranean Area of Turkey. *Journal of Plant Research*, *129*, 1021-1032.

Am Lee, S., Lee, S. H., Kim, J. Y. and Lee, W. S., 2019. Effects of glycyrrhizin on lipopolysaccharide-induced acute lung injury in a mouse model. *Journal of Thoracic Disease*, *11*(4), 1287.

Anagha, K., Manasi, D., Priya, L. and Meera, M., 2012. Comprehensive review on historical aspect of Yashtimadhu-*Glycyrrhiza glabra* L. *Global Journal Research on Medicinal Plants and Indigenous Medicines*, *1*(12), 687-693.

Anderson, D. M. and Smith, W. G., 1961. The antitussive activity of glycyrrhetinic acid and its derivatives. *Journal of Pharmacy and Pharmacology*, *13*(1), 396-404.

Anis, M. and Faisal, M., 2005. *In vitro* regeneration and mass multiplication of *Psoralea corylifolia*—an endangered medicinal plant. *Indian Journal of Biotechnology, 4,* 261–264.

Arias-Castro, C., Scragg, A. H., Stafford, A. and Rodriguez-Mendiola, M., 1993. Growth characteristics of *Glycyrrhiza glabra* cell suspension cultures. *Plant cell, tissue and organ culture*, *34*, 77-82.

Arya, S., Rathi, N. and Arya, I. D., 2009. Micropropagation Protocol for *Glycyrrhiza glabra* L. *Phytomorphology*, *59*(1), 71.

Ashfaq, U. A., Masoud, M. S., Nawaz, Z. and Riazuddin, S., 2011. Glycyrrhizin as antiviral agent against Hepatitis C Virus. *Journal of Translational Medicine*, *9*(1), 1-7.

Asl, M. N. and Hosseinzadeh, H., 2008. Review of pharmacological effects of *Glycyrrhiza sp*. and its bioactive compounds. *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives*, *22*(6), 709-724.

Ayangla, N. W., Dwivedi, P., Dey, A. and Pandey, D. K., 2022. *In vitro* propagation, genetic and phytochemical fidelity in *Glycyrrhiza glabra* L., a potent glycyrrhizin yielding endangered plant. *The Nucleus*, *65*(2) 1-9.

Aydar, A. Y., 2018. Utilization of response surface methodology in optimization of extraction of plant materials. *Statistical Approaches with Emphasis on Design of Experiments Applied to Chemical Processes*, 157-169.

Azmir, J., Zaidul, I. S. M., Rahman, M. M., Sharif, K. M., Mohamed, A., Sahena, F., Jahurul, M. H. A., Ghafoor, K., Norulaini, N. A. N. and Omar, A. K. M., 2013. Techniques for extraction of bioactive compounds from plant materials: A review. *Journal of Food Engineering*, *117*(4), 426-436.

Azwanida, N. N., 2015. A review on the extraction methods use in medicinal plants, principle, strength and limitation. *Medicinal and Aromatic Plants*, *4*(196), 2167-0412.

Badam, L., 1997. In vitro antiviral activity of indigenous glycyrrhizin, licorice and glycyrrhizic acid (Sigma) on Japanese encephalitis virus. *The Journal of Communicable Diseases*, *29*(2), 91-99.

Badkhane, Y., Yadav, A. S. and Bajaj, A., 2016. Effect of explant source and different concentrations of plant growth regulators on in vitro micropropagation of *Glycyrrhiza glabra* L. *Indo American Journal of Pharmaceutical Research*, *6*, 5830-5840.

Bakhshipour, M., Mafakheri, M., Kordrostami, M., Zakir, A., Rahimi, N., Feizi, F. and Mohseni, M., 2019. *In vitro* multiplication, genetic fidelity and phytochemical potentials of *Vaccinium arctostaphylos* L.: An endangered medicinal plant. *Industrial Crops and Products*, *141*, 111812.

Bala, M., Pratap, K., Verma, P. K., Singh, B. and Padwad, Y., 2015. Validation of ethnomedicinal potential of *Tinospora cordifolia* for anticancer and immunomodulatory activities and quantification of bioactive molecules by HPTLC. *Journal of Ethnopharmacology*, *175*, 131-137.

Balamithra, S., Girija, S. and Priyadharsini, J.V., 2020. An in silico analysis of protein targeted by glycyrrhizin in common dental pathogens. *Journal of Pharmaceutical Research International*, *32*(15), 170-178.

Barazani, O., Perevolotsky, A. and Hadas, R., 2008. A problem of the rich: prioritizing local plant genetic resources for *ex situ* conservation in Israel. *Biological Conservation*, *141*(2), 596-600.

Basar, N., Oridupa, O. A., Ritchie, K. J., Nahar, L., Osman, N. M. M., Stafford, A., Kushiev, H., Kan, A. and Sarker, S. D., 2015. Comparative cytotoxicity of *Glycyrrhiza glabra* roots from different geographical origins against immortal human keratinocyte (HaCaT), lung adenocarcinoma (A549) and liver carcinoma (HepG2) cells. *Phytotherapy Research*, *29*(6), 944-948.

Baskaran, P., Kumari, A. and Van Staden, J., 2018. In vitro propagation via organogenesis and synthetic seeds of *Urginea altissima* (Lf) Baker: a threatened medicinal plant. *3 Biotech*, *8*, 1-8.

Begna, T., 2021. Role and economic importance of crop genetic diversity in food security. *International Journal of Agricultural Science and Food Technology*, *7*(1), 164-169.

Behdad, A., Mohsenzadeh, S. and Azizi, M., 2020. Comparison of phytochemical compounds of two *Glycyrrhiza glabra* L. populations and their relationship with the ecological factors. *Acta Physiologiae Plantarum*, *42*, 1-18.

Behera, S., Kamila, P. K., Rout, K. K., Barik, D. P., Panda, P. C. and Naik, S. K., 2018. An efficient plant regeneration protocol of an industrially important plant, *Hedychium coronarium* J. Koenig and establishment of genetic & biochemical fidelity of the regenerants. *Industrial Crops and Products*, *126*, 58-68.

Bernardi, M., D'Intino, P. E., Trevisani, F., Cantelli-Forti, G., Raggi, M. A., Turchetto, E. and Gasbarrini, G., 1994. Effects of prolonged ingestion of graded doses of licorice by healthy volunteers. *Life Sciences*, *55*(11), 863-872.

Bhan, M., Satija, S., Garg, C., Dureja, H. and Garg, M., 2017. A novel approach towards green extraction for glycyrrhitinic acid by ionic liquid based microwave assisted extraction and optimization through response surface methodology. *Pharmacognosy Journal*, *9*(6).

Bi, D., Chen, D., Khayatnezhad, M., Hashjin, Z.S., Li, Z. and Ma, Y., 2021. Molecular identification and genetic diversity in *Hypericum* L.: A high value medicinal plant using RAPD markers markers. *Genetika*, *53*(1), 393-405.

Bishoyi, A. K., Pillai, V. V., Geetha, K. A. and Maiti, S., 2014. Assessment of genetic diversity in *Clitoria ternatea* populations from different parts of India by RAPD and ISSR markers. *Genetic Resources and Crop Evolution*, *61*(8), 1597-1609.

Bishoyi, A. K., Pillai, V. V., Geetha, K. A. and Maiti, S., 2014. Assessment of genetic diversity in *Clitoria ternatea* populations from different parts of India by RAPD and ISSR markers. *Genetic Resources and Crop Evolution*, *61*(8), 1597-1609.

Biswas, P., Nandy, S., Dey, A., Tikendra, L. and Nongdam, P., 2022. Molecular markers in assessing genetic clonal fidelity for i*n vitro* propagated endangered medicinal plants. *Molecular Genetics and Genomics Tools in Biodiversity Conservation*, 97-149.

Borges, T. H., López, L. C., Pereira, J. A., Cabrera–Vique, C. and Seiquer, I., 2017. Comparative analysis of minor bioactive constituents (CoQ10, tocopherols and phenolic

compounds) in *Arbequina* extra virgin olive oils from Brazil and Spain. *Journal of Food Composition and Analysis*, *63*, 47-54.

Bornet, B. and Branchard, M., 2001. Nonanchored inter simple sequence repeat (ISSR) markers: reproducible and specific tools for genome fingerprinting. *Plant Molecular Biology Reporter*, *19*, 209-215.

Chandra, S., Bandopadhyay, R., Kumar, V. and Chandra, R., 2010. Acclimatization of tissue cultured plantlets: from laboratory to land. *Biotechnology Letters*, *32*, 1199-1205.

Chandrika, M. and Rai, V. R., 2009. Genetic fidelity in micropropagated plantlets of *Ochreinauclea missionis* an endemic, threatened and medicinal tree using ISSR markers. *African Journal of Biotechnology*, *8*(13).

Charpe, T.W. and Rathod, V.K., 2012. Extraction of glycyrrhizic acid from licorice root using ultrasound: process intensification studies. *Chemical Engineering and Processing: Process Intensification*, *54*, 37-41.

Cheel, J., Tůmová, L., Areche, C., Van Antwerpen, P., Nève, J., Zouaoui-Boudjeltia, K., San Martin, A., Vokřál, I., Wsól, V. and Neugebauerová, J., 2013. Variations in the chemical profile and biological activities of licorice (*Glycyrrhiza glabra* L.), as influenced by harvest times. *Acta Physiologiae Plantarum*, *35*, 1337-1349.

Chen, H. R. and Sheu, S. J., 1993. Determination of glycyrrhizin and glycyrrhetinic acid in traditional Chinese medicinal preparations by capillary electrophoresis. *Journal of chromatography. A*, *653*(1), 184-188.

