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Hairy Root Cultures: A Comprehensive Review of Biological Principles, Metabolic Engineering Strategies, and Bioreactor-Based Production Systems

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ABSTRACT

Hairy root culture, induced through *Agrobacterium rhizogenes*–mediated genetic transformation, has emerged as one of the most powerful platforms in plant biotechnology for the production of high-value secondary metabolites. This technology combines rapid growth, genetic and biosynthetic stability, and synthesis under controlled conditions. In recent years, limitations associated with conventional extraction from natural plants have intensified global interest in vitro systems. Hairy roots offer an efficient alternative, while also enabling metabolic reprogramming and the accumulation of unique phytochemicals not detected in non-transformed tissues. This review summarizes the biological principles of hairy root induction and the metabolic advantages of transformed roots. In addition, the article highlights technological strategies for yield enhancement. Special emphasis is placed on bioreactor-based cultivation, with a focus on reactor designs, engineering constraints, and challenges related to oxygen transfer, shear sensitivity, and biomass aggregation. Key findings highlight that hairy root cultures can surpass wild plants in yield for specific metabolites when combined with elicitation strategies. However, major challenges such as shear sensitivity in bioreactors, biomass aggregation, and scale-up complexities remain significant hurdles for industrial adoption. Continued advances in molecular engineering and bioreactor design are expected to accelerate the commercial deployment of this technology.

Keywords: Hairy roots, Bioreactor systems, Secondary metabolites, Metabolic engineering, Elicitors

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Introduction

According to the definition provided by the World Health Organization (WHO, 2013), traditional medicine is described as "the sum of knowledge, skills, and practices based on the theories, beliefs, and experiences indigenous to different cultures, used in the maintenance of health as well as in the prevention, diagnosis, improvement, or treatment of physical and mental illnesses." Today, the utilization of traditional medicine has grown on a global scale, serving as the primary or complementary component of healthcare systems in many countries (Chandran et al., 2020). It is estimated that nearly 80% of the global population relies on medicinal plants for their primary healthcare needs (WHO, 2013).

Plant tissue culture, as an advanced technology, represents a valuable tool for developing innovative biotechnological approaches and accessing therapeutically substantial chemical compounds. In this technique, plant cells, tissues, or organs are maintained under completely sterile and controlled conditions, where all environmental parameters, including light, temperature, humidity, and the optimized composition of the cultivation medium, are tightly regulated (De Schutter et al., 2022). The fundamental idea of culturing isolated plant cells to reveal their latent potential was first proposed by Haberlandt (1902) in the early twentieth century. Although he was not successful in inducing cell division in practice, his visionary insight laid the foundation for one of the most influential fields in plant sciences: plant cell and tissue culture. Haberlandt believed that such a simplified biological system could provide an unparaleled platform for understanding the intrinsic characteristics and hidden potential of individual cells, opening a window into the complex interactions and cooperative behaviors within multicellular organisms (Haberlandt, 1902).

Today, not only has the maintenance and propagation of isolated plant cells under in vitro conditions become a routine reality, but the complete regeneration of a whole plant from a single cell is considered a remarkable achievement of plant biotechnology. The superiority of cell-culture–based systems in studying metabolic pathways and cellular responses, compared to experiments performed on whole plants, is now well established (Singh et al., 2023). These systems enable researchers to apply chemical treatments with high precision, rapidly modify culture medium compositions, and conduct detailed biochemical analyses. Moreover, the clonal propagation of single cells provides a platform for the genetic improvement of crop plants by employing principles similar to microbial genetics. At the industrial scale, plant tissue culture is now regarded as a powerful and cost-effective approach for the mass production of valuable plant phytochemicals with high commercial importance (Wilczańska et al., 2023).

Interest in medicinal herbs has deep historical roots, as these plants have been used for centuries as natural remedies for a wide range of human ailments. They display diverse bioactivities, including anti-inflammatory, anxiolytic, antipyretic, antiviral, and antibacterial effects, serving as valuable reservoirs of life-saving compounds and playing a pivotal role in global healthcare systems (Chandran et al., 2020). These therapeutic properties primarily arise from the rich diversity of chemical constituents found in plants, widely known as secondary metabolites (Singh et al., 2023).

Despite their significance, the accumulation of valuable specialized metabolites in herbal plants faces numerous challenges. Traditional extraction from natural plants, which has long been the main source of pharmacologically active compounds, pigments, and flavoring agents, is constrained by climatic dependency, depletion of natural genetic resources, pesticide contamination, and production instability (Yamamoto et al., 2022). In contrast, plant cell culture, although offering considerable promise, encounters obstacles such as high cellular water content, foaming issues in bioreactors, and metabolic instability (Murthy et al., 2023). In this context, in vitro culture of differentiated plant systems has emerged as a promising strategy for diverse plant species (Table 1). Among these systems, hairy root culture, induced through co-cultivation with *Agrobacterium rhizogenes*, holds a special position. This system possesses unique benefits, including accelerated growth, long-term genetic and metabolic stability, and the capacity to produce root-specific secondary metabolites, making it one of the most promising biotechnological platforms to support sustainable biosynthesis of economically important medicinal compounds (Biswas et al., 2023).

Table 1. Applications of *in vitro* plant tissue and cell culture in multiple species

Plant	Culture	Reference
Silybum marianum	Callus	Ehsani et al., 2025
Salvia apiana	Hairy roots	Krol et al., 2025
Urena lobata L.	Hairy roots	Cao et al., 2024
Melia azedarach	Callus	Ahmadpoor et al., 2022
Taxus cuspidata	Cell suspension	Yamamoto et al., 2022
Curcuma amada	Micropropagated plants	Behera et al., 2022
Gynostemma pentaphyllum	Cell suspension	Quang et al., 2022
Salvia dominica	Hairy roots	Boccia et al., 2022
Solanum lycopersicum	Callus	Gogliettino et al., 2022
Ocimum sp	Hairy roots	Pandey et al., 2022
Taxus × *media*	Hairy roots	SykłowskaBaranek et al., 2022
Hyoscyamus muticus	Cell suspension	Abdelmaksood et al., 2022
Atropa acuminata	Regenerated plants	Dar et al., 2022

This table underscores the versatility of in vitro culture techniques, ranging from undifferentiated callus to organized hairy roots, for producing metabolites across diverse plant families.

Despite the demonstrated potential of transformed root cultures, challenges such as metabolic instability under submerged conditions and limitations in process scale-up have hindered their widespread industrial application (Biswas et al., 2023). This review is based on a comprehensive survey of peer-reviewed literature retrieved from major scientific databases, including Scopus, Web of Science, and Google Scholar, with a particular focus on studies published over the past two decades related to hairy root cultures, secondary metabolite production, and bioreactor-based systems. Therefore, the primary objectives of this review are: (1) to elucidate the biological principles and metabolic advantages of hairy root cultures; (2) to evaluate current technological strategies, including elicitation and permeabilization, for enhanced metabolite production; (3) to critically analyze bioreactor designs and scale-up challenges; and (4) to provide a analysis that identifies key barriers and future opportunities for the commercial deployment of this platform.

Hairy Roots: A Novel Technique

Genetic engineering, as an advanced technology in plant breeding, enables the targeted transfer of genes encoding desirable traits into the genomes of various organisms. In this process, DNA fragments are excised from the donor genome using restriction endonucleases and cloned into a suitable genetic vector. These vectors are capable of delivering foreign DNA into host cells, ultimately generating fully transgenic plants (Gutierrez-

Valdes et al., 2020). Among the different gene delivery systems, *Agrobacterium rhizogenes* holds a special position. This soil-borne bacterium is naturally capable of inducing "hairy root" syndrome in numerous dicotyledonous plants (Ying et al., 2023). The underlying mechanism involves the genomic integration of Ri plasmid T-DNA sequences within the host plant genome, resulting in the development of transgenic root tissues with rare characteristics (Lin et al., 2025).

Hairy roots generated under in vitro conditions exhibit numerous advantages. These roots demonstrate

accelerated growth, stable genetic traits, and the capacity to sustain development without the need for exogenous growth regulators. Furthermore, due to their differentiated structure and fully developed vascular network, the capacity for biosynthesis of bioactive compounds in these roots is often reported to be significantly higher than that observed in conventional cell cultures (Mirmazloum et al., 2024).

Agrobacterium rhizogenes, a soil-dwelling Gram-negative bacterium, was first identified in the 1930s under the name Rhizobium rhizogenes and received its current designation in 1942. This bacterium has the ability to initiate hairy root syndrome involving excessive root proliferation at the site of infection (Zheng, 2025). Agrobacterium species are widely distributed worldwide, among these, pathogenic species including *Agrobacterium tumefaciens* and *A. rhizogenes* are responsible for inducing crown gall and hairy root diseases in plants, respectively. Despite its long history as a plant pathogen, *A. rhizogenes* has now been established as an efficient tool in plant biotechnology (Ying et al., 2023). Its natural ability to stably transfer genes into plant cells enables the generation of diverse lines of transgenic hairy roots, which are widely used for the production of valuable bioactive compounds as well as in fundamental plant science studies (Lin et al., 2025). Morphological variations among hairy roots include differences in primary root thickness, lateral root density, spontaneous callus formation, and other traits (Figure 1). The phenotype of hairy roots can vary depending on the specific transformation event, the integration site of T-DNA within the plant genome, the copy number of T-DNA, and ultimately the level of suppression or expression of aux and rol-related genes (Vinterhalter et al., 2019). Transformed roots are characterized by neoplastic growth in hormone-free media (non-geotropic growth) and extensive branching, features that facilitate the successful production of a broad variety of secondary metabolites (Phuong et al., 2018). Overall, hairy root culture represents an effective biotechnological approach for the conservation of unique, valuable, endangered, or endemic medicinal plant species. It not only contributes to biodiversity preservation but also enables the in vitro biosynthesis of high-value secondary metabolites under controlled conditions (Gutierrez-Valdes et al., 2020).

The quality and quantity of metabolites extracted from hairy roots are generally comparable to those found in natural herbs; however, their chemical profile can be optimized to enhance the levels of specific target compounds. Furthermore, in many cases, hairy roots are capable of accumulating novel compounds that are not detected in non-transgenic tissues (Wilczańska et al., 2023). Plant species have been successfully infected with *Agrobacterium rhizogenes*, and a variety of plant organs have served as suitable explants for infection (Table 2). The induction of hairy roots is not solely dependent on the genotype of the host plant; bacterial strain, infection method, and culture conditions also play critical roles. In general, the transfer of bacterial DNA into the plant cell genome occurs through four main steps:

1. Induction of vir gene expression and production of single-stranded DNA: Agrobacterium senses chemical compounds released from wounded plant tissues (e.g., wound-derived phenolics), which activate the bacterial vir genes.
2. Covalent attachment of T-DNA to VirD2 protein and formation of an effector protein complex: This complex, encoded by the vir genes, is excised from the bacterium and delivered into the plant cell.
3. Targeting of T-DNA and VirD2 proteins to the plant cell nucleus: Upon entry into the cytoplasm, the T-DNA strands, along with VirD2, are directed toward the nucleus of the host cell.

4. Integration of T-DNA into the plant genome: VirD2 and certain host proteins are removed from the T-DNA strands, and ultimately, the T-DNA is stably integrated into the host plant genome (Mirmazloum et al., 2024)

A schematic overview of this Agrobacterium-mediated transformation process is depicted in Figure 2. The detection of gene integration is typically performed using polymerase chain reaction (PCR) targeting rol genes. In addition, some studies employ reporter genes to assess the success of the transformation process (Ahmadpoor et al., 2022). The β-glucuronidase (GUS) reporter gene is commonly used to detect DNA integration in the plant genome, as it can be introduced into the host plant via Agrobacterium-mediated transformation (Banihashemi et al., 2020). However, the GUS assay is destructive, since the tested cells or tissues are destroyed during the reaction, preventing subsequent propagation or regeneration of the identified transgenic lines (Alcalde et al., 2022). This limitation is considered a major drawback of using GUS as a reporter gene. In contrast, the green fluorescent protein (GFP), derived from the jellyfish Aequorea victoria, is now recognized as the most widely used and advanced reporter gene in cellular and biochemical research. The key advantage of GFP is that it allows non-destructive, live visualization of transgenic tissues, enabling their recovery and propagation (Boccia et al., 2022).

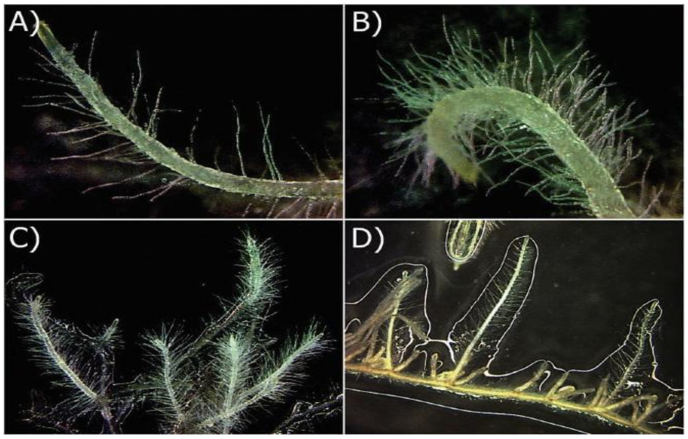

Figure 1. Phenotypic features of hairy root systems. A), B), and C) hairy roots; D) non-transgenic roots (Villar-Martínez et al., 2023).

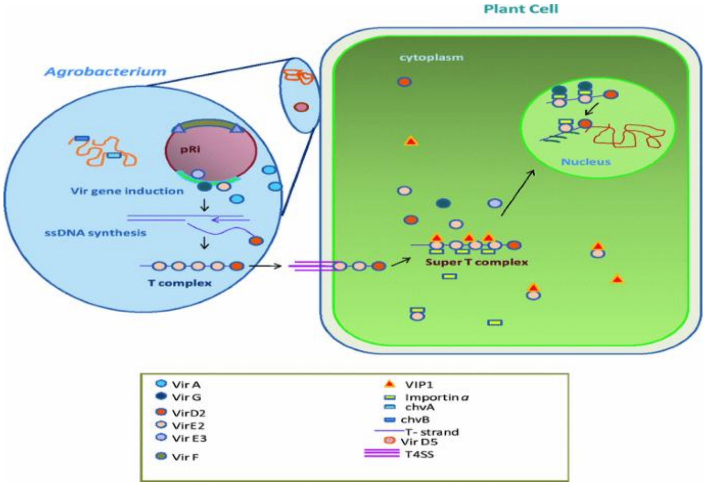

Figure 2. Overview of *Agrobacterium*-mediated plant cell transformation (Chandra, 2012).

Regeneration of plants from Ri plasmid-transformed hairy roots has been reported in numerous plant species. Morphologically, Ri-transformed plants are characterized by dwarfism, reduced stem length and internode elongation, and an increased number of nodes and leaves (Aghaali et al., 2024). The hairy root syndrome in these plants results from the integration and expression of rol genes located in the TL-DNA of the Ri plasmid. Among these, rolB and rolC genes are known as plast genes due to their ability to alter the growth and development of regenerated plants derived from hairy roots. The biosynthesis of secondary metabolites is not always confined to roots or underground tissues. In many plants, precursors of medicinal compounds are produced in the roots, while the final products are synthesized in the leaves or aerial parts. In the ornamental plant industry, Ri-transformed plants have attracted increasing interest due to specific morphological traits, including dwarfing, enhanced branching, leaf curling, reduced apical dominance, and increased root growth, all of which are associated with the Ri phenotype (Kumar et al., 2025).

Table 2. Induction of hairy roots by diverse *Agrobacterium rhizogenes* strains in multiple plant explants.

Species	Explant	Strains	Reference
Salvia apiana	Hypocotyl	A4, LBA9402	Krol et al., 2025
Urena lobata L.	Leaves	ATCC 15834	Cao et al., 2024
Atropa komarovii	Leaves	ATCC 15834	Banihashemin et al., 2020
Rhodiola rosea	Leaves, stems, and rhizomes	ATCC43057	Martínez et al., 2020
Salvia bulleyana	Leaves and shoots	A4	Wojciechowska et al., 2020
Trigonella foenum-graecum	Hypocotyl	ATCC15834, R1000, A4 and C58	Zolfaghari et al., 2020
Gentiana urticulosa	Shoots	A4	Vinterhalter et al., 2019
Dracocephalum kotschyi	Leaves	ATCC 15834	Nourozi et al., 2019
Lemna minor	Leaves and root tips	MSU 440 harboring pBIN-YFP	Kanchanamala et al., 2019

As evident from the compiled studies, leaf and hypocotyl explants, along with strains like ATCC 15834 and A4, are among the most frequently and successfully utilized combinations for hairy root induction.

Agrobacterium rhizogenes as a Tool for Metabolite Engineering

Agrobacterium rhizogenes is widely used as an efficient biotechnological tool for enhancing secondary metabolite biosynthesis. Strains of this bacterium harbor a root-inducing (Ri) plasmid containing a segment of transferred DNA (T-DNA) that carries genes essential for initiating and sustaining root differentiation. Among these, the *rol* gene family plays a central role in root morphogenesis and the biosynthesis of bacterial-specific metabolites known as opines (Martínez et al., 2020). Extensive research has demonstrated that only a small subset of open reading frames (ORFs) within the T-DNA is indispensable for hairy root induction, proliferation, and the characteristic hairy root phenotype. Based on the features of their Ri plasmids, *A. rhizogenes* strains are classified into several types, including agropine, mannopine, octopine, and cucumopine (Ying et al., 2023).

Agropine-type strains possess Ri plasmids in which the T-DNA is divided into two functional regions: left T-DNA (TL-DNA) and right T-DNA (TR-DNA). TL-DNA contains the *rol* genes responsible for inducing hairy root formation, while TR-DNA carries genes associated with auxin metabolism and opine biosynthesis (Martínez et al., 2020). Deletions may occur near either T-DNA border during transfer, complicating precise identification of the integrated region (Phuong et al., 2028). Importantly, although TL-DNA and TR-DNA can independently integrate into the plant genome, TL-DNA incorporation is essential to initiate hairy root development. Opines are low-molecular-weight compounds formed by the condensation of amino-containing molecules with keto acids or sugars. Once produced in transformed tissues, they create a specialized biochemical niche that supports selective bacterial metabolism and growth (Zolfaghari et al., 2020). Growing evidence of bioactive compounds responsible for the therapeutic properties of medicinal plants has intensified efforts to explore new plant species and develop strategies to enhance the accumulation of valuable phytochemicals (Wojciechowska et al., 2020).

Hairy root cultures provide a rapid, genetically stable, and hormone-independent source of biomass, making them an effective system for producing metabolites naturally synthesized and stored in root tissues (Malarz et al., 2023). The transformation process itself can reprogram plant metabolism, sometimes inducing the production of compounds absent in non-transformed roots. Notably, each hairy root clone originates from an independent transformation event, enabling the establishment of multiple transgenic lines, each with potentially distinct metabolic profiles (Liu et al., 2025).

Secondary Metabolites: Valuable Bioactive Compounds

Secondary metabolites (SMs) are small organic molecules produced by plants through specialized biosynthetic pathways. Although they are not directly required for basic growth and development, these compounds play essential roles in plant adaptation and survival under environmental stresses such as pathogen attack, UV radiation, and oxidative stress (Abdelazeez et al., 2022). Owing to their structural diversity and derivation from multiple branches of primary metabolism, secondary metabolites encompass major classes, including terpenes, phenolics, nitrogen-containing compounds (e.g., alkaloids), and glycosides. These metabolites are synthesized and accumulated in specific tissues or organs depending on the plant species, developmental stage, and environmental cues (Chandran et al., 2020).

Substantial evidence indicates that the health-promoting properties of plant-derived secondary metabolic products are largely attributed to their antioxidant, anti-inflammatory, antimicrobial, and anticancer activities. Higher plants produce a broad spectrum of these bioactive compounds, including alkaloids, quinones, flavonoids, steroids, lignans, and terpenoids, which are widely used in pharmaceuticals, crop protection agents, cosmetics, flavorings, natural dyes, and food additives (Singh et al., 2022). Despite their biological and industrial significance, the mass production of these valuable compounds remains challenging due to several constraints:

- Environmental dependence: Their synthesis is tightly regulated by abiotic and biotic signals.
- Low natural abundance: Many high-value metabolites occur at very low concentrations in wild plants.
- Complex biosynthetic pathways: Production often requires multi-step enzymatic reactions.
- Threats to natural populations: Overexploitation of medicinal plants may endanger their survival (Wojciechowska et al., 2020).

In hairy root cultures, the accumulation of secondary metabolites is governed by two fundamental phases: biomass growth and metabolite biosynthesis. The first phase, biomass accumulation, directly affects the overall yield and is largely influenced by factors controlling cell and organ proliferation. The second phase involves the biosynthesis and accumulation of secondary metabolic products within the biomass, which is regulated by various physiological and biochemical parameters affecting secondary metabolic pathways (Zolfaghari et al., 2020). Hairy root cultures have been successfully employed for the enhanced production of numerous high-value secondary metabolites. Notable examples include tropane alkaloids in Atropa and Datura species (Banihashemi et al., 2020), diosgenin (a precursor for steroid hormones) in Trigonella foenum-graecum (Zolfaghari et al., 2020), rosmarinic acid (a potent antioxidant) in Salvia species (Krol et al., 2025), and paclitaxel (a chemotherapeutic agent) in Taxus (Sykłowska-Baranek et al., 2022) cultures. The capacity of hairy roots to produce these compounds at levels comparable to or exceeding those of intact plants, especially when optimized with elicitors or metabolic engineering, underscores their practical and commercial relevance.

Technological Strategies for Sustainable Production

Several technological strategies have been developed to enable the sustainable, large-scale production of pharmacologically important bioactive compounds using hairy root cultures. These approaches include optimization of culture media, supplementation with pathway precursors, elicitor treatments, membrane permeabilization, and metabolic engineering (Sykłowska-Baranek et al., 2022).

Elicitors are generally categorized as abiotic or biotic. Abiotic elicitors mainly include mineral salts and physical stimuli, whereas biotic elicitors encompass plant-derived signaling molecules—such as methyl jasmonate (MJ), salicylic acid (SA), and ethylene-releasing compounds—as well as microbial components, including chitosan, yeast or bacterial polysaccharides, mycelial extracts, and glycoconjugates. The regulatory effects of SA and MJ on hairy root development are illustrated in Figure 3. Certain amino acids, such as phenylalanine or cysteine, can also serve as metabolic precursors to enhance specific biosynthetic pathways (Vergara-Martínez et al., 2021).

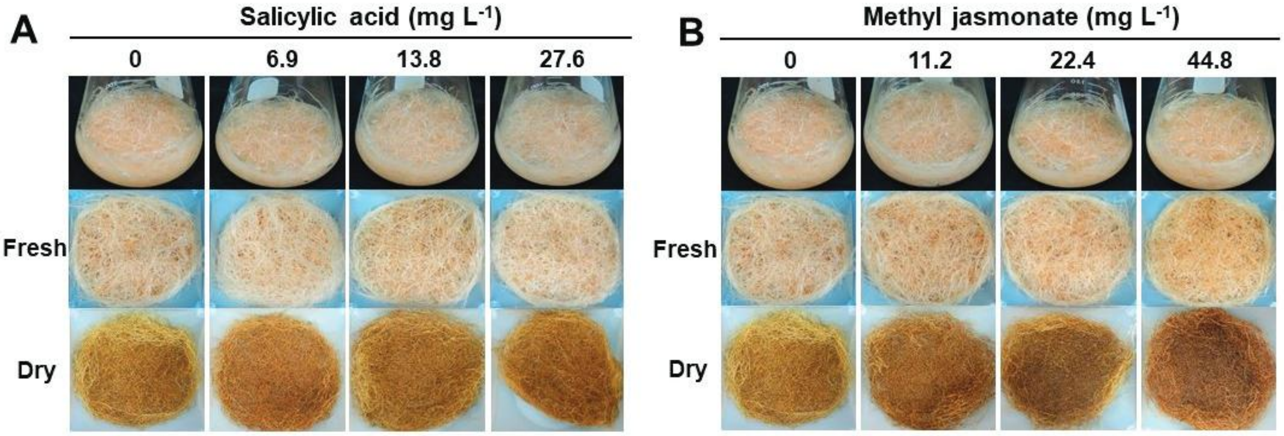

Figure 3. Regulatory effects of SA and MJ on hairy root development in *A. macrocephala* (Paek and Murthy, 2024).

