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DEVELOPMENT OF HIGH-EFFICIENCY IN VITRO REGENERATION SYSTEM FOR RAPID CLONAL PROPAGATION OF *GLYCYRRHIZA GLABRA L.*

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ABSTRACT
Glycyrrhiza glabra L. (licorice) is a high-value medicinal plant prized for its major bioactive triterpenoid saponin, glycyrrhizin. However, commercial exploitation is severely bottlenecked by low seed viability, prolonged root maturation, and destructive wild harvesting. This study established an efficient, highly reproducible *in vitro* regeneration protocol for rapid clonal propagation using nodal explants and shoot tips. Surface-sterilised explants were cultured on Murashige and Skoog (MS) medium supplemented with different concentrations and combinations of cytokinins and auxins. Maximum shoot induction frequency (66%) and superior shoot length (7.50±0.12 cm) were achieved on MS medium supplemented with 2.0 mg L^{-1} 6-benzylaminopurine (BAP) and 1.5 mg L^{-1} indole-3-butyric acid (IBA). Multiple shoot proliferation reached its peak (75% multiplication frequency) on MS medium containing 2.0 mg L^{-1} BAP and 1.5 mg L^{-1} (KN). Optimal adventitious rooting (100% response) was induced on half-strength MS medium fortified with 1.5 mg L^{-1} α-naphthaleneacetic acid (NAA) and 1.0 mg L^{-1} BAP. Fully rooted plantlets were successfully acclimatized *ex vitro* in a hardened substrate mix of cocopeat, garden soil, and sand (2:2:1 v/v), exhibiting high survival rates without phenotypic somaclonal variation. This high-frequency direct organogenesis protocol offers a robust technological platform for mass clonal multiplication, germplasm conservation, and sustainable phytopharmaceutical raw material supply.

KEYWORDS: *Glycyrrhiza glabra*, Micropropagation, Plant growth regulators, Nodal explants, Clonal propagation.

1. INTRODUCTION
Glycyrrhiza glabra L., commonly known as licorice, is an economically and therapeutically vital perennial herb in the family Fabaceae (Leguminosae) (He et al. 2023). The generic name Glycyrrhiza originates from the Greek words *glykos* (sweet) and *rhiza* (root) (Quattrocchi, 2012). These subterranean organs have formed a cornerstone of ethnopharmacological systems worldwide, maintaining a continuous record of therapeutic application in Traditional Chinese Medicine (TCM), Ayurveda, and classical European medicine for over four millennia.
The genus *Glycyrrhiza* comprises approximately 30 species, of which *G. glabra, G. uralensis* Fisch., *and G. inflata* are the principal medicinal species used worldwide. However, the global pharmaceutical marketplace and large-scale supply chains are heavily dominated by a core triad (Batalin; Li et al., 2022). Within this context, precise morphological profiling and definitive species authentication remain critical tenets of contemporary herbal quality control (Zhao et al. 2022; Avula et al. 2022). Because wild populations face escalating overexploitation, the unintentional or fraudulent substitution of authentic *G. glabra* raw material with morphologically similar but chemically distinct, or potentially toxic, wild congeners poses a severe threat to consumer safety (Ashurmetov et al. 2023). Such adulteration introduces significant clinical discrepancies, altering the bioavailability of core bioactive triterpenoid saponins and flavonoids, underscoring the urgent need for standardized propagation and verification frameworks (Chen et al. 2022; Wang et al. 2022).
Although several micropropagation protocols for *G. glabra* have been documented in the literature, most conventional systems are often bottlenecked by high rates of microbial contamination, severe tissue necrosis due to phenolic browning, low *ex vitro* acclimatization survival rates, and the lack of reliable long-term germplasm conservation frameworks (Sishu et al. 2024; Müller et al. 2025; El-Dawayati et al. 2022; Chmielarz et al. 2023; Naaz et al. 2023; Beruto et al. 2024; Ruta et al. 2021; Verma et al. 2022). To overcome these commercial limitations, the present study introduces a highly optimized, cost-effective, and rapid direct organogenesis pipeline utilising nodal explants (Xu et al. 2022; Ikeuchi et al. 2024). The novelty of this work lies in the strategic optimization of specific cytokinin-auxin ratios that could facilitate axillary bud break while simultaneously preventing hyperhydricity Werner and Schmülling 2023; Polivanova and Bedarev 2022; Schaller et al. 2021). Furthermore, this study bridges the gap between large-scale propagation and germplasm

protection by establishing a highly successful, six-month slow-growth conservation protocol under minimal growth kinetics (Ferdousi et al. 2022). This dual-action framework offers a practical approach to satisfy the escalating commercial demand for uniform, high-quality licorice raw materials while actively preventing the over-exploitation and extinction of natural *G. glabra* habitats (Wu et al. 2025).

Despite its well-documented pharmacological importance and escalating global demand, the large-scale production of *G. glabra* L. remains constrained by several biological and agronomic limitations. The fundamental bottleneck lies in the biological and logistical constraints inherent to conventional field cultivation, prolonged root maturation period, low and unpredictable seed germination rates (poor and inconsistent seed germination under uncontrolled field conditions), extreme vulnerability to soil-borne fungal pathogens during early vegetative establishment, and the near-total dependence of large-scale raw material supply on environmentally destructive wild-harvesting operations concentrated across the fragile semi-arid ecosystems of Central Asia, the Mediterranean basin, and the Indian subcontinent (United Nations Environment Programme 2023). These compounding constraints have created a widening structural gap between raw material supply and the sharply accelerating global demand driven by the pharmaceutical, nutraceutical, cosmeceutical, and functional food industries (Future Market Insights 2024). Plant tissue culture and *in-vioffers*icropropagation offers a scientifically validated and industrially scalable solution to this crisis (Kulus et al. 2023). By harnessing the totipotent regenerative capacity of meristematic nodal tissues under precisely controlled aseptic and phytohormonal environments, it is possible to produce thousands of genetically uniform, pathogen-free *G. glabra* plantlets within a fraction of the time required by conventional seed-based or vegetative propagation systems (Mishra et al. 2023). The present investigation is therefore specifically designed to address these critical production gaps by optimizing a complete, end-to-end *in vitro* regeneration protocol from surface sterilization and explant selection through phytohormone-mediated shoot organogenesis, adventitious rhizogenesis, and successful *ex vitro* acclimatization, thereby establishing a reproducible and commercially viable micropropagation framework for the sustainable large-scale production and long-term germplasm conservation of this irreplaceable medicinal species.

Although several regeneration protocols have been reported for *G. glabra*, most studies focused primarily on shoot induction and rooting, with limited emphasis on minimizing contamination and phenolic browning, optimizing rapid direct organogenesis from nodal ex vitro plants, establishing reproducible *ex vitro* acclimatization, and integrating medium-term germplasm conservation into a single protocol. Therefore, a reproducible regeneration system suitable for conservation and large-scale propagation is still required.

1.1 Active compounds in Glycyrrhiza glabra

The secondary metabolome of *G. glabra* L. is characterized by a complex array of bioactive principles that dictate its multifaceted therapeutic efficacy (Khan et al. 2023). The phytochemical profile of *G. glabra* is dominated by triterpenoid saponins and diverse flavonoids, including glycyrrhizin, liquiritin, liquiritigenin, isoliquiritigenin, glabridin, glabrene, and licichalcones, which occur in trace quantities to provide mild supportive metabolic modulation; the chief pharmacological marker is glycyrrhizin (glycyrrhizic acid) (Bakr et al. 2022; Zhang et al. 2024). This major triterpenoid saponin constitutes 2.0% to 25.0% of the root's dry biomass and exhibits a relative sweetness index roughly 50-fold greater than sucrose (Pastorino et al. 2018). Upon oral administration (or ingestion), glycyrrhizin is hydrolysed by intestinal microbiota to glycyrrhetinic acid, the principal bioactive metabolite (Nascimento and Araújo 2022). In parallel, the dense internal yellow pigmentation characteristics of the rhizospheric tissues are imparted by an abundant fraction of polyphenols, principally flavonoids, isoflavones, and chalcones, including liquiritin, isoliquirtin, and the highly prized phytoestrogen Glabridin (Nascimento and Araújo 2022).