Chen, S. L., Yu, H., Luo, H. M., Wu, Q., Li, C. F., and Steinmetz, A., 2016. Conservation and sustainable use of medicinal plants: problems, progress, and prospects. *Chinese medicine*, *11*(1), 1-10.

Chen, Y. and Sørensen, L. K., 2000. Determination of marker constituents in radix *Glycyrrhizae* and radix Notoginseng by near infrared spectroscopy. *Fresenius' Journal of Analytical Chemistry*, *367*, 491-496.

Chester, K., Tamboli, E. T., Parveen, R. and Ahmad, S., 2013. Genetic and metabolic diversity in *Stevia rebaudiana* using RAPD and HPTLC analysis. *Pharmaceutical Biology*, *51*(6), 771-777.

Chigurupati, H., Auddy, B., Biyani, M. and Stohs, S.J., 2016. Hepatoprotective effects of a proprietary glycyrrhizin product during alcohol consumption: a randomized, double-blind, placebo-controlled, crossover study. *Phytotherapy Research*, *30*(12), 1943-1953.

Chirumamilla, P., Gopu, C., Jogam, P. and Taduri, S., 2021. Highly efficient rapid micropropagation and assessment of genetic fidelity of regenerants by ISSR and SCoT markers of *Solanum khasianum* Clarke. *Plant Cell, Tissue and Organ Culture*, *144*, 397-407.

Chunhua, F., Lei, C., Gan, L., Li, M., Yang, Y. and Yu, L., 2010. Optimization of embryogenic-callus induction and embryogenesis of *Glycyrrhiza glabra*. *African Journal of Biotechnology*, *9*(36).

Ciganović, P., Jakimiuk, K., Tomczyk, M. and Zovko Končić, M., 2019. Glycerolic licorice extracts as active cosmeceutical ingredients: Extraction optimization, chemical characterization, and biological activity. *Antioxidants*, *8*(10), 445.

Cinatl, J., Morgenstern, B., Bauer, G., Chandra, P., Rabenau, H. and Doerr, H., 2003. Glycyrrhizin, an active component of liquorice roots, and replication of SARS-associated coronavirus. *The Lancet*, *361*(9374), 2045-2046.

Cirillo, G., Curcio, M., Parisi, O. I., Puoci, F., Iemma, F., Spizzirri, U. G., Restuccia, D. and Picci, N., 2011. Molecularly imprinted polymers for the selective extraction of glycyrrhizic acid from liquorice roots. *Food Chemistry*, *125*(3), 1058-1063.

Cragg, G. M. and Newman, D. J., 2001. Natural product drug discovery in the next millennium. *Pharmaceutical Biology*, *39*(1), 8-17.

Craig, W.J., 1999. Health-promoting properties of common herbs. *The American Journal of Clinical Nutrition*, *70*(3), 491-499. https://doi.org/10.1093/ajcn/70.3.491s

Crance, J. M., Lévêque, F., Biziagos, E., van Cuyck-Gandré, H., Jouan, A. and Deloince, R., 1994. Studies on mechanism of action of glycyrrhizin against hepatitis A virus replication *in vitro*. *Antiviral Research*, *23*(1), 63-76.

Croteau, R., Kutchan, T. M. and Lewis, N. G., 2000. Natural products (secondary metabolites). *Biochemistry and Molecular Biology of Plants*, *24*, 1250-1319.

Cui, S., Fu, B., Lee, F. S. C. and Wang, X., 2005. Application of microemulsion thin layer chromatography for the fingerprinting of licorice (*Glycyrrhiza* spp.). *Journal of Chromatography B*, *828*(1-2), 33-40.

Dagar, J. C., Yadav, R. K., Dar, S. R. and Ahamad, S., 2015. Liquorice (*Glycyrrhiza glabra*): a potential salt-tolerant, highly remunerative medicinal crop for remediation of alkali soils. *Current Science*, 1683-1688.

Dang, H., Zhang, T., Li, Y., Li, G., Zhuang, L. and Pu, X., 2022. Population evolution, genetic diversity and structure of the medicinal legume, glycyrrhiza uralensis and the effects of geographical distribution on leaves nutrient elements and photosynthesis. *Frontiers in plant science*, *12*, 708709.

Dashzeveg, N., Vornam, B., Nergui, S. and Buerkert, A., 2018. Differentiation of *Glycyrrhiza uralensis* Fisch. Ex DC. Populations in the Bulgan river basin, western Mongolia. *Genetic Resources and Crop Evolution*, *65*, 1857-1865.

Dastagir, G. and Rizvi, M. A., 2016. *Glycyrrhiza glabra* L. (Liquorice). *Pakistan Journal of Pharmaceutical Sciences*, *29*(5).

Davis, E.A. and Morris, D.J., 1991. Medicinal uses of licorice through the millennia: the good and plenty of it. *Molecular and Cellular Endocrinology*, *78*(1-2), 1-6.

De Clercq, E., 2000. Current lead natural products for the chemotherapy of human immunodeficiency virus (HIV) infection. *Medicinal Research Reviews*, *20*(5), 323-349.

De, A. K., Datta, S. and Mukherjee, A., 2012. Quantitative analysis of glycyrrhizic acid from a polyherbal preparation using liquid chromatographic technique. *Journal of Advanced Pharmaceutical Technology & Research*, *3*(4), 210.

Dhingra, D., Parle, M. and Kulkarni, S. K., 2004. Memory enhancing activity of *Glycyrrhiza glabra* in mice. *Journal of Ethnopharmacology*, *91*(2-3), 361-365.

Dimitrova, D., Varbanova, K., Peeva, I., Angelova, S. and Guteva, Y., 1994. A study on *in vitro* cultivation of *Glycyrrhiza glabra*. *Elena Hanes*.

Doyle, J. J., 1990. Isolation of plant DNA from fresh tissue. *Focus*, *12*, 13-15.

Duran, C., Appleby, N., Edwards, D. and Batley, J., 2009. Molecular genetic markers: discovery, applications, data storage and visualisation. *Current Bioinformatics*, *4*(1), 16-27.

Earl, D. A., and VonHoldt, B. M., 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetics Resources*, *4*(2), 359-361.

Eghlima, G., Kheiry, A., Sanikhani, M., Hadian, J., Aelaei, M. and Nejad Ebrahimi, S., 2020. Investigation of phytochemical variability, antioxidant activity and ecological conditions of native Iranian *Glycyrrhiza glabra* L. *International Journal of Horticultural Science and Technology*, *7*(4), 387-400.

El-Senduny, F. F., Zidane, M. M., Youssef, M. M. and Badria, F.A., 2019. An approach to treatment of liver cancer by novel glycyrrhizin derivative. *Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents)*, *19*(15), 1863-1873.

Eng, A. T. W., Heng, M. Y. and Ong, E.S., 2007. Evaluation of surfactant assisted pressurized liquid extraction for the determination of glycyrrhizin and ephedrine in medicinal plants. *Analytica Chimica Acta*, *583*(2), 289-295.

Erayman, M., İlhan, E., Güzel, Y. and Eren, A. H., 2014. Transferability of SSR markers from distantly related legumes to *Glycyrrhiza* species. *Turkish Journal of Agriculture and Forestry*, *38*(1), 32-38.

Esmaeili, H., Karami, A., Hadian, J., Ebrahimi, S. N. and Otto, L.G., 2020. Genetic structure and variation in Iranian licorice (*Glycyrrhiza glabra* L.) populations based on morphological, phytochemical and simple sequence repeats markers. *Industrial Crops and Products*, *145*, 112140.

Esmaeili, H., Karami, A., Hadian, J., Saharkhiz, M. J. and Ebrahimi, S. N., 2019. Variation in the phytochemical contents and antioxidant activity of *Glycyrrhiza glabra* populations collected in Iran. *Industrial Crops and Products*, *137*, 248-259.

Evanno, G., Regnaut, S. and Goudet, J., 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology*, *14*(8), 2611-2620.

Farnsworth, N. R. and Soejarto, D. D., 1985. Potential consequence of plant extinction in the United States on the current and future availability of prescription drugs. *Economic Botany*, *39*(3), 231-240.

Fenwick, G. R., Lutomski, J. and Nieman, C., 1990. Liquorice, *Glycyrrhiza glabra* L.—Composition, uses and analysis. *Food Chemistry*, *38*(2), 119-143.

Fiore, C., Eisenhut, M., Krausse, R., Ragazzi, E., Pellati, D., Armanini, D. and Bielenberg, J., 2008. Antiviral effects of *Glycyrrhiza* species. *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives*, *22*(2), 141-148.

Future Market Insights, 2022. Licorice root market by product form (Roots, extracts, blocks, powder, paste), end-use (food and beverage industry, cosmetic industry, dietrary supplementary) and region-forecast 2022-2023. *Food and Beverage*. https://www.futuremarketinsights.com/reports/licorice-root-market

Gamborg, O. L., Miller, R. and Ojima, K., 1968. Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research*, *50*(1), 151-158.

Gao, W. Y., Fu, R. Z., Fan, L., Zhao, S. P. and Paek, K. Y., 2000. The effects of spaceflight on soluble protein, isoperoxidase, and genomic DNA in ural licorice (*Glycyrrhiza uralensis* Fisch.). *Journal of Plant Biology*, *43*, 94-98.

Gaurav, N., Juyal, P., Tyagi, M., Chauhan, N. and Kumar, A., 2018. A review on in vitro propagation of medicinal plants. *Journal of Pharmacognosy andPphytochemistry*, *7*(6), 2228-2231.