In most plant tissue culture systems, secondary metabolites are retained within the cells, with only trace amounts secreted into the culture medium. This intracellular accumulation complicates downstream processing, as product extraction typically requires cell lysis, resulting in biomass loss and preventing continuous production (Quang et al., 2022). Therefore, strategies that enable metabolite secretion while maintaining cell viability are both technically and economically essential (Alcalde et al., 2022).

Permeabilization is an effective method to increase cellular permeability, facilitating the diffusion of intracellular metabolites into the culture medium. This can be achieved through physical treatments, such as heat shock, osmotic shock, ultrasound, or microwave irradiation, or via chemical agents, including surfactants and organic solvents. Chemical permeabilizers are widely preferred due to their operational simplicity and high efficiency (Quang et al., 2022). Compounds such as dimethyl sulfoxide (DMSO), Tween-20/80, Triton X-100, polyethylene glycol, and unsaturated fatty acids induce temporary changes in membrane structure, promoting metabolite release (Boitel-Conti et al., 1995). In some cases, low-polarity solvents like hexane or isooctane are applied to continuously extract secreted molecules (Liu et al., 2025). The effect of these agents depends on concentration and exposure time: low concentrations generally induce reversible increases in permeability, whereas high concentrations can cause irreversible membrane damage and cell death. An especially effective approach combines permeabilization with adsorbent resins, such as Amberlite XAD-2 or XAD-7. These resins capture secreted metabolites, simplify product recovery, and prevent the accumulation of toxic compounds in the medium (Kamiński et al., 2024).

The major advantage of controlled permeabilization is the potential for continuous metabolite production without destroying biomass. Secretion also alleviates feedback inhibition from intracellular product accumulation, often enhancing overall yields. Nevertheless, the selection of permeabilizing agents requires careful evaluation of toxicity, stability, and biocompatibility, as well as assessment of long-term effects on cell viability before industrial application (Liu et al., 2025). In summary, controlled membrane permeabilization, especially when integrated with stabilization techniques and adsorbent phases, represents a key strategy for establishing efficient and sustainable bioprocesses for bioactive compound biosynthesis in plant cell and hairy root cultures (Kamiński et al., 2024).

Bioreactor-Based Cultivation

Large-scale in vitro cultivation of plant cells and hairy roots has emerged as a practical alternative for producing a wide range of high-value, low-volume phytochemicals. Consequently, over the past two decades, significant efforts have been devoted to designing plant-specific bioreactors (Jin, 2025).

Bioreactors—typically made of glass or stainless steel—are vessels in which living cells are cultured. Ideally, bioreactors are equipped with sensors to monitor pH, temperature, and dissolved oxygen, and allow sterile sampling, fresh medium addition, pH adjustment, aeration, agitation, and temperature control (Murthy et al., 2023). Compared with flask cultures, bioreactors provide more precise control and monitoring of culture conditions (Jin, 2025). Although basic requirements for plant cell cultures are similar to those for microbial submerged cultures, fermenters designed for microbial cells are generally unsuitable for plant cells due to substantial differences in growth characteristics. Therefore, modifications are applied to achieve more effective plant cell growth (Kim et al., 2003).

Efficient mixing of plant cells at a large scale is critical to maintain uniform physiological conditions within the culture (Kim et al., 2003). Proper mixing improves nutrient and gas transfer and facilitates oxygen delivery by breaking and dispersing air bubbles. While plant cells are mechanically stronger than microbial cells, their large size, thick cell walls, and large vacuoles make them sensitive to shear stress, limiting the use of high agitation rates (Jin, 2025). Thus, plant cells are typically agitated at very low speeds in modified stirred-tank bioreactors. Air-lift bioreactors may provide more homogeneous conditions with lower shear stress compared to stirred-tank systems (Kim et al., 2003).

All plant cells are aerobic and require a continuous oxygen supply. However, plant cells consume oxygen at a slower rate than microorganisms due to slower metabolism, and high oxygen concentrations may even be toxic. Air is usually sparged from the base of the bioreactor (Kim et al., 2003).

Hairy roots in liquid cultures tend to form aggregates. In late exponential growth, increased polysaccharide secretion makes roots sticky, causing adhesion to reactor walls, sensors, and impellers, eventually forming large clumps. These aggregates hinder mixing, create stagnant zones, interfere with sensor performance, and may block inlet and outlet ports. Aggregation can negatively affect growth and metabolite production, but some degree of aggregation and cellular differentiation is necessary for secondary metabolite synthesis. Measuring culture conductivity is used as an indirect method to estimate biomass growth (Murthy et al., 2023). Therefore, controlling hairy root aggregation is a major process engineering consideration (Chandran et al., 2020).

Currently, bioreactors used for transformed roots are broadly classified into two types: liquid-phase (immersed) systems, where roots are fully submerged, and gas-phase systems, where roots are primarily exposed to air or gas mixtures. Researchers have successfully optimized secondary metabolite production in various reactor configurations. The selection of a bioreactor design depends on whether the target metabolite is intracellular or extracellular. Based on mixing type, the main reactor types for plant cultures include stirred-tank, bubble-column, air-lift, and rotating-drum reactors (Jin, 2025) (Figure 4).

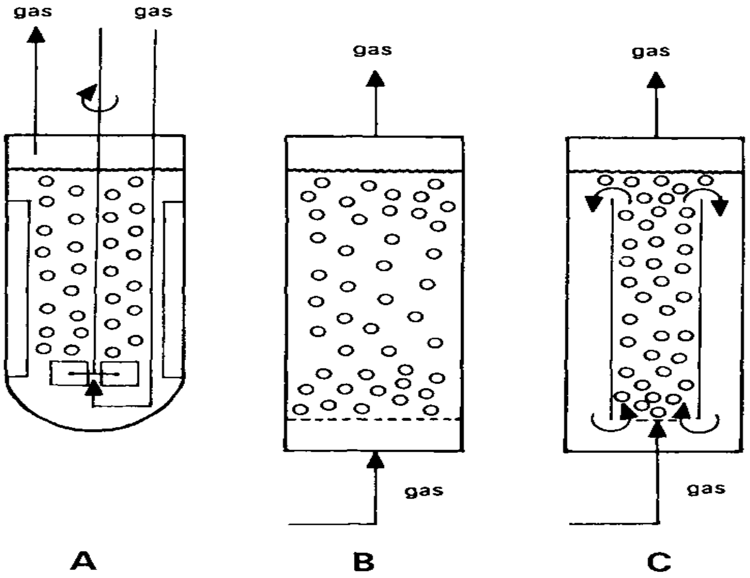

Figure 4. Major bioreactor configurations for large-scale hairy root production (Paek and Murthy, 2024).

Stirred-Tank Bioreactors

The stirred-tank reactor (Figure 4. A) represents the classical aerobic system, where air is dispersed by mechanical agitation. Its hydrodynamics are well-studied, allowing precise control of temperature, dissolved oxygen, pH, and nutrient concentrations (Jin, 2025).

A major disadvantage of STRs is shear stress generated by impellers, which can damage plant tissues (Murthy et al., 2023). Most laboratory and industrial STRs have been adapted for plant cultures by reducing agitation speeds (50–150 rpm), replacing Rushton blades with marine or paddle impellers, removing baffles, eliminating unnecessary sensors, and enlarging sampling ports (~1 cm) to prevent clogging by root aggregates. Recent studies indicate hairy roots can tolerate shear rates up to ~1000 s^{-1}, much higher than previously assumed. STRs also require high energy, are complex to construct, and can be challenging to scale up (Kim et al., 2003).

Bubble-Column Bioreactors

Bubble-column reactors (Figure 4. B) are among the simplest gas–liquid systems, used for aerobic cultivation (Jin, 2025). Air is injected at the base and dispersed pneumatically through nozzles or perforated plates. In small-scale reactors (<1 L), inadequate mixing can reduce growth rates. Advantages include easy sterilization due to the absence of moving parts, high mass and heat transfer without mechanical energy, suitability for shear-sensitive cells, scalability, and low maintenance. Limitations include poorly defined flow patterns and uneven mixing (Kim et al., 2003).

Air-Lift Bioreactors

Air-lift reactors circulate culture medium using density differences created by rising air bubbles (Jin, 2025). Injected air reduces medium density, causing upward flow in the riser tube, generating circulation (Figure 4. C). Air-lift reactors provide more uniform mixing than bubble-column systems, low shear stress, efficient oxygen transfer, absence of moving parts, reduced contamination risk, and lower operating costs. However, at high biomass densities, stagnant zones may form (Kim et al., 2003).

Conclusion

Hairy root in vitro cultures have emerged as a powerful biotechnological approach for the production of secondary metabolites and have been extensively investigated over recent decades. Many plant-derived compounds exhibit potent bioactivities, including cytotoxic, antimicrobial, antifungal, anti-inflammatory, and antiviral effects. Secondary metabolites, however, are often confined to specific tissues or developmental stages, making their extraction from natural sources challenging and inefficient. Hairy root cultures offer an attractive alternative, providing a genetically stable, fast-growing, and hormone-independent system capable of producing high accumulation of root-specific metabolites under controlled conditions.

Transformation mediated by *Agrobacterium rhizogenes* can also induce the biosynthesis of novel secondary metabolic products absent in non-transformed tissues. Since each hairy root line originates from an independent transformation event, substantial metabolic diversity can be achieved among lines. Extraction yields from hairy root cultures can match or even surpass those of field-grown plants, particularly when combined with strategies such as elicitation. Hairy roots also enable the regeneration of whole plants, which may display altered morphological traits that can have ornamental value. Despite these advantages, the industrial-scale implementation of hairy root cultures remains limited due to challenges in bioreactor design, large-scale productivity, and process scalability. Nevertheless, this technology remains a highly promising approach for producing metabolites primarily synthesized in roots. Whereas many medicinal plants require 10–15 years to reach a metabolically active stage, hairy root cultures can achieve comparable biomass and metabolite accumulation within months. A SWOT analysis of hairy root culture technology reveals its distinct profile for industrial application, including strengths **(**Rapid growth, genetic stability, hormone-independent culture, ability to produce novel metabolites), weaknesses (Shear sensitivity, biomass aggregation, challenges in *Agrobacterium* elimination, complex downstream processing), opportunities (Integration with synthetic biology, advanced bioreactor designs, market demand for plant-based pharmaceuticals), and threats (Competition from microbial fermentation and synthetic biology platforms, regulatory hurdles, high initial investment costs). Consequently, this platform holds considerable potential for pharmaceutical, agricultural, and food applications and is expected to gain broader commercial adoption in the near future.

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Gene Network Modeling and Promoter Motif Analysis of Hub Genes in Skeletal Muscles of Young Bulls Treated with Steroid Growth Promoters

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ABSTRACT

Skeletal muscle growth, regulated by complex genetic and hormonal factors, is crucial for livestock productivity. This study identified key hub genes and promoter elements in the skeletal muscle of young bulls treated with dexamethasone (± 17β-estradiol) using the GEO dataset GSE12179. Differentially expressed genes were identified, and a protein-protein interaction network was analyzed using Cytoscape, revealing 11 hub genes as dexamethasone-responsive molecular targets. Promoter motif analysis via MEME Suite identified conserved elements potentially regulated by transcription factors such as SP2, ZEB1, and TFAP2C. Functional enrichment showed these hubs genes are involved in IGF-1/Akt/mTOR and TGF-β/Smad signaling, extracellular matrix remodeling, and ribosome biogenesis. Although glucocorticoid treatments are widely used, these findings suggest that genetic-based approaches such as genomic selection and marker-assisted breeding may provide realistic alternatives for enhancing muscle development. These findings provide novel insights into the molecular mechanisms underlying dexamethasone-associated muscle transcriptional responses and offer a framework for improving livestock traits through targeted genetic strategies rather than hormonal interventions.

Keywords: Skeletal muscle growth, Dexamethasone, Hub genes, Promoter motif analysis.

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Introduction

Body growth and size, particularly skeletal muscle mass, are critical traits in domestic animals because they are directly associated with productivity, meat quality, and economic profitability in the livestock industry. Consequently, numerous programs have been implemented to improve growth rate and body composition in farm animals, including selective breeding, targeted nutrition, and environmental optimization (Devlin et al., 2009).

In addition to these approaches, the use of bioactive compounds such as growth hormones has emerged as an effective tool for stimulating anabolic pathways and increasing muscle mass. In this context, steroid growth promoters, both natural and synthetic, play a key role in regulating gene expression and growth-related signaling pathways (Gharahdaghi et al., 2021). Although the use of these compounds is permitted in some countries, it is entirely prohibited in the European Union due to food safety and ethical considerations (Combes and Balls, 2007). Nonetheless, reports of illegal application of these compounds in the livestock industry, particularly in beef cattle, continue to raise concerns regarding public health and production chain monitoring (Nebbia et al., 2011).

Investigating the molecular effects of these compounds can provide deeper insights into the biological mechanisms and regulatory pathways influencing muscle growth. In this regard, gene network modeling, as a systems biology approach, enables the identification of key or hub genes that play central roles in biological interactions and regulatory networks associated with muscle growth. These hub genes are typically located at the core of protein–protein interaction networks and their expression is directly influenced by intracellular signals and environmental factors (Zhang et al., 2023). Identifying such central genes can enhance our understanding of the molecular mechanisms controlling growth and the response to hormonal stimulants.

Promoter motif analysis of hub genes is a useful approach for identifying conserved DNA motifs and transcription factors involved in gene regulation under different physiological and treatment conditions. This approach facilitates the exploration of upstream transcriptional regulatory mechanisms and helps reveal shared regulatory patterns associated with steroid exposure. Dexamethasone (Dex), a synthetic glucocorticoid, has been reported to influence skeletal muscle gene expression and, when administered at subtherapeutic doses, has occasionally been used illegally as a growth promoter in beef cattle (Nebbia et al., 2011).

Although several studies have investigated the effects of steroid compounds on gene expression and muscle physiology, an integrated analysis combining protein–protein interaction (PPI) networks of differentially expressed genes with promoter motif analysis of hub genes in response to Dex treatment has not yet been comprehensively explored, particularly in young bulls. In the present study, we analyzed hub genes identified in the skeletal muscle of young bulls treated with Dex and investigated their promoter regions to identify common regulatory motifs and potential transcription factors using network-based analysis and motif discovery approaches. This study aims to improve understanding of the transcriptional regulatory mechanisms associated with Dex-induced responses in bovine skeletal muscle and to highlight key genes involved in these processes.

Materials and Methods

To identify differentially expressed genes (DEGs) associated with muscle growth in response to glucocorticoid treatment, gene expression data were retrieved from the Gene Expression Omnibus (GEO) database hosted by NCBI (https://www.ncbi.nlm.nih.gov/gds), a widely used resource for storing and accessing genomic datasets in bioinformatics studies. In this study, the microarray dataset GSE12179 was selected which was originally generated and published by Carraro et al., (20009), and examines gene expression in skeletal muscle of young bulls treated with dexamethasone (Dex) and a combination of dexamethasone with 17β-estradiol (Estr) at sub-therapeutic doses, compared to a control group (untreated). Differential expression analysis was performed using the GEO2R tool, based on the limma package. To correct for multiple testing, the Benjamini–Hochberg method was applied. Genes with $|\log_2 FC| > 1$ and $P\text{-value} < 0.01$ were considered significantly differentially expressed.

The identified DEGs were further subjected to functional annotation and biological clustering using the DAVID Bioinformatics Resources (version 6.8). Functional enrichment analysis included Gene Ontology (GO) categories for Biological Process and Molecular Function, as well as KEGG pathways, with a significance threshold of $P\text{-value} < 0.05$. Subsequently, to explore interactions among genes related to muscle growth and development, a protein–protein interaction (PPI) network was constructed using data from STRING v10 (http://string-db.org), with a minimum interaction confidence score of 0.15 (low confidence). A low confidence threshold was applied to prevent the exclusion of potential interactions and to maintain network comprehensiveness, as commonly practiced in exploratory network analyses. The extracted interactions were imported into Cytoscape v3.9.1 as TSV files to construct the PPI network. To identify hub genes, the CytoHubba plugin v0.1 was used. Four computational algorithms Maximal Clique Centrality (MCC), Degree, Density of Maximum Neighborhood Component (DMNC), and Maximum Neighborhood Component (MNC) were employed to rank genes and select hub genes in the PPI subnetwork. These algorithms provide complementary analytical approaches, reducing bias that may arise from using a single method. Specifically, MNC and DMNC are based on the cluster structure of the network, Degree relies on the number of edges connected to each node, and MCC evaluates shortest paths between nodes (Elasbali et al., 2024). For promoter motif analysis of hub genes, the 1,000 bp upstream flanking regions (UFRs) of the selected genes were retrieved from Ensembl. The MEME Suite v5.4.1 (https://meme.nbcr.net) was used to identify regulatory motifs. In MEME analysis, up to 10 motifs of 6–50 nucleotides were identified under the Zero or One Occurrence per Sequence (ZOOPS) model, with a significance threshold of P-value < 0.01; all other parameters were set to default. Identified motifs were further analyzed using Tomtom (http://meme-suite.org/tools/tomtom) to match them against known motif databases and remove redundant patterns. Additionally, GOMo (http://meme-suite.org/tools/gomo) was employed to predict the biological functions of motifs and assess their potential roles in regulating genes responsive to steroid treatment.

Results

Analysis of gene expression profiles between young bulls treated with dexamethasone (± 17β-estradiol) and the control group revealed a substantial number of genes exhibiting significant differential expression. In total, 445 genes were upregulated ($|\log_2 FC| > 1$, P-value < 0.01) and 580 genes were downregulated in the treated group compared to controls. These expression changes indicate the broad effects of glucocorticoid treatment (dexamethasone) on gene regulation in bovine muscle tissue.

To identify key genes within the protein–protein interaction (PPI) network, network analysis was performed using the CytoHubba plugin. Based on the results from four algorithms MNC, DMNC, Degree, and MCC a total of 11 hub proteins with the highest levels of connectivity and central roles in the network were identified (Figure 1). The characteristics and biological functions of these hub proteins are summarized in Table 1.

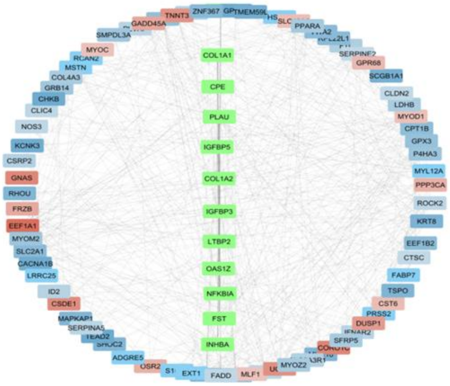


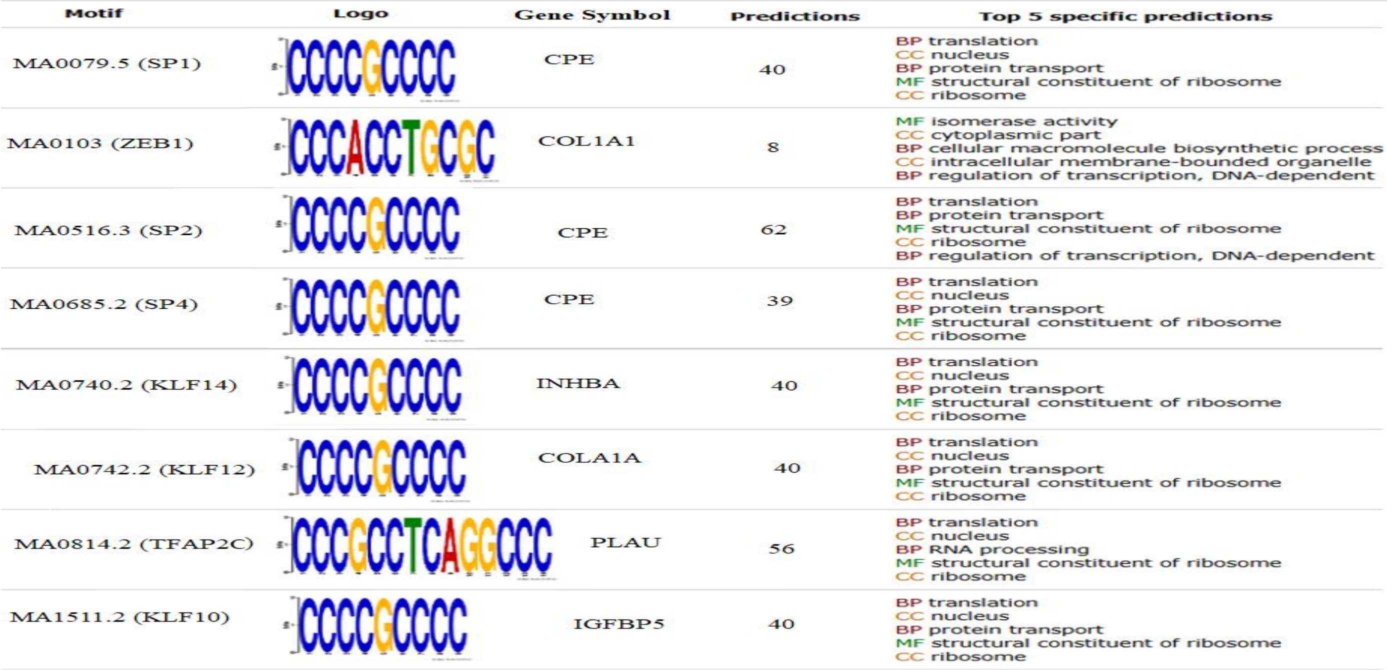
Figure 1. Gene co-expression network of hub genes in skeletal muscles of young bulls treated with steroid growth promoters.

In this study, to investigate the regulatory mechanisms of the identified hub genes and to identify potential transcriptional regulatory elements, the promoter regions of these genes were analyzed. Motif analysis revealed that among the promoter sequences of the hub genes, eight conserved motifs ranging from 8 to 62 base pairs in length were identified (Table 2). These motifs may represent potential transcription factor binding sites and could be associated with the regulation of genes responsive to dexamethasone, as well as biological processes related to muscle growth, development, and tissue remodeling. The identification of these conserved motifs suggests the presence of shared regulatory patterns among key genes in the protein–protein interaction network that may contribute to the transcriptional response to dexamethasone treatment.

Table 1. Hub genes identified in the gene co-expression network of skeletal muscles in young bulls treated with steroid growth promoters.

Rank	Gene symbol	Method	(|logFC| > 1	P-value < 0.01	Gene discription
1	OAS1Z	DMNC	2.155456	0.000607	ENSBTAG00070032649
1	NFKBIA	MNC	1.644063	2.99E-05	ENSBTAG00070002974
2,1	PLAU	DMNC- MCC	-1.03886	0.032873	ENSBTAG00070028590
4	FST	DMNC	-1.94367	0.004001	ENSBTAG00070019948
2	CPE	MNC	-3.76218	0.001029	ENSBTAG00070006277
3,1,4	IGFBP5	Degree-MNC-MCC	1.365756	0.015781	ENSBTAG00070001801
2	LTBP2	Degree	-1.46046	0.006171	ENSBTAG00070023359
1,1,2	INHBA	Degree-DMNC- MCC	-1.06888	0.011863	ENSBTAG00070016353
4,1	IGFBP3	DMNC- MCC	-1.26046	0.001539	ENSBTAG00070016065
1,2,4	COL1A1	Degree-MNC-MCC	2.465887	0.000668	ENSBTAG00070016905
3,2	COL1A2	Degree-MNC	1.818516	0.014556	ENSBTAG00070001070

Table 2. Promoter motifs and associated transcription factors identified in hub genes of skeletal muscles in young bulls treated with steroid growth promoters.