Functionally, these phytochemical constituents exhibit potent cytoprotective effects in the gastrointestinal tract (Singh et al. 2022). By forming a protective barrier across the gastric mucosal lining, they mitigate irritation triggered by endogenous HCl and alleviate peptic ulceration with greater physiological compatibility than conventional chemical antacids (Shipa et al. 2022). Structurally, the pentacyclic triterpenoid saponin glycyrrhizin displays significant homology with endogenous adrenal steroids. Its active metabolite glycyrrhetinic acid predominantly inhibits 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), with weaker effects on 11β-HSD1, thereby increasing local glucocorticoid activity (Feng et al. 2015). In addition, isolated hydrophobic flavonoids like Glabridin demonstrate exceptional free-radical scavenging profiles and broad-spectrum antimicrobial actions; its lipid peroxidation quenching capacity is documented to be significantly superior to that of α-tocopherol (vitamin E), making it a premium target for natural preservation and therapeutic bioprospecting.

1.2. Glycyrrhizin content in G. glabra

Glycyrrhizin (glycyrrhizic acid) is the principal bioactive compound, a triterpenoid saponin of *G. glabra* L., and serves as the primary pharmacological and quantity marker governing its pharmaceutical and industrial value. It is biosynthesized predominantly within the cortical and phloem tissues of the root and rhizome through the mevalonate pathway, where it accumulates predominantly in roots and rhizomes as glycyrrhizin, a highly water-soluble triterpenoid saponin. The glycyrrhizin content in commercially harvested *G. glabra* roots varies considerably, typically ranging from approximately 2-9% 2.0% dry weight, although higher values have occasionally been reported depending on species, genotype, and cultivation conditions (Huang et al. 2022). Commercial cultivation generally recommends harvesting mature roots after several years to maximize glycyrrhizin accumulation, while pharmacopoeias specify minimum glycyrrhizic acid content for quality control, including those of the European Pharmacopoeia and the United States Pharmacopoeia. Upon ingestion, the intestinal microbial communities, particularly intestinal microbiota, hydrolyse glycyrrhizin to 18*β*-glycyrrhetinic acid as the biologically active aglycone metabolite responsible for most of the compound's systemic pharmacological actions.

This biotransformation step is critical because intact glycyrrhizin has limited membrane permeability, whereas glycyrrhetinic acid is rapidly absorbed through intestinal epithelia and achieves effective systemic bioavailability. Accurate quantification of glycyrrhizin content in the plant material and commercial extracts is routinely performed using High-Performance Liquid Chromatography (HPLC), particularly HPLC-UV methods described in international pharmacopoeias, and remains the standard analytical technique for glycyrrhizin quantification (Kamata et al. 2023).

The bioactive architecture of *G. glabra* L. serves as a critical dual paradigm, bridging traditional ethnopharmacological systems with contemporary cosmetic, food, and pharmaceutical manufacturing pipelines. Mechanistically, the primary triterpenoid saponin, glycyrrhizin, operates as a potent demulcent and expectorant (Jahan et al. 2023). It exhibits demulcent and expectorant properties that promote mucus secretion and facilitate mucociliary clearance, thereby helping to relieve cough and throat irritation (Morice et al. 2022). Beyond respiratory care, these secondary metabolites exhibit targeted gastroprotective properties; they promote local mucosal prostaglandin synthesis to create an acid-resistant cytoprotective barrier, thereby contributing to the prevention and healing of gastric ulcers through enhancement of mucosal defence mechanisms (Sharma and Agarwal 2023).

Concurrently, the hydrophobic polyphenolic fractions of *G.* glabra demonstrate profound antimicrobial efficacy (Chen et al. 2024). Crude extracts and isolated components exhibit specialised bactericidal and bacteriostatic dynamics against key Gram-positive and Gram-negative pathogenic baselines. Crude extracts and isolated phytochemicals exhibit bacteriostatic and bactericidal activities against a broad spectrum of Gram-positive and Gram-negative bacteria through multiple mechanisms, including disruption of membrane integrity and inhibition of microbial growth (Saroj et al. 2023; Sultana et al. 2024). In the realm of neuroprotection, these molecules act as powerful endogenous free-radical scavengers. These phytochemicals exhibit potent antioxidant activity by scavenging reactive oxygen species (ROS), reducing oxidative stress, and modulating antioxidant signalling pathways, thereby contributing to cellular protection (Zhang et al. 2023; Qiu et al. 2024). Within modern dermatology and demo-cosmetics industries, the prenylated isoflavonoid Glabridin has emerged as a premium bioactive ingredient for hyperpigmentation therapies (Hassan et al. 2023). Glabridin targets melanogenesis by downstream competitive inhibition of tyrosinase enzymes. Glabridin suppresses melanogenesis primarily through tyrosinase inhibition and has shown promising efficacy with a favourable safety profile in cosmetic formulations (Chang et al. 2023). Also, *G. glabra* derivatives manifest robust hepatoprotective capabilities. By downregulating pro-inflammatory cytokine cascades and maintaining cellular glutathione (GSH) reserves, these phytochemicals effectively mitigate chemical-induced hepatic injuries and have attracted considerable scientific interest owing to their broad-spectrum antiviral and immunomodulatory activities. Beyond hepatoprotection, bioactive constituents of *G. glabra* L. have demonstrated remarkable antiviral and immunomodulatory properties that have gained significant renewed scientific attention have gained significant renewed scientific attention in the post-pandemic era (Katiyar et al. 2021; Fiore et al. 2022). Glycyrrhizin has been extensively documented to exert a broad-spectrum antiviral activity against a diverse range of enveloped and non-enveloped viruses, including Influenza A, Herpes Simplex Virus (HSV-1 and HSV-2), Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV), and Hepatitis C Virus (HCV). The antiviral activity has been attributed to multiple mechanisms, including inhibition of viral attachment, membrane fusion, replication, and modulation of host immune responses. Furthermore, isolated flavonoid fractions, particularly liquiritigenin and isoliquiritigenin, function as selective modulators of T-lymphocyte proliferation and cytokine secretion profiles and have demonstrated immunomodulatory effects by regulating T-lymphocyte activity and reducing the production of pro-inflammatory cytokines, including IL-6, TNF-α, and IL-1β, suggesting potential therapeutic value in inflammatory disorders (Bordbar et al. 2022).

1.3. Synthetic Chemical Alternatives in comparison with Glycyrrhizin:

A critical evaluation of contemporary medicine highlights the therapeutic divergence between synthetic chemical agents and the natural triterpenoid saponin, glycyrrhizin, across several pathological indications. In the treatment of chronic inflammatory disorders, synthetic glucocorticoids such as prednisolone and hydrocortisone bind directly to cytosolic glucocorticoid receptors to exert potential anti-inflammatory and immunosuppressive effects; prolonged systemic therapy may increase gastrointestinal adverse effects, particularly when combined with NSAIDs (Wilson and Webb 2022; Liu et al. 2022; Freedberg et al. 2022). Conversely, glycyrrhizin modulates the endocrine axis indirectly; it acts as a competitive inhibitor of 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), the enzyme responsible for converting active cortisol into its inactive metabolite, cortisone. By extending the biological half-life of endogenous cortisol, glycyrrhizin prolongs endogenous cortisol activity through inhibition of 11β-HSD2, thereby contributing to anti-inflammatory effects. Additionally, glycyrrhizin has been reported to exhibit gastroprotective properties through stimulation of mucus secretion and enhancement of mucosal defense mechanisms; it exerts an independent gastroprotective effect by stimulating local mucus synthesis, thereby mitigating the risk of mucosal ulceration.