Ge, S., Li, G., Ma, Z., Wu, X., Meng, Y. and Huo, Y., 2009. Analysis on genetic diversity of wild populations of licorice (*Glycyrrhiza uralensis* Fisch.) with AFLP markers. *Scientia Agricultura Sinica*, *42*(1), 47-54.

George, E. F., Hall, M. A. and Klerk, G. J. D., 2008. Plant tissue culture procedure-background. *Plant Propagation by Tissue Culture*, *1*, 1-28.

Giacometti, J., Kovačević, D. B., Putnik, P., Gabrić, D., Bilušić, T., Krešić, G., Stulić, V., Barba, F.J., Chemat, F., Barbosa-Cánovas, G. and Jambrak, A. R., 2018. Extraction of bioactive compounds and essential oils from mediterranean herbs by conventional and green innovative techniques: A review. *Food Research International*, *113*, 245-262.

Giahi, E., Jahadi, M. and Khosravi-Darani, K., 2021. Enzyme-assisted extraction of glycyrrhizic acid from licorice roots using heat reflux and ultrasound methods. *Biocatalysis and Agricultural Biotechnology*, *33*, 101953.

Ginwal, H. S., Mittal, N., Tomar, A. and Varshney, V. K., 2011. Population genetic structure and diversity of high value vulnerable medicinal plant *Acorus calamus* in India using RAPD and chloroplast microsatellite markers. *Journal of Forestry Research, 22*, 367-377.

Goel, B., Sharma, A., Tripathi, N., Bhardwaj, N., Sahu, B., Kaur, G., Singh, B. and Jain, S. K., 2021. *In-vitro* antitumor activity of compounds from *Glycyrrhiza glabra* against C6 glioma cancer cells: identification of natural lead for further evaluation. *Natural Product Research*, *35*(23), 5489-5492.

Gopitha, K., Bhavani, A.L. and Senthilmanickam, J., 2010. Effect of the different auxins and cytokinins in callus induction, shoot, root regeneration in sugarcane. *International Journal of Pharmaceutical and Biological Sciences, 1*, 1-7.

Govindaraju, D. R., 1989. Variation in gene flow levels among predominantly self-pollinated plants. *Journal of Evolutionary Biology*, *2*(3), 173-181.

Gupta, V., Kak, A. and Singh B. B., 1997. Studies on seed germination and seedling vigour in liquorice (*Glycyrrhiza glabra*). *Journal of Medicinal and Aromatic Plant Sciences,* 2, 412-413.

Gupta, R., 2016. Yastimadhu (*Glycyrrhiza glabra*)–an important plant of Ayurvedic system of medicine. *Journal of Medicinal Plants Studies, 4*, 30-31.

Gupta, S., Pandotra, P., Gupta, A. P., Verma, M. K., Ahuja, A. and Vishwakarma, R. A., 2013. Direct rhizogenesis, *in vitro* stolon proliferation and high-throughput regeneration of plantlets in *Glycyrrhiza glabra*. *Acta Physiologiae Plantarum*, *35*, 2699-2705.

Gupta, S., Sharma, R., Pandotra, P., Jaglan, S. and Gupta, A. P., 2012. Chromolithic method development, validation and system suitability analysis of ultra-sound assisted extraction of glycyrrhizic acid and glycyrrhetinic acid from *Glycyrrhiza glabra*. *Natural Product Communications*, *7*(8), 991-994.

Gustafsson, L. and Gustafsson, P., 1994. Low genetic variation in Swedish populations of the rare species *Vicia pisiformis* (Fabaceae) revealed with RFLP (rDNA) and RAPD. *Plant Systematics and Evolution*, *189*(3-4), 133-148.

Hamrick, J. L., Godt, M. J. W. and Sherman-Broyles, S. L., 1992. Factors influencing levels of genetic diversity in woody plant species. *Population Genetics of Forest Trees*, 95-124.

Hantemirova, E. V., Belyaev, A. Y., Korchagina, O. S. and Laletina, I. A., 2020. Genetic differentiation and phylogenetic relationships of *Glycyrrhiza glabra* L., *G. uralensis* Fisch., and *G. korshinskyi* Grig. inferred from chloroplast DNA variation. *Russian Journal of Genetics*, *56*, 810-821.

Hayashi, H. and Sudo, H., 2009. Economic importance of licorice. *Plant Biotechnology*, *26*(1), 101-104.

Hayashi, H., Fukui, H. and Tabata, M., 1988. Examination of triterpenoids produced by callus and cell suspension cultures of *Glycyrrhiza glabra*. *Plant Cell Reports*, *7*, 508-511.

Hedayati, A. and Ghoreishi, S. M., 2015. Supercritical carbon dioxide extraction of glycyrrhizic acid from licorice plant root using binary entrainer: experimental optimization via response surface methodology. *The Journal of Supercritical Fluids*, *100*, 209-217.

Hedayati, A. and Ghoreishi, S. M., 2016. Artificial neural network and adaptive neuro-fuzzy interface system modeling of supercritical CO2 extraction of glycyrrhizic acid from *Glycyrrhiza glabra* L. *Chemical Product and Process Modeling*, *11*(3), 217-230.

Heikrujam, N., Thoungamba, A., Leimapokpam, T., Hamidur, R. and Potshangbam, N., 2017. ISSR marker based population genetic study of *Melocanna baccifera* (Roxb.) Kurz: A commercially important bamboo of Manipur, North-East India. *Scientifica*.

Henry, M., Edy, A. M., Desmarest, P. and Manoir, J. D., 1991. *Glycyrrhiza glabra* L. (Licorice): cell culture, regeneration, and the production of glycyrrhizin. In *Medicinal and Aromatic Plants*, *3*, 270-282.

Hoareau, L. and DaSilva, E. J., 1999. Medicinal plants: a re-emerging health aid. *Electronic Journal of Biotechnology*, *2*(2), 3-4.

Hossein, S., Ibrahim, R. and Ling, P. K. A., 2006. Somatic embryogenesis: an alternative method for *in vitro* micropropagation.156-161.

Hosseini, M.S., Ebrahimi, M., Samsampour, D., Abadía, J., Khanahmadi, M., Amirian, R., Ghafoori, I.N., Ghaderi-Zefrehei, M. and Gogorcena, Y., 2021. Association analysis and molecular tagging of phytochemicals in the endangered medicinal plant licorice (*Glycyrrhiza glabra* L.). *Phytochemistry*, *183*, 112629.

Hosseini, S. M. A., Souri, M. K., Farhadi, N. A. S. R. I. N., Moghadam, M. and Omidbaigi, R. E. Z. A., 2014. Changes in glycyrrhizin content of Iranian licorice (*Glycyrrhiza glabra* L.)

affected by different root diameter and ecological conditions. *Agricultural Communication*, *2*, 27-33.

Huang, R. Y., Chu, Y. L., Jiang, Z. B., Chen, X. M., Zhang, X. and Zeng, X., 2014. Glycyrrhizin suppresses lung adenocarcinoma cell growth through inhibition of thromboxane synthase. *Cellular Physiology and Biochemistry*, *33*(2), 375-388.

Huie, C.W., 2002. A review of modern sample-preparation techniques for the extraction and analysis of medicinal plants. *Analytical and Bioanalytical Chemistry*, *373*, 23-30.

Huo, X., Meng, X., Zhang, J. and Zhao, Y., 2020. Hepatoprotective effect of different combinations of 18α-and 18β-Glycyrrhizic acid against CCl4-induced liver injury in rats. *Biomedicine and Pharmacotherapy*, *122*, 109354.

Hurst, W. J., McKim, J. M. and Martin Jr, R. A., 1983. High-performance liquid chromatographic determination of glycyrrhizin in licorice products. *Journal of Agricultural and Food Chemistry*, *31*(2), 387-389.

Hussain, M. S., Fareed, S., Ansari, S., Rahman, M. A., Ahmad, I. Z. and Saeed, M., 2012. Current approaches toward production of secondary plant metabolites. *Journal of Pharmacy and Bioallied Sciences*, *4*(1), 10.

Hussain, R. A., Lin, Y. M., Poveda, L. J., Bordas, E., Chung, B. S., Pezzuto, J. M., Soejabto, D. D. and Kinghorn, A. D., 1990. Plant-derived sweetening agents: saccharide and polyol constituents of some sweet-tasting plants. *Journal of Ethnopharmacology*, *28*(1), 103-115.

Isbrucker, R. A. and Burdock, G. A., 2006. Risk and safety assessment on the consumption of Licorice root (*Glycyrrhiza* sp.), its extract and powder as a food ingredient, with emphasis on the pharmacology and toxicology of glycyrrhizin. *Regulatory Toxicology and Pharmacology*, *46*(3), 167-192.

Ismail, N. A., Rafii, M. Y., Mahmud, T. M. M., Hanafi, M. M. and Miah, G., 2019. Genetic diversity of torch ginger (*Etlingera elatior*) germplasm revealed by ISSR and SSR markers. *BioMed Research International*.

Ito, M., Sato, A., Hirabayashi, K., Tanabe, F., Shigeta, S., Baba, M., De Clercq, E., Nakashima, H. and Yamamoto, N., 1988. Mechanism of inhibitory effect of glycyrrhizin on replication of human immunodeficiency virus (HIV). *Antiviral Research*, *10*(6), 289-298.

Itokawa, H., Morris-Natschke, S. L., Akiyama, T. and Lee, K. H., 2008. Plant-derived natural product research aimed at new drug discovery. *Journal of Natural Medicines*, *62*(3), 263-280.