Motif	Logo	Gene Symbol	Predictions	Top 5 specific predictions
MA0079.5 (SP1)	CCCCGCCCC	CPE	40	BP translation CC nucleus BP protein transport MF structural constituent of ribosome CC ribosome
MA0103 (ZEB1)	CCCACCTGCGC	COL1A1	8	MF isomerase activity CC cytoplasmic part BP cellular macromolecule biosynthetic process CC intracellular membrane-bounded organelle BP regulation of transcription, DNA-dependent
MA0516.3 (SP2)	CCCCGCCCC	CPE	62	BP translation BP protein transport MF structural constituent of ribosome CC ribosome BP regulation of transcription, DNA-dependent
MA0685.2 (SP4)	CCCCGCCCC	CPE	39	BP translation CC nucleus BP protein transport MF structural constituent of ribosome CC ribosome
MA0740.2 (KLF14)	CCCCGCCCC	INHBA	40	BP translation CC nucleus BP protein transport MF structural constituent of ribosome CC ribosome
MA0742.2 (KLF12)	CCCCGCCCC	COLA1A	40	BP translation CC nucleus BP protein transport MF structural constituent of ribosome CC ribosome
MA0814.2 (TFAP2C)	CCCGCCTCAGGCCC	PLAU	56	BP translation CC nucleus BP RNA processing MF structural constituent of ribosome CC ribosome
MA1511.2 (KLF10)	CCCCGCCCC	IGFBP5	40	BP translation CC nucleus BP protein transport MF structural constituent of ribosome CC ribosome

Discussion

In this study, network analysis highlighted several hub genes associated with the transcriptional response to dexamethasone in bovine skeletal muscle. These findings suggest that the response to dexamethasone involves coordinated changes in gene regulatory networks, rather than the regulation of individual genes alone, potentially influencing processes related to muscle growth and tissue remodeling.

Given the increasing demand for improved production efficiency in the livestock industry, various hormonal compounds have been investigated for their effects on muscle growth and metabolism. Dexamethasone, a synthetic glucocorticoid, has been reported to influence muscle physiology and metabolic regulation in cattle (Vascellari et al., 2012). Unlike anabolic agents, dexamethasone primarily exerts catabolic effects, modulating gene expression pathways in skeletal muscle that are involved in protein turnover, tissue remodeling, and metabolic adaptation. Understanding the transcriptional networks responsive to dexamethasone may therefore provide insights into glucocorticoid-associated muscle regulation in bovine species.

In recent years, genomic and transcriptomic approaches have enabled the identification of key or hub genes within co-expression networks. These hub genes, acting as central nodes in regulatory networks, play pivotal roles in controlling cellular functions and thus represent valuable targets for investigating the molecular mechanisms underlying hormonal responses (Tian et al., 2024). In the present study, protein–protein interaction network analysis led to the identification of a set of hub genes associated with pathways involved in muscle growth, differentiation, and tissue remodeling, which are likely critical for mediating the physiological effects of dexamethasone in beef cattle.

Among these genes, IGFBP5 exhibited a significant upregulation ($\log_2 FC = 1.365756$) in response to dexamethasone treatment, highlighting its central role in regulating skeletal muscle growth in young bulls. Increased IGFBP5 expression may modulate the bioavailability of insulin-like growth factors (IGFs), fine-tuning the IGF-1/Akt/mTOR signaling pathway. By binding to IGF-1, IGFBP5 not only protects it from degradation but also spatially and temporally regulates its distribution in muscle tissue, thereby maintaining the balance between myoblast proliferation and differentiation (Sadkowski et al., 2009).

Furthermore, elevated IGFBP5 expression has been reported to enhance myogenic differentiation under certain conditions, promoting the maturation of muscle fibers a process essential for increasing functional muscle mass. Therefore, changes in IGFBP5 expression in response to dexamethasone likely reflect an adaptive regulation of the muscle gene network to steroid signals, optimizing muscle growth through the modulation of IGF activity (Foulstone et al., 2003). Consequently, IGFBP5 can be considered a key indirect transcriptional regulator in the dexamethasone response of bovine skeletal muscle, with its upregulation linked to enhanced myogenesis and increased muscle mass.

The INHBA gene, another key gene identified in this study, is a member of the TGF-β superfamily and plays a crucial role in regulating muscle cell proliferation and differentiation, tissue remodeling, and muscle homeostasis (Huang et al., 2024). The present results showed that the expression of this gene significantly decreased in response to dexamethasone treatment ($\log_2FC = -1.06888$). Reduced INHBA expression may lead to weakened Smad signaling, a pathway typically associated with inhibition of muscle growth and induction of anti-myogenic signals. This change may create a permissive environment for the activation of anabolic pathways and enhanced muscle growth. In this context, the relative suppression of INHBA may act as a regulatory mechanism that diminishes the inhibitory effects of the TGF-β pathway on myoblast differentiation, thereby facilitating increased muscle growth and hypertrophy. Previous evidence has shown that inhibition of TGF-β signaling can promote anabolic pathways and enhance the muscle response to growth factors (Huang et al., 2024). Therefore, the observed downregulation of INHBA in this study likely reflects a skeletal muscle gene network reprogramming toward reduced growth-inhibitory signaling. Overall, these findings suggest that dexamethasone not only promotes growth through the activation of growth pathways but also shifts the balance of cellular signaling toward increased muscle mass by selectively inhibiting anti-myogenic genes such as INHBA in young beef cattle.

In addition, the COL1A1 and COL1A2 genes, which play key roles in type I collagen synthesis and extracellular matrix (ECM) organization, were identified with significantly increased expression, showing $\log_2FC = 2.465887$ and 1.818516, respectively. The upregulation of these genes may indicate ECM remodeling and enhanced structural integrity of skeletal tissue in response to dexamethasone, a process essential for mechanical support of muscle growth. Since dysregulation of these genes is associated with disorders such as Osteogenesis Imperfecta, their upregulation is critical for maintaining skeletal tissue integrity (Rauch et al., 2020). It has also been reported that COL1A1 and COL1A2 expression is regulated by the TGF-β pathway, suggesting potential interactions between steroid signaling and ECM regulatory pathways (Kimoto et al., 2004).

Alongside these genes, PLAU, which plays an important role in ECM degradation, cell migration, and tissue remodeling, was identified with reduced expression in this study ($\log_2FC = -1.03886$). Decreased PLAU expression may indicate reduced proteolytic activity associated with ECM remodeling and consequently the stabilization of tissue structure during later stages of muscle and bone growth. Considering PLAU's role in facilitating tissue repair and early growth, its relative suppression may reflect a transition from active remodeling to structural stabilization in response to dexamethasone (Mahmood et al., 2018). Additionally, since PLAU is involved in regulating immune responses and interactions with viral agents such as PPRV, its downregulation may reflect steroid-mediated modulation of the immune response, indirectly contributing to tissue homeostasis and sustainable growth in livestock (Connolly et al., 2010; Wu et al., 2024).

Overall, these findings indicate that dexamethasone, by modulating key molecular pathways including IGF-1/Akt/mTOR, TGF-β/Smad, and ECM remodeling (through COL1A1/2 and PLAU), can influence skeletal muscle growth in beef cattle. These genes not only serve as molecular markers of the response to dexamethasone treatment but may also represent potential targets for improving growth efficiency and enhancing livestock health in the animal husbandry industry.

One of the key approaches to understanding the regulatory mechanisms of hub gene expression is the analysis of their promoter regions and the identification of regulatory motifs. Motifs are short, conserved DNA sequences that serve as binding sites for transcription factors and play a critical role in determining gene expression patterns. Identifying these motifs can provide a more precise understanding of the regulatory pathways activated by growth stimuli such as dexamethasone and reveal the key transcription factors involved in these processes (Bussemaker et al., 2000). For instance, studies have shown that specific motifs in promoter regions can interact with transcription factors that regulate gene expression in response to hormonal treatments (Wang et al., 2022).

The analysis of motifs identified in the promoter regions of hub genes indicates the involvement of key transcription factors in regulating muscle growth pathways in response to dexamethasone treatment. One of the most prominent motifs is MA0516.3, 62 base pairs in length, which is associated with the transcription factor SP2. Although direct studies on SP2's role in skeletal muscle are limited, its membership in the Sp/KLF family, which regulates multiple structural and metabolic genes, suggests that SP2 may play a role in controlling growth, differentiation, or muscle regeneration under growth-stimulating conditions such as dexamethasone treatment (Cai et al., 2024).

Another identified motif, MA0814.2, 56 base pairs long, is associated with the transcription factor TFAP2C. TFAP2C is involved in various cellular processes, including differentiation and hormonal responses, and has been shown to regulate the expression of estrogen receptor alpha (ERα) and other genes related to hormone signaling pathways. This suggests that TFAP2C may influence muscle growth and differentiation in response to steroid treatments such as dexamethasone (Woodfield et al., 2010). Although its direct role in skeletal muscle is not well-defined, its involvement in hormone signaling and cellular differentiation supports the idea that it may contribute to the regulation of muscle growth. For example, TFAP2C has been implicated in controlling genes critical for cell growth and differentiation, processes essential for muscle development (Zhang et al., 2024).

The motif MA0103.3, related to ZEB1, was also observed among the identified elements. ZEB1 acts as a regulator of the balance between differentiation and self-renewal in muscle cells, playing a key role in muscle growth dynamics and tissue regeneration (Elasbali et al., 2024). The presence of this factor in the promoters of hub genes may indicate precise regulation of muscle repair and remodeling pathways in response to injury or hormonal stimulation.

Beyond identifying transcription factors, GOMo analysis revealed that the identified motifs are associated with diverse biological functions, classified into the three main GO categories: Biological Process (BP), Molecular Function (MF), and Cellular Component (CC). Regarding biological processes, these motifs were associated with protein transport, macromolecular biosynthesis, and DNA-dependent transcription. From a molecular function perspective, these elements were linked to ribosomal structural activity and enzymatic functions such as isomerase activity. At the cellular component level, the nucleolus and ribosomes were identified as enriched sites in the GO analysis.

Protein transport plays a particularly crucial role in skeletal muscle growth regulation, especially in response to dexamethasone, as it not only mediates the trafficking of growth factors and hormone receptors but also supports the delivery of structural proteins to functional regions of the muscle (Biolo et al., 2002). The presence of this process in the transcriptomic data underscores that muscle growth in young bulls is directly influenced by the regulation of pathways related to protein transport and translation. Structural ribosomal activity, as an indicator of cellular protein synthesis capacity, is also increased under growth-stimulating treatments, reflecting the readiness of muscle cells to enter hypertrophy. This upregulation is likely mediated through the activation of signaling pathways such as mTOR and IGF-1(Yoshida and Delafontaine, 2020).

Finally, the enrichment of the nucleolus and ribosomes, as key components of protein synthesis, directly contributes to muscle hypertrophy. Increased nucleolar activity enhances ribosome biogenesis, providing the cellular machinery required to expand translational capacity in response to growth stimuli (Wen et al., 2016).

Conclusion

The results of this study demonstrate that dexamethasone, a synthetic glucocorticoid, induces broad transcriptional changes in skeletal muscle of young bulls. The identification of 11 hub genes through protein–protein interaction (PPI) network analysis underscores the importance of systems-level approaches for understanding the molecular responses triggered by glucocorticoid exposure. Promoter motif analysis further revealed conserved regulatory elements associated with transcription factors such as SP2, ZEB1, and TFAP2C, suggesting their potential involvement in coordinating muscle-related molecular responses to dexamethasone. Functional enrichment results highlighted key biological processes, including protein transport, ribosome biogenesis, and nucleolar activity, which may contribute to cellular adaptation under glucocorticoid influence. Collectively, these findings provide insight into the regulatory architecture underlying the glucocorticoid-responsive gene network in bovine skeletal muscle. Future studies should focus on experimental validation of the implicated transcription factors and on evaluating temporal gene expression dynamics following dexamethasone treatment.

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Effect of Gamma-Aminobutyric Acid (GABA) Pretreatment on Antioxidant Responses and Alleviation of Salinity Damage in *Prosopis koelziana* Seedlings

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ABSTRACT

Prosopis koelziana belongs to the Fabaceae family (formerly Mimosaceae) and grows in arid and semi-arid regions. It is used for the reclamation of dry forests with low fertility and high salt content. This study was conducted to investigate the effect of gamma-aminobutyric acid (GABA) at concentrations of 0.25 and 0.5 mM on enhancing salinity tolerance in *Prosopis koelziana* seedlings under 400 and 600 mM NaCl. The results showed that salinity stress induced oxidative stress, and malondialdehyde (MDA) content increased in both shoots and roots. Salinity also increased the activities of antioxidant enzymes, including catalase (CAT), guaiacol peroxidase (GPX), and ascorbate peroxidase (APX). However, pretreatment with GABA reduced MDA content and antioxidant enzyme activity. Under 400 and 600 mM salt stress, root sodium (Na^+) content increased by 55% and 62%, and shoot sodium content increased by 73% and 100%, respectively, compared to controls. GABA (0.25 and 0.5 mM) reduced sodium levels in both organs. Salt stress decreased potassium (K^+) content in roots and shoot, whereas GABA pretreatment increased potassium levels. The findings indicate that pretreating plants with GABA significantly reduced membrane lipid peroxidation and lowered the activity of antioxidant enzymes. This stress alleviation appears to be primarily due to reduced sodium uptake and transport into plant tissues, along with increased potassium content. Consequently, antioxidant enzyme activity decreased in GABA pretreated plants under stress conditions.

Keywords: Salinity stress, Gamma Amino-butyric Acid, *Prosopis koelziana,* Antioxidant enzymes

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Introduction

Salinity is a major limiting factor for plant growth and one of the most significant environmental stresses. It is caused by an excessive amount of salt due to high evapotranspiration and a lack of quality water in arid and semi-arid regions (Ahmed and Khalid, 2023). Salty, alkaline soils typically form in these areas, which disrupt plant growth. Salt stress leads to various problems for plants, primarily resulting from ionic toxicity and osmotic stress (Ali et al., 2023).

Prosopis koelziana is widely used in the restoration of deserts, forests, and degraded soils characterized by low fertility and high salinity (Figure 1). Native to Iran, this species naturally grows in arid and desert regions, where it withstands low rainfall and limited water availability (Bhojvaid and Timmer 1998). Given its native status and its diverse economic and ecological benefits, Iranian Prosopis species represent the optimal choice for conserving biodiversity and protecting the environment in the country's arid and desert areas. Prosopis koelziana is a hardy plant that thrives in dry and saline conditions, making a significant contribution to the improvement and conservation of soil and water resources. Its key roles within its natural habitats include enhancing water supply, preserving biodiversity, protecting soil, and providing positive economic and social impacts on local communities (Zare and Tavili 2011; Emtahani and Elmi 2006; Saadatfar et al., 2023; Mousavi et al., 2025). The preservation and restoration of Prosopis koelziana are crucial for maintaining the natural balance and sustainability of the environment. For this reason, its application in soil stabilization projects is particularly valuable in sandy soil areas, such as deserts (Mousavi et al., 2025; Saadatfar et al., 2023). This approach contributes significantly to sustainable development and effective natural resource management programs.

Figure 1. Image of the Prosopis koelziana plant and the morphology of its leaves.

GABA is a four-carbon (C4) non-protein amino acid having a chemical formula of $C_4H_9NO_2$ and is an important component of free amino acids (Beuve et al., 2004). In plants and animals, GABA is mainly metabolized through a short pathway called GABA shunt, which is able to bypass the two steps of Krebs cycle (Bown et al., 2006). The Shunt pathway consists of the cytosolic Glutamate Decarboxylase (GAD) and mitochondrial enzymes such as Gaba Trans Aminase (GAD) and Succinic Semi Aldehyde Dehydrogenase (SSADH) (Kinnersley and Lin, 2010). This compound has metabolic effects such as, protection against oxidative stress, osmotic regulation, and protection against insects, which is found in Arabidopsis, for instance (Bown et al., 2006; Islam et al., 2024). The impact of the exogenous application of GABA on the increment of plant tolerance to biological and non-biological stresses has been reported in certain studies (Alen et al., 2014). As an example, it has been reported that GABA increases tolerance of peach fruit against the cold stress (Yang et al., 2011) and the tomato plant under the salt stress (Zhang et al., 2011). Furthermore, the exogenous application of GABA has led to an increase in activity of antioxidant enzymes such as catalase and ascorbate peroxidase, and non-enzymatic antioxidants namely, ascorbate and glutathione in rice plant at high temperature (Nayyar et al., 2014). Moreover, it has been reported that GABA retains the osmotic potential of plants exposed to osmotic stress and prevents the disruption of normal cell activities (Xia et al., 2011). Also, some reports show the effect of exogenous application of GABA on ions absorption (Xing et al., 2007). Given the importance of conserving plant species such as Prosopis koelziana and preventing their extinction, scientific studies are essential to enhance seedling resistance and facilitate the successful transfer of resilient seedlings to natural conditions. Due to the role of gamma-aminobutyric acid (GABA) in increasing tolerance to environmental stresses such as salinity, this study aimed to investigate the potential use of GABA to improve stress tolerance in Prosopis koelziana seedlings. By applying this compound during the early stages of plant development, it may be possible to produce seedlings capable of withstanding salinity and being successfully established in saline environments.

Materials and methods

The seeds used for this experiment were obtained from native Prosopis koelziana plants in Shahdad (Estehkam), located close to the Dasht-E-Lut desert in the Kerman province of Iran (Herbarium number: MIR-4752). This area is not protected, so seed collection is allowed. A small quantity of seeds from this plant was collected for research purposes. The seeds were treated with sulfuric acid for 2 minutes and washed three times with distilled water and soaked in distilled water for 24 hours. After this treatment, the seeds were germinated in petri dishes at 27°C for 24 hours before being planted into plastic pots filled with sand, which had been washed with distilled water prior to use. The seedlings were irrigated daily with half-strength Hoagland solution. After 10 days of growth under a 16/8-hour light/dark photoperiod at 27 °C and 40% humidity, uniform seedlings were selected for treatment. The plants were divided into three groups, each with six replicates (pot) (four plants per pot). Two groups were pretreated with 0.25 mM and 0.5 mM GABA (20 ml of solution per pot), while the control group received only distilled water for five days. On the sixth day, one set of plants that had been treated with GABA solutions and distilled water was exposed to either 400 mM or 600 mM NaCl for six days, while the other set was treated with distilled water only. These NaCl concentrations were optimized in a preliminary experiment. After six days of treatment, the shoots and roots of the plants were harvested, immediately frozen in liquid nitrogen, and stored at -80 for future analysis.

This study was conducted as a completely randomized design (CRD) with three replications. Statistical analyses were performed using SPSS version 23. Treatment effects were evaluated using analysis of variance (ANOVA), and mean comparisons were carried out using Duncan's multiple range test at a 95% confidence level. Graphs were prepared using Microsoft Excel 2019.

Lipid peroxidation

The level of lipid peroxidation, as an indicator of cellular damage, was measured in terms of malondialdehyde (MDA) content according to Heath and Packer (1968). Shoot and root samples (0.1g) were homogenized in 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 4000 × g for 10 min. The supernatant (0.5 ml) was mixed with 1.5 ml of 20% (w/v) TCA containing 0.5% (w/v) 2-thiobarbituricacid (TBA). The mixtures were heated at 95 °C for 30 min and then quickly cooled in an ice bath. The mixtures were centrifuged at 10000 × g for 5 min and their supernatant absorbance was measured at 532nm. The value of non-specific absorption at 600 nm was subtracted from the 532 nm reading. The MDA content was calculated using the Lambert-Beer law, with extinction coefficient of 155 $mM^{-1}cm^{-1}$ and expressed as micromole MDA per g fresh weight.

Enzyme extraction and activity determination

Shoot and root tissues (500 mg) were homogenized in 50 mM potassium phosphate buffer (pH 7.0) containing 1% soluble PVP and 1 mM EDTA. The homogenate was centrifuged at 20,000 × g for 20 min and the supernatant used for assay of the activity of enzymes.

Catalase (CAT) activity (EC 1.11.1.6)

Catalase activity was assayed by measuring the initial rate of H_2O_2 disappearance at 240nm using the extinction coefficient of 40 mM^{-1} cm^{-1} for H_2O_2 (Dhindsa et al., 1981). The enzyme activity was reported as U per milligram of protein (equivalent to 1 μmol of H_2O_2 reduction per minute per milligram of protein).

Guaiacol peroxidase (GPX) activity (EC1.11.1.7)

The GPX activity was determined using the method of Plewa et al., (1999). The oxidation of guaiacol (formation of tetraguaiacol) was assessed by measuring the absorbance at 470 nm one minute after the addition of H_2O_2. The extinction coefficient of tetraguaiacol (25.5 mM^{-1} cm^{-1}) was applied for unit determination. Enzyme activity was expressed as units of enzyme activity per milligram of protein content.

Ascorbate peroxidase (APX) activity (EC 1.11.1.11)

Ascorbate peroxidase was determined spectrophotometrically according to the oxidation of ASA. The reaction solution contained 50mM potassium phosphate buffer (pH 7.0), 0.5mM ascorbate, 0.1mM H_2O_2 and 150μl enzyme extract. H_2O_2-dependent oxidation of ASA was followed by measuring the decrease in absorbance within 1min at 290 (extinction coefficient of 2.8 mM^{-1} cm^{-1}) (Nakano and Asada., 1981). The amount of enzyme that decomposed 1 μmol of ascorbate per minute was defined as one unit (U) of APX activity. The enzyme activity was reported in units per milligram of protein.

Total soluble proteins

Protein content was determined according to the method of Bradford (1976) using bovine serum albumin as standard.

Measurement of K^+ and Na^+ content in root and shoots of plants

Dried samples (100 mg) were digested in 67% (v/v) HNO_3. Samples were maintained overnight in 10 ml HNO_3. The samples were heated for 45 min at 90°C, and then the temperature was increased to 150°C, at which the samples were boiled for at least three h until a clear solution was obtained. Digestion continued until the volume was reduced to about one ml. Finally, these extracts were filtered, diluted with distilled water, and the ion contents were determined using flame photometer. Standard curve was used for the calculation of each ion concentration.

Result

The results showed that the rate of membrane lipid peroxidation, as one of the important stress indicators in the shoot of Prosopis koelziana plants under 400 and 600 mM salt stress, increased by 56% and 70%, respectively in compared to control. This increase and the intensity of stress were greater in the root tissue, such that the rate of membrane lipid peroxidation in the root tissue of plants under 400 and 600 mM salt stress was 2.5 and 5 times that of the control plants, respectively. Under 400 mM salt stress conditions, the application of GABA at concentrations of 0.25 and 0.5 mM in the shoots caused a 23% and 30% reduction in lipid peroxidation, respectively in comparison with control plants. At 600 mM salinity, the application of concentrations of 0.25 and 0.5mM also reduced peroxidation by 28% and 32%. In the roots, GABA application reduced the rate of peroxidation in both salinity treatments, but there was not a significant difference between the different concentrations of GABA (Figure 2). The highest MDA level was observed in plants under 600 mM NaCl stress without GABA pretreatment.