A simple paradigm is observed in antitussive therapies. Conventional synthetic antitussives, such as dextromethorphan and codeine phosphate, function as central nervous system (CNS) depressants that chemically suppress the medullary cough centre in the brainstem. These central-acting agents frequently induce somnolence, gastrointestinal hypomotility, and carry a pronounced risk of chemical dependency and tolerance. In contrast, bioactive fractions of G. glabra alleviate upper respiratory tract irritation via peripheral mechanisms. Glycyrrhizin acts as a physical demulcent, forming a protective, soothing film over the inflamed pharyngeal mucosa while upregulating tracheobronchial mucociliary clearance without inducing neurological deficits or central respiratory depression (Ganesan et al. 2022; Healthcare 2023).

In the management of peptic ulcer disease, proton pump inhibitors (PPIs) such as omeprazole induce a profound, unmitigated reduction in gastric acid secretion through irreversible inhibition of the parietal cell H+/K+-ATPase (Shin and Sachs 2022; Scarpignato et al. 2023). While highly effective in acid-related disorders, long-term PPI therapy has been

associated with disturbances in gastric physiology, alterations of the intestinal microbiota, impaired absorption of minerals including calcium (Ca2+) and magnesium (Mg2+), and other adverse effects requiring appropriate clinical monitoring (Imhann et al. 2023). In contrast, glycyrrhizin and its deglycyrrhizinated derivatives (DGL) do not primarily suppress physiological gastric acid secretion. Instead, they enhance gastric mucosal defense by stimulating mucus production, promoting epithelial repair, increasing prostaglandin-mediated cytoprotection, and facilitating mucosal healing, thereby providing gastroprotective effects while maintaining normal physiological gastric function.

1.4. Crisis of Glycyrrhiza glabra L. Biomass and its inefficiency

Despite the profound therapeutic and physiological efficacy of G. glabra L. compared with synthetic chemical alternatives, the industrial supply chain for this species faces a severe, compounding global biomass crisis. The vulnerability stems from a critical intersection of destructive harvesting methods, socioeconomic pressures in sourcing regions, and escalating market demand across the pharmaceutical, nutraceutical, cosmeceutical, and confectionery sectors.

Unlike primary agricultural commodities managed via standardized agronomic pipelines, the global licorice trade remains heavily dependent on wild-harvested populations indigenous to the arid and semi-arid steppes of Central Asia and the Middle East. Historically, rural populations practiced low-impact, manual root excavation, removing only the superficial rhizomatous tissue while leaving the deeper root architecture intact to facilitate vegetative regeneration. However, intensified commercial pressures have driven a shift toward destructive, mechanized uprooting. The deployment of heavy machinery turns over massive expanses of fragile topsoil, tearing out the entire subterranean root network (2.5-3.0 m depth) and leaving residual tissue fragments to desiccate and die, resulting in severe genetic erosion and localized population collapses (European Directorate for the Quality of Medicines & HealthCare 2023; Jha and Halder 2023).

The multi-year lifecycle creates an unsustainable lag phase that cannot keep pace with modern market expansion. This stark industrial bottleneck underlines the urgent necessity of developing advanced biotechnology alternatives, specifically. The optimization of rapid in-vitro micropropagation and long-term ex-situ germplasm conservation protocols to address this overexploitation triggers profound ecological consequences. G. glabra functions as a vital ecological "pioneer species" within fragile dryland ecosystems. Its extensive, interlocking root networks are essential for soil stabilization, protecting against desertification, wind-driven soil erosion, and macro-hydrological disturbances like flash flooding (Phillips and Garda 2019; George et al. 2008).

From a production standpoint, G. glabra exhibits extreme biological and logistical inefficiencies when compared to the rapid, high-throughput manufacturing turnaround achieved in synthetic chemistry laboratories. The operational timeline required for commercially viable biomass accumulation follows a highly constrained chronological trajectory. Like in years 1-2 (Vegetative establishment Phase), the initial root system development occurs with minimal secondary metabolite allocation. In years 3-5 (Phytochemical Maturation Window), the underground biomass must grow undisturbed for a minimum of 3-5 years to achieve both the physical volume and the threshold concentration of glycyrrhizin required for industrial extraction pipelines. In years 4-6 (Mandatory rhizosphere recovery period) following harvest, the local ecosystem or cultivated plot requires a prolonged fallow window of 4 to 6 years to recover soil organic carbon balances and allow any remaining propagules to re-establish vegetative viability before a subsequent harvest can be safely executed, decoupling high-value glycyrrhizin production from the destructive exploitation of wild botanical resources (Hesami and Jones 2021).

1.5. Recent Medical Success of G. glabra L.

The contemporary clinical validation of G. glabra L. represents a fundamental transition from traditional ethnobotanical applications to targeted, evidence-based pharmacotherapy. This transition has been largely accelerated by advanced molecular isolation techniques and the strategic formulation of modified extracts engineered to eliminate inherent systemic risks (Yuan et al. 2022).

The most prominent therapeutic milestone of G. glabra is gastroenterology, which centres on Deglycyrrhizinated Licorice (DGL). By selectively removing the major triterpenoid saponin glycyrrhizin while maintaining the integrity of cytoprotective polyphenols, such as liquiritin, isoliquiritin, and isoliquiritigenin, DGL provides a highly effective therapeutic framework for peptic ulcer disease, acute gastritis, and gastroesophageal reflux disease (GERD). Mechanistically, DGL accelerates natural mucosal healing by upregulating endogenous prostaglandin synthesis, expanding the life span of gastric epithelial cells, and augmenting local hexosamine secretion. Crucially, because the glycyrrhizin fraction is absent, DGL stimulates this protective barrier without disrupting systemic electrolyte balances or provoking mineralocorticoid anomalies. In oral medicine and maxillofacial therapeutics, standardized aqueous extracts of G. glabra have demonstrated considerable clinical efficacy in managing recurrent aphthous stomatitis (canker sores) (Dorsareh et al. 2023). Recent randomized controlled trials confirm that topical licorice formulations significantly attenuate localized pro-inflammatory cytokine cascades, resulting in an immediate reduction in visual analog scale pain scores and an accelerated re-epithelialization of mucosal ulcers (Hoseinsalari et al. 2024; Zhao et al. 2024).

Coincidentally, dermo-cosmetic pipelines have heavily integrated Glabridin, a hydrophobic isoflavone isolated from the rhizospheric tissues. Glabridin acts as a potent, non-toxic, competitive inhibitor of tyrosinase enzymes, successfully suppressing melanogenesis. This molecule has emerged as a gold-standard natural therapeutic for cutaneous hyperpigmentation disorders, melasma, and chronic inflammatory dermatoses like rosacea, achieving exceptional clinical clearance without triggering the secondary dermal toxicity or cellular apoptosis associated with synthetic alternatives like hydroquinone (Pastorino et al. 2018).

Within clinical oncology, the isolated chalcone, liquiritigenin (ISL), has achieved clinical success as a potent adjuvant chemoprotective agent. Preclinical studies suggest that isoliquiritigenin (ISL) exhibits chemoprotective, anti-

inflammatory, and antioxidant activities through modulation of NF-κB signaling and oxidative stress pathways (Qiu et al. 2024).