Izzatullayeva, V., Akparov, Z., Babayeva, S., Ojaghi, J. and Abbasov, M., 2014. Efficiency of using RAPD and ISSR markers in evaluation of genetic diversity in sugar beet. *Turkish Journal of Biology*, *38*(4), 429-438.

Jadhav, V. M., Kedar, U. S., Gholve, S. B. and Kadam, V.J., 2009. Development and validation of HPTLC method for determination of glycyrrhizin in herbal extract and in herbal gel. *Development*, *1*(4), 826-831.

Jafari-Sales, A. and Bolouri, P., 2018. Evaluation of the antimicrobial effects of *Glycyrrhiza glabra* L. on some gram positive and gram negative pathogenic bacteria in laboratory conditions. *Jorjani Biomedicine Journal*, *6*(4), 78-84.

Jain, C., Khatana, S. and Vijayvergia, R., 2019. Bioactivity of secondary metabolites of various plants: a review. *International Journal of Pharmaceutical Sciences and Research*, *10*(2), 494-504.

Jain, M., Johnson, T. S. and Krishnan, P., 2012. Biotechnological approaches to conserve the wealth of nature: endangered and rare medicinal plant species, a review. *Journal of Natural Remedies*, *12*(2), 93-102.

Jaiswal, N., Verma, Y. and Misra, P., 2021. High frequency *in vitro* callogenesis and plant regeneration of *Glycyrrhiza glabra* L. *Vegetos*, *34*(3), 495-504.

Jalilzadeh-Amin, G., Najarnezhad, V., Anassori, E., Mostafavi, M. and Keshipour, H., 2015. Antiulcer properties of *Glycyrrhiza glabra* L. extract on experimental models of gastric ulcer in mice. *Iranian Journal of Pharmaceutical Research*, *14*(4), 1163.

Jang, S., Lee, A. Y., Lee, A. R., Choi, G. and Kim, H. K., 2017. Optimization of ultrasound-assisted extraction of glycyrrhizic acid from licorice using response surface methodology. *Integrative Medicine Research*, *6*(4), 388-394.

Jiang, Y., Lu, H.T. and Chen, F., 2004. Preparative purification of glycyrrhizin extracted from the root of liquorice using high-speed counter-current chromatography. *Journal of Chromatography A*, *1033*(1), 183-186.

Kabe, Y., Koike, I., Yamamoto, T., Hirai, M., Kanai, A., Furuhata, R., Tsugawa, H., Harada, E., Sugase, K., Hanadate, K. and Yoshikawa, N., 2021. Glycyrrhizin derivatives suppress cancer chemoresistance by inhibiting progesterone receptor membrane component 1. *Cancers*, *13*(13), 3265.

Kadam, S. T. and Pawar, A. D., 2020. Conservation of medicinal plants: A review. *International Ayurvedic Medicine Journal*, *8*, 3890-3895.

Kakutani, K., Ozaki, K., Watanabe, H. and Tomoda, K., 1999. Somatic embryogenesis and plantlet regeneration on several species of licorice. *Plant Biotechnology*, *16*(3), 239-241.

Kala, C. P., Dhyani, P. P. and Sajwan, B. S., 2006. Developing the medicinal plants sector in northern India: challenges and opportunities. *Journal of Ethnobiology and Ethnomedicine*, *2*(1), 1-15.

Karkanis, A., Martins, N., Petropoulos, S.A. and Ferreira, I.C., 2018. Phytochemical composition, health effects, and crop management of liquorice (*Glycyrrhiza glabra* L.): A medicinal plant. *Food Reviews International*, *34*(2), 182-203.

Kaur, R., Kaur, H. and Dhindsa, A. S., 2013. *Glycyrrhiza glabra*: a phytopharmacological review. *International Journal of Pharmaceutical Sciences and Research*, *4*(7), 2470.

Khan, S., Mirza, K. J., Tayaab, M. and Abdin, M. Z., 2009. RAPD profile for authentication of medicinal plant *Glycyrrhiza glabra* Linn. *Internet Journal of Food Safety*, *11*, 24-28.

Khanuja, S.P.S., Shasany, A.K., Srivastava, A. and Kumar, S., 2000. Assessment of genetic relationships in Mentha species. *Euphytica*, *111*, 121-125.

Kim, H.S., Lee, S.Y., Kim, B.Y., Lee, E.K., Ryu, J.H. and Lim, G.B., 2004. Effects of Modifiers on the Supercritical CO_2 extraction of glycyrrhizin from Licorice and the morphology of licorice tissue after extraction. *Biotechnology and Bioprocess Engineering*, *9*(6), 447-453.

Kim, N. C. and Kinghorn, A. D., 2002. Highly sweet compounds of plant origin. *Archives of Pharmacal Research*, *25*, 725-746.

Kohjyouma, M., Kohda, H., Tani, N., Ashida, K., Sugino, M., Yamamoto, A. and Horikoshi, T., 1995. *In vitro* propagation from axillary buds of *Glycyrrhiza glabra* L. *Plant Tissue Culture Letters*, *12*(2), 145-149.

Komalavalli, N. and Rao, M. V., 2000. In vitro micropropagation of *Gymnema sylvestre*–A multipurpose medicinal plant. *Plant Cell, Tissue and Organ Culture*, *61*, 97-105.

Kordrostami, M. and Rahimi, M., 2015. Molecular markers in plants: concepts and applications. *Genetics in the 3rd Millennium*, *13*, 4024-4031.

Kozhuharova, A. and Stanilova, M., 2017. *In vitro* cultures initiation from seeds of Bulgarian localities of *Glycyrrhiza glabra* L. (Fabaceae). *Journal of BioScience and Biotechnology*.

Kukreja, A. K., 1998. *In vitro* propagation of liquorice (*Glycyrrhiza glabra* L.) through multiple shoot formation. *Journal of Spices and Aromatic Crops*, *7*(1), 13-17.

Kushiev, H., Noble, A. D., Abdullaev, I. and Toshbekov, U., 2005. Remediation of abandoned saline soils using *Glycyrrhiza glabra*: A study from the Hungry Steppes of Central Asia. *International Journal of Agricultural Sustainability*, *3*(2), 102-113.

Kvasnička, F., Voldřich, M. and Vyhnálek, J., 2007. Determination of glycyrrhizin in liqueurs by on-line coupled capillary isotachophoresis with capillary zone electrophoresis. *Journal of Chromatography A*, *1169*(1-2), 239-242.

Lanjekar, K. J. and Rathod, V. K., 2021. Application of ultrasound and natural deep eutectic solvent for the extraction of glycyrrhizic acid from *Glycyrrhiza glabra*: Optimization and kinetic evaluation. *Industrial & Engineering Chemistry Research*, *60*(26), 9532-9538.

Lanjekar, K. J. and Rathod, V. K., 2021. Green extraction of glycyrrhizic acid from *Glycyrrhiza glabra* using choline chloride based natural deep eutectic solvents (NADESs). *Process Biochemistry*, *102*, 22-32.

Lanteri, S., Saba, E., Cadinu, M., Mallica, G. M., Baghino, L. and Portis, E., 2004. Amplified fragment length polymorphism for genetic diversity assessment in globe artichoke. *Theoretical and Applied Genetics*, *108*, 1534-1544.

Laribi, B., Rouatbi, N., Kouki, K. and Bettaieb, T., 2012. *In vitro* propagation of *Stevia rebaudiana* (Bert.)-A non caloric sweetener and antidiabetic medicinal plant. *International Journal of Medicinal and Aromatic Plants*, *2*(2), 333-339.

Lata, H., Chandra, S., Techen, N., Wang, Y. H. and Khan, I. A., 2013. Molecular analysis of genetic fidelity in micropropagated plants of *Stevia rebaudiana* Bert. using ISSR marker.

Li, T., Zhou, H., Ma, J., Dong, L., Xu, F. and Fu, X., 2021. Quality assessment of licorice based on quantitative analysis of multicomponents by single marker combined with HPLC fingerprint. *Evidence-Based Complementary and Alternative Medicine*, 1-12.

Liu, Y., Geng, Y., Song, M., Zhang, P., Hou, J. and Wang, W., 2019. Genetic structure and diversity of *Glycyrrhiza* populations based on transcriptome SSR markers. *Plant Molecular Biology Reporter*, *37*, 401-412.

Liu, Y., Zhang, P., Song, M., Hou, J., Qing, M., Wang, W. and Liu, C., 2015. Transcriptome analysis and development of SSR Molecular Markers in *Glycyrrhiza uralensis* Fisch. *PloS one*, *10*(11), 143017.

Luo, P., Liu, D. and Li, J., 2020. Pharmacological perspective: glycyrrhizin may be an efficacious therapeutic agent for COVID-19. *International Journal of Antimicrobial Agents*, *55*(6), 105995.

Ma, S., Khayatnezhad, M. and Minaeifar, A. A., 2021. Genetic diversity and relationships among *Hypericum* L. species by ISSR Markers: A high value medicinal plant from Northern of Iran. *Caryologia*, *74*(1), 97-107.

Mahboobeh, A., Mahmood, G. and Majid, M., 2020. A study on the genetic variation in licorice (*Glycyrrhiza glabra*) in Iran by molecular DNA markers (RAPD). *Health, Food & Biotechnology*, *2*(4), 89-97.

Mallavadhani, U.V., Aparna, Y., Mohapatra, S. and Mane, D.V., 2019. A fast isolation method for glycyrrhizic acid, the bioactive marker of *Glycyrrhiza glabra*, and its quantitative evaluation in some single and multiherbal formulations using high-performance thin-layer chromatography. *Journal of Planar Chromatography-Modern TLC*, *32*(2), 81-87.