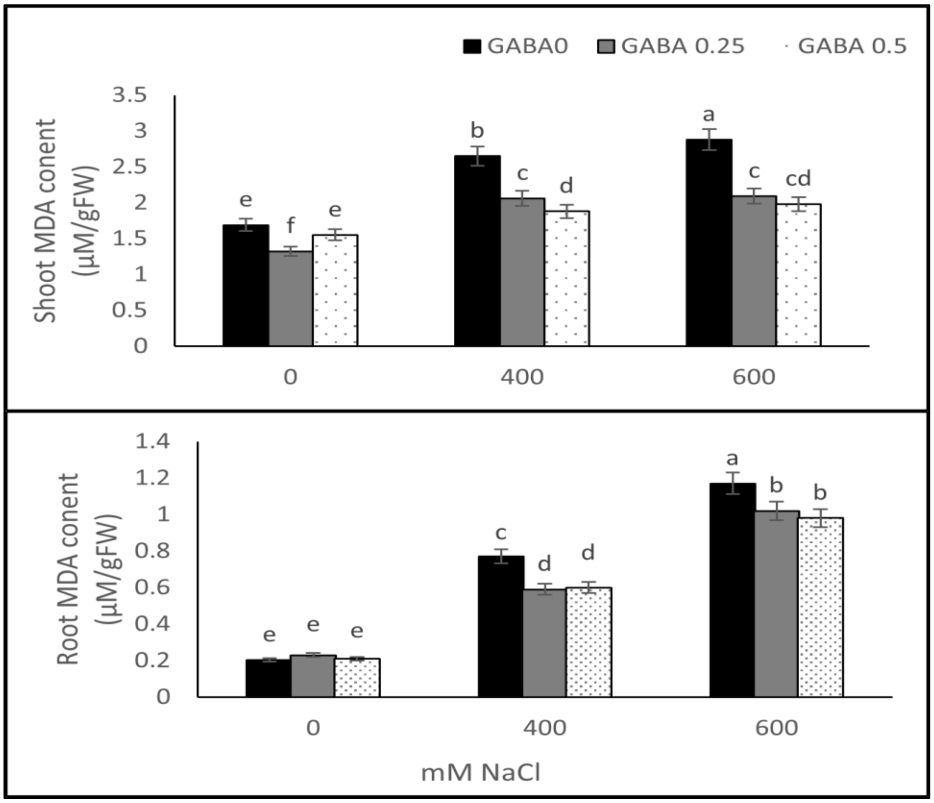

Figure 2. Effect of GABA pretreatment (0, 0.25, and 0.5 mM) on lipid peroxidation of membranes in the shoots and roots of *Prosopis koelziana* seedlings under salinity stress (0, 400, and 600 mM NaCl). Data are means ± SD (n= 3 independent pots). Different lowercase letters above bars indicate significant differences according to Duncan's multiple range test ($p < 0.05$).

The assay of ascorbate peroxidase enzyme showed that salt stress increased the activity of this enzyme in both roots and shoots. The application of 0.25 and 0.5 mM of GABA reduced the activity of this enzyme in the shoot and roots. The results indicated that under 400 mM salinity, the 0.25 mM of GABA was more effective in reducing stress and consequently decreasing enzyme activity; however, under 600 mM salinity, the higher concentration of GABA was more effective (Figure 3). The highest ascorbate peroxidase activity under salinity stress conditions was observed in plants that were not pretreated with GABA.

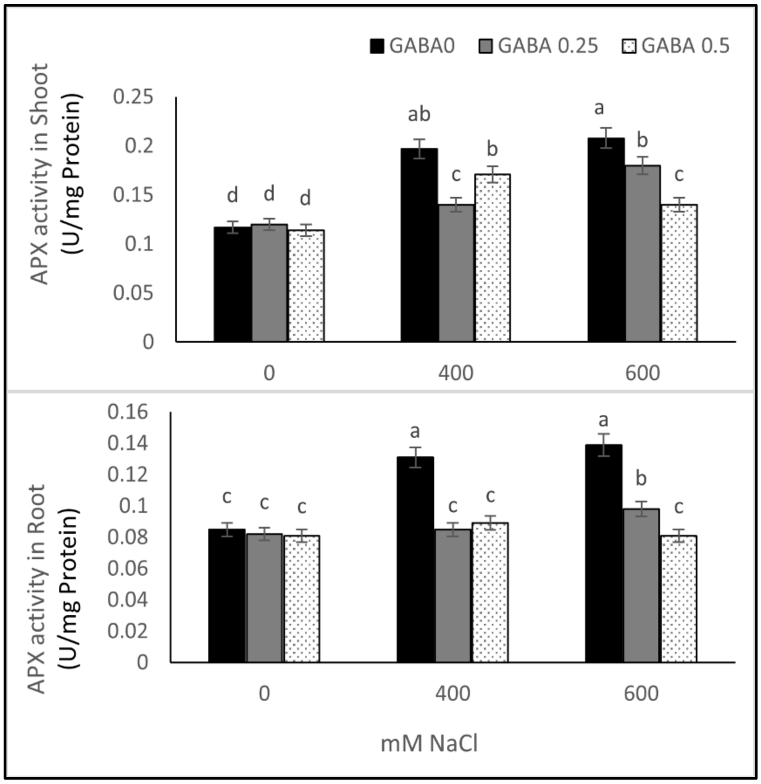

Figure 3. Effect of GABA pretreatment (0, 0.25, and 0.5 mM) on the ascorbate peroxidase activity in the shoots and roots of *Prosopis koelziana* seedlings under salinity stress (0, 400, and 600 mM NaCl). Data are means ± SD (n = 3 independent pots). Different lowercase letters above bars indicate significant differences according to Duncan's multiple range test ($p < 0.05$).

The results of measuring catalase enzyme activity in the roots and shoot of the *Prosopis koelziana* plant showed that this enzyme increased significantly under salt stress, and the application of GABA reduced the activity of this enzyme in both the roots and shoots of the plant. Almost no significant difference was observed between the effects of the two GABA concentrations on the activity of this enzyme, and both concentrations reduced enzyme activity. The application of GABA in the 400 mM salinity treatment did not have a significant effect on catalase enzyme activity in the roots (Figure 4).

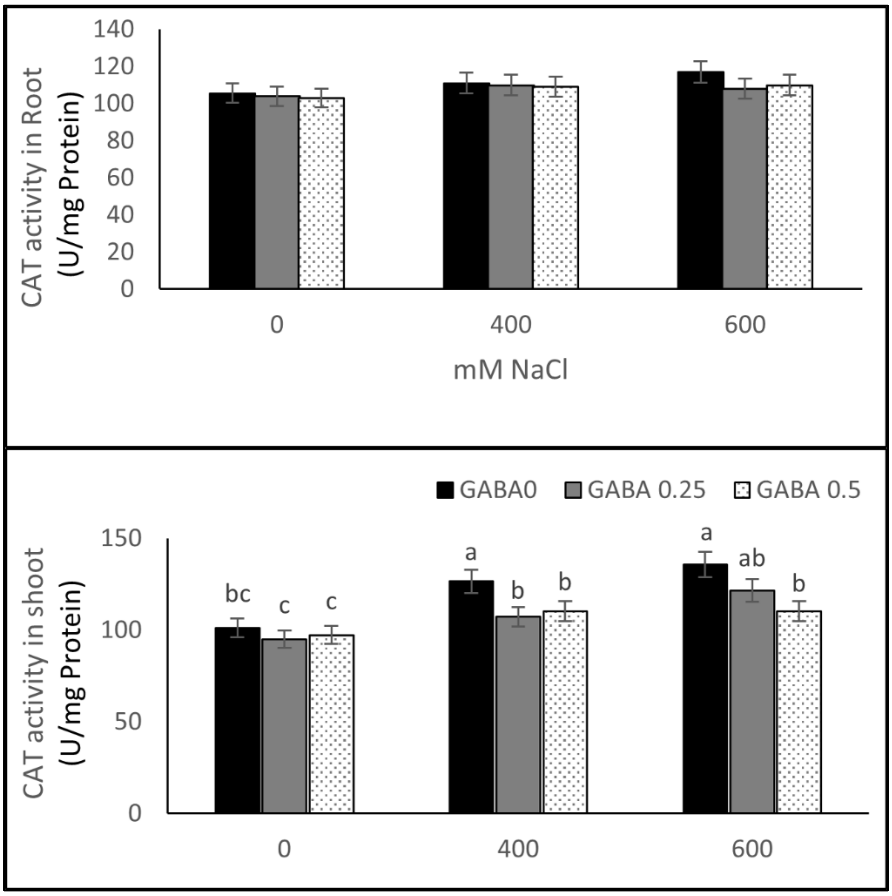

Figure 4. Effect of GABA pretreatment (0, 0.25, and 0.5 mM) on the catalase activity in the shoots and roots of *Prosopis koelziana* seedlings under salinity stress (0, 400, and 600 mM NaCl). Data are means ± SD (n= 3 independent pots). Different lowercase letters above bars indicate significant differences according to Duncan's multiple range test ($p < 0.05$).

Measurement of GPX enzyme activity also showed an increase in its activity under salt stress. As the salinity level increased, the activity of this enzyme, like other enzymes, increased. In the shoot of plants affected by 400 mM salinity, only the concentration of 0.25 mM GABA reduced enzyme activity, and the concentration of 0.5 mM had no significant effect on enzyme activity. However, in plants under 600 mM salt stress, both applied concentrations of GABA reduced enzyme activity, and the effect of the 0.5 mM concentration in reducing enzyme activity was greater than that of 0.25 mM. In the roots of *Prosopis koelziana* plants, both concentrations of GABA under 400 mM salinity and the concentration of 0.5 mM under 600 mM salinity had a significant effect in reducing the activity of this enzyme (Figure 5). The highest guaiacol peroxidase activity was observed in plant samples that were under 600 mM salinity stress and were not pretreated with GABA.

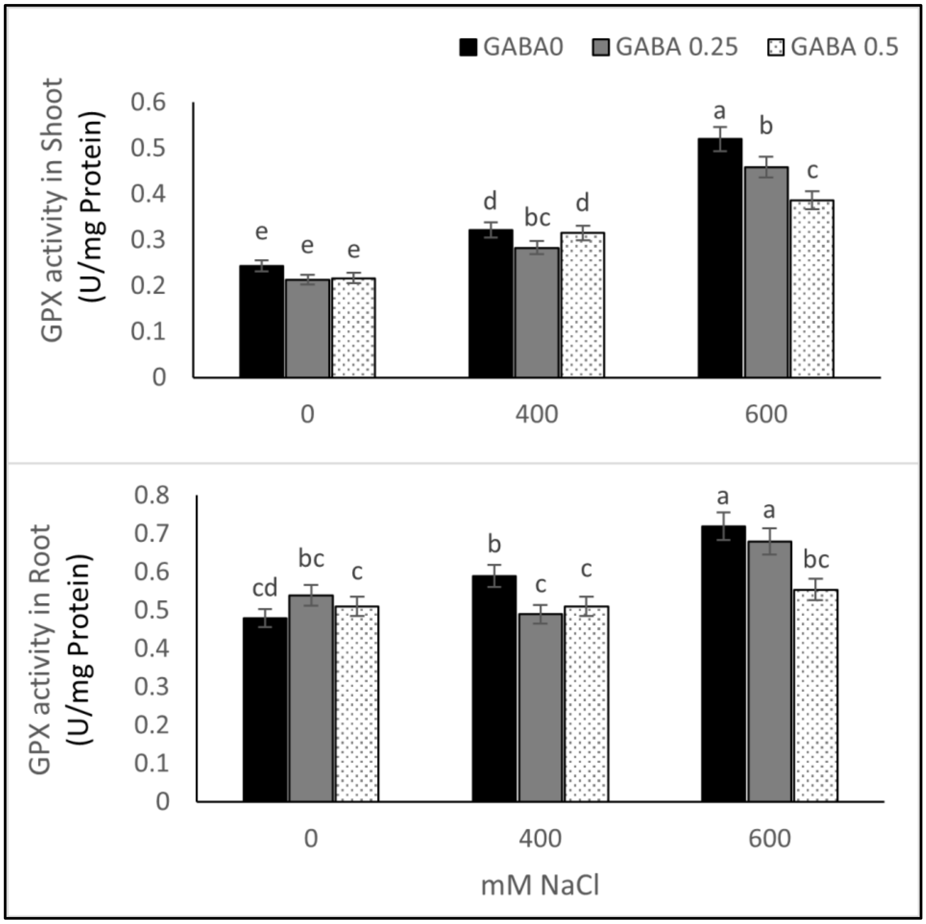

Figure 5. Effect of GABA pretreatment (0, 0.25, and 0.5 mM) on the guaiacol peroxidase activity in the shoots and roots of *Prosopis koelziana* seedlings under salinity stress (0, 400, and 600 mM NaCl). Data are means ± SD (n = 3 independent pots). Different lowercase letters above bars indicate significant differences according to Duncan's multiple range test ($p < 0.05$).

Data obtained from measuring sodium and potassium ions showed that in the roots of plants subjected to 400 and 600 mM salt stress, the sodium ion content increased by 55% and 62%, respectively, compared to control plants, while in the leaves, the sodium ion content in the 400 and 600 mM treatments showed a 73% and 100% increase, respectively. Application of GABA at concentrations of 0.25 and 0.5 mM reduced the sodium ion content in both roots and shoot.

Potassium ion assay showed that the content of this ion decreased under salt stress in both shoot and roots, and pretreatment with GABA increased this ion in root and shoot tissues (Table 1).

Table 1. Effect of GABA pretreatment on sodium and potassium content of shoot and root of *Prosopis koelziana* seedling under the control and stress conditions. Means were compared using Duncan's multiple range test. Differences were considered significant at $p < 0.05$. Means denoted by different letters represent significant difference.

Pretreatment and treatment	Root Na^+ (mg/gFW)	Shoot Na^+ (mg/gFW)	Root K^+ (mg/gFW)	Shoot K^+ (mg/gFW)
Control	43.22^{cd}	10.93^{e}	28.01^{a}	19.42^{b}
GABA 0.25 mM	44.81^{c}	11.26^{de}	25.57^{ab}	19.83^{b}
GABA 0.5 mM	45.09^{c}	13.12^{d}	32.39^{a}	17.72^{c}
Salinity 400 mM	66.94^{ab}	18.98^{b}	20.96^{b}	17.33^{c}
Salinity 400mM + GABA 0.25mM	58.04^{b}	15.36^{c}	24.85^{ab}	26.41^{a}
Salinity 400mM + GABA 0.5 mM	60.85^{b}	16.27^{c}	28.05^{a}	19.88^{b}
Sainity600mM	70.44^{a}	25.35^{a}	21.82^{b}	17.65^{c}
Salinity 600mM + GABA 0.25mM	59.97^{b}	23.82^{ab}	20.53^{b}	23.88^{a}
Salinity 600mM + GABA 0.5 mM	56.81^{c}	21.01^{b}	20.67^{b}	20.57^{ab}

Discussion

Seed germination and early seedling growth are critical stages of plant development that are particularly sensitive to abiotic stresses (Firoozkouhi et al., 2025). One significant issue caused by salinity is oxidative stress, which often results in increased lipid peroxidation. This process involves the oxidation of membrane lipids, ultimately compromising cell integrity and function. Elevated lipid peroxidation is a well-known indicator of increased oxidative stress under saline conditions. During salt stress, this heightened lipid peroxidation is a direct consequence of increased production of reactive oxygen species (ROS) (Golestani et al., 2025). This investigation emphasizes the benefits of GABA pretreatment in reducing lipid peroxidation in seedlings of *Prosopis koelziana,* suggesting that this approach enhances the plants' resilience to oxidative stress. The ameliorative effect of GABA under abiotic stress conditions has been reported in previous studies (Islam et al., 2024). Similar studies conducted on plants such as Prosopis (Soleimani et al., 2011), Haloxylon (Wang et al., 2014), and bean (Palma et al., 2009) under salt stress conditions have shown an increase in oxidative stress, alongside a rise in malondialdehyde levels, leading to a decrease in the membrane stability index. These findings are consistent with the results of this study on Prosopis seedlings. Additionally, the role of GABA in reducing membrane lipid peroxidation has been documented in tomato

(Malekzadeh et al., 2014) under cold stress, *Stellaria longipes*, lemon, Suger beet and mung bean (Kathiresan et al., 1998; Kinnersley and Lin, 2000; Ullah et al., 2023; Yu et al., 2024) under salt stress, as well as in black pepper (Shi et al., 2010) under osmotic stress. To manage oxidative stress, it is crucial to enhance the effectiveness of a plant's antioxidant defense system. The activity levels of antioxidant enzymes in plants serve as good indicators of the cell's redox state, which is vital for developing resistance to stress (Firoozkouhi et al., 2025). As these enzymes act as scavengers for reactive oxygen species (ROS), they play a significant role in protecting cells and the photosynthetic apparatus (Shi et al., 2010). Research data indicated that increased salinity heightened the activity of antioxidant enzymes, suggesting the onset of oxidative stress and the activation of the plant's antioxidant system. However, this study found that pretreating Prosopis seedlings with GABA reduced the activity of these antioxidant enzymes compared to non-pretreated plants. It appears that in seedlings pretreated with GABA, stress levels were lower, meaning the plants did not have to increase their antioxidant enzyme activity. Additionally, examining the levels of sodium and potassium ions showed that these ions were reduced in the pretreated plants, further supporting this hypothesis.

Salinity can lead to nutrient depletion or imbalance by competing with essential nutrients such as potassium (K^+) and calcium (Ca^{2+}) for chloride (Cl^-) and sodium (Na^+) ions (Hu et al., 2007). Research indicates that salt stress affects certain plants, including Guar (Firoozkouhi et al., 2025; Golestani et al., 2025), *Prosopis Koelziana* (Mousavi et al., 2025) and cotton (Dong et al., 2014), leading to alterations in the distribution of ions within their aerial parts. Potassium is crucial for various physiological processes and plant growth, including protein and starch synthesis, the activation of numerous photosynthetic and respiratory enzymes, the maintenance of photosynthetic system integrity, and osmotic regulation (Hu et al., 2005; Rahnama et al., 2004; Cardon et al., 2003). Research has shown that increased levels of sodium chloride (NaCl) can depolarize the cell membrane and create an antagonistic effect with sodium, which ultimately reduces potassium (K^+) uptake (Turan et al., 2010). Research has shown that one of the beneficial effects of GABA under salt stress conditions is its ability to enhance the absorption of essential mineral ions. For instance, treating seedlings with GABA resulted in increased absorption of minerals such as manganese, potassium, and iron (Kinnersley et al., 2010). Similarly, in wheat plants, the application of exogenous GABA led to a higher uptake of minerals crucial for plant metabolism, including manganese and calcium (Xing et al., 2007). One of the most significant effects of salinity is the increasing ratio of sodium to potassium, which has been reported in numerous studies (Juan et al., 2005; Abdel, 2010; Farsaraei et al., 2020). In the present experiment, the data indicated that GABA pretreatment decreased the absorption and transport of sodium into plant tissues while simultaneously increasing potassium content the $Na+$ absorption and increased the ratio in the leaves of plants subjected to salt stress.

Conclusions

The data from this study indicate that GABA can play an effective role in mitigating salt stress in *Prosopis koelziana* plants. In this research, it was observed that the mechanism of action of GABA was primarily through reducing the absorption and translocation of sodium ions from the roots to the shoots. It seems that, this function of GABA alleviated the intensity of the stress and resulted in decreased stress indicators and reduced antioxidant enzyme activity in plants pretreated with GABA.

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Eco-Friendly Pest Management: Exploring the Biocontrol Power of Bacillus Species

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ABSTRACT

Bacillus species are widely recognized as effective biological control agents due to their multifaceted mechanisms for suppressing plant pathogens and promoting plant growth. These mechanisms include the formation of resistant endospores, high adaptability to environmental stresses, and the production of antimicrobial compounds such as lipopeptides, volatile organic compounds, and hydrolytic enzymes. In addition, Bacillus spp. can induce systemic resistance in plants, contribute to nutrient solubilization, and modulate the rhizosphere microbiome, making them key components of sustainable and organic agriculture. Despite these advantages, several challenges limit the consistent performance of Bacillus-based products under field conditions. Environmental factors such as ultraviolet radiation, soil pH, temperature, and moisture can reduce bacterial survival and bioactivity. Moreover, certain strains, including *B. velezensis*, may exhibit phytopathogenic potential under specific conditions, highlighting the need for careful strain selection and strict regulatory oversight. Recent advances in genomics, bioinformatics, and synthetic biology have facilitated the identification and manipulation of biosynthetic gene clusters, enabling improvements in strain performance. These developments support the design of optimized formulations and more targeted delivery strategies. Furthermore, combining Bacillus-based products with other biological agents or organic amendments can enhance their efficacy. Incorporation of Bacillus into Integrated Pest Management and Integrated Crop Management programs may further increase their effectiveness and sustainability.

Keywords: Pesticides, Antimicrobial Compounds, Lipopeptides, bacteria

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Introduction

Excessive use of pesticides causes chemical contamination of agricultural lands and wastewater, creating a serious environmental problem. It also leads to the loss of non-target species such as beneficial insects, birds, aquatic organisms, and soil invertebrates (Wu and Chen, 2004, Tazunoki et al., 2022). Furthermore, the accumulation of pesticide residues in the food chain at elevated concentrations can cause severe health problems (Sharma et al., 2024), such as acute poisoning, cancer, neurological impairments, and endocrine disruptions (Bhatia et al., 2024, Zhou et al., 2025). Infants and children are particularly susceptible to these adverse effects due to their greater physiological vulnerability.

To reduce the adverse impacts of pesticide, use and foster sustainable agricultural practices, alternative strategies such as organic farming and biological control have been developed. Among these, the application of biological control agents (particularly beneficial microorganisms such as bacteria and fungi) plays a pivotal role in pest management and plant health without causing environmental harm (Manzar et al., 2022). In recent years, biological control products, especially those based on *Bacillus* species, have gained considerable attention as effective and environmentally safe alternatives to chemical pesticides. Their effectiveness stems from their strong ability to colonize the plant rhizosphere, enhance tolerance to environmental stresses, and produce a wide range of antimicrobial compounds (Pirttilä et al., 2021, Hussaini, 2014, Mongkolthanaruk, 2012). Moreover, these bacteria generate heat- and drought-resistant spores, which provide remarkable stability and persistence under natural conditions, thereby contributing significantly to reducing pesticide dependence and improving crop productivity (Mongkolthanaruk, 2012).

Biological and Physiological Characteristics of *Bacillus*

Bacillus species are Gram-positive, rod-shaped microorganisms that are aerobic or facultatively anaerobic and capable of forming endospores. Most members of this genus are saprophytic, contributing significantly to the decomposition of organic matter (Muthulakshmi et al., 2023, Soltani and Ringø, 2024). Owing to their distinct physiological traits, *Bacillus* species can colonize diverse ecological niches, including freshwater and saline environments, marine sediments, desert sands, hot springs, and even polar soils (Soltani and Ringø, 2024).

Many *Bacillus* species possess the ability to degrade a wide range of organic compounds derived from plant and animal sources, such as cellulose, starch, proteins, agar, and hydrocarbons. In addition, several species are recognized for their functional roles as antibiotic producers, heterotrophic nitrifiers, denitrifiers, nitrogen fixers, iron precipitators, selenium oxidizers, manganese reducers, and acidophilic or alkaliphilic microorganisms (Soltani and Ringø, 2024).

Beyond their metabolic versatility, *Bacillus* species establish interactions with plants both in the rhizosphere and within internal tissues (as endophytes), thereby influencing the expression of key genes and promoting plant growth and health (Muthulakshmi et al., 2023). These bacteria synthesize a broad spectrum of secondary metabolites with diverse structures and biological functions, which substantially enhance their potential as biological control agents (Aqel et al., 2024, Chaabouni et al., 2012). Their remarkable adaptability to fluctuating environmental conditions (including elevated temperatures, wide pH ranges, variable oxygen levels, and their inherent metabolic and genetic plasticity) further underscores their role as effective microbial agents under adverse environmental conditions (Aqel et al., 2024).