Historically, the systemic clinical utility of unrefined G. glabra extracts has been strictly capped by the distinct risk of apparent mineralocorticoid excess. When unrefined glycyrrhizin is ingested in high doses, its active metabolite glycyrrhrtinic acid induces a severe metabolic bottleneck by inhibiting the 11β-hydroxysteroid dehydrogenase type 2(11β-HSD2) enzyme, provoking renal cortisol accumulation, severe hypokalemia, pathological water retention, and secondary hypertension. To bypass this physiological constraint, modern nanomedicines and targeted drug delivery systems, including liposomes, polymeric nanoparticles, and bio-engineered carrier complexes, have been successfully developed. These structural advancements isolate and guide targeted bioactive fractions directly to afflicted tissues while completely minimizing peripheral systemic exposure. Consequently, these novel formulations establish G. glabra derivative compounds as highly safe, successful, and globally integrated agents in contemporary precision pharmacotherapy (Zhao et al. 2024).

2. MATERIALS AND METHODS

2.1. Selection of Explant

The establishment of high-fidelity, commercially scalable micropropagation is driven by escalating global demand across the pharmaceutical, nutraceutical, and functional food sectors; the global G. glabra root extract has entered a phase of highly accelerated valuation. Market forecasting indicates that the global licorice extract sector is projected to expand significantly from its established baseline valuation. This commercial expansion is sustained by massive B2B commercial ingredient agreements and structured long-term procurement frameworks. Large-scale industrial supply chains process substantial quantities of licorice roots annually to meet the growing demand of pharmaceutical, food, cosmetic, and nutraceutical industries.

From an agronomic perspective, the G. glabra crop demands an uncompromised, strict 3-to-4-year developmental cultivation period before mechanical harvest. This prolonged duration is chronologically essential to ensure that subterranean secondary metabolic deposition progresses beyond juvenile thresholds and reaches optimal commercial and clinical benchmarks. Specifically, the cultivation cycle targets a minimum glycyrrhizin concentration of 6.0% to 25.0% within the dry rhizospheric mass, alongside the critical accumulation of downstream polyphenolic components like liquiritin and isoliquirtigenin.

Harvested roots are subjected to specialised high-pressure washing systems to eliminate geographic field particulates without fracturing the bark tissues. This is followed immediately by rapid, low-temperature forced-air drying protocols (typically regulated between 50°C and 60°C) designed to prevent thermal denaturation of thermosensitive chalcones. Industrial drying operations must systematically reduce internal root moisture thresholds strictly below 12.0%. Achieving this critical dry-weight equilibrium is mandatory to arrest localized enzymatic self-degradation and inhibit the post-harvest proliferation of mycotoxin-producing saprophytic fungi, thereby fulfilling international phytosanitary and pharmacopeial compliance standards for global export.

The increasing global demand for plant-derived therapeutics has shifted the focus of medicinal plant research from simple conservation strategies toward the development of scalable propagation systems capable of supporting a sustainable phytopharmaceutical supply chain. Despite its economic significance, commercial exploitation of G. glabra continues to rely predominantly on root harvesting, a destructive practice that reduces plant populations, limits biomass availability, and creates an imbalance between industrial demand and natural regeneration. Therefore, developing an efficient regeneration system is no longer merely a propagation strategy but an essential step toward establishing a sustainable production platform for glycyrrhizin-rich planting material.

The present investigation was undertaken to develop a reliable, reproducible, and high-frequency in vitro regeneration protocol for Glycyrrhiza glabra L. that can facilitate rapid clonal multiplication of elite genotypes suitable for large-scale cultivation. The study aimed to optimize the morphogenic response of suitable explants through the precise regulation of plant growth regulators, establish an efficient rooting and acclimatization system for maximum field survival, and generate genetically uniform propagules that can serve as reliable planting material for commercial cultivation. By improving the availability of high-quality planting stock, this regeneration strategy is expected to strengthen the cultivation-based production of G. glabra, reduce dependence on destructive harvesting of natural populations, and provide a sustainable biological resource for enhanced glycyrrhizin production to meet the growing industrial demand.

Protocols for G. glabra L. depend entirely on identifying an optimal explant type capable of initiating efficient in-vitro organogenic or embryogenic regeneration pathways. While a diverse array of donor tissues, including apical meristems, leaf laminas, petiole fragments, zygotic embryos, and root/stolon segments, possess clear totipotent regenerative capacities, nodal segments containing pre-existing axillary buds remain vastly superior to alternative somatic tissues. Nodal explants exhibit significantly enhanced genetic stability, bypass callus phases that can induce soma clonal variation, and a lower mortality threshold during structural initiation, rendering them the gold-standard source material for cloning this economically vital medicinal species.

For rigorous experimental validation, true-to-type mother plants must be collected from native eco-geographical zones known to harbour robust chemotypes, such as the hilly, semi-arid regions of Dehradun (Uttarakhand, India). Following collection, these wild-sourced specimens are systematically acclimatized within standard greenhouse environments, potted in structured soil-potting mixtures to stimulate uniform vegetative development, and carefully monitored under controlled irrigation profiles to minimize initial endophyte or fungal surface pathogens. Healthy, actively growing three-month-old G. glabra donor plants are selected to guarantee a highly active cambial zone and low tissue lignification, which are physiological prerequisites for efficient nutrient uptake during early tissue culture phases.

To execute micro-inoculation, target nodal segments are carefully excised from the donor plants using sharp, sterilised scalpel blades. The explants are then mechanically micro-trimmed to specialised architectural dimensions, measuring precisely 2-3 cm in total length and 1-2 cm in width at the tissue base. The explants were trimmed to uniform dimensions (2-3 cm) to ensure consistent exposure to the culture medium, facilitate aseptic handling, and minimize tissue injury during surface sterilization.

2.2. Preparation for Explant

2.2.1. Surface Sterilization

Healthy nodal segments and shoot tips of G. glabra L. were excised into approximately 2 cm long segments and washed thoroughly under running tap water for 5-10 min with a few drops of Tween-20 to remove adhering dust and surface debris. The explants were subsequently treated with 0.1% (w/v) Bavistin solution for 25 min to eliminate surface-associated fungal contaminants. To minimize oxidative browning caused by the release of phenolic compounds, the explants were immersed in 0.2% (w/v) polyvinylpyrrolidone (PVP) solution for 15 min. Under aseptic conditions in a laminar airflow cabinet, the explants were immersed in 70% (v/v) ethanol for 2 min, followed by surface sterilization with 0.1 % (w/v) mercuric chloride ($HgCl_2$) for 1 min. Thereafter, the explants were rinsed seven times with sterile distilled water to remove residual traces of the sterilizing agents completely. Finally, the sterilized nodal segments were trimmed to approximately 1.5 cm in length and inoculated vertically onto full-strength semi-solid MS medium for culture initiation (Toivonen & Rosenqvist, 1995).

2.2.2. Preparation of Culture Medium and Inoculation of Explant

Full-strength MS basal nutrient medium was employed as the foundational substrate for callogenesis, de novo shoot bud induction, and subsequent multiple shoot branching from nodal segments, whereas a shifted half-strength (1/2) MS macronutrient configuration was reserved for adventitious rooting assays, following established morphogenic baselines for the species. The basal media formulation was fortified with 3% (w/v) sucrose as the primary carbon source and amended with variable, treatment-specific concentrations of exogenous phytohormones. Before gelling with 0.4% (w/v) agar, the pH of the media matrices was meticulously adjusted to a fixed threshold of 5.8 ± 0.02 using either 0.1 N HCl or 0.1 N NaOH to optimize nutrient bioavailability. Approximately 30-50 mL of liquefied medium was systematically dispensed into individual 250 mL Erlenmeyer culture flasks.