Maroufi, A., 2016. Selection of reference genes for real-time quantitative PCR analysis of gene expression in *Glycyrrhiza glabra* under drought stress. *Biologia plantarum*, *60*, 645-654.

Martin, K.P., 2002. Rapid propagation of *Holostemma ada-kodien* Sehult., a rare medicinal plant, through axillary bud multiplication and indirect organogenesis. *Plant Cell Reports, 21*, 112–117.

Martins, N., Barros, L., Dueñas, M., Santos-Buelga, C. and Ferreira, I. C., 2015. Characterization of phenolic compounds and antioxidant properties of *Glycyrrhiza glabra* L. rhizomes and roots. *RSC Advances*, *5*(34), 26991-26997.

Mauricio, I., Francischetti, B., Monteiro, R.Q. and Guimarães, J.A., 1997. Identification of glycyrrhizin as a thrombin inhibitor. *Biochemical and biophysical research communications*, *235*(1), 259-263.

Meena, A. K., Kaur, R., Singh, B., Yadav, A. K., Singh, U., Sachan, A., Pal, B. and Rao, M. M., 2010. Review on antifungal activities of ayurvedic medicinal plants. *Drug Invention Today*, *2*(2), 146-148.

Mehrotra, S., Khwaja, O., Kukreja, A. K. and Rahman, L., 2012. ISSR and RAPD based evaluation of genetic stability of encapsulated micro shoots of *Glycyrrhiza glabra* following 6 months of storage. *Molecular Biotechnology, 52,* 262–268.

Mendes-Silva, W., Assafim, M., Ruta, B., Monteiro, R. Q., Guimarães, J. A. and Zingali, R. B., 2003. Antithrombotic effect of glycyrrhizin, a plant-derived thrombin inhibitor. *Thrombosis Research*, *112*(1-2), 93-98.

Milbourne, D., Meyer, R., Bradshaw, J.E., Baird, E., Bonar, N., Provan, J., Powell, W. and Waugh, R., 1997. Comparison of PCR-based marker systems for the analysis of genetic relationships in cultivated potato. *Molecular Breeding*, *3*, 127-136.

Mishra, S. and Aeri, V., 2017. Biotransformation of lignan glycoside to its aglycone by *Woodfordia fruticosa* flowers: Quantification of compounds using a validated HPTLC method. *Pharmaceutical Biology*, *55*(1), 360-366.

Mohammad, N. and Rehman, S., 1985. Performance of *Glycyrrhiza glabra* in Mastung valley, Baluchistan. *Pakistan Journal of Agricultural Research*, *6*(3), 176-179.

Mondini, L., Noorani, A. and Pagnotta, M.A., 2009. Assessing plant genetic diversity by molecular tools. *Diversity*, *1*(1), 19-35.

Moraes, R. M., Cerdeira, A. L., and Lourenço, M. V., 2021. Using micropropagation to develop medicinal plants into crops. *Molecules*, 26, 1752.

Morinaga, O., Fujino, A., Tanaka, H. and Shoyama, Y., 2005. An on-membrane quantitative analysis system for glycyrrhizin in licorice roots and traditional Chinese medicines. *Analytical and Bioanalytical Chemistry*, *383*(4), 668-672.

Mousa, N., Siaguru, P., Wiryowidagdo, S. and Wagih, M.E., 2006. Rapid clonal propagation of licorice (*Glycyrrhiza glabra*) by *in vitro* shoot culture. *Sugar Tech*, *8*, 292-298.

Muhammed, A. ed., 2012. *Genetic diversity in plants* (Vol. 8). Springer Science & Business Media.

Mukhopadhyay, M. and Panja, P., 2008. A novel process for extraction of natural sweetener from licorice (*Glycyrrhiza glabra*) roots. *Separation and Purification Technology*, *63*(3), 539-545.

Mukhopadhyay, M. and Panja, P., 2010. Pressurised hot water as a novel extractant of natural products: a review. *Indian Chemical Engineer*, *51*(4), 311-324.

Muluvi, G. M., Sprent, J. I., Soranzo, N., Provan, J., Odee, D., Folkard, G., McNicol, J. W. and Powell, W., 1999. Amplified fragment length polymorphism (AFLP) analysis of genetic variation in *Moringa oleifera* Lam. *Molecular Ecology*, *8*(3), 463-470.

Murashige, T. and Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, *15*, 473-497.

Mustafa, A. and Turner, C., 2011. Pressurized liquid extraction as a green approach in food and herbal plants extraction: A review. *Analytica Chimica Acta*, *703*(1), 8-18.

Mustafa, S.B., Akram, M., Muhammad Asif, H., Qayyum, I., Hashmi, A.M., Munir, N., Khan, F.S., Riaz, M. and Ahmad, S., 2019. Antihyperglycemic activity of hydroalcoholic extracts of selective medicinal plants *Curcuma longa*, *Lavandula stoechas*, *Aegle marmelos*, and *Glycyrrhiza glabra* and their polyherbal preparation in alloxan-induced diabetic mice. *Dose-Response*, *17*(2), 1-6.

Mutegi, E., Snow, A.A., Rajkumar, M., Pasquet, R., Ponniah, H., Daunay, M.C. and Davidar, P., 2015. Genetic diversity and population structure of wild/weedy eggplant (*Solanum insanum*, Solanaceae) in southern India: Implications for conservation. *American Journal of Botany*, *102*(1), 140-148.

Nadeem, M., Palni, L.M.S., Purohit, A.N., Pandey, H. and Nandi, S.K., 2000. Propagation and conservation of *Podophyllum hexandrum* Royle: an important medicinal herb. *Biological Conservation*, *92*(1), 121-129.

Newman, D.J. and Cragg, G.M., 2020. Natural products as sources of new drugs over the nearly four decades from 01/1981 to 09/2019. *Journal of Natural Products*, *83*(3), 770-803.

Nezamabadi, N., Rahimiyan Mashhadi, H., Zand, A. and Alizadeh, H., 2007. Investigation of some ecophysiological aspects of licorice (*Glycyrrhiza glabra*) rhizomes. *Applied Entomology Phytopathology*, *74*(2), 45-62.

Ngo, T. V., Scarlett, C. J., Bowyer, M. C., Ngo, P. D. and Vuong, Q. V., 2017. Impact of different extraction solvents on bioactive compounds and antioxidant capacity from the root of *Salacia chinensis* L. *Journal of Food Quality*, 1-8. https://doi.org/10.1155/2017/9305047.

Noori, W. O., Waisi, B. I. and Alhassani, M. H., 2018. Extraction of glycyrrhizin from licorice (Glycyrrhiza glabra L.) by bulk liquid membrane. *Environmental Technology & Innovation*, *12*, 180-188.

Nybom, H., 2004. Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants. *Molecular ecology*, *13*(5), 1143-1155.

Nybom, H. and Bartish, I. V., 2000. Effects of life history traits and sampling strategies on genetic diversity estimates obtained with RAPD markers in plants. *Perspectives in Plant Ecology, Evolution and Systematics*, *3*(2), 93-114.

Oloumi, H. and Hassibi, N., 2011. Study the correlation between some climate parameters and the content of phenolic compounds in roots of *Glycyrrhiza glabra*. *Journal of Medicinal Plants and Research*, *5*(25), 6011-6016.

Omogbadegun, Z., Uwadia, C., Ayo, C., Mbarika, V., Omoregbe, N., Otofia, E. and Chieze, F., 2011. Multimedia-based medicinal plants sustainability management system. *International Journal of Computer Science*, *8*(5), 492-502.

Ong, E.S., 2004. Extraction methods and chemical standardization of botanicals and herbal preparations. *Journal of Chromatography B*, *812*(1-2), 23-33.

Pagare, S., Bhatia, M., Tripathi, N., Pagare, S. and Bansal, Y. K., 2015. Secondary metabolites of plants and their role: Overview. *Current Trends in Biotechnology and Pharmacy*, *9*(3), 293-304.

Palkar, R. S. and Sellappan, K., 2019. Genetic diversity between and within the natural populations of *Garcinia indica* (Thouars) Choisy: A high value medicinal plant from Northern Western Ghats of India using ISSR markers. *Journal of Applied Research on Medicinal and Aromatic Plants*, *15*, 100219.

Pan, X., Liu, H., Jia, G. and Shu, Y.Y., 2000. Microwave-assisted extraction of glycyrrhizic acid from licorice root. *Biochemical Engineering Journal*, *5*(3), 173-177.

Panda, S., Naik, D. and Kamble, A., 2015. Population structure and genetic diversity of the perennial medicinal shrub *Plumbago*. *Annals of Botany Plants*, *7*, 1-7.

Pandey, A. and Tripathi, S., 2014. Concept of standardization, extraction and pre phytochemical screening strategies for herbal drug. *Journal of Pharmacognosy and Phytochemistry*, *2*(5), 115-119.

Pandey, D. K. and Ayangla, N.W., 2018. Biotechnological aspects of the production of natural sweetener glycyrrhizin from *Glycyrrhiza* sp. *Phytochemistry Reviews*, *17*(2), 397-430.

Pandey, D. K. and Kaur, P., 2018. Optimization of extraction parameters of pentacyclic triterpenoids from *Swertia chirata* stem using response surface methodology. *3 Biotech*, *8*(3), 152.

Parvaiz, M., Hussain, K., Khalid, S., Hussnain, N., Iram, N., Hussain, Z. and Ali, M. A., 2014. A review: Medicinal importance of *Glycyrrhiza glabra* L.(Fabaceae family). *Global Journal of Pharmacology*, *8*(1), 8-13.