Biocontrol Mechanisms of *Bacillus* Spp.

Bacillus species play a vital role in the biological control of plant pathogens through diverse mechanisms. One of the most prominent strategies is the production of antimicrobial compounds, including lipopeptides such as iturin, surfactin, and fengycin, which exhibit strong antifungal and antibacterial activities (Chaabouni et al., 2012, Zeriouh et al., 2014, Dimkić et al., 2022). Additionally, the synthesis of secondary metabolites like subtilosin A, bacilysin, and bacillomycin contributes significantly to the direct suppression of plant pathogens (Ji et al., 2023).

Volatile organic compounds (VOCs) produced by Bacillus not only inhibit pathogen growth but also enhance plant defense against biotic stress through the induction of systemic resistance (ISR) (Dimkić et al., 2022, Ji et al., 2023, Zhang et al., 2020). These bacteria also compete with pathogens for nutrients and space, thereby preventing their growth and colonization (Dimkić et al., 2022, Miljaković et al., 2020). Moreover, *Bacillus* forms stable biofilms around plant roots, enabling the sustained release of antimicrobial substances (Ganchev, 2021).

Another notable feature of *Bacillus* is its ability to activate induced systemic resistance in plants via jasmonic acid (JA) and salicylic acid (SA) signaling pathways, leading to the expression of pathogenesis-related (PR) proteins and other defense-related proteins (Dimkić et al., 2022, Miljaković et al., 2020). Furthermore, Bacillus disrupts the quorum sensing systems of pathogens, interfering with processes such as biofilm formation, sporulation, and the expression of virulence factors (Miljaković et al., 2020, Bais et al., 2004).

Additional Roles of Bacillus in Soil Microbiota and Plant Health

Another prominent role of *Bacillus* species is their impact on soil microbiota. These bacteria modify the microbial community composition, creating favorable conditions for the growth of beneficial (Zhang et al., 2023). Moreover, Bacillus enhances plant growth and development by solubilizing and making nutrients (such as phosphate) more bioavailable (Hashem et al., 2019, Miljaković et al., 2020), and by producing plant growth hormones like auxin and gibberellic acid (Hashem et al., 2019, Muthulakshmi et al., 2023). In addition, the production of antibiotics (Nowocień and Sokołowska, 2022) and hydrolytic enzymes such as chitinases and proteases (Dobrzyński et al., 2023) contributes to the degradation of pathogen cell walls and suppression of their growth.

Antimicrobial Compounds

One of the most important strategies employed by Bacillus species in the biological control of plant pathogens is the production of diverse antimicrobial compounds. These include lipopeptides, secondary metabolites, and volatile organic compounds (VOCs), which exhibit significant antifungal, antibacterial, and antiviral activities (Al-Mutar et al., 2023, Cawoy et al., 2015). The antimicrobial agents produced by Bacillus play a key role in suppressing the growth of pathogens, enhancing plant immunity, and improving soil health.

Diversity of Antimicrobial Metabolites Produced by *Bacillus* Spp.

Bacillus spp. is recognized for producing an exceptionally wide array of antimicrobial metabolites, including lipopeptides, ribosomal peptides (bacteriocins), non-ribosomal peptides, polyketides, and volatile organic compounds (VOCs), which play important roles in the inhibition of plant pathogens, as summarized in Figure 1. According to a review by Tran et al., (2022), more than 47 antimicrobial compounds with distinct mechanisms have been identified from different *Bacillus* spp., mainly targeting vital pathways of microbial cells, including the cell wall, membrane, intracellular processes, and emerging molecular pathways (Tran et al., 2022). Lantibiotics such as subtilin, mersacidin, clausin, and haloduracin bind to cell wall precursors (lipid II), thereby disrupting peptidoglycan synthesis and ultimately causing cell death (Tran et al., 2022).

On the other hand, lipopeptides such as iturin, surfactin, and fengycin exhibit potent antifungal and antibacterial activities by disrupting the cell membranes of fungi and bacteria, while bacteriocins and bacteriocin-like inhibitory substances (BLISs) inhibit a wide range of pathogens, particularly species of *Listeria, Staphylococcus*, and *Pseudomonas* (Stoica et al., 2019). In addition, recent studies have reported that more than 89 antimicrobial peptides produced by *Bacillus* spp. have been identified, which are capable of targeting the DNA, membrane, and cell wall of pathogens and can also enhance plant immune responses (Zhang et al., 2025).

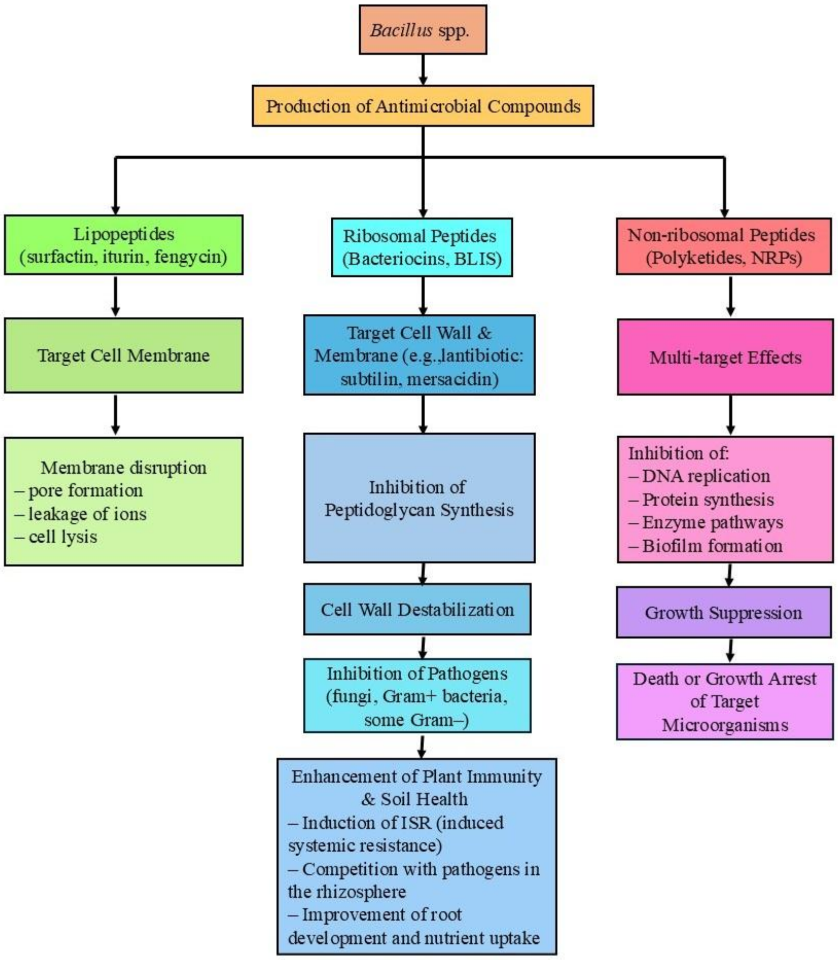

Figure 1: Schematic overview of the major classes of antimicrobial compounds produced by *Bacillus* spp. and their mechanisms of action in suppressing plant pathogens and enhancing plant health.

Biofilm Formation

Certain epiphytic microorganisms possess the ability to form biofilms by aggregating and adhering to surfaces. A biofilm is defined as a structured community of microorganisms embedded within an extracellular matrix and attached to living or non-living surfaces (Fig. 2). Many bacteria are capable of forming biofilms under various environmental conditions, including on the surface of stems, leaves, the plant rhizosphere, soil particles, fungi, and organic compost (Pandin et al., 2017).

It has been shown that root exudates can stimulate biofilm formation by biocontrol agents (Pandin et al., 2017). Biofilm formation is closely linked to the efficiency of rhizosphere colonization (in rhizobacteria) or plant tissue colonization (in endophytes). Effective colonization enhances nutrient accessibility and strengthens biocontrol performance. However, factors such as pH, temperature, moisture, oxygen concentration, and the presence of metal ions in the rhizosphere or plant tissues can significantly influence the formation and stability of biofilms (Dobrzyński et al., 2023).

Biofilms enhance the ability of *Bacillus* spp to suppress plant pathogens. *B. velezensis* PG12, for instance, forms biofilms that improve its colonization on apple fruits and enhance its biocontrol efficacy against apple ring rot disease (Zhang et al., 2025). Similarly, *Bacillus amyloliquefaciens* WS-10 forms biofilms that help suppress *Ralstonia solanacearum*, the causative agent of bacterial wilt in tobacco (Ahmed et al., 2022).

Biofilm formation is often associated with the production of antimicrobial compounds. For example, *B. subtilis* ASAG 010 produces surfactin within its biofilm, enhancing its antifungal activity against *Fusarium graminearum* (Jia et al., 2025).

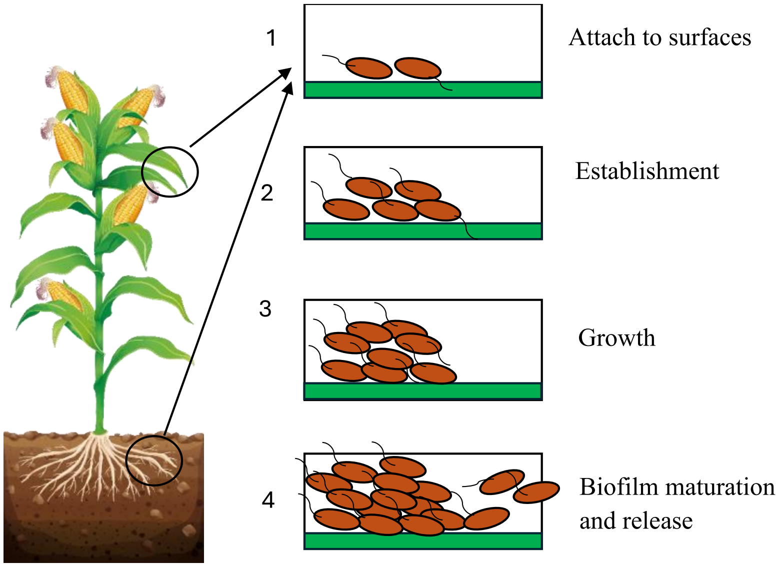

Figure 2: Schematic model of bacterial biofilm development on plant surfaces

Induced Systemic Resistance Triggered by *Bacillus* Spp.

Induced Systemic Resistance (ISR) is an enhanced physiological state in plants that is activated in response to specific environmental stimuli. In this state, the plant's innate defense mechanisms are strengthened against future biotic stresses. Bacillus species can induce long-lasting systemic resistance in plants through interactions with the root system (Dobrzyński et al., 2023). A key feature of ISR is its persistence, providing continuous protection against pathogens (Dobrzyński et al., 2023, Choudhary and Johri, 2009). This process is mediated by signaling pathways such as jasmonic acid and salicylic acid, which lead to the expression of a suite of defense-related genes in the plant (Choudhary and Johri, 2009)

Key Bacillus Spp. Used in Biocontrol

Several Bacillus species are widely utilized in the development of biocontrol products due to their ability to produce diverse bioactive compounds, their high adaptability to various environmental conditions, and their multifunctional role in promoting plant health (Table 1).

Table 1: Effectiveness of Bacillus Species in Plant Disease Management and Their Role as Eco-Friendly Alternatives to Chemical Pesticides

Species	Application	Example Product(s)	Mode of Action	Reference(s)
B. thuringiensis	Biopesticide	Dipel®, Xentari®	Cry proteins, antibiosis	(Balderas-Ruíz et al., 2021, Sena da Silva et al., 2021, Turanli et al., 2012)
B. subtilis	Biocontrol, plant growth promotion	Serenade	Antimicrobial compounds, antibiosis, biofilm formation, ISR	(Rahman, 2017)
B. amyloliquefaciens	Biocontrol, plant growth promotion	Various formulations	Lipopeptides, antibiosis, systemic resistance	(Zheng et al., 2013, Jin et al., 2018)
B. velezensis	Fungal disease control	Fungifree AB™	Antibiosis, systemic resistance	(Balderas-Ruíz et al., 2021)
B. cereus	Plant disease control	Various formulations	Antimicrobial compounds	(Seo et al., 2012)

Below is an overview of the most important species within this genus:

B. thuringiensis (Bt)

This species is one of the most well-known biological control agents with insecticidal properties. Bt produces crystalline proteins known as δ-endotoxins or Cry proteins, which interact with specific receptors in the insect gut, leading to feeding disruption, paralysis, and ultimately death (Sanchis and Bourguet, 2009, Sanchis, 2011). Bt-based products occupy a significant portion of the biopesticide market (Berini et al., 2024).

B. subtilis

A soil-dwelling bacterium with strong capabilities in protecting plants and promoting their growth. B. subtilis plays a significant role in sustainable agriculture through mechanisms such as the production of diverse antimicrobial compounds, biofilm formation, induction of systemic resistance in plants, and secretion of hydrolytic enzymes (Sidorova et al., 2018, Ganchev, 2021, Blake et al., 2021).

B. velezensis

This species is recognized as an effective biological control agent due to its ability to suppress a wide range of plant pathogens, including fungi, bacteria, and nematodes. *B. velezensis* produces cyclic lipopeptides, polyketides, and enzymes such as protease and chitosanase, which degrade fungal cell walls. It also exhibits strong biofilm formation and induces systemic resistance in plants (Rabbee et al., 2019, Han et al., 2024, Li et al., 2024).

B. amyloliquefaciens

This species plays a key role in plant growth and development by enhancing soil nutrient availability, modifying the rhizosphere microbial community, secreting plant growth hormones and volatile organic compounds (VOCs), and inducing resistance against both biotic and abiotic stresses (Zhang et al., 2017, Ilyas et al., 2024, Kröber et al., 2016).

B. cereus

B. cereus is capable of forming biofilms that enhance plant resistance to environmental stresses and pathogens through the secretion of metabolites, surfactants, and enzymes (Majed et al., 2016). Additionally, by producing growth-promoting compounds such as indole-3-acetic acid (IAA), ACC deaminase, and siderophores, it improves seed germination, seedling growth, and overall plant health (Zhou et al., 2021).

Main Pests Targeted by *Bacillus* spp.

Bacillus species, especially *B. Thuringiensis* and *Bacillus subtilis*, are widely used as biological control agents against different agricultural pests. These bacteria have shown effectiveness against several important insect pests and plant pathogens, as reported in many scientific studies.

B. Thuringiensis is mainly applied for the control of insect pests. It is particularly effective against lepidopteran species such as *Helicoverpa armigera*, where *B. Thuringiensis* strains have demonstrated strong insecticidal activity and significant larval mortality (Pinheiro and Valicente, 2021). Similarly, high mortality rates have been reported for *Spodoptera litura* following treatment with *B. Thuringiensis* and *B. subtilis* fusants (Revathi et al., 2014). *B. Thuringiensis* toxins are also commonly used to manage *Plutella xylostella* (diamondback moth), a major pest of cruciferous crops (Sayyed et al., 2008). *B. Thuringiensis* strains have also shown activity against coleopteran pests. Notable examples include *Anthonomus grandis* (cotton boll weevil), which causes major economic losses in cotton production (Sauka et al., 2024), and *Diabrotica virgifera virgifera* (western corn rootworm), for which *B. Thuringiensis* maize has been widely adopted, although resistance has been reported in some cases (Gassmann et al., 2020). In addition, *B. Thuringiensis* strains have demonstrated toxicity toward hemipteran pests such as *Bemisia tabaci* (whitefly), suggesting their potential use in integrated pest management programs (Cabra and Fernandez, 2019, Mensah and Young, 2017).

Bacillus subtilis is mainly recognized for its biocontrol activity against plant pathogens. Several studies have shown that *B. subtilis* strains are effective in controlling fungal pathogens such as *Fusarium oxysporum*, the causal agent of Fusarium wilt (Boulahouat et al., 2023), and *Rhizoctonia solani*, where high biocontrol efficacy has been reported, particularly in tomato plants (Ma et al., 2015). In addition to fungi, *B. subtilis* has shown antagonistic effects against bacterial pathogens, including *Ralstonia solanacearum*, which causes bacterial wilt in a wide range of crops (Kadhim and Matloob, 2025). Moreover, *B. subtilis* strains have been reported to cause high mortality in certain insect pests such as *Pieris brassicae*, a pest of brassica crops, and *Dendroctonus micans*, an important forest pest (Usta et al., 2025).

Limitations of Bacillus-Based Biological Control

Although Bacillus-based biocontrol agents often perform successfully under laboratory or controlled conditions, they frequently fail to demonstrate the same level of efficacy in field environments (Serrão et al., 2024). This discrepancy can be attributed to various environmental factors; for instance, exposure to ultraviolet (UV) radiation may reduce the viability of bacterial cells or the stability of their active compounds (Idris et al., 2024). Additionally, harsh climatic conditions or unfavorable physicochemical properties of the soil (such as unsuitable pH or poor texture) can diminish the effectiveness of these agents (Mahapatra et al., 2022).

Moreover, certain *Bacillus* strains, have been associated with disease outbreaks in specific crops. For example, *B. velezensis* has been reported as the causal agent of bulb rot in onion and soft rot in potato (Rabbee et al., 2023) and rot in peach fruits (Zeng et al., 2022). In addition, other *Bacillus* species have also been implicated in plant and animal diseases; for example, *B. pumilus* has been associated with fruit rot of muskmelon (*Cucumis melo*) in China (Song et al., 2018). Although *B. subtilis* is widely regarded as a safe and efficient microbial cell factory, certain strains have exhibited pathogenic potential in animals (Zhu et al., 2017), and *B. cereus* was historically considered harmless for decades before being recognized as a human pathogen (Tuipulotu et al., 2021).

Therefore emphasized that strains intended for biocontrol applications must be carefully evaluated at the strain level to avoid unintended phytopathogenic effects (Rabbee et al., 2023). The development, registration, and commercialization of *Bacillus*-based biocontrol products also require navigating complex regulatory procedures, including efficacy evaluation, safety assessments, and risk analysis, which can be both costly and time-consuming (Vedamurthy et al., 2020).

Recent Advances and Future Directions

Significant progress in DNA sequencing technologies and bioinformatics has enabled precise identification and characterization of gene clusters responsible for secondary metabolite production in Bacillus strains, playing a crucial role in the development of more effective biocontrol agents (Su et al., 2020, Toymentseva et al., 2019). Moreover, modern technologies such as genetic engineering and synthetic biology have been employed to optimize the performance of these bacteria in biocontrol and biofertilizer applications (Pal et al., 2024, Liu et al., 2013). Today, Bacillus-based products are increasingly integrated into sustainable agricultural strategies, contributing to reduced use of chemical fertilizers and pesticides (Pal et al., 2024, Liu et al., 2013). The growth of the global market for these products, along with numerous patents and extensive research activities, reflects the dynamic nature of this field and strong interest from the private sector (Piedra-Buena et al., 2015, Sales and Rigobelo, 2024).

Conclusion

Bacillus Spp. have gained a prominent position in sustainable agriculture and organic crop production due to their diverse and effective mechanisms in pest control and plant growth promotion. Their ability to form resistant spores, survive under harsh environmental conditions, produce antimicrobial compounds, induce systemic resistance in plants, and enhance nutrient availability makes them one of the most important biological control agents.

Nevertheless, challenges such as reduced efficacy under real field conditions, complexity in developing effective formulations, and the need for precise strain selection and continuous monitoring remain obstacles to their widespread development and commercialization.

Recent advances in areas such as genomic sequencing, synthetic biology, and genetic engineering have paved the way for optimizing the performance of Bacillus strains and developing innovative biological products. Ultimately, integrating these products into Integrated Pest Management (IPM) and Integrated Crop Management (ICM) programs not only enhances agricultural system productivity but also plays a vital role in environmental protection and the provision of safe and sustainable food.

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Functional Diversity on the effect of Grazing Intensity in Golestan National Park

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ABSTRACT

Ruminants and vegetation in natural ecosystems are inherently interdependent, and grazing, as a major ecological disturbance, exerts profound impacts on plant functional traits. This study conducted to evaluate the effects of sheep grazing intensity and topographic aspect on plant functional traits and functional components of grasslands in Golestan National Park. Based on the distance from a watering point, three grazing sites were selected: Site 1 (nearest to the watering point) to Site 3 (farthest), across both western and eastern aspects (six sites in total). From each plant species, five plants were sampled, and two leaves per each species were collected to measure functional traits including leaf area, leaf thickness, leaf length, leaf width, leaf dry matter content (LDMC), and specific leaf area (SLA). At each site, ten 4m^2 quadrats were established to estimate vegetation cover, and functional indices including community-weighted mean (CWM) traits, functional diversity (FD), and Rao's quadratic entropy were calculated. Results indicated that grazing significantly affected Rao's index, functional diversity, and CWM of SLA, LDMC, leaf area, thickness, width, and length. Topographic aspect had no significant effect on any trait; however, the magnitude of variation differed between aspects. Overall, livestock grazing exerted relatively lower effects on Rao's index, while its impact on FD was more pronounced under moderate grazing intensity. The CWM of leaf traits revealed divergent strategies, with plants tending to increase leaf area rather than leaf thickness as an adaptive response to grazing pressure.

Keywords: watering point, grazing intensity, leaf thickness, Rao's index, functional diversity

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Introduction

Global environmental changes, including land use, climate warming, grazing, and fire, strongly affect vegetation dynamics and ecosystem services. This concept was initially emphasized by Díaz et al. (2007) and has been further elaborated in recent global assessments (e.g., Díaz et al., 2019). Grazing influences the morphology and physiology of plants as well as the structure and functioning of rangeland ecosystems, such that plants adopt various strategies to minimize the negative effects of herbivory (Gordon et al., 2008). Vegetation is a key component of the natural environment, shaping the visual character of landscapes and serving as a direct reflection of ecological conditions. Because of this, plant cover is widely recognized as one of the most effective indicators for assessing environmental and ecological factors within a given region (Cui et al., 2023). Therefore, by measuring the amount, variation, and distribution of plant traits within plant communities, it is possible to examine ecosystem functioning (Lefcheck et al., 2015). Plant functional traits serve as reliable tools for predicting the effects of grazing on ecosystems and the resulting changes in ecosystem functioning. Recent studies highlight that grazing intensity modifies trait-dependent community composition and functional diversity, which in turn drive ecosystem productivity and multifunctionality (Guo et al., 2024; Zhang et al., 2023). Nowadays, functional components are used to describe functional traits (Butterfield and Suding, 2013), to represent the overall processes of communities (Mason et al., 2013), and to quantify them (Lavorel et al., 2008). They are considered key tools for understanding the relationship between ecosystem functioning and biodiversity (Schmera et al., 2009).

Recent studies have highlighted the importance of grazing intensity and environmental factors in shaping plant functional traits and ecosystem functioning. For instance, in the steppic rangelands of Nodoushan, Yazd, Gholami and Fakhimi (2023) reported that functional groups such as annuals and geophytes declined under heavy grazing, while hemicryptophytes increased, emphasizing that plant traits can serve as reliable indicators for dryland rangeland management. Jalili (2023) stressed the necessity of adopting an ecosystem-based approach in rangeland management in Iran, warning that focusing solely on grazing without considering ecological structural diversity accelerates rangeland degradation. A study in Mazandaran (2024) demonstrated that species diversity indices (richness, Shannon, Simpson) and functional indices were directly correlated with aboveground biomass, suggesting that appropriate grazing management can simultaneously sustain biodiversity and ecosystem functioning. On a broader scale, Zhang et al. (2023) showed that the effects of grazing on biodiversity and ecosystem multifunctionality are strongly dependent on aridity, with severe reductions in functional diversity in dry regions compared to milder impacts in more humid areas.