Thermal sterilization of the medium was executed via autoclaving at a saturated steam pressure of 1.05 kg cm-2 (0.103 MPa) at 121°C for a strict duration of 20 min. Following aseptic inoculation of the surface-sterilized explants within a horizontal laminar airflow bench, all treatment cohorts were transferred to a climate-controlled phytotron chamber. The culture environments were continuously maintained at a regulated ambient temperature of 25 ± 2 °C under a strict 16-hour light/8- hour dark photo period. Illumination was provided by cool-white, fluorescent tubes at a photosynthetic photon flux density (PPFD) of 40-50 µmol-2 s -1 to support normal in vitro shoot growth and morphogenesis.

2.2.3. Used Reagents, Chemicals, and PGRs:

All macro elements, microelements, vitamins, and PGRs of Hi-Media Laboratories Pvt. Ltd. utilized to compile the nutrient substrates were purchased from a scientific supplier in Dehradun, Uttarakhand, India, ensuring high-fidelity chemical profiles. All chemicals and plant growth regulators were procured from HiMedia Laboratories Pvt. Ltd. (India) and were of analytical grade. To facilitate smooth logistics and ensure the raw materials did not experience temperature-induced degradation during transit, all chemical inventories, including tissue-culture grade sucrose, agar-agar, and specialised growth adjuvants, were procured directly through Dehradun's prominent authorised scientific laboratory distributor, Siddhartha Distributors, a well-reputed supplier network serving premier scientific institutes across Uttarakhand, India.

The standardized base formulation relied on pre-formulated MS Plant Tissue Culture Media (Product Code: PT021, HiMedia), integrating a globally validated hyper-concentrated matrix of macro-nutrients and micro-nutrients. The basal salt mixture was precisely measured at a concentration range of 70-80 g per total preparation batch. The specialised PGRs used to trigger axillary shoot proliferation and downstream rhizogenesis included pure cytokinins, specifically BAP and KN, alongside target inductive auxins, including NAA and IBA, all purchased in crystalline form from HiMedia and prepared into stock solutions using analytical-grade solvents.

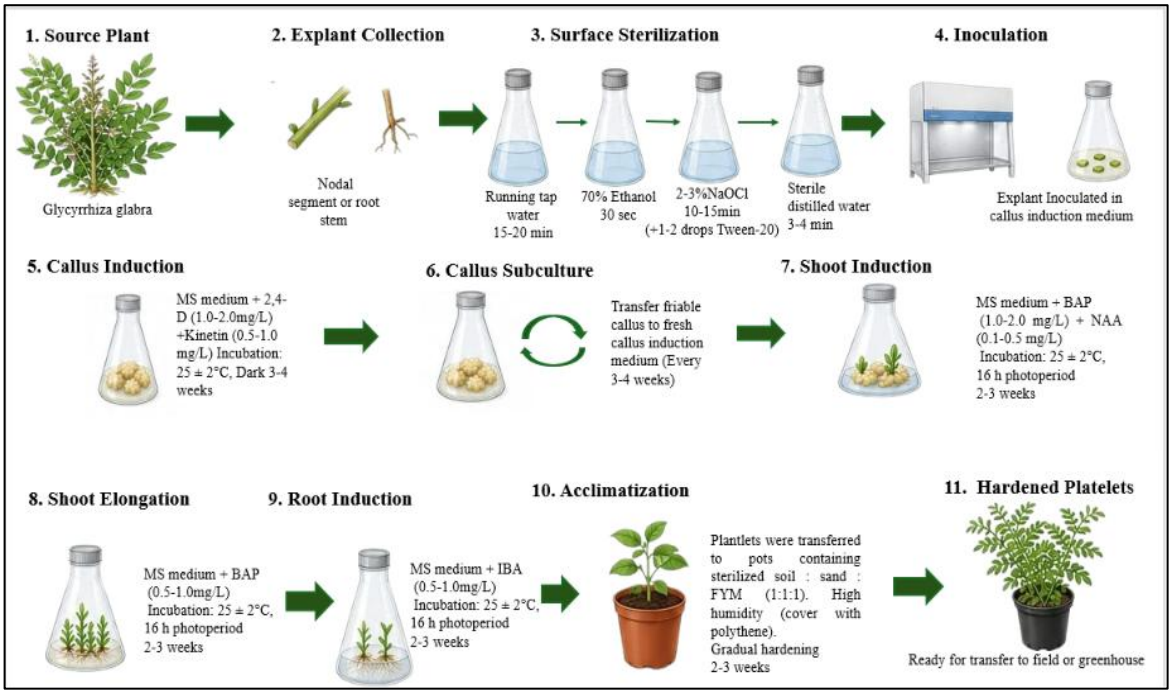

Fig 1: "A Stepwise Flowchart" In vitro propagation Protocol for G. glabra (Mulethi).

2.3. Shoot Regeneration

Phytohormones were used for shoot induction; MS medium + (BAP + IBA) in different concentrations was used, in combination with BAP and KN, for shoot Proliferation [86]. After shoot induction in the plant, different combinations of cytokinin and MS medium (BAP+KN) were used for further proliferation and growth of the plantlets. The culture medium without plant growth promoters served as controls in all cases. Data collection occurred at regular intervals, within 15 days after the cultures were initiated.

2.4. Root Regeneration

For root formation, shoots measuring 2-5 cm in length were isolated and transferred to a half-strength MS medium containing different concentrations of BAP and NAA in various combinations. The number of roots, shoot length, and root length were recorded after 22 days of culture. Control cultures, which were grown in the same medium without phytohormones, were used as a comparison for both shoot and root development (Mehrotra et al., 2008).

2.5. Preparation of Soil Mixtures

To transition the in-vitro regenerated plantlets of G. glabra L. from heterotrophic glass enclosures to autotrophic field conditions, three distinct substrate matrices varying in porosity, moisture retention, and nutrient availability were formulated. Mixture 1 (M1) consisted of a homogenized combination of sterile garden soil, fine river sand, and vermiculite in an equal volumetric ratio (1:1:1, v/v/v) to establish a baseline equilibrium between drainage and moisture suspension. Mixture 2 (M2) was a highly porous, synthetic substrate composed entirely of sterile soil rite (a 1:1 v/v blend of peat moss and expanded perlite) designed to optimize rhizosphere aeration and minimize mechanical impedance for delicate secondary roots. Mixture 3 (M3) was formulated using a rich organic blend of autoclaved field soil and well-decomposed farmyard manure (FYM) in a 2:1 (v/v) ratio to evaluate the morphogenic impact of early organic nutrient loading. Well-developed, rooted plantlets of G. glabra were carefully divested into residual carbon-rich agar matrices using lukewarm sterile distilled water to remove residual culture medium and reduce the risk of microbial contamination during acclimatization. The plantlets were transferred into uniform plastic cups filled with the respective soil mixtures (M1, M2, M3), which were immediately covered with transparent micro-perforated polythene bags to maintain a high relative humidity during the initial acclimation period (Patil et al., 2026).

3. RESULTS AND DISCUSSION

3.1 Planting of plantlets

Each experiment comprised three independent, parallel replicate cohorts designated as Set A, Set B, and Set C. Within each set, a minimum of ten uniform explants were inoculated across individual culture flasks (250 ml), establishing a robust technical and biological replication framework (N=30 explants per treatment) (Polivanova & Bedarev, 2022). The multiplication and shoot induction stages were executed in three sequential experimental runs, whereas the downstream adventitious rooting experiment was replicated twice. Due to the persistent vulnerability of in-vitro culture to microbial contamination, a multistep surface sterilization protocol was deployed. Extracted nodal explants were initially submerged in 70% (v/v) ethanol for 1-2 min, followed by an aqueous solution of mercuric chloride ($HgCl_2$, 0.1%w/v) fortified with 2-3 drops of surfactant, Tween-20, for 5-10 min under continuous agitation. This optimization generated a 90% axenic culture survival rate. To actively counter the oxidative exudation of endogenous phenolic compounds, which typically

triggers media discoloration and tissue necrosis, the medium was amended with polyvinylpyrrolidone (PVP 0.1% w/v) as an antioxidant clipping agent (Bakhtiar et al., 2026).