Pastorino, G., Cornara, L., Soares, S., Rodrigues, F. and Oliveira, M.B.P., 2018. Liquorice (*Glycyrrhiza glabra*): A phytochemical and pharmacological review. *Phytotherapy research*, *32*(12), 2323-2339.

Patel, H. K., Fougat, R. S., Kumar, S., Mistry, J. G. and Kumar, M., 2015. Detection of genetic variation in *Ocimum* species using RAPD and ISSR markers. *3 Biotech*, *5*(5), 697-707.

Pathak, H. and Dhawan, V., 2012. ISSR assay for ascertaining genetic fidelity of micropropagated plants of apple rootstock Merton. *In Vitro Cellular & Developmental Biology - Plants*, *793*(48), 137–143.

Peakall, R. O. D., and Smouse, P.E., 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, *6*(1), 288-295.

Pence, V. C., Ballesteros, D., Walters, C., Reed, B. M., Philpott, M., Dixon, K. W., Pritchard, H. W., Culley, T. M. and Vanhove, A.C., 2020. Cryobiotechnologies: Tools for expanding long-term ex situ conservation to all plant species. *Biological Conservation*, *250*, 108736.

Petramfar, P., Hajari, F., Yousefi, G., Azadi, S. and Hamedi, A., 2020. Efficacy of oral administration of licorice as an adjunct therapy on improving the symptoms of patients with Parkinson's disease, A randomized double blinded clinical trial. *Journal of Ethnopharmacology*, *247*, 112226.

Polyák, É., Gombos, K., Hajnal, B., Bonyár-Müller, K., Szabó, S., Gubicskó-Kisbenedek, A., Marton, K. and Ember, I., 2010. Effects of artificial sweeteners on body weight, food and drink intake. *Acta Physiologica Hungarica*, *97*(4), 401-407.

Potawale, S. E., Gabhe, S. Y. and Mahadik, K. R., 2013. HPTLC Method development and validation for densitometric analysis of 18 β-Glycyrrhetinic acid and β-Sitosterol in polyherbal drug formulation. *International Journal of Pharmacy and Pharmaceutical Sciences*, *5*(1), 278-282.

Powell, W., Morgante, M., Andre, C., Hanafey, M., Vogel, J., Tingey, S. and Rafalski, A., 1996. The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. *Molecular Breeding*, *2*, 225-238.

Pradeep Reddy, M., Sarla, N. and Siddiq, E.A., 2002. Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding. *Euphytica*, *128*, 9-17.

Pratama, M. R. F. and Gusdinar, T., 2019. Docking study of secondary metabolites from *Glycyrrhiza glabra* as PPAR-γ agonist. *Biointerface Research in Applied Chemistry*, *9*(4).

Prevost, A. and Wilkinson, M.J., 1999. A new system of comparing PCR primers applied to ISSR fingerprinting of potato cultivars. *Theoretical and Applied Genetics*, *98*, 107-112.

Pritchard, J. K., Stephens, M. and Donnelly, P., 2000. Inference of population structure using multilocus genotype data. *Genetics*, *155*(2), 945-959.

Priya, K., Gupta, V.R.M. and Srikanth, K., 2011. Natural sweeteners: A complete review. *Journal of Pharmacy Research*, *4*(7), 2034-2039.

Radušienė, J., Karpavičienė, B. and Stanius, Ž., 2012. Effect of external and internal factors on secondary metabolites accumulation in St. John's worth. *Botanica Lithuanica*, *18*(2), 101-108.

Raja, M.S., Khan, I. and Perumal, P., 2010. Quantitative analysis of glycyrrhizic acid in crude drug and its herbal formulation by HPLC. *Asian Journal of Research in Chemistry*, *3*(3), 703-706.

Ramak, P., Sharifi, M., Osaloo, S. K., Ebrahimzadeh, H. and Behmanesh, M., 2011. Studies on seed germination and in vitro shoot multiplication of *Satureja khuzistanica* Jamzad, an important medicinal plant. *African Journal of Biotechnology*, *10*(83), 19407-19414.

Rameshkumar, R., Pandian, S., Rathinapriya, P., Selvi, C. T., Satish, L., Gowrishankar, S., Leung, D.W. and Ramesh, M., 2019. Genetic diversity and phylogenetic relationship of *Nilgirianthus ciliatus* populations using ISSR and RAPD markers: Implications for conservation of an endemic and vulnerable medicinal plant. *Biocatalysis and Agricultural Biotechnology*, *18*, 101072.

Rani, R., Dahiya, S., Dhingra, D., Dilbaghi, N., Kim, K.H. and Kumar, S., 2017. Evaluation of anti-diabetic activity of glycyrrhizin-loaded nanoparticles in nicotinamide-streptozotocin-induced diabetic rats. *European Journal of Pharmaceutical Sciences*, *106*, 220-230.

Rao K.V.S., 1993. A review on licorice. *Ancient science of life*, *13*(1-2), 57.

Rezaei, S., Nejadsattari, T., Assadi, M., Khavarinejad, R.A. and Mehregan, I., 2017. Study of glycyrrhizic acid contents from *Glycyrrhiza glabra* populations in Iran and their relation with environmental factors. *Biodiversitas Journal of Biological Diversity*, *18*(1).

Rhodes, M. J. C., 1994. Physiological roles for secondary metabolites in plants: some progress, many outstanding problems. *Plant molecular biology*, *24*(1), 1-20.

Riaz, U., Iqbal, S., Sohail, M.I., Samreen, T., Ashraf, M., Akmal, F., Siddiqui, A., Ahmad, I., Naveed, M., Khan, N.I. and Akhter, R.M., 2021. A comprehensive review on emerging

importance and economical potential of medicinal and aromatic plants (MAPs) in current scenario. *Pakistan Journal of Agricultural Research*, *34*(2), 381-392.

Rout, G.R., Mohapatra, A. and Jain, S.M., 2006. Tissue culture of ornamental pot plant: A critical review on present scenario and future prospects. *Biotechnology advances*, *24*(6), 531-560.

Sabbioni, C., Ferranti, A., Bugamelli, F., Forti, G.C. and Raggi, M.A., 2006. Simultaneous HPLC analysis, with isocratic elution, of glycyrrhizin and glycyrrhetic acid in liquorice roots and confectionery products. *Phytochemical Analysis: An International Journal of Plant Chemical and Biochemical Techniques*, *17*(1), 25-31.

Salunke, C., Chittam, K., Pawar, A. and Chaudhari, H., 2020. Development of an Analytical Method to Determine Extracts of Different Species of Glycyrrhizin by HPTLC. *Research Journal of Pharmaceutical Dosage Forms and Technology*, *12*(2), 89-92.

Santelli, R.E., Oliveira, E.P., Villar, L.S. and Escaleira, L.A., 2008. Response surface methodology (RSM) as a tool for optimization in analytical chemistry. *Talanta*, *76*, 965-977.

Sarkar, M. and Roy, S.C., 2015. Influences of medium parameters on somatic embryogenesis from leaf explants of *Glycyrrhiza glabra*. *Indian Journal of Scientific Research*, *10*(1), 63-67.

Sarwat, M., Das, S. and Srivastava, P.S., 2008. Analysis of genetic diversity through AFLP, SAMPL, ISSR and RAPD markers in *Tribulus terrestris*, a medicinal herb. *Plant Cell Reports*, *27*, 519-528.

Savitikadi, P., Jogam, P., Rohela, G.K., Ellendula, R., Sandhya, D., Allini, V.R. and Abbagani, S., 2020. Direct regeneration and genetic fidelity analysis of regenerated plants of *Andrographis echioides* (L.) - An important medicinal plant. *Industrial Crops and Products*, *155*, 112766.

Sawaengsak, W., Saisavoey, T., Chuntaratin, P. and Karnchanatat, A., 2011. Micropropagation of the medicinal herb *Glycyrrhiza glabra* L., through shoot tip explant culture and glycyrrhizin detection. *International Research Journal of Plant Science*, *2*(5), 129-136.

Schippmann, U., Leaman, D.J. and Cunningham, A.B., 2002. Impact of cultivation and gathering of medicinal plants on biodiversity: Global trends and issues. *Biodiversity and the ecosystem approach in agriculture, forestry and fisheries*.

Schippmann, U.W.E., Leaman, D. and Cunningham, A.B., 2006. A comparison of cultivation and wild collection of medicinal and aromatic plants under sustainability aspects. *Frontis*, 75-95.

Schlag, E.M. and McIntosh, M.S., 2012. RAPD-based assessment of genetic relationships among and within American ginseng (*Panax quinquefolius* L.) populations and their implications for a future conservation strategy. *Genetic Resources and Crop Evolution*, *59*, 1553-1568.

Selyutina, I. Y., Konichenko, E. S., Zibzeev, E. G. and Kuban, I. N., 2021. Genetic diversity of the endangered endemic species *Hedysarum sangilense Krasnoborov* ET Timokhina (Fabaceae). *Botanica Pacifica: A Journal of Plant Science and Conservation*, *10*(1), 29-35.

Sen, S., Chakraborty, R. and De, B., 2011. Challenges and opportunities in the advancement of herbal medicine: India's position and role in a global context. *Journal of Herbal medicine*, *1*(3-4), 67-75.

Seth, S. and Panigrahi, J., 2019. In vitro organogenesis of *Abutilon indicum* (L.) Sweet from leaf derived callus and assessment of genetic fidelity using ISSR markers. *The Journal of Horticultural Science and Biotechnology*, *94*(1), 70-79.