Complementary international findings reinforce these patterns. Bahreini et al. (2024), working in the mountainous grasslands of northern Iran, reported that high grazing intensity reduced species richness but increased functional diversity, indicating a shift in plant strategies toward stress-tolerant forms. Zhang et al. (2023) demonstrated that the long-term impacts of grazing on biodiversity and ecosystem multifunctionality are strongly mediated by aridity, with more severe negative effects observed in drier regions. In semiarid rangelands, Taylor et al., (2025) found that soil health indicators such as saturated hydraulic conductivity and mineralizable carbon declined under heavy grazing, emphasizing the link between grazing pressure and soil ecosystem services. In Golestan National Park, Madadi et al., (2024) identified distance from roads and water sources as key drivers of illegal grazing, while Alinaghizadeh and Aslani Kordkandi (2024) showed that climate change and drought significantly reduced vegetation cover, as reflected in declining NDVI values.

Altogether, these studies suggest that grazing intensity, environmental stressors, and human activities interact to influence vegetation dynamics, functional diversity, and ecosystem resilience in rangelands. Despite the various effects of grazing on plant traits (Nikan et al., 2010; Díaz et al., 2007) and on species diversity and richness (Gholami et al., 2012), functional components are considered new and useful tools for predicting changes in rangeland communities and enhancing biodiversity (Komac et al., 2015). It is hoped that by considering the effects of grazing on vegetation cover and ecosystem functioning, more effective information will be provided for rangeland management. Moreover, these components provide better insights into environmental constraints within community structure (Ricotta and Moretti, 2011). Environmental factors also influence the extent of grazing effects; therefore, the impact of topography must be considered as environmental stress conditions change (Collins et al., 2012). Given the limited knowledge regarding species responses to livestock grazing and vegetation degradation, while accounting for aspect and functional components in the protected rangelands of Golestan National Park, this study was conducted to achieve the above objectives.

Methodology

Study Area and Sampling

The study area is located in the northeastern part of Golestan National Park. Its geographical coordinates range from 37°21′50″ N to 37°56′11″ N latitude and from 50°50′52″ E to 51°11′50″ E longitude. The average elevation of the sites is 1,070 meters above sea level. The area consists of small rolling hills, with high plant diversity and a considerable presence of stones and calcareous gravel. The dominant vegetation cover is composed of *Artemisia* (sagebrush) and *Salsola* shrubs. To investigate the effects of sheep grazing intensity, a campsite and a permanent watering point located outside the park were selected as reference sites. Based on the distance from the watering point, three sites were selected: Site 1 with the shortest distance to the watering point, and Site 3 located approximately 2 km away, representing the farthest distance. Site 3 was situated inside the park and subjected to light grazing by gazelles. In each of the three study sites, both eastern and western aspects were considered to account for the effect of topography. Thus, a total of six sites were selected to examine grazing intensity (Figure 1).

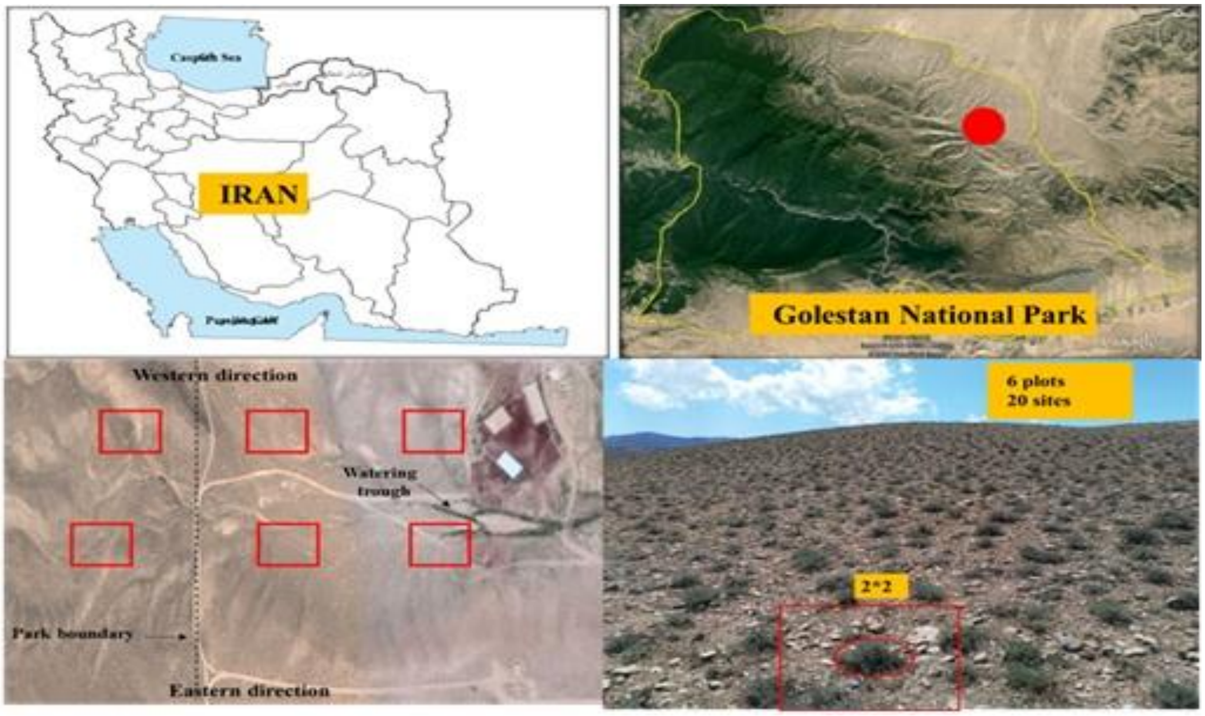

Figure 1. Location of the study area in Iran and the position of the sampling sites

Trait Measurements

For analyzing the effects of grazing intensity, five plant species were randomly selected in each site (a total of 6 sites were randomly selected, with 5 plant stands), and two leaves were collected from each plant. For measuring leaf thickness, area, width, length, dry matter content, and specific leaf area, the leaves were immediately placed in zip-lock bags after collection to preserve fresh weight and transported to the laboratory. Leaf thickness was determined using a micrometer (Mitutoyo 3109A). To assess other leaf traits, leaves were scanned with a flatbed scanner (Canon LiDE 120), and leaf area was calculated using ImageJ software (Version 1.51k 1) following the protocol of Pérez-Harguindeguy et al., (2013). Specific leaf area (SLA) is the one-sided area of a fresh leaf, divided by its oven-dry mass. Note that leaf mass per area (LMA), specific leaf mass (SLM) and specific leaf weight (SLW), are simply 1/SLA).

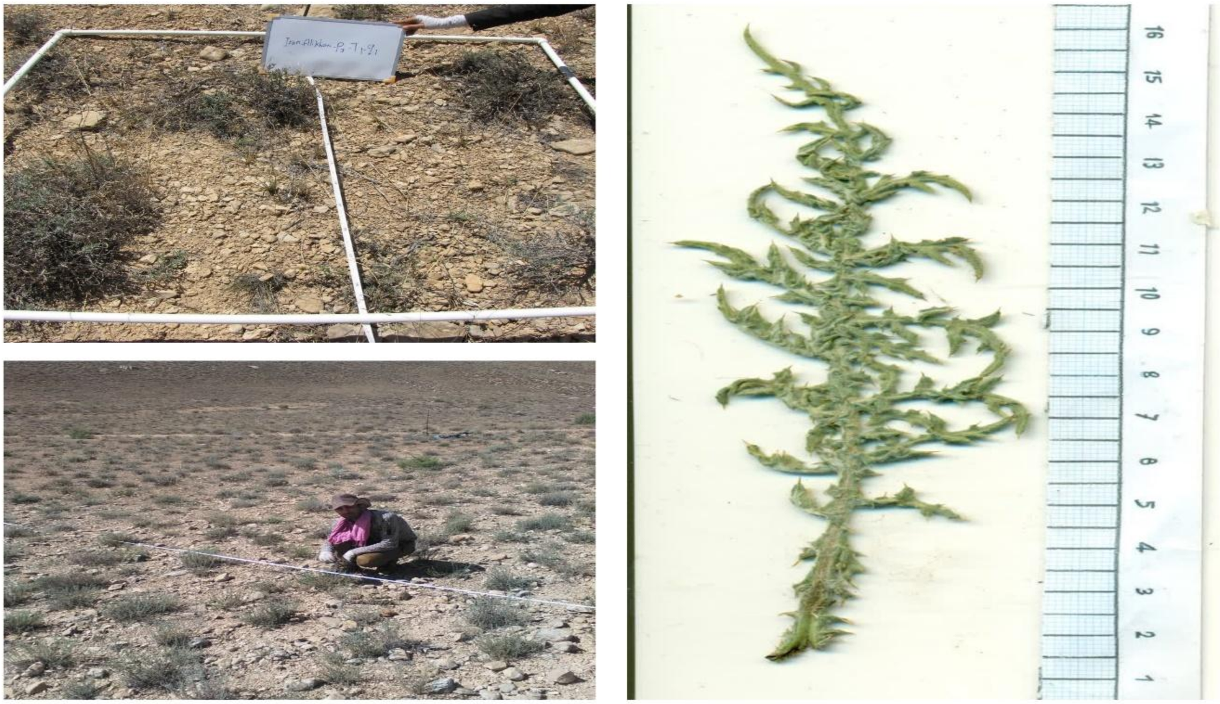

Figure 2. Leaf sampling and measurement of morphological traits

Specific Leaf Area (SLA)

SLA = Leaf area (cm^2) ÷ Leaf dry weight (g)

Leaf Dry Matter Content (LDMC)

LDMC = Mean leaf dry weight (mg) ÷ Mean leaf fresh weight (mg)

Leaf Volume

Leaf Volume = Leaf thickness × Leaf area

Calculation of Components

The components calculated in this study included the community-weighted mean traits (CWM), Rao's quadratic entropy (Rao's index) (Carmona et al., 2015), and functional diversity (FD) (Valencia et al., 2015). Community-Weighted Mean Traits (CWM) The community-weighted mean of traits is calculated as the percentage cover of each species divided by the total percentage cover of all species, where Xi represents the trait value and Wi denotes the percentage cover of the species.

Community-Weighted Mean Traits (CWM) CWM = Σ (Wi × Xi)

- Xi: trait value of species i

- Wi: percentage cover of species i

Rao's Quadratic Entropy (Rao's Index) $\text{Rao} = \Sigma\,\Sigma\, d_{ij} \times p_i \times p_j$

- dij: Euclidean distance between species i and j
- pi, pj: relative abundance (percentage cover) of species i and j

Functional Diversity (FD) Functional diversity represents the dispersion of traits within plant communities and is analogous to variance. It is derived from a weighted distribution based on the frequency of each trait in the community. The formula is expressed as:

Functional Diversity (FD) $FD_j = \Sigma\, p_{ij} \left(|T_{ij} - CWM_j| \,/\, \Sigma\, |T_{ij} - CWM_j| \right)$

- FDj: functional diversity for community j
- pij: relative abundance of species i in community j
- Tij: trait value of species i in community j
- CWMj: community-weighted mean traits in community j

Vegetation Cover Measurement

To assess the cumulative effects of livestock grazing at each study site, vegetation changes a long distance-from-water gradients was used as an indicator. Along these gradients, four groups of variables were recorded: (1) measures of grazing pressure, including consumption, utilization, fecal density, and a defoliation index; (2) primary productivity, which reflects a core ecosystem response to grazing; (3) plant community composition, providing insight into population- and community-level responses; and (4) soil organic matter, used as an indicator of grazing effects on long-term productivity potential. For this study, we selected One distinct permanent water point within each of the six study areas, typically situated in separate pastures. We specifically chose grazing lands where management practices, including stocking rates and the spatial arrangement of fences and water points, had been stable for two decades. At each location, we established five to seven sampling sites at different distances from the water. Beyond the direct assessments of herbivory and grazing pressure conducted via temporary exclosures, we also employed two indirect methods to estimate grazing intensity across all study locations. The primary indirect measure involved quantifying livestock fecal density. Specifically, within the PAT study area, we calculated the density of individual fecal pellets using two 40 × 0.2 m belt transects per site, a methodology consistent with the approach described by Fernández et al. After selecting the focal species, individuals were chosen that were healthy and growing under adequate ambient light conditions. This is an especially important consideration for leaf-related functional traits. Leaves were collected from plant species experiencing minimal shading and showing no signs of discoloration or stress. To assess vegetation cover at each site, a total of 6 sites were randomly selected, with 5 plant stands, totaling 6 plots. At each site, all encountered species were pressed for herbarium preparation and subsequently identified using the herbarium of Golestan National Park.

Statistical Analysis

To determine the most important factors influencing species distribution across different aspects under grazing, a generalized linear mixed model (GLMM) was applied. In the model, plots were considered as random factors, while aspect and grazing were treated as fixed effects. A Gaussian distribution and a log-link function were used (5). Random effects were specified based on the number of plots within each site. Model evaluation was conducted using Fisher's F-statistic. For treatment mean comparisons, least squares means and independent t-tests were applied. All statistical analyses were performed in R software (Version 2.2.3). The *lme4* package was used for computations, as it is specifically designed for fitting mixed models. A Gaussian distribution was applied in the modeling since the data were continuous. For mean comparisons, the lsmean package was employed, which calculates least squares means for pairwise comparisons.

Results

According to Table 2, a total of 79 plant species belonging to 27 families were identified under different grazing intensities in the eastern and western aspects of the study area. The most important plant families included: Poaceae (33.33%), Asteraceae (29.62%), Caryophyllaceae (29.62%), Berberidaceae (25.92%), and Brassicaceae (22.22%). The dominant vegetation type in the study area was Artemisia–Salsola. Species richness was lower in the heavily grazed site, whereas the lightly grazed site exhibited higher species richness. Results of the analysis of variance indicated that grazing had a significant effect on Rao's index, functional diversity, community-weighted mean specific leaf area, dry matter content, leaf area, leaf width, leaf thickness, and leaf length. Geographic aspect showed no significant effect on any of these traits. However, the interaction between grazing and aspect had a significant effect on the community-weighted mean of specific leaf content and leaf area (Table 1).

Table 1. Analysis of variance results for the interaction effect of grazing and direction on functional components

Component		Grazing		direction		Grazing and direction	
	df	P value	F value	P value	F value	P value	F value
Functional Diversity Indicators	1	0	6.41	0.28	1.13	0.22	1.44
Rao's quadratic entropy (Rao)	1	0.03	2.77	0.72	0.12	0.83	0.31
Community-weighted mean specific leaf area (CWM-SLA)	1	0.00	34.58	0.24	1.38	0.63	0.56
Community-weighted mean leaf dry matter content (CWM-LDMC)	1	0.00	35.66	0.58	0.30	0.00	4.63
Community-weighted mean leaf area (CWM-LA)	1	0.00	4.06	0.14	2.12	0.30	1.23
Community-weighted mean leaf length (CWM-LL)	1	0.00	3.93	0.34	0.61	0.41	0.99
Community-weighted mean leaf width (CWM-LW)	1	0.00	10.58	0.80	0.05	0	3.82
Community-weighted mean leaf thickness (CWM-LT)	1	0.00	16.14	0.33	0.92	0.25	1.36

The results of the mean comparison indicate that:

1. Geographical Orientation Effects on Traits and Indices

Geographical orientation exerted significant effects only under heavy grazing, specifically on functional diversity, Rao's quadratic entropy (Rao's Q), leaf dry matter content (LDMC), and leaf area. In contrast, community-weighted mean specific leaf area (SLA), leaf thickness, leaf length, and leaf width consistently varied across all sites, irrespective of grazing intensity (Figure 3).

2. Influence of Grazing Intensity on Leaf Morphological Traits

Grazing intensity markedly altered leaf morphological attributes. SLA, LDMC, leaf area, and leaf width increased with grazing pressure, whereas leaf thickness and leaf length decreased. These trait shifts reflect adaptive strategies of plant communities in response to herbivory along the grazing gradient (Figure 3 A).

3. Functional Diversity and Rao's Q Across the Grazing Gradient

Functional diversity declined progressively with increasing grazing intensity, with the most pronounced reduction observed at the moderately grazed site. Similarly, Rao's Q decreased under moderate grazing, while changes were comparatively minor under low and heavy grazing. These findings highlight the sensitivity of community functional structure to intermediate levels of disturbance (Figure 3 B).

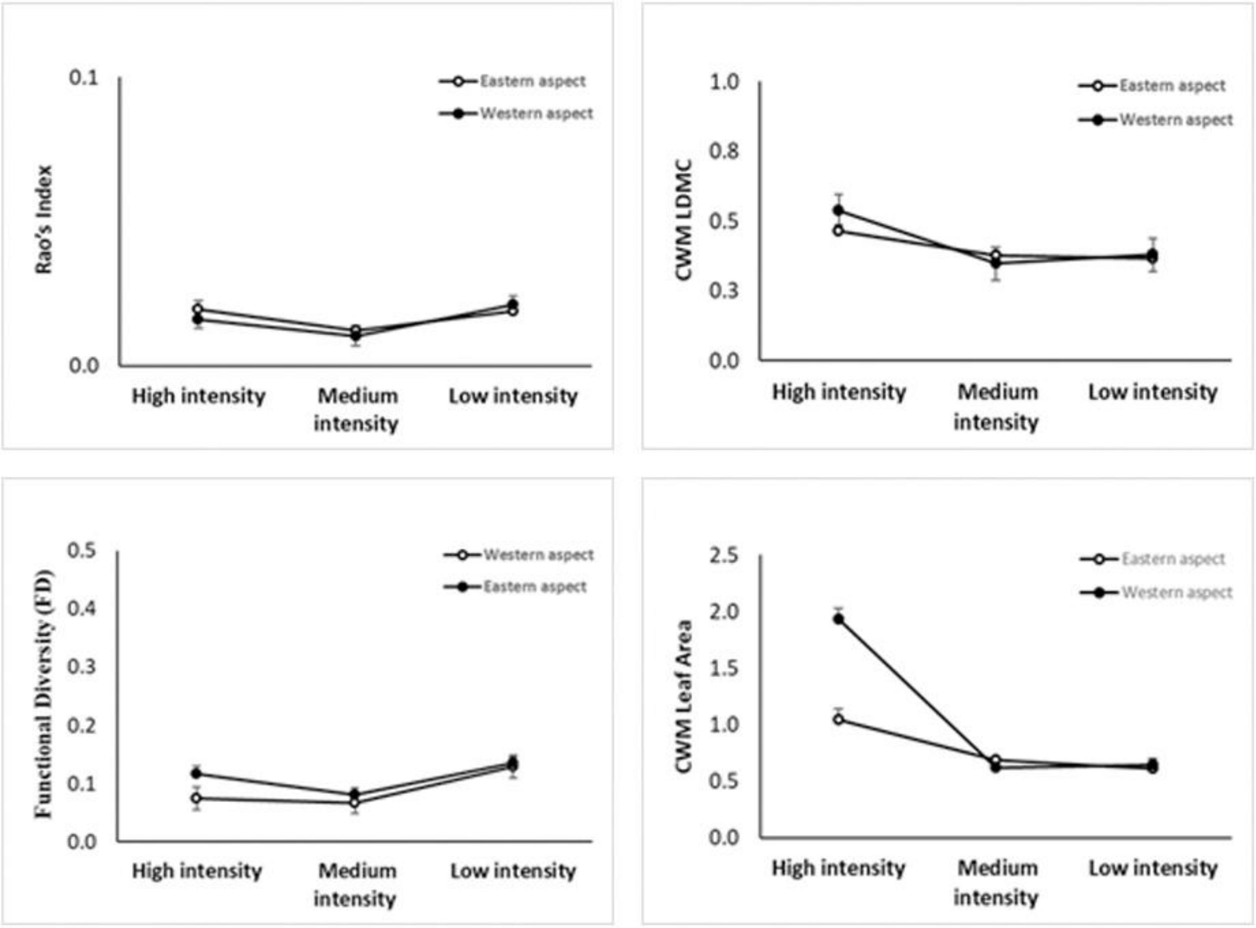

Figure 3 A. Variation of functional components across grazing intensities

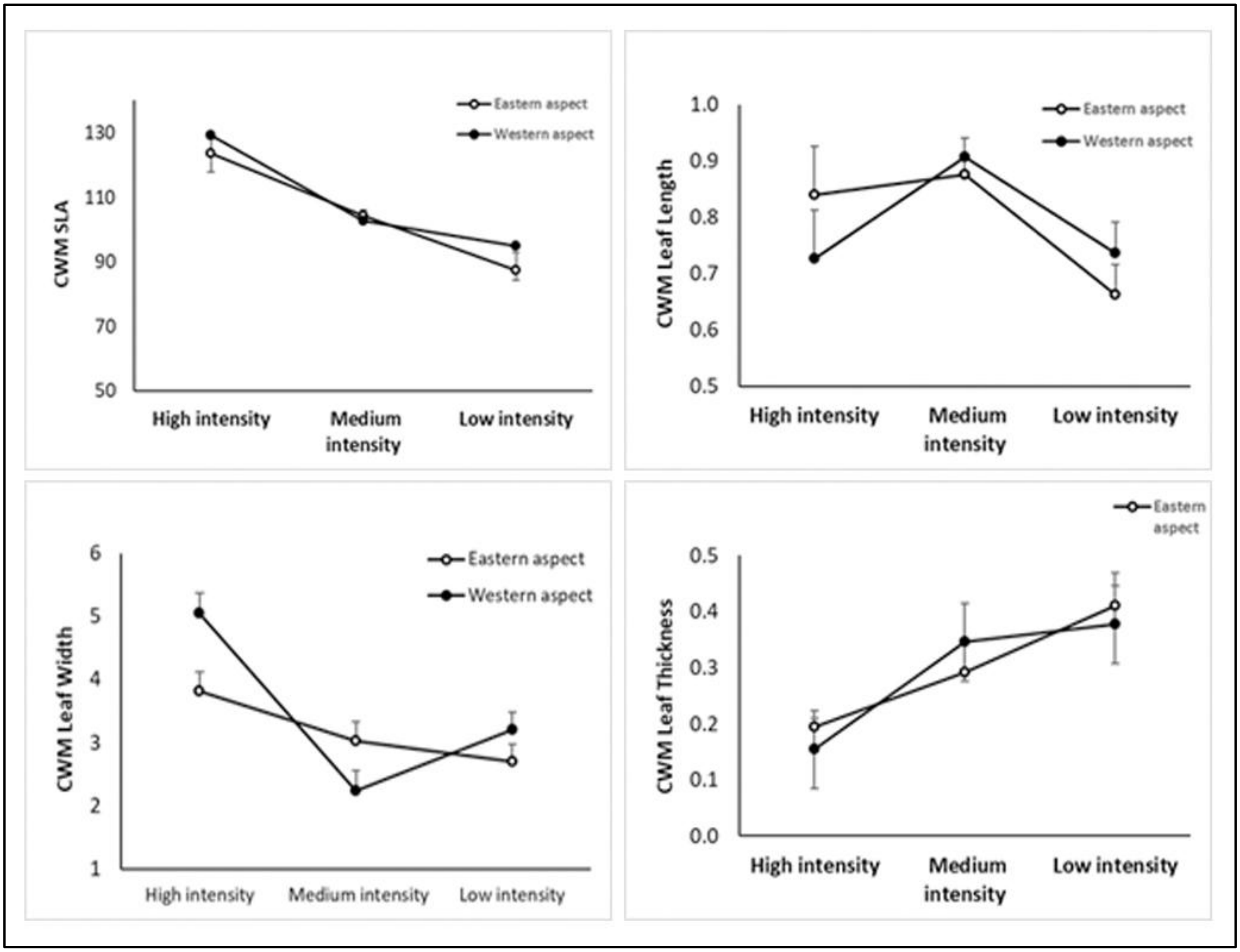

Figure 3 B. Variation of functional components across grazing intensities

Table 2. Annex – List of Species Coverage Values in the Sites

Functional Group	**Family**	**Species Name**	**Functional Group**	**Family**	**Species Name**
PH	Apiaceae	*Ferula* L.	L	Fabaceae	*Trigonella monantha* C.A.Mey.
PH	Asteraceae	*Achillea tenuifolia* Lam.	AH	Geraniaceae	*Erodium ciconium* (L.) L'Hér.
S	Asteraceae	*Artemisia* L.	G	Iridaceae	*Iris acutiloba* C.A.Mey.
PH	Asteraceae	*Cousinia arctotidifolia* Bunge	Cu	Juglandaceae	*Teucrium polium* L.
PH	Asteraceae	*Cousinia decipiens* Boiss. & Buhse	PH	Juglandaceae	*Ziziphora* L.
PH	Asteraceae	*Cousinia turcomanica* Bunge	G	Lamiaceae	*Eremostachys macrophylla* Montbret & Aucher ex Benth.
AH	Asteraceae	*Crepis sancta* (L.*)* Babe	Cu	Lamiaceae	*Lagochilus cabulicus* Benth.