3.2 Influence of Phytohormones on Shoot Bud Induction and Proliferation

In-vitro shoot bud induction, axillary caulogenesis, and downstream macro-shoot elongation in G. glabra L. were heavily modulated by the specific architectural configurations and concentration gradients of exogenous cytokinin paired with auxins (Xiao et al., 2022). To establish an optimized micropropagation index, uniform nodal explants were systematically evaluated across variable treatment matrices of BAP administered in combination with either IBA, KN, or NAA (Fig. 2a, 2b, 2c). The enhanced morphogenic response obtained in the present investigation is likely associated with a balanced interaction between cytokinin-mediated activation of the axillary meristem and auxin-regulated cellular differentiation (Hasnain et al., 2022). Such hormonal equilibrium promotes rapid bud break while maintaining normal shoot architecture and reducing the occurrence of physiological abnormalities frequently observed under supra-optimal cytokinin concentrations.

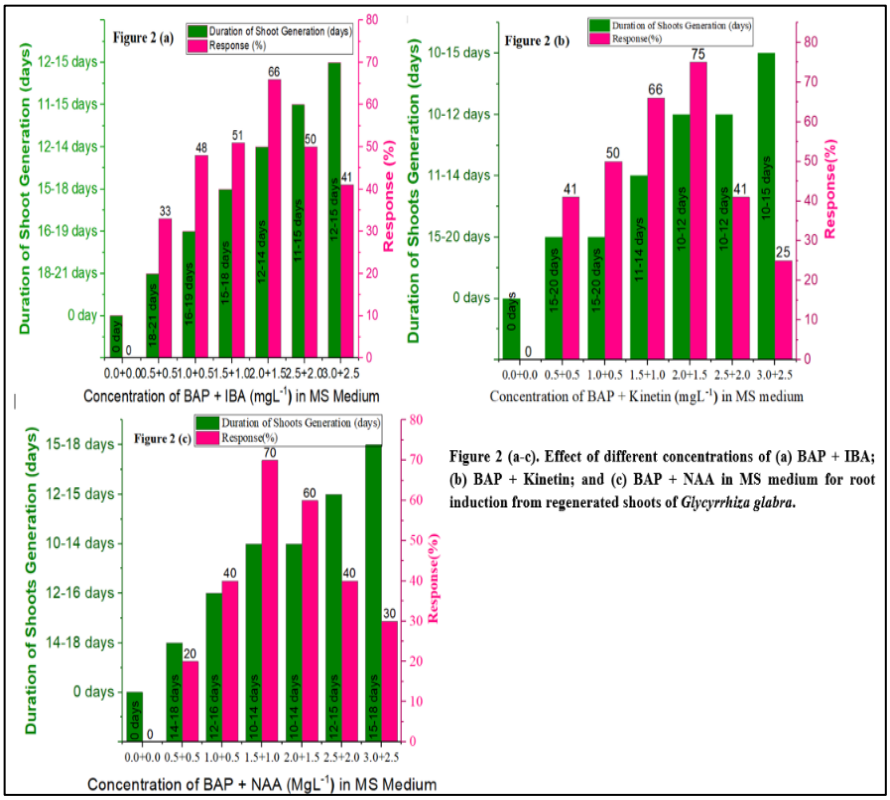

Figure 2 (a-c). Effect of different concentrations of (a) BAP + IBA; (b) BAP + Kinetin; and (c) BAP + NAA in MS medium for root induction from regenerated shoots of *Glycyrrhiza glabra*.

3.2.1. Effect of BAP and IBA concentrations

Fig. 2(a) delineates that the interaction between the principal cytokinin BAP and the auxin IBA exerted a profound, concentration-dependent influence on caulogenic initiation. In the absolute absence of phytohormones (0.0+0.0 mg L-1 control), the morphogenic response remained at 0% with no bud break over 0 days. Bud-break frequency scaled progressively with increasing hormone concentrations, initiating at a 33% response with an extensive developmental lag of 18-21 days under low-dose environments (0.5+0.5 mg L-1).

The premier morphogenic response within this matrix was explicitly achieved on MS basal medium supplemented with a synergistic blend of 2.0 mg L-1 BAP and 1.5 mg L-1 IBA (Fig. 2a), which induced high-frequency axillary bud break in 66.0% of the inoculated cultures within an accelerated timeline of 11-15 days. This optimal treatment profile yielded an impressive mean shoot length of 7.50±0.12 shoots per explant across parallel replicate Sets A, B, and C.

When the BAP and IBA concentration was pushed to a higher threshold (2.5 + 2.0 mg L-1), a strict physiological decline was triggered, reducing the response to 50% while stretching the generation duration back to 12-15 days due to supra-optimal chemical toxicity. A secondary optimal response was recorded at the lower gradient of 1.5 mg L-1 BAP and 1.0 mg L-1 IBA, displaying a 51% response rate over 12-14 days, culminating in a mean shoot length of 2.45±0.03 cm but generating a dense, localised multi-shoot proliferation rate of 6.50±0.29 shoots per explant. This combination produced the highest shoot induction frequency and maximum shoot proliferation among all treatments, indicating that a balanced cytokinin-auxin interaction effectively promoted axillary meristem activation while minimizing vitrification.

3.2.2. Effect of Dual-Cytokinin Blends (BAP + KN)

The introduction of a dual-cytokinin matrix combining BAP and KN targeted the elimination of apical dominance to maximize lateral branching density, Fig. 2(b). This hormonal configuration yielded the highest overall percentage of responsive explants observed across the entire study. The apex of vegetative caulogenesis was reached at a combination of 2.0 mg L-1 BAP + 1.5mg L-1 KN, which generated a peak micropropagation response of 75.0% in a highly compressed time frame of just 10-12 days.

The biological mechanism driving this trend is the direct synergistic interaction of two distinct adenylated cytokinins; BAP actively stimulates primary cell division within axillary meristem clusters, while KN enhances longitudinal node generation and tissue elongation kinetics. However, maintaining this high-dose cytokinin profile beyond its optimal equilibrium point led to sharp developmental setbacks. Increasing the treatment matrix to 3.0 + 2.5 mg L-1 BAP + KN dramatically depressed the morphogenic response down to 41%, while forcing a severe developmental delay of 10-15 days, marked by localized hyperhydricity and a significant reduction in micro-shoot leaf area.

3.2.3. Effect of BAP and NAA Combinations

The combination of BAP with the strong synthetic auxin NAA demonstrated a rapid capacity for early organogenic bud break but was bound by strict dose-response limits Fig. 2(c). The maximum regeneration profile within this block was recorded on MS medium fortified with 1.5 mg L-1 BAP + NAA, driving a reliable morphogenic response of 70.0% within 10-14 days.

While this treatment proved highly effective at initiating rapid caulogenesis, its developmental efficiency was statistically counterbalanced by a persistent, unwanted side effect: the high stability of NAA at the base of the explants regularly induced significant callusing. This unorganised basal callus mass acted as a metabolic sink, absorbing nutrient resources and reducing long-term shoot elongation rates compared to the BAP-IBA groups. Further increases in NAA concentration up to 3.0+2.5 mg L-1 severely disrupted cellular differentiation pathways, dropping the response rate to 30% and causing extensive tissue browning and necrosis within 15-18 days due to the toxic hyper-accumulation of ethylene triggered by excessive auxin activity (Vaseghi Baba et al., 2026).