Sevindik, E., Paksoy, M.Y., Topseçer, F. and Aydoğan, M., 2019. Phylogenetic Analysis of Chrysochamela (Fenzl) Boiss. (Brassicaceae) Taxa Growing in Turkey. *Turkish Journal of Agriculture-Food Science and Technology*, *7*(12), 2129-2132.

Shabkhiz, M.A., Eikani, M.H., Sadr, Z.B. and Golmohammad, F., 2016. Superheated water extraction of glycyrrhizic acid from licorice root. *Food Chemistry*, *210*, 396-401.

Shaheen, A., Ali, M., Ahmad, N., Hassan Dewir, Y., El-Hendawy, S., Gawad, A.E. and Ahmed, M., 2020. Micropropagation of licorice (*Glycyrrhiza glabra* L.) by using intermediate nodal explants. *Chilean Journal of Agricultural Research*, *80*(3), 326-333.

Shams, K.A., Abdel-Azim, N.S., Kamal, S.A., Husseiny, H.A. and Hammouda, F.M., 2015. Green extraction: enhanced extraction yield of glycyrrhizic acid from *Glycyrrhiza glabra* L. *Journal of Chemical and Pharmaceutical Research*, *7*(4), 725-728.

Shams-Ardakani, M., Mohagheghzadeh, A., Ghannadi, A. and Barati, A., 2007. Formation of glycyrrhizin by *in vitro* cultures of *Glycyrrhiza glabra*. *Chemistry of Natural Compounds*, *43*(3), 353-354.

Sharma, P. R. A. G. Y. A., Tiwari, G., Tripathi, M. K., Mishra, S. N., Baghel, B. S. and Tiwari, S., 2008. Morphogenesis and plantlet regeneration from leaf disc explants of liquorice (*Glycyrrhiza glabra* L.). *Plant Cell Biotechnology &. Molecular Biol*ogy, *9*, 117-126.

Sharma, P., Tiwari, G., Tripathi, M.K., Mishra, S.N., Baghel, B.S. and Tiwari, S., 2008. Morphogenesis and plantlet regeneration from leaf disc explants of liquorice (*Glycyrrhiza glabra* L.). *Plant Cell Biotechnology and Molecular Biology*, *9*, 117-126.

Sharma, P., Tripathi, M.K., Tiwari, G., Tiwari, S. and Baghel, B.S., 2010. Regeneration of liquorice (*Glycyrrhiza glabra* L.) from cultured nodal segments. *Indian Journal of Plant Physiology*, *15*(1), 1.

Sharma, S., Rathi, N., Kamal, B., Pundir, D., Kaur, B. and Arya, S., 2010. Conservation of biodiversity of highly important medicinal plants of India through tissue culture technology-a review. *Agriculture and Biology Journal of North America*, *1*(5), 827-833.

Sharma, V.A.R.S.H.A. and Agrawal, R.C., 2013. *Glycyrrhiza glabra*-A plant for the future. *Mintage Journal of Pharmaceutical and Medical Sciences*, *2*(3), 15-20.

Sheikhi, S., Khamesipour, A., Radjabian, T., Ghazanfari, T. and Miramin Mohammadi, A., 2022. Immunotherapeutic effects of Glycyrrhiza glabra and Glycyrrhizic Acid on Leishmania major-infection BALB/C mice. *Parasite Immunology*, *44*(1-2), 12879.

Shetty, K., 1997. Biotechnology to harness the benefits of dietary phenolics; Focus on Lamiaceae. *Asia Pacific Journal of Clinical Nutrition*, *6*, 162-171.

Shilpha, J., Silambarasan, T., Karutha Pandian, S. and Ramesh, M., 2013. Assessment of genetic diversity in *Solanum trilobatum* L., an important medicinal plant from South India using RAPD and ISSR markers. *Genetic Resources and Crop Evolution*, *60*, 807-818.

Siddiqui, N.A., Alam, P., Parvez, M.K., Basudan, O.A., Al-Dosari, M.S., Al-Rehaily, A.J., Al-Ajmi, M.F. and Shakeel, F., 2015. Quantification of glycyrrhizin in anti-stress herbal formulations by validate HPTLC method: A rational paradigm towards quality control of herbals. *Pakistan Journal of Pharmaceutical Sciences*, *28*(1), 353-357.

Simić, V. M., Rajković, K. M., Stojičević, S. S., Veličković, D. T., Nikolić, N. Č., Lazić, M. L. and Karabegović, I. T., 2016. Optimization of microwave-assisted extraction of total polyphenolic compounds from chokeberries by response surface methodology and artificial neural network. *Separation and Purification Technology*, *160*, 89-97.

Sorkheh, K., Zolfaghari, M. and Ercisli, S., 2019. Reliability authentication of *Glycyrrhiza glabra* L. populations from south Iran using SSR and SNP-based markers. *Proceedings of the National Academy of Sciences, India Section B: Biological Sciences*, *89*, 1283-1294.

Souza, S. G. H. D., Carpentieri-Pípolo, V., Ruas, C. D. F., Carvalho, V. D. P., Ruas, P. M. and Gerage, A. C., 2008. Comparative analysis of genetic diversity among the maize inbred lines (*Zea mays* L.) obtained by RAPD and SSR markers. *Brazilian Archives of Biology & Technology*, *51*, 183-192.

Srivastava, V., Yadav, A. and Sarkar, P., 2020. Molecular docking and ADMET study of bioactive compounds of *Glycyrrhiza glabra* against main protease of SARS-CoV2. *Materials Today: Proceedings*.

Starmans, D.A. and Nijhuis, H.H., 1996. Extraction of secondary metabolites from plant material: a review. *Trends in Food Science & Technology*, *7*(6), 191-197.

Subositi, D., Kurnianingrum, N., Mujahid, R., and Widiyastuti, Y., 2020. *Kaempferia galanga* L. a medicinal plant used by Indonesian ethnic groups: genetic diversity based on inter-simple sequence repeats (ISSR). *AGRIVITA, Journal of Agricultural Science*, *42*(1), 45-52.

Subramanyam, K., Muralidhararao, D. and Devanna, N., 2009. Genetic diversity assessment of wild and cultivated varieties of J*atropha curcas* (L.) in India by RAPD analysis. *African Journal of Biotechnology*, *8*(9).

Susila, T., Reddy, G.S. and Jyothsna, D., 2013. Standardization of protocol for in vitro propagation of an endangered medicinal plant *Rauwolfia serpentina* Benth. *Journal of Medicinal Plants and Research*, *7*(29), 2150-2153.

Suyal, R., Jugran, A. K., Bhatt, I. D. and Rawal, R. S., 2022. Assessment of genetic diversity, population structure and phytochemical variations in *Polygonatum cirrhifolium* (Wall.) Royle: an endangered medicinal herb. *Genetic Resources and Crop Evolution*, 1-15.

Takahara, T., Watanabe, A. and Shiraki, K., 1994. Effects of glycyrrhizin on hepatitis B surface antigen: A biochemical and morphological study. *Journal of Hepatology*, *21*(4), 601-609.

Talebpour, Z., Ghassempour, A., Abbaci, M. and Aboul-Enein, H.Y., 2009. Optimization of microwave-assisted extraction for the determination of glycyrrhizin in menthazin herbal drug by experimental design methodology. *Chromatographia*, *70*, 191-197.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, *30*(12), 2725-2729.

Tan, S.P., Kha, T.C., Parks, S., Stathopoulos, C. and Roach, P.D., 2015. Optimising the encapsulation of an aqueous bitter melon extract by spray-drying. *Foods*, *4*(3), 400-419.

Taşcıoğlu, T., Sadıkoğlu, N., Doğanlar, S. and Frary, A., 2018. Molecular genetic diversity in the *Origanum* genus: EST-SSR and SRAP marker analyses of the 22 species in eight sections that naturally occur in Turkey. *Industrial Crops and Products*, *123*, 746-761.

Thengane, S.R., Kulkarni, D.K. and Krishnamurthy, K.V., 1998. Micropropagation of licorice (*Glycyrrhiza glabra* L.) through shoot tip and nodal cultures. *In Vitro Cellular & Developmental Biology-Plant*, *34*, 331-334.

Tian, M., Yan, H. and Row, K.H., 2008. Extraction of glycyrrhizic acid and glabridin from licorice. *International Journal of Molecular Sciences*, *9*(4), 571-577.

Tianwei, T., Qing, H. and Qiang, L., 2002. Purification of glycyrrhizin from *Glycyrrhiza uralensis* Fisch with ethanol/phosphate aqueous two phase system. *Biotechnology Letters*, *24*, 1417-1420.

Tiwari, G., Singh, R., Singh, N., Choudhury, D.R., Paliwal, R., Kumar, A. and Gupta, V., 2016. Study of arbitrarily amplified (RAPD and ISSR) and gene targeted (SCoT and CBDP) markers for genetic diversity and population structure in Kalmegh [*Andrographis paniculata* (Burm. f.) Nees]. *Industrial Crops and Products*, *86*, 1-11.

Tripathi, N., Chouhan, D. S., Saini, N. and Tiwari, S., 2012. Assessment of genetic variations among highly endangered medicinal plant *Bacopa monnieri* (L.) from Central India using RAPD and ISSR analysis. *3 Biotech*, *2*, 327-336.

Um, Y., Jin, M.L., Lee, Y., Hur, M., Cha, S.W., Jung, C.S., Kim, S.M. and Lee, J.H., 2016. Genetic diversity analysis of *Glycyrrhiza uralensis* using 8 novel polymorphic microsatellite markers. *Journal of Plant Biotechnology*, *43*(2), 174-180.