AH	Asteraceae	*Crupina vulgaris* Cass	PH	Lamiaceae	*Phlomis cancellata* Bunge
AG	Asteraceae	*Koelpinia linearis* Pall	Cu	Lamiaceae	*Stachys turcomanica* Trautv
PH	Berberidaceae	*Berberis integerrima* Bunge	AH	Lamiaceae	*Ziziphora tenuior* L.
AH	Boraginaceae	*Arnebia decumbens (*Vent.*)* Coss	G	Liliaceae	*Allium rubellum* M.Bieb.
AH	Boraginaceae	*Lappula Moench* L.	G	Liliaceae	*Muscari caucasicum* (Griseb.)
PH	Boraginaceae	*Lappula barbata* (M.Bieb.)	G	Liliaceae	*Tulipa* L.
AH	Boraginaceae	*Nonea caspica* (Willd.) G.Don	AH	Malvaceae	*Gypsophila bicolor* (Freyn & Sint.)
AH	Boraginaceae	*Nonea lutea (*Desr.*)*	PH	Malvaceae	*Malva neglecta* Wallr.
PH	Boraginaceae	*Onosma* L.	AH	Papaveraceae	*Roemeria hybrida* (L.) DC.
AH	Brassicaceae	*Aethionema carneum* (Banks & Sol.) B.Fedtsch.	Cu	Plumbaginaceae	*Acantholimon pterostegium*
AH	*Brassicaceae*	*Alyssum desertorum* Stapf	AG	Poaceae	*Bromus danthoniae* Trin.
AH	Brassicaceae	*Alyssum meniocoides* Boiss	AG	poaceae	*Bromus tectorum* L.
AH	Brassicaceae	*Conringia* Heist. ex Fabr.	AG	Poaceae	*Eremopyrum triticeum (*Gaertn.*)*
S	Brassicaceae	*Matthiola farinosa* Bunge *ex* Boiss	AG	Poaceae	*Hordeum murinum* L.
AH	Brassicaceae	*Rapistrum rugosum* (L.) All.	AG	poaceae	*Loliolum subulatum* (Banks & Sol.) Eig
Cu	Caryophyllaceae	*Acanthophyllum pungens (*Bunge*)* Boiss	PG	Poaceae	*Piptatherum holciforme* (M. Bieb.*)*
Cu	Caryophyllaceae	*Ferula* L.	PG	Poaceae	*Poa bulbosa* L.
PH	Caryophyllaceae	*Gypsophila bicolor (*Freyn & Sint.*)*	PG	Poaceae	*Stipa barbata* Desf.
Cu	Caryophyllaceae	*Herniaria incana* Lam.	AH	poaceae	*Taeniatherum caput-medusae* L.
AH	Caryophyllaceae	*Holosteum glutinosum (*M. Bieb.*)*	G	Podophyllaceae	*Bongardia chrysogonum* L.
AH	Caryophyllaceae	*Minuartia hamata* (Hausskn. & Bornm.) Mattf.	AH	Primulaceae	*Androsace maxima* L.
AH	Caryophyllaceae	*Minuartia meyeri (*Boiss.*)*	AH	Ranunculaceae	*Adonis aestivalis* L.
AH	Caryophyllaceae	*Silene conoidea* L.	AH	Ranunculaceae	*Ceratocephala falcata (*L.) Pers.
Cu	Chenopodiaceae	*Krascheninnikovia ceratoides* L.	PH	Rosaceae	*Potentilla recta* L.
S	Chenopodiaceae	*Salsola arbusculiformis* Drobnick	AH	Rubiaceae	*Callipeltis cucullaris* L.
PG	Cistaceae	*Festuca valesiaca* Schleich. ex-Gaudin	AH	Rubiaceae	*Galium ghilanicum* Stapf

S	Cistaceae	*Fumana procumbens* (Dun.)	S	*Rubiaceae*	*Rubia tinctorum L.*
AH	Cistaceae	*Helianthemum salicifolium* L.	PH	Rutaceae	*Haplophyllum obtusifolium* (Ledeb.*)*
AH	Convolvulaceae	*Convolvulus commutatus* Boiss.	Cu	Tamaricaceae	*Reaumuria alternifolia (*Labill.*)*
AH	Dipsacaceae	*Scabiosa rotata* M.Bieb.	AG	Typhaceae	*Vulpia myuros* L.
PH	Euphorbiaceae	*Andrachne telephioides* L.	PH	Zygophyllaceae	*Malva neglecta* L.
L	Fabaceae	*Astragalus* L.			
L	Fabaceae	*Lathyrus inconspicus* L.			

Discussion

Effects of Sheep Grazing on Rao's Index and Functional Diversity

The diversity of plant communities and ecosystem functioning depends on competition among species for various resources. In the present study, Rao's quadratic entropy and functional diversity changed primarily under moderate grazing intensity, whereas light and heavy grazing did not lead to significant dispersion in plant traits. These results suggest that moderate grazing promotes trait differentiation and species coexistence, while heavy grazing reduces aboveground biomass and diversity, and light grazing allows annual species to establish. Similar findings were reported by Lv et al., (2024), who showed that species diversity in meadow steppes followed a unimodal curve under grazing intensity, consistent with the intermediate disturbance hypothesis. The observed changes in the moderately grazed site are likely due to the disappearance of annual species prior to sampling.

This finding contrasts with previous studies that reported increases in Rao's index and functional diversity under higher grazing intensities (Niu et al., 2016; Komac et al., 2015). However, the decline in functional diversity along the grazing gradient observed here aligns with Bahreini et al., (2024), who found that heavy grazing reduced species richness and phylogenetic diversity in mountainous grasslands of northern Iran. Meta-analyses further support these results: Yu Zheng et al., (2025) demonstrated that heavy grazing consistently reduces soil organic carbon and aboveground biomass, while light grazing may enhance soil organic matter in certain grassland types. Similarly, Zhang et al., (2023) highlighted that grazing impacts on biodiversity and ecosystem multifunctionality are strongly mediated by aridity, with dry ecosystems showing sharper declines in functional diversity under grazing pressure.

Effects of Sheep Grazing on Community-Weighted Leaf Traits

Livestock grazing is an abiotic disturbance that alters plant species traits and functional strategies (Bahreini et al., 2024). In this study, specific leaf area (SLA), leaf dry matter content (LDMC), leaf area, leaf length, leaf width, and leaf thickness were all affected by grazing. Heavy grazing reduced leaf thickness while increasing SLA, LDMC, and leaf area. According to Gillison et al., (2013), such trait shifts reflect plant strategies to cope with grazing pressure and competition for light following biomass removal. These findings are consistent with studies reporting that grazing increases SLA and LDMC as plants attempt to balance nutrient limitation and water stress (Zheng et al., 2010; 2015With increasing grazing intensity, plant species richness declines, while the proportion of stress-tolerant and prostrate species increases (Bahreini et al., 2024). Moreno García et al., (2014), studying grazing gradients in semi-arid African rangelands, found that plants near watering points (heavy grazing) exhibited higher nitrogen content, lower cell wall components, greater SLA, and consequently absorbed light more rapidly and efficiently, resulting in higher leaf surface and biomass efficiency. Furthermore, selective grazing by livestock alters leaf functional traits, as grazing intensity shifts specific leaf area (SLA) and leaf dry matter content (LDMC), with shorter species exhibiting higher SLA values compared to taller plants (Caram et al., 2024; Ronk et al., 2024).

Effects of Slope Aspect

According to the results of the analysis of variance, slope aspect had no significant effect on most measured traits. However, under heavy grazing, differences between eastern and western slopes became more pronounced, particularly for leaf area. This may be explained by differences in solar radiation, soil properties, and grazing behavior, since the watering point was located only on one side Shokrollahi et al., (2012). In mountainous regions, variations in snowmelt timing, slope aspect, and soil heterogeneity strongly influence ecological stress, often resulting in shifts in species composition and potential species loss (Schaefer et al., 2024). Functional diversity is an essential component of biodiversity and is considered a key factor for understanding ecosystem processes and the functioning of plant communities (Song et al., 2014). Plant functional diversity plays a critical role in predicting ecosystem permeability, nutrient cycling, and ecosystem stability (Zhang et al., 2023; Guo et al., 2024). Livestock grazing has detrimental effects not only on plant structural characteristics such as species composition but also on soil properties among plant communities (Arzani and Abedi, 2006). Therefore, integrating plant trait responses with soil ecosystem changes provides valuable insights for rangeland managers in estimating and forecasting ecosystem resilience under varying grazing.

Under heavy grazing, greater changes were observed in Rao's index, FD, LDMC, and leaf area, while other traits exhibited variable responses across sites. Overall, livestock grazing exerted relatively lower effects on Rao's index, while its impact on FD was more pronounced under moderate grazing intensity.

Conclusion

The results showed that livestock grazing had a significant effect on all leaf components, including RAO, functional diversity, and the community weighted mean of specific leaf area, dry matter content, leaf area, thickness, width, and length. Geographical aspect had no significant effect on any of these components. However, in the heavily grazed site, RAO, functional diversity, leaf dry matter content, and leaf area differed between the two aspects, while the remaining traits varied across all sites. Overall, it can be concluded that the effect of livestock grazing on RAO was relatively weak, whereas its effect on functional diversity was stronger, particularly in the moderately grazed site. The community weighted means of leaf traits also responded differently: plants tended to increase leaf area rather than leaf thickness as a compensatory strategy under grazing pressure.

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Effects of Abscisic Acid (ABA) and Benzylaminopurine (BAP) Derived Minitubers on Germination and Yield of Two Potato Cultivars (*Solanum tuberosum* L.)

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ABSTRACT

Potato, the world's fourth most important food crop, is primarily vegetatively propagated, making the production of high-quality, virus-free seed tubers essential. This experiment was conducted in two stages at the University of Jiroft during the years 2022–2023. In the first stage, the plantlets were subjected to sprinkler (foliar) application of growth regulators to the mother plants. The treatments were applied in two sequential steps: stolon induction and tuberization, using Abscisic Acid (ABA), Benzylaminopurine (BAP), and the combined treatment BAP + ABA, under controlled conditions. In the second stage, the tubers produced from the first stage were evaluated for germination characteristics, seedling establishment, and subsequent performance under field conditions. The measured traits included the number of sprouts on minitubers, sprout length, days to seedling emergence, seedling emergence percentage, number of stems per plant, morphological characteristics, and yield and yield components. Results showed that use of BAP during the tuberization stage increased sprout length by 41% and 52% in cv. Santé and cv. Colomba, respectively, compared with the control plants. Moreover, the shortest days to seedling emergence were observed in this treatment compared with the control. The highest seedling emergence percentage was recorded with ABA application during the stolon induction stage, showing an average increase of 40% over the control. Additionally, cv. Colomba exhibited a 10% higher seedling emergence than cv. Santé. In cv. Santé, the combined treatment BAP + ABA during the tuberization stage increased the number of sprouts by 50% compared with the control. In contrast, the greatest number of sprouts on tubers in cv. Colomba was obtained with BAP applied during the tuberization stage, with an increase of 33% over the control. In both cultivars, plants treated with BAP + ABA during the tuberization stage produced the highest number of tubers per plant.

Keywords: Hairy roots, Bioreactor systems, Secondary metabolites, Elicitors

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Introduction

Potato (*Solanum tuberosum* L.) with an annual production of 400 million tons and an average yield of 40 tons per hectare, is recognized as the fourth most important food crop in the world (Anonymous, 2023). Potato is predominantly propagated vegetatively through tubers. Minitubers are small tubers potatoes produced from micropropagated plantlets under in vitro conditions, which are subsequently planted at high densities in greenhouses using culture media or pots containing various substrates. Minituber size ranges from 5 to 25 mm, and their weight varies between 0.1 and 10 g (Struik, 2007). The production of Minitubers from in vitro plantlets allows for more rapid multiplication of seed tubers, better planning, and a reduction in the number of field generations (Rentzsch et al., 2012). Minitubers generally exhibit a longer dormancy period compared to conventional potato tubers, which can be critical for field establishment. For seed tuber producers, rapid and uniform tuber sprouting is a prerequisite for proper establishment of healthy plants. Sprout growth can be stimulated by various chemical, hormonal, and physical treatments. Depending on the timing of application, these substances can directly terminate tuber dormancy and induce sprout growth (Suttle, 2008). Cytokinins and gibberellins are known to break dormancy and initiate sprout growth (Hartmann et al., 2011). Struik (2007) reported that application of synthetic cytokinin terminates tuber dormancy and initiates active sprout growth. Hartmann et al., (2011) found that benzylaminopurine application induced dormancy break in tubers but did not affect sprout growth. application of plant growth regulators is widely used to modify sprouting characteristics and break tuber dormancy (Kaya et al., 2023). Plant Growth Regulators (PGRs) are chemical compounds that regulate a wide range of processes in plants (Ahmadi Lahijani et al., 2018). Among various hormones, abscisic acid (ABA) and cytokinins (CK) play fundamental roles not only in regulating senescence-related processes but also in establishing source-sink strength and modulating dormancy initiation and release (Bednarek et al., 2021). There is evidence regarding the effect of cytokinins on enhancing tuberization (Rossouw, 2008). Cytokinins are recognized as inducers of cell division (Suttle, 1995). Low concentrations of cytokinins have been shown to be more effective in promoting sprout growth in potato tubers compared to higher concentrations (Rossouw, 2008). Cytokinins are more effective than gibberellins in shortening the dormancy period of sprouts; however, they stimulate sprout growth to a lesser extent than gibberellins (Rossouw, 2008). Abscisic acid plays a role in the initiation and maintenance of tuber dormancy (Blauer et al., 2013). Although ABA content in tubers declines during storage, no consistent correlation has been observed between ABA levels and sprouting behavior across different potato cultivars (Biemelt et al., 2000). As a plant growth inhibitor, abscisic acid positively influences the onset and induction of tuberization (Farran et al., 2006). ABA promotes the mobilization of carbohydrates stored in stems and leaves, facilitating their translocation and accumulation in reproductive organs such as seeds and storage organs, thereby accelerating the process of carbohydrate deposition in these tissues (Mahajan et al., 2024). Sprouting can be stimulated by the application of chemical agents either before or after harvest (Dutta et al., 2024). Once tuber dormancy is broken, the sprouts on the seed tuber begin to grow. Upon sprouting initiation, tubers transform into a source organ that supports the growth and development of sprouts, accompanied by structural and metabolic changes as well as alterations in gene expression patterns (Davies, 2010). This sprout growth is dependent on the supply of energy, nutrients, and other essential resources provided by the mother tuber (Struik, 2007). Within each tuber, sprouts compete for available resources, particularly when the mother tuber is small, as is the case with minitubers. The growth of potato plants derived from Minitubers under field conditions is largely influenced by the sprouting pattern established during tuber storage (Suttle et al., 2012). Numerous studies have investigated the effects of chemical treatments on dormancy break and subsequent sprout characteristics (Suttle et al., 2012). Furthermore, there is little knowledge concerning the impact of maternal plant foliar application on the growth traits of the resulting tubers (Suttle et al., 2012). The objective of this study was to evaluate the effects of Abscisic Acid (ABA) and Benzylaminopurine (BAP) applications during stolon induction and tuberization stages on the subsequent germination characteristics and field performance of Colomba and Sante potato cultivars.

Materials and Methods

This experiment was conducted in two stages at the University of Jiroft during the years 2022–2023. Plantlets of two Potato cultivars (Solanum tuberousum L. cvs. Sante and Colomba) were used in this study.The first experiment was arranged as a factorial experiment in a completely randomized design (CRD). The second experiment was also factorial, but arranged in a randomized complete block design (RCBD). In the first stage, the plantlets were subjected to sprinkler (foliar) application of growth regulators to the mother plants.

First Experiment: Micropropagation and Maternal Plant Treatment

Using in vitro tissue culture techniques, micropropagation was performed, and uniform plantlets were produced at Partikan Bazar Gostar Company. Murashige and Skoog (MS) basal medium was employed for this purpose (Suttle et al., 2012). The MS culture medium was prepared based on micro and macronutrient salts and vitamins, without hormones, supplemented with 30 g/L sucrose, and pH was adjusted to approximately 5.8. The plantlets were transplanted into cubic containers (50 × 50 × 50 cm) filled with a 1:1 (v/v) mixture of perlite and cocopeat. Cultivation was carried out in a representative greenhouse in Anbarabad in early January 2022. The photoperiod was set at 12/12 h (light/dark), day and night temperatures were maintained at 27 ± 20 C and 20 ± 2 C, respectively, and relative humidity was adjusted to 40 ± 5% (Bhaskara, 2017). Hoagland nutrient solution was applied weekly to supply plant nutritional requirements (Bhaskara, 2017). During the growth period, hilling was performed twice, at 30 and 50 day after planting, to cover the lower stem nodes. No pests or diseases were observed throughout the experimental period. The experiment was arranged as a factorial based on a completely randomized design with three replications. Each replicate consisted of 12 plantlets (four plantlets per container). The first factor included two potato) Colomba and Sante) , and the second factor comprised seven hormonal treatments. Tubers were harvested manually 120 days after planting. Following harvest, Minitubers were washed with water, air-dried for 24 hours, and subsequently stored in darkness at 5 ± 1 C with a relative humidity of 85 ± 5%. After 15 weeks of storage, tubers were removed from darkness and exposed to artificial light at ambient temperature for two weeks prior to planting. The presence of at least one sprout with a minimum length of five millimeters in at least 80% of tubers was considered as the criterion for sprouting and termination of tuber dormancy. The number of activated sprouts and sprout length were recorded.

Second Experiment: Field Evaluation

Tubers obtained from the first experimental phase were planted in research farm in Anbarabad County, located at 57 58' 30" E longitude, 28 47' 43" N latitude, and an altitude of 601 meters above sea level, in late October 2023. This study was arranged as a factorial experiment based on a randomized complete block design with three replications. The first factor consisted of two potato cultivars (Colomba and Sante), and the second factor comprised tubers derived from the seven hormonal treatments applied in the first experiment. uniform tubers (15–25 mm in diameter) were planted in plots of 2.3 m^2 in three lines with a distance of 75 cm, a distance between the lines of 20 cm, and a depth of 8 cm. Fertilizer requirements were determined based on soil test results. Nitrogen was applied at 300 kg ha^{-1} from urea source, half at planting and the remainder as topdressing four weeks after emergence and prior to hilling. Phosphorus was applied at 90 kg ha^{-1} as diammonium phosphate ($(NH_4)_2HPO_4$) at planting, and potassium was applied at 100 kg ha^{-1} as potassium nitrate at planting. The experimental field had been fallowed for two years prior to planting. Selected physicochemical properties of the soil are presented in Table 1. For hormonal treatments, both plant growth regulators, BAP (6-Benzylaminopurine, Sigma) and ABA (2-cis, 4-trans-Abscisic acid, Sigma), were applied at a concentration of 50 µM via foliar spraying. Tween® (Riedel-de Haen) at a concentration of 0.5% (v/v) was used as a surfactant. Control plants were sprayed with distilled water containing 0.5% (v/v) Tween®. To ensure adequate solution uptake, foliar application was carried out until complete wetting of the plants was achieved. Hormone spraying was performed at the end of the light period under indirect sunlight to prevent rapid photodegradation. Plant growth regulator treatments were applied at seven levels and at two growth stages, including: BAP at early stolonization, ABA at early stolonization, BAP + ABA at early stolonization, BAP at early tuberization, ABA at early tuberization, BAP + ABA at early tuberization, and control (sprayed with distilled water).

Table 1. Physicochemical characteristics of the soil in depth of 0- 30 cm.

Texture	Available Potassium (mg.kg-1)	Available Phosphorus (mg.kg-1)	Total nitrogen (%)	Organic carbon (%)	Electrical conductivity (EC) (dS.m^{-1})	pH
Clay loam	130	10	0.1	1.2	1.11	7.21

Irrigation was carried out using a drip irrigation system. Weed control was performed through a single application of the herbicide metribuzin (sencor) (70% WP) at a rate of 1 kg ha^{-1} before weed seed germination, supplemented by two rounds of hand weeding. No pests or diseases were observed during the experimental period. Day to emergence were calculated as the number of days from planting to the emergence of at least 50% of seedlings. Emerged plants relative to the total number of planted tubers per treatment were considered as the percentage of germinated and established seedlings. At physiological maturity, plants were manually harvested from a 1 m^2 area within each plot, excluding border rows, for yield assessment. Three plants per replication were selected to measure leaf area, shoot dry weight, and tuber-related traits. Green leaf area was determined using a leaf area meter (Li-3100 area meter, LI-COR, Lincoln, NE). For dry weight determination, shoot samples were oven-dried at 70 °C for 24 hours until constant weight was achieved. Tuber number per plant, tuber yield, and average tuber weight were recorded. Average tuber weight per plant was calculated as the ratio of total tuber weight per plant to the number of tubers per plant.

Statistical analysis

After confirming normality, data were analyzed using SAS software (9.1). Means were compared using the Least Significant Difference (LSD) test at the 5% probability level.

Results and Discussion

Sprout Number per Minituber

Analysis of variance revealed that sprout number per minituber was significantly affected ($P \leq 0.01$) interaction cultivar at plant growth regulator (Table 2). The response of cultivars to growth regulator application varied. In both Sante and Colomba cultivars, Minitubers derived from BAP + ABA application at tuberization and stolonization stages did not show statistically significant differences in sprout number; however, both treatments produced significantly more sprouts per minituber compared to control plants. In the Sante cultivar, application of ABA or BAP alone at either stolonization or tuberization stages did not significantly differ from each other (Table 3). In contrast, BAP treatment at the tuberization stage significantly increased sprout number per minituber in Colomba compared to the control, with the highest sprout number (4.6 sprouts per minituber) recorded in this cultivar under this treatment, although no such positive effect was observed in Sante (Table 3). Therefore, it can be concluded that BAP + ABA application effectively promotes sprout production on minitubers through dormancy release and stimulation of cell division. However, cultivar differences in response to growth regulator applications were evident. These findings are consistent with those reported by Kumar et al., (2007) and Mahajan et al., (2024).

Sprout Length

According to the analysis of variance, the effect of cultivar was significant at the 5% probability level, and the effect of growth regulators was significant at the 1% probability level on sprout length (Table 2). Colomba cultivar produced longer sprouts compared to Sante cultivar. The highest sprout length in Sante (8 mm) and Colomba (8.8 mm) was recorded under BAP treatment at the stolonization stage (Table 3). BAP application at the tuberization stage increased sprout length in both Sante and Colomba cultivars by 41% and 52%, respectively, compared to control plants (Table 3). The stimulation of sprout elongation by BAP application is likely due to the induction of cell division and subsequent growth. Newly formed cells probably act as novel sinks for carbohydrates, thereby enhancing sprout growth. The differential responses observed between cultivars may be attributed to differences in morphology, endogenous physiological mechanisms, and varying sensitivity to plant growth regulators. These findings are consistent with those reported by Kuluev et al., (2016), Bhaskara (2017), and Kumar et al., (2011). Recent findings have highlighted the complex interplay of phytohormones such as Abscisic Acid (ABA) and cytokinins like Benzylaminopurine (BAP) in regulating critical stages of potato development, from dormancy release to tuber initiation (Saidi and Hajibarat., 2021).