3.3. Preparation of replicate Sets

To ensure statistical stringency and eliminate microclimatic vessel variations during the in-vitro propagation steps, a completely randomized experimental hierarchy was strictly maintained. Each distinct phytohormone treatment group (for instance, MS basal medium supplemented with 2.0 mg L-1 BAP + 1.5 mg L-1 IBA) was partitioned into a tripartite replicate architecture consisting of three parallel, independent experimental cohorts designated as Set A, Set B, and Set C. Within each discrete cohort, exactly 10 uniform, surface-sterilized nodal explants were inoculated across individual culture flasks, establishing a rigorous biological and technical replication framework (n = 10 explants per set; cumulative sample N = 30 explants per treatment). At the termination of the 16–18-day incubation cycle, quantitative morphogenic measurements from all three independent sets were compiled and mathematically pooled. This converged dataset was used to compute a single consolidated statistical mean expressed with its corresponding variation metric (e.g., 7.50 ± 0.09 cm), where the standard error directly signifies the high level of reproducibility across the independent technical replicates. Plant tissue culture methodologies remain a cornerstone for the clonal propagation, germplasm conservation, and mass scaling of high-value ornamental, herbaceous, and medicinal plant species. In the present study, a highly efficient, direct organogenic regeneration protocol was successfully established using nodal segments containing axillary buds of G. glabra L., confirming that the selection of structurally organized meristematic tissue is fundamental for preserving elite clonal uniformity. The physiological state and seasonal timing of the maternal explant collection emerged as critical determinants governing initial in-vitro stabilization and axenic establishment. Following an incubation window of 12-16 days, axillary bud break and microshoot elongation were significantly influenced by the specific configurations and concentrations of exogenous PGRs. The optimal morphogenic frequency for multiple shoot induction was achieved on full-strength MS basal medium amended with a synergistic combination of 2.0 mg L-1 BAP and IBA. This specific cytokinin-auxin balance yielded a premier shoot elongation of 7.50 ± 0.09 cm alongside an exceptional multiplication rate of 2.77 ± 0.29 shoots per explant. Comparative evaluations using variable concentrations of BAP paired with KN confirmed that while both adenylated cytokinins successfully induced axillary bud proliferation, a lower hormonal threshold consisting of 2.0 mg L-1 BAP and 1.5 mg L-1 KN was required to optimize multiple shoots branching and prevent hyperhydricity or tissue verification during subculturing phases.

For downstream rhizogenesis, full-strength media proved less effective due to high osmotic concentrations. Consequently, a shift to half-strength (1/2) MS micronutrient medium fortified with a combination of 1.5 mg L-1 NAA and 1.0 mg L-1 proved highly effective, stimulating rapid root primordia differentiation and robust secondary root branching within 16-18 days. These findings demonstrate that the micropropagation efficacy of G. glabra is closely dependent on precise exogenous phytohormone ratios, providing a reliable and reproducible pipeline for the rapid commercial scaling of this overexploited medicinal resource.

4. Acclimatization and Field Establishment

Successful acclimatization represents one of the most critical stages of any micropropagation protocol because in vitro-grown plantlets develop under highly controlled environmental conditions characterized by high humidity, low irradiance, continuous carbohydrate availability, and limited gas exchange. Consequently, regenerated plantlets possess thin cuticular layers, poorly developed epicuticular wax, partially functional stomata, and a heterotrophic mode of growth, making them highly susceptible to rapid water loss and transplant shock immediately after transfer to ex vitro conditions (Loyola-Vargas & Ochoa-Alejo, 2022).

Following successful adventitious root induction, healthy and well-rooted plantlets of G. glabra were carefully removed from the culture vessels. Residual agar adhering to the root system was gently washed away with sterile distilled water to minimize microbial contamination and facilitate direct contact between the roots and the potting substrate. Removal of agar also improves root aeration and prevents excessive moisture accumulation around the rhizosphere, thereby reducing the risk of fungal infection during the early stages of acclimatization.

The regenerated plantlets were transferred to plastic pots containing a sterilized substrate composed of cocopeat, garden soil, and river sand (2:2:1, v/v/v). The selection of this substrate was based on its complementary physical properties. Cocopeat provided high water-holding capacity and maintained adequate moisture around the developing roots; garden soil supplied essential mineral nutrients required for early autotrophic growth, while river sand improved substrate porosity and drainage, ensuring sufficient oxygen diffusion to the root system (Xiao et al., 2022). The balanced substrate composition created a favorable rhizospheric environment that promoted root establishment while minimizing waterlogging and root decay.

Immediately after transplantation, plantlets were maintained under controlled growth chamber conditions with a relative humidity of approximately 85–90%, a temperature of 25 ± 2°C, and a 16 h light/8 h dark photoperiod. High humidity during the initial hardening period reduced excessive transpiration and prevented rapid desiccation before complete recovery of stomatal regulation. Gradual reduction of humidity over the subsequent two weeks facilitated progressive development of epicuticular wax deposition, restoration of normal stomatal function, and activation of photosynthetic metabolism (Chandra et al., 2022). This physiological transition enabled regenerated plantlets to shift successfully from heterotrophic dependence on exogenous sucrose supplied by the culture medium to complete autotrophic growth under natural environmental conditions.

Following primary acclimatization, vigorous plantlets were transferred to nursery conditions for secondary hardening. During this stage, exposure to ambient environmental fluctuations promoted further strengthening of the root system, stem tissues, and leaf morphology. Intermittent mist irrigation was provided during the initial 48 h to minimize transplant stress and maintain leaf water potential. Progressive exposure to natural light and lower humidity stimulated normal photosynthetic activity and enhanced the physiological adaptation of regenerated plants.

The successful establishment of regenerated G. glabra plantlets under ex vitro conditions demonstrates the effectiveness of the developed regeneration protocol beyond in vitro shoot multiplication and rooting. Efficient acclimatization is an essential prerequisite for commercial micropropagation because it determines the practical field transferability of regenerated plants. The healthy establishment of regenerated plantlets obtained in the present study indicates that the validated regeneration protocol can generate planting material capable of successful adaptation under greenhouse and nursery conditions, thereby supporting sustainable cultivation and ex situ conservation of elite G. glabra germplasm.

4.1 Novel Contribution of the Present Study

Unlike previously published regeneration studies that primarily refined individual stages such as shoot multiplication or rooting, the present investigation establishes a complete regeneration pipeline encompassing optimized explant sterilization and medium-term slow-growth germplasm conservation within a single reproducible protocol (Hasnain et al., 2022). The integration of these sequential stages reduces procedural complexity and provides a practical framework for both commercial clonal propagation and conservation of elite G. glabra germplasm. Collectively, the protocol demonstrates that regeneration efficiency should be evaluated not only by shoot multiplication frequency but also by successful plant establishment and conservation potential, thereby expanding its practical relevance beyond laboratory-scale micropropagation.

In contrast to previously published protocols that generally terminate after successful rooting or greenhouse acclimation, the present study extends the regeneration workflow to include medium-term slow-growth conservation, thereby integrating propagation and germplasm preservation into a single experimental framework (Ruta et al., 2021; Verma et al., 2022). This comprehensive strategy increases the practical applicability of the protocol for long-term conservation programmes as well as commercial nursery production.