Van de Sand, L., Bormann, M., Alt, M., Schipper, L., Heilingloh, C.S., Steinmann, E., Todt, D., Dittmer, U., Elsner, C., Witzke, O. and Krawczyk, A., 2021. Glycyrrhizin effectively inhibits SARS-CoV-2 replication by inhibiting the viral main protease. *Viruses*, *13*(4), 609.

Varsha, S., Agrawal, R.C. and Sonam, P., 2013. Phytochemical screening and determination of anti-bacterial and anti-oxidant potential of *Glycyrrhiza glabra* root extracts. *Journal of Environmental Research and Development*, *7*(4A), 1552.

Vasanth, M. P., Purushotham, K. G., Sathish, M. and Venkatesh, M., 2020. In-vitro anti-inflammatory activity of liquorice (*Glycyrrhiza glabra*) using aqueous extract. *International Journal of Research in Pharmaceutical Sciences*, *11*(1), 657-662.

Vasconsuelo, A. and Boland, R., 2007. Molecular aspects of the early stages of elicitation of secondary metabolites in plants. *Plant Science*, *172*(5), 861-875.

Vemula, S., Koppula, T., Jogam, P. and Mohammed, M., 2020. In vitro high frequency multiplication and assessment of genetic fidelity of *Corallocarpus epigaeus*: an endangered medicinal plant. *Vegetos*, *33*, 63-73.

Verma, P., Mathur, A.K., Jain, S.P. and Mathur, A., 2012. *In vitro* conservation of twenty-three overexploited medicinal plants belonging to the Indian sub-continent. *The Scientific World Journal*.

Verma, S. and Singh, S.P., 2008. Current and future status of herbal medicines. *Veterinary world*, *1*(11), 347.

Virk, P.S., Zhu, J., Newbury, H.J., Bryan, G.J., Jackson, M.T. and Ford-Lloyd, B.V., 2000. Effectiveness of different classes of molecular marker for classifying and revealing variation in rice (*Oryza sativa*) germplasm. *Euphytica*, *112*, 275-284.

Von Arnold, S., Sabala, I., Bozhkov, P., Dyachok, J. and Filonova, L., 2002. Developmental pathways of somatic embryogenesis. *Plant cell, Tissue and Organ culture*, *69*, 233-249.

Wang, J., Gao, W., Zhang, L. and Huang, L., 2013. Establishment and quality assessment of tissue cultures in *Glycyrrhiza uralensis* Fisch. *Applied Biochemistry & Biotechnology*, *169*, 588–594.

Wang, Q.E., Ma, S., Fu, B., Lee, F.S. and Wang, X., 2004. Development of multi-stage countercurrent extraction technology for the extraction of glycyrrhizic acid (GA) from licorice (*Glycyrrhiza uralensis* Fisch). *Biochemical engineering journal*, *21*(3), 285-292.

Wang, Y.C. and Yang, Y.S., 2007. Simultaneous quantification of flavonoids and triterpenoids in licorice using HPLC. *Journal of Chromatography B*, *850*(1-2), 392-399.

Wei, L., Zhang, W., Yin, L., Yan, F., Xu, Y. and Chen, F., 2015. Extraction optimization of total triterpenoids from *Jatropha curcas* leaves using response surface methodology and evaluations of their antimicrobial and antioxidant capacities. *Electronic Journal of Biotechnology*, *18*(2), 88-95.

Wesolowski, M. and Suchacz, B., 2012. Artificial neural networks: theoretical background and pharmaceutical applications: A review. *Journal of AOAC International*, *95*(3), 652-668.

Wongwicha, W., Tanaka, H., Shoyama, Y., Tuvshintogtokh, I. and Putalun, W., 2008. Production of glycyrrhizin in callus cultures of licorice. *Zeitschrift für Naturforschung C*, *63*(5-6), 413-417.

Xie, J., Zhang, Y. and Wang, W., 2010. HPLC analysis of glycyrrhizin and licochalcone a in *Glycyrrhiza inflata* from Xinjiang (China). *Chemistry of Natural Compounds*, *46*, 148-151.

Xu, W., Chu, K., Li, H., Zhang, Y., Zheng, H., Chen, R. and Chen, L., 2012. Ionic liquid-based microwave-assisted extraction of flavonoids from *Bauhinia championii* (Benth.) Benth. *Molecules*, *17*(12), 14323-14335.

Yadav, A.R., Dewal, M.L., Anand, R.S. and Gupta, S., 2013. Classification of hardwood species using ANN classifier. In *2013 Fourth national conference on computer vision, pattern recognition, image processing and graphics*, 1-5.

Yadav, K. and Singh, N., 2012. Factors influencing in vitro plant regeneration of Liquorice (*Glycyrrhiza glabra* L.). *Iranian Journal of Biotechnology*, *10*(3), 161-167.

Yamazaki, M., Sato, A., Shimomura, K., Saito, K. and Murakoshi, I., 1994. Genetic relationships among *Glycyrrhiza* plants determined by RAPD and RFLP analyses. *Biological and Pharmaceutical Bulletin*, *17*(11), 1529-1531.

Yaneva, I., Balabanski, V., Karanesheva, T. and Ignatov, I., 2020. Some endangered healings plants in Bulgaria-legislative regulation, protection, characteristic description, application, agricultural cultivation. *Bulgarian Journal of Agricultural Science*, *26*(4), 847-852.

Yang, L., Chao, L., Su, X., Wang, C., Dong, C. and Chen, S., 2021. High-frequency in vitro plantlet regeneration in *Lilium davidii* var. unicolour Salisb, an important edible and medicinal plant, and confirmation of genetic fidelity of regeneration plantlets using ISSR markers. *Plant Biotechnology Reports*, *15*(4), 435-446.

Yang, R., Li, W., Yuan, B., Ren, G., Wang, L., Cheng, T. and Liu, Y., 2018. The genetic and chemical diversity in three original plants of licorice, *Glycyrriza uralensis* Fisch., *Glycyrrhiza inflata* Bat. and *Glycyrrhiza glabra* L. *Pakistan Journal of Pharmaceutical Sciences*, *31*(2).

Yao, H., Zhao, Y., Chen, D.F., Chen, J.K. and Zhou, T.S., 2008. ISSR primer screening and preliminary evaluation of genetic diversity in wild populations of *Gycyrrhiza uralensis*. *Biologia Plantarum*, *52*, 117-120.

Yeh, F.C., Yang, R.C. and Boyle, T., 1999. POPGENE version1. 32, Microsoft window-base software for population genetic analysis: a quick user's guide. University of Alberta. *Center for International Forestry Research, Alberta, Canada*.

Zakrzewski, P. A., 2002. Bioprospecting or biopiracy? the pharmaceutical industry's use of indigenous medicinal plants as a source of potential drug candidates. *University of Toronto Medical Journal*, *79*(3), 252-254.

Zalapa, J.E., Cuevas, H., Zhu, H., Steffan, S., Senalik, D., Zeldin, E., McCown, B., Harbut, R. and Simon, P., 2012. Using next-generation sequencing approaches to isolate simple sequence repeat (SSR) loci in the plant sciences. *American Journal of Botany*, *99*(2), 193-208.

Zhang, H.F., Yang, X.H. and Wang, Y., 2011. Microwave assisted extraction of secondary metabolites from plants: Current status and future directions. *Trends in Food Science & Technology*, *22*(12), 672-688.

Zhang, Z., Xu, M., Sun, X. and Guo, X., 2020. Naturally occurring glycyrrhizin triterpene exerts anticancer effects on colorectal cancer cells via induction of apoptosis and autophagy and suppression of cell migration and invasion by targeting MMP-9 and MMP-2 expression. *Journal of B.U. ON*, *25*(1), 188-193.

Zhang, Z.F., Wang, R., Ye, F., Wang, H. and Zhao, W., 2019. Extraction of glycyrrhizic acid by aqueous two-phase system formed by PEG and two environmentally friendly organic acid salts-sodium citrate and sodium tartrate. *Green Processing and Synthesis*, *8*(1), 551-556.

Zhao, Y., Li, W. and Zhang, D., 2021. Gycyrrhizic acid alleviates atherosclerotic lesions in rats with diabetes mellitus. *Molecular Medicine Reports*, *24*(5), 1-8.

PUBLICATIONS

- Pandey, D. K. and Ayangla, N. W., 2018. Biotechnological aspects of the production of natural sweetener glycyrrhizin from *Glycyrrhiza* sp. *Phytochemistry Reviews*, *17*(2), 397-430.

- Ayangla, N. W., Kumar, V., Gupta, R. C., Dey, A., Dwivedi, P. and Pandey, D. K., 2022. Response surface methodology and artificial neural network modelling for optimization of solid-liquid extraction and rapid HPTLC analysis of glycyrrhizin in *Glycyrrhiza glabra* root. *South African Journal of Botany*, *148*, 11-20.

- Ayangla, N. W., Dwivedi, P., Dey, A. and Pandey, D. K., 2022. *In vitro* propagation, genetic and phytochemical fidelity in *Glycyrrhiza glabra* L., a potent glycyrrhizin yielding endangered plant. *The Nucleus*, *65*(2) 1-9.

CONFERENCES

National Symposium on Plant Biotechnology: Current Perspectives on Medicinal and Crop Plants & 38th Annual Meeting of Plant Tissue Culture Association (India).

Held from 3rd-5th March, 2017

At CSIR-Indian Institute of Chemical Biology, Kolkata.

International Conference on Global Environmental Challenges Human Health and Sustainable Development.

Held from 11th-13th January, 2019

At Jawaharlal National University, New Delhi.