5. CONCLUSION

This study establishes a reproducible regeneration and conservation protocol for G. glabra L. that combines efficient direct organogenesis, rooting, ex vitro acclimatization, and medium-term germplasm conservation within a single workflow. Because regeneration occurred through direct organogenesis without an intervening callus phase, the protocol is expected to reduce the likelihood of somaclonal variation. Nevertheless, molecular marker analysis and phytochemical profiling are recommended in future studies to confirm the genetic and biochemical uniformity of regenerated plants. Our architectural optimization demonstrates that precise phytohormonal stoichiometry, rather than absolute concentration, dictates morphogenic efficiency (Pérez-Molphe-Balch et al., 2022). For shoot development, full-strength MS basal medium supplemented with 2.0 mg L-1 BAP and 1.5 mg L-1 IBA optimised axillary bud break, yielding a 66.0% morphogenic response within 11-15 days and a peak shoot length of 7.50 ± 0.12 cm. Lateral branching density was maximized via a dual-cytokinin blend (2.0 mg L-1 BAP and 1.5 mg L-1 KN), driving the highest overall proliferation response of 75.0%. Downstream adventitious rhizogenesis was governed by osmotic manipulation; shifting to half-strength (1/2) MS medium fortified with 1.5 mg L-1 NAA and $10\ \mathrm{mg\ L^{-1}}$ BAP triggered rapid root primordia differentiation within 16-22 days. When coupled with a sequential, two-phase ex vitro acclimatization pathway, this robust root architecture ensured exceptional post-transplantation survival (Ahmad et al., 2021; Batool et al., 2023).

Crucially, this work introduces a successful medium-term ex situ conservation protocol using minimal growth kinetics (Duta-Cornescu et al., 2023). By reducing ambient temperature, light irradiance, and hormonal inputs, shoot apices were successfully maintained in a metabolically suppressed state for six continuous months without mid-cycling subculturing (Neelakandan & Wang, 2012). The 100% phenotypic and physiological recovery of preserved microshoots upon re-culture validates this method as a reliable strategy for safeguarding elite wild chemotypes against habitat-driven genetic erosion. Although the refined protocol demonstrated efficient regeneration and acclimatization, molecular genetic fidelity and

phytochemical equivalence of regenerated plants were not evaluated in the present study and should be investigated in future research.

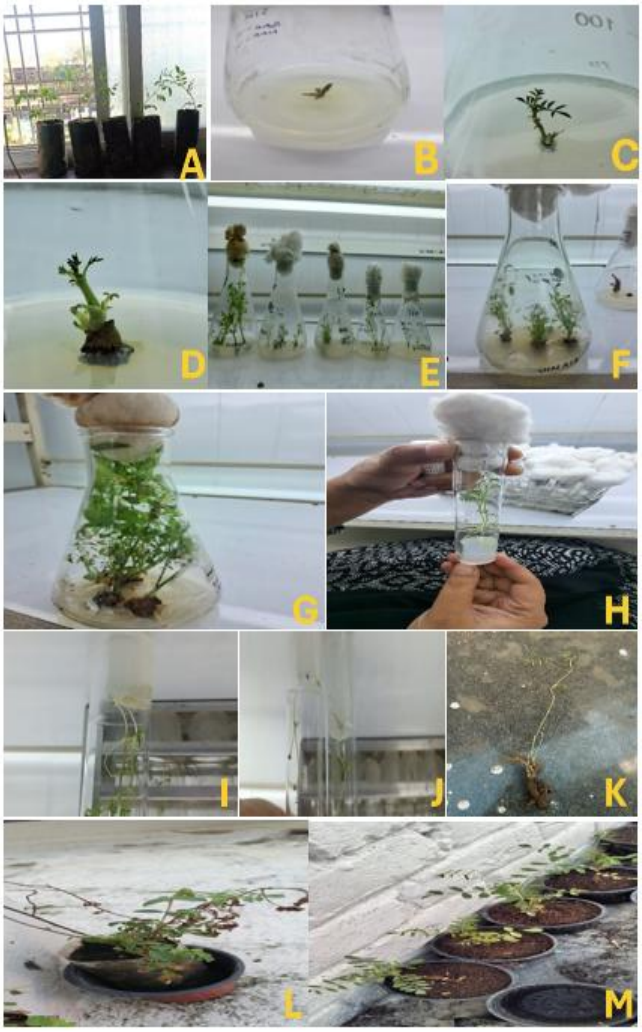

Fig 3 (A-M). In vitro regeneration from nodal explants and shoot tip of G. glabra.

A. G. glabra from which nodal explants were taken; (B) Shoots regenerated from the shoot tip and nodal explant on MS medium supplemented with BAP and IBA; (C-G) Multiple shoots formed on MS medium fortified with BAP and KN after the first subculture; (H-K) Roots were produced on in vitro shoots grown on half-strength MS medium; (L-M) Hardening of rooted plants with mixture of cocopeat, garden soil and sand in ratio of 2:2:1.

6. Prospects

Our findings suggest a reproducible and economically viable in vitro clonal propagation framework for G. glabra L. via direct organogenesis; it also lays the foundational groundwork for several high-impact biotechnological, genomic, and industrial scaling avenues. Transforming this micropropagation protocol into a high-throughput, commercially disruptive platform requires addressing the following key scientific horizons.

The high-fidelity, direct organogenesis baseline established in this study provides a highly reproducible platform expected to minimize the risk of somaclonal variation because regeneration was achieved through direct organogenesis. This framework opens several critical, high-impact avenues for translating G. glabra from a vulnerable wild resource into a precision-engineered biotechnological classis. Future investigations should exploit this synchronized in vitro system to elucidate the transcriptional and metabolic networks governing glycyrrhizin and specialised polyphenolic biosynthetic pathways. Integrating high-throughput transcriptomics and untargeted metabolomics will allow us to map how targeted abiotic inputs (e.g., osmotic stress via polyethylene glycol, heavy metal elicitors, or narrow-band UV-B irradiation) and biotic signalling molecules (e.g., methyl jasmonate, salicylic acid) trigger the up-regulation of key rate-limiting enzymes like squalene synthase andβ-amyrin synthase (Bairu et al., 2011; Krishna et al., 2016; Thorpe, 2007).

To artificially drive superior agronomic fitness and high-yielding chemotypes, future work will focus on optimizing colchicine-mediated mitotic inhibition during the early axillary bud break phase (Hazarika, 2003). Inducing stable autopolyploidy (chromosome doubling) offers a proven genetic strategy to expand cellular volume, alter gene dosage, and exponentially upregulate secondary metabolite accumulation profiles beyond standard wild-type capacities.

Scaling the protocol to meet global pharmaceutical demand requires transitioning from static glass vessels to automated bioreactor architectures, such as Temporary Immersion Bioreactor Systems (TIBS) (Engelmann, 2011). Optimizing the

immersion-to-dryness cycles within TIBS may facilitate labor overhead, eliminate hyperhydricity (glassiness), optimize gas exchange (O_2 and CO_2), and could improve biomass accumulation and root primordial multiplication rates (Pence, 2010).

The use of direct organogenesis is generally considered to reduce the likelihood of somaclonal variation; however, molecular marker-based validation would further strengthen genetic fidelity assessment. Applying CRISPR/Cas9 ribonucleoprotein complexes to silence competitive metabolic pathways could strategically re-route metabolic flux directly toward the overproduction of ultra-pure glycyrrhizin (Rai et al., 2017).

Future optimization of this regeneration platform, combined with metabolic engineering and bioreactor-based scale-up, may facilitate sustainable production of glycyrrhizin-rich planting material (Wasternack & Song, 2017). Although the present study established an efficient regeneration protocol, molecular genetic fidelity and phytochemical equivalence of regenerated plants were not evaluated. These analyses would further strengthen the commercial application of the protocol (Seki et al., 2015).

Abbreviations: BAP - 6-Benzylaminopurine, KN - Kinetin, PGRs- Plant growth regulators, NAA - Naphthalene Acetic Acid, IBA - Indole-3-butyric acid, MS - Murashige and Skoog medium, IAA - Indole-3-acetic acid, CNS – Central Nervous System, DGL - Deglycyrrhizinated Licorice, GERD- Gastroesophageal reflux disease

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Conflict of interest

The authors declare no conflicts of interest related to this article.

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