Day to Emergence

Analysis of variance revealed that day to emergence was significantly affected only by the application of plant growth regulators at the 1% probability level (Table 2). BAP application at both tuberization and stolonization stages, on average, reduced the number of days to emergence in both cultivars compared to ABA treatment (Table 3). No statistically significant differences were observed between BAP and BAP + ABA treatments at either stolonization or tuberization stages and the control plants in both Sante and Colomba cultivars (Table 3). BAP application at the stolonization stage resulted in seedling emergence at 7 and 8 days after planting in Colomba and Sante cultivars, respectively, which was on average five day earlier in Colomba and four day earlier in Sante compared to control plants. BAP application removes callose deposits from blocked plasmodesmata, allowing assimilates to move toward dormant meristematic cells, thereby stimulating growth and accelerating seedling emergence under field conditions. Li et al., (2019) reported that the application of growth-promoting hormones enhances the translocation of photosynthetic assimilates to meristematic cells, thereby increasing the rate of seedling emergence. Cytokinins and gibberellins may facilitate the removal of callose deposits from blocked plasmodesmata, permitting photosynthates to reach dormant meristematic cells and stimulating the initiation of their growth (Reinoso et al., 2011). Sensitivity to applied plant hormones depends on processes such as uptake, metabolism, and perception of received signals (Salimi et al., 2010). Postharvest management strategies, including the exogenous application of ABA, have been shown to significantly enhance the germination rate of mini-tuber seeds, with studies reporting rates as high as 97.33% under optimal conditions (Zhu et al., 2023).

Table 2. Analysis of variance for effect of application of abscisic acid and benzylaminopurine on number of sprouts, length of sprouts and day to emergence of two potato cultivar in second experiment

		MS		
S.O. V	df	Number of sprouts per Minitubers	Length of sprouts	Day to emergence
Replication (Block)	2	0.48	0.38	0.11
Cultivar (C)	1	9.5**	3.85*	2.91^{ns}
Plant growth regulator (PGR)	6	4.02**	27.61**	30.03**
Cultivar (C) × Plant growth regulator (PGR)	6	2.2^{**}	0.75^{ns}	1.34^{ns}
Error	26	0.63	0.42	0.77
C.V. (%)		18.5	11.9	7.7

Table 3. Mean comparison of effect of application of abscisic acid and benzylaminopurine on number of sprouts, length of sprouts and day to emergence of two potato cultivars in first experiment

Plant growth regulator	Number of sprouts per Minitubers		Length of sprouts		Day to emergence	
	Sante	Colomba	Sante	Colomba	Sante	Colomba
Control	2.2^{b}	3.1^{b}	4.1^{c}	4.3^{d}	13^{a}	13^{ab}
BAP S	2.5^{bc}	2.8^{b}	8^{a}	8.8^{ab}	8^{c}	7^{c}
ABA S	2.7^{bc}	2.1^{c}	3.5^{d}	4.3^{d}	14^{a}	14^{a}
BAP + ABA S	4^{b}	4.4^{a}	6^{b}	7^{bc}	13^{a}	14^{a}
BAP T	1.5^{c}	4.6^{a}	8.2^{a}	9.5^{a}	9^{c}	9^{cb}
ABA T	1.6^{c}	2.3^{c}	4^{c}	4^{d}	12^{b}	11^{b}
BAP + ABA T	4.1^{a}	4.5^{a}	6.3^{b}	6^{cd}	11^{b}	11^{b}

In each row, mean followed by similar letters are not significantly different ($p > 0.05$) using LSD test.

Seedling Emergence Percentage

Based on the analysis of variance, the effects of cultivar and plant growth regulators on seedling emergence percentage were significant at the 1% probability level (Table 4). According to the mean comparison results, the highest seedling emergence percentage in both Sante and Colomba cultivars was observed in the ABA treatment applied at the stolonization stage, on average, it was 30% higher in the Columba cultivar and 20% higher in the Sante cultivar than in the control plants. (Table 5). Additionally, seedling emergence percentage in the Colomba cultivar was 10% higher than in Sante (Table 5). Mean comparisons also showed no statistically significant differences among ABA, BAP, and BAP + ABA treatments applied at the tuberization stage (Table 5). In this study, tubers harvested from plants treated with ABA exhibited a higher seedling emergence percentage compared to other treatments. Abscisic acid, by inducing a longer dormancy period during storage, aligned bud break more closely with the planting time, thereby preventing damage, breakage, and decay of sprouts on seed tubers during storage. Moreover, it increased sprout length after planting, contributing to improved seedling emergence. Bednarek et al., (2006) reported that ABA induces dormancy in seeds, preventing precocious germination under stress conditions and protecting both seeds and sprouts (Tanaka et al., 2006). Furthermore, ABA stimulates the translocation of assimilates toward tubers, increasing their starch and carbohydrate content (Travaglia et al., 2007). Consequently, these tubers, with higher energy reserves, support greater seedling emergence percentage, higher stem and plant number, and improved plant establishment. Minitubers with longer sprout length (up to 8 mm) exhibit shorter emergence times when planted (Dutta et al., 2024).

Number of Plants

The results of the analysis of variance showed that the effects of cultivar and plant growth regulators on the number of plants per square meter were significant at the 1% probability level (Table 4). Mean comparison results indicated that the ABA treatment at the stolonization stage in the Colomba cultivar produced the highest number of plants, with 7.2 plants per square meter, representing a 37% increase over the control plants (Table 5). In the Sante cultivar, the highest number of plants was observed with ABA application at the tuberization stage, which was 41% higher than the control. Moreover, the number of plants in the Colomba cultivar was 10% higher than in Sante (Table 5). Based on the findings of this study, it can be reported that the application of abscisic acid results in the formation of thicker and more robust sprouts. These vigorous sprouts, due to reduced damage and breakage, contribute to an increased number of plants. Suttle et al., (2012) reported that, unlike gibberellin-which promotes excessive sprout growth and increases the risk of damage and breakage-abscisic acid induces the development of shorter, thicker, and sturdier sprouts, thereby enhancing plant establishment in the field. Abscisic acid application reduced sprout fragility and susceptibility to damage, increased plant number, and decreased the number of days to sprout emergence. Gibberellin application significantly increased sprout length but led to greater brittleness and vulnerability, especially at planting time, thereby reducing the quality of micro-tubers. In contrast, sprouts resulting from ABA application at both the stolonization and tuberization stages exhibited reduced sprout length on tubers, increased plant number per square meter, and improved seedling establishment (Davies, 2010).

Number of Stems

Analysis of variance showed that the number of stems per plant was significantly affected by cultivar, plant growth regulators, and their interaction at the 1% probability level (Table 3). According to the mean comparison results, the Colomba cultivar produced an average of 35% and 15% more stems per plant than Sante at the stolonization and tuberization stages, respectively. In both Sante and Colomba cultivars, the application of BAP + ABA at the tuberization stage resulted in an average of 3.1 and 4.1 stems per plant, respectively, corresponding to 29% and 15% more stems compared to the control plants (Table 5). Thus, it can be stated that micro-tubers with a higher number of sprouts produce more stems in the subsequent generation. Haverkort et al., (2012) observed a significant linear relationship between the number of sprouts on seed tubers and the number of stems, stolons, tubers, and also between sprout number and tuber yield. They attributed differences in sprout number per seed tuber primarily to variations in pre-sprouting conditions or cultivar differences. Soaking tubers in a 20 ppm benzyladenine solution broke apical dominance and increased the number of sprouts per tuber (Mingo-Castel et al., 1976). Haverkort et al., (2012) further reported that the number of potato stems increased with a higher number of sprouts on seed tubers.

Table 4. Analysis of variance for effect of application of abscisic acid and benzylaminopurine on Emergence Percentage, Plant Per and Stem Per Plant of two potato cultivars in second experiment

		MS		
S.O. V	Df	Emergence Percentage	Plant Per m^{2}	Stem Per Plant
Replication (Block)	2	825**	5.05**	0.40^{ns}
Cultivar (C)	1	1362.5**	8.95**	8.13**
Plant growth regulator (PGR)	6	1066.3**	5.31**	6.03**
Cultivar (C) × Plant growth regulator (PGR)	6	113.2^{ns}	0.72^{ns}	1.75**
Error	26	111.5	0.50	0.30
C.V. (%)		15.5	12.8	16.5

Table 5. Mean comparison of effect of application of abscisic acid and benzylaminopurine on Emergence Percentage, Per Plant and Stem Per Plant of two potato cultivars in first experiment

Plant growth regulator	Emergence Percentage		Plant Per m^{2}		Stem Per Plant	
	Sante	Colomba	Sante	Colomba	Sante	Colomba
Control	70.1^{b}	70.2^{b}	3.8^{c}	4.5^{c}	2.5^{ab}	3.5^{a}
BAP S	85.5^{ab}	88.2^{ab}	4.8^{b}	6.2^{ab}	1.8^{ab}	1.8^{b}
ABA S	89.9^{ab}	100^{a}	6^{ab}	7.2^{a}	1.8^{ab}	2.2^{b}
BAP + ABA S	86.2^{ab}	91.2^{ab}	6.1^{ab}	6.5^{ab}	2.7^{ab}	4^{a}
BAP T	70.5^{b}	70.2^{b}	4.2^{bc}	5^{b}	1.3^{b}	2^{b}
ABA T	85.1^{ab}	84.1^{ab}	6.4^{a}	6.1^{ab}	1.4^{b}	2.5^{a}
BAP + ABA T	73.2^{b}	75.7^{b}	4.3^{ab}	5.5^{bc}	3.5^{a}	4.1^{a}

In each row, mean followed by similar letters are not significantly different ($p > 0.05$) using LSD test.

Leaf Area

Based on the analysis of variance, leaf area was significantly affected by cultivar and plant growth regulators at the 1% probability level, and by their interaction at the 5% probability level (Table 6). Mean comparison results showed that the highest leaf area per plant was observed in the Colomba cultivar with the application of BAP + ABA at the tuberization stage, reaching 1200 cm^{2} per plant-39% higher than the control plants. In contrast, for the Sante cultivar, the highest leaf area was recorded with ABA application at the tuberization stage, with 998 cm^{2} per plant, representing a 38% increase over the control (Table 7). In this study, the leaf area of the Colomba cultivar was generally greater than that of Sante. This difference between cultivars in response to plant growth regulator application may be attributed to variations in plant morphology, such as leaf area as a surface for hormone absorption, or to differences in the mechanisms of uptake and internal metabolism. These findings are consistent with the results of other researchers (Knowles and Knowles, 2006; Kaya et al., 2023; Kumar et al., 2007). Application of BAP + ABA at tuber initiation stage increased soluble carbohydrate content of both cultivars (Ahmadi Lahijani et al., 2018). Yadav et al., (1997) also reported that application of BAP stimulated accumulation of soluble sugar, proline and amino acids in Cicer plants.

Shoot Dry Weight

Analysis of variance showed that shoot dry weight was significantly affected by plant growth regulators and their interaction with cultivar at the 1% probability level (Table 6). Mean comparison results indicated that the highest shoot dry weight in Colomba and Sante cultivars, with values of 2.31 and 2.28 g per plant, respectively, was obtained from the BAP + ABA treatment applied at the tuberization stage. This represented an average increase of 15% in Colomba and 3% in Sante compared to the control plants. Furthermore, shoot dry weight at the tuberization stage was 2% higher in Colomba than in Sante (Table 7). Based on these findings, it can be concluded that the application of plant growth regulators enhances shoot dry weight by improving seedling establishment in the field, increasing canopy cover, and boosting the number of stems and plants per square meter. The differential response observed between potato cultivars following growth regulator application may be attributed to morphological factors and differences in internal uptake and metabolic mechanisms. These results are consistent with the findings of other researchers (Mahajan et al., 2024; Kaya et al., 2023; Viola et al., 2007). Roosta et al., (2015) reported that plant dry weight, diameter of tubers and tuber yield of potato plants were increased by application of BAP in vitro.

Table 6. Analysis of variance for effect of application of abscisic acid and benzylaminopurine on Leaf area and shoot dry weight of two potato cultivars in second experiment

		MS	
S.O.V	Df	Leaf area	Shoot dry weight
Replication (Block)	2	55854^{ns}	38.20**
Cultivar (C)	1	50489**	15.21^{ns}
Plant growth regulator (PGR)	6	48998**	174.98**
C × PGR	6	20047*	92.40**
Error	26	21995	11.20
C.V. (%)		19.2	16.1

Table 7. Mean comparison of effect of application of abscisic acid and benzylaminopurine on Leaf area and shoot dry weight of two potato cultivars in first experiment

Plant growth regulator	Leaf area (Cm2 Plant $^{-1}$)		Shoot dry weight (g Plant^{-1})	
	Sante	Colomba	Sante	Colomba
Control	611^{d}	735^{bc}	2.11^{a}	1.98^{b}
BAP S	720^{c}	549^{d}	1.54^{bc}	1.58^{bc}
ABA S	415^{e}	389^{c}	1.11^{c}	0.92^{c}
BAP + ABA S	552^{ce}	521^{d}	1.01^{c}	1.55^{bc}
BAP T	819^{b}	845^{b}	1.91^{ab}	1.61^{bc}
ABA T	998^{a}	862^{b}	1.75^{b}	1.97^{b}
BAP + ABA T	982^{a}	1002^{a}	2.28^{a}	2.31^{a}

In each row, mean followed by similar letters are not significantly different ($p > 0.05$) using LSD test.

Number of Tubers

Analysis of variance revealed that the effect of cultivar on tuber number was significant at the 1% probability level, while the effect of plant growth regulators was significant at the 5% probability level (Table 8). Mean comparison results showed that, in both Sante and Colomba cultivars, the BAP + ABA treatment applied at the tuberization stage produced the highest number of tubers per plant, with averages of 6 and 7 tubers, respectively. This represented increases of 37% in Sante and 22% in Colomba compared to the control plants (Table 9). Overall, the Colomba cultivar produced an average of 15% more tubers per plant than Sante (Table 9). Application of BAP + ABA at the tuberization stage increased the number of sprouts on mother tubers, stem number, and tuber number per plant in both potato cultivars. These findings suggest that a higher number of sprouts on the mother tuber leads to increased tuber production in the subsequent generation. Reinoso et al., (2011) reported that an increase in stem number per plant was associated with higher tuber number but a decrease in average tuber weight. The application of growth regulators accelerates seedling emergence, increases stem and tuber number per plant, and reduces average tuber weight in potato (Bednarek et al., 2021). Following dormancy break, sprout growth depends on the mother tuber for the supply of energy, nutrients, and other compounds required for development (Haverkort et al., 2012). The genotypic-specific response to PGRs, as seen in our cvs. Santé and Colomba, has also been documented in other recent studies. For instance, García-García et al., (2019) reported differential responses to BAP and ABA between the 'Atlantic' and 'Alpha' potato varieties during in vitro tuberization, indicating that endogenous hormonal balances play a decisive role.

Average Tuber Weight

Analysis of variance revealed significant effects of cultivar, plant growth regulators, and their interaction on average tuber weight at the 1% probability level. Mean comparison results showed that the Sante cultivar produced an average single tuber weight of 66.7 g, which was 25% higher and 16.5 g heavier per tuber than the Colomba cultivar. In the Sante cultivar, the application of BAP at the stolonization stage resulted in the highest average tuber weight (66.7 g per tuber), representing an 18% increase over the control (Table 9). In the Colomba cultivar, plants treated with BAP + ABA at the stolonization stage produced the highest average tuber weight (50.2 g per tuber), which was 29% higher than the control plants (Table 9). It appears that the application of BAP and BAP + ABA at the stolonization stage stimulates cell division in tubers, leading to heavier tubers with higher carbohydrate content, thereby increasing tuber yield (Bhaskara, 2017). Furthermore, some researchers have reported that cytokinin activates starch biosynthesis enzymes during the onset of tuberization, resulting in starch accumulation and enhanced sink capacity of developing potato tubers (Mingo-Castel et al., 1976). Cytokinin application to mother plants stimulates cell division in tubers, producing more vigorous tubers with greater carbohydrate reserves. These tubers provide better support for sprouts, leading to the development of more and stronger sprouts, which in turn results in more robust plants. This contributes to improved plant establishment and increased tuber yield (Kaya et al., 2023). Xu et al., (1998) found that ABA/GA ratio has a determining role in the initiation of tuberization in potato. Liu and Xie (2001) reported that different cytokinin concentrations increased minituber size and weight, and a linear relationship was observed between these two traits.

Tuber Yield

Analysis of variance showed that tuber yield was significantly affected by plant growth regulators at the 5% probability level (Table 8). Mean comparison results indicated that the highest tuber yield was observed in the Colomba cultivar (2282 g/m^2) and the Sante cultivar (2120 g/m^2) under BAP + ABA and BAP treatments, respectively, both applied at the stolonization stage. These represented increases of 36% and 33% compared to the control plants, respectively. At the stolonization stage, the Colomba cultivar produced the highest tuber yield, which was 7% greater than that of the Sante cultivar (Table 9). The findings of this study demonstrate that the application of BAP + ABA and BAP at the stolonization stage enhances tuber yield in potato plants by increasing the number of sprouts per minituber, subsequently increasing stem and plant number, and ultimately improving tuber number and weight. These results are consistent with those reported by Dalla Rizza, Vilaro, and Izquierdo (2019); Pospisilova and Batkova (2004); and Li et al., (2007), who stated that growth regulators such as BAP + ABA and BAP increase final potato yield by enhancing yield components (e.g., tuber weight, tuber number, etc.). Application of BAP, ABA, or their combination at tuber initiation improved key physiological traits-enhancing photosynthesis and chlorophyll via higher soluble carbohydrate accumulation-and significantly increased tuber yield components (yield per plant, mean tuber weight, and tuber number), with a stronger response in Agria Fontane cultivar (Ahmadi Lahijani et al., 2018). The positive effect of BAP application observed in our study aligns with recent findings by Thinakaran et al., (2025), who demonstrated that BAP, particularly when combined with optimal sucrose concentrations, is a highly significant factor for improving in vitro tuberization and tuber weight in potato cultivars.

Harvest Index

Based on the analysis of variance, the effect of plant growth regulators on harvest index was significant at the 1% probability level, and the interaction effect of cultivar and growth regulators was significant at the 5% probability level (Table 8). Mean comparison results showed that in the Sante cultivar, application of BAP + ABA at the stolonization stage resulted in a harvest index of 80%, which was on average 35% higher than that of the control plants. In the Colomba cultivar, ABA treatment at the stolonization stage also produced a harvest index of 80%, representing an 11% increase compared to the control (Table 9). Based on these findings, it can be concluded that the positive effects of the combined application of BAP + ABA on the number of stems per plant, yield, yield components, and ultimately harvest index are attributed to the synergistic effects of these growth regulators when applied together. These results are consistent with the findings of Dutta et al., (2024), Suttle (1995), and Travaglia et al., (2007).

Table 8. Analysis of variance for effect of application of abscisic acid and benzylaminopurine on Tuber per plant, Mean tuber weight, Tuber yield and Harvest index of two potato cultivars in second experiment

		MS			
S.O. V	Df	Tuber per plant	Mean tuber weight	Tuber yield	Harvest index
Replication (Block)	2	3.01	55.01	15251	0.002
Cultivar (C)	1	18.75**	808.5**	32217 ^{ns}	0.006 ^{ns}
Plant growth regulator (PGR)	6	4.5*	620.3**	74187*	0.059**
Cultivar (C) × Plant growth regulator (PGR)	6	1.5^{ns}	152.7**	11995.75 ^{ns}	0.015*
Error	26	0.85	75.20	105521	0.002
C.V. (%)		15.3	19.2	17.4	8.5

Table 9. Mean comparison of effect of application of abscisic acid and benzylaminopurine on Tuber per plant, Mean tuber weight, Tuber yield and Harvest index of two potato cultivars in first experiment

Plant growth regulator	Tuber per plant		Mean tuber weight (g)		Tuber yield (g m-^{2})		Harvest index (%)	
	Sante	Colomba	Sante	Colomba	Sante	Colomba	Sante	Colomba
Control	3.8^{b}	5.5^{ab}	55.2^{b}	35.5^{ab}	1420^{bc}	1459^{bc}	52^{c}	71^{b}
BAP S	4.0^{b}	5.6^{ab}	66.7^{a}	42.2^{ab}	2120^{a}	1890^{ab}	73^{a}	75^{ab}
ABA S	3.4^{c}	4.4^{b}	46.3^{bc}	47.8^{a}	1151^{c}	1325^{c}	67^{ab}	80^{a}
BAP + ABA S	5.8^{a}	5.7^{ab}	42.8^{bc}	50.2^{a}	2120^{ab}	2282^{a}	80^{a}	72^{b}
BAP T	3.7^{b}	6.5^{ab}	45.2^{bc}	34.4^{ab}	1052^{c}	1620^{bc}	55^{bc}	61^{c}
ABA T	5.1^{ab}	6.3^{ab}	39.2^{bc}	25.5^{b}	1400^{bc}	1282^{c}	60^{b}	50^{d}
BAP + ABA T	6.0^{a}	7.0^{a}	30.5^{c}	25.2^{b}	1350^{bc}	1310^{c}	54^{c}	60^{c}

In each row, mean followed by similar letters are not significantly different ($p > 0.05$) using LSD test.

Conclusion

The results demonstrated that the application of BAP and ABA can effectively improve the sprouting process on seed tubers and enhance plant establishment in the field. Overall, BAP appears to increase the number and length of sprouts, by reducing seedling emergence time, while ABA reduces sprout length on tubers and improves the percentage and uniformity of seedling emergence and establishment under field conditions. Since sprout growth largely depends on the energy supply from the mother tuber, both BAP and ABA seem to enhance the physiological sink strength of the tubers. Stronger tubers provide greater energy reserves to support sprout development during subsequent growth stages in the field. These effects are cultivar-dependent, and further research is recommended to better understand the advantages and limitations of using each growth regulator to improve tuber sprouting, plant establishment, and minituber production.

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The effect of *Ferula assa-foetida* Hydroalcoholic Extract on Pentylenetetrazole-Induced Seizures in Male Rats

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ABSTRACT

Epilepsy is a common neurological disorder with treatment resistance remaining a challenge. *Ferula assa-foetida* (asafoetida) is traditionally used in Persian medicine for neurological conditions, but its effects on seizure parameters are rarely investigated. This study evaluated the effect of *F. assa-foetida* hydroalcoholic extract on pentylenetetrazole (PTZ)-induced seizures in male rats. 35 adults male Wistar rats were divided into five groups ($n = 7$): control (saline + PTZ), three groups receiving 50, 100, and 200 mg/kg extract (i.p., 30 min before PTZ), and diazepam (1 mg/kg) as positive control. Seizures were induced by PTZ (85 mg/kg, i.p.). Parameters included latency to onset, duration of tonic, clonic, and tonic-clonic phases, total seizure duration, Racine scores, and 24-hour mortality. Data were analyzed by one-way ANOVA with LSD post-hoc test. Doses of 100 and 200 mg/kg extract significantly increased seizure latency versus control ($p < 0.001$). However, contrary to classical anticonvulsant profiles, these doses also significantly increased tonic, clonic, tonic-clonic, and total seizure durations ($p < 0.05$). The 200 mg/kg dose showed comparable latency to diazepam ($p > 0.05$) but differed significantly on seizure durations ($p < 0.05$). Racine scores were not significantly reduced by the extract. The 24-hour survival rate increased dose-dependently ($p < 0.01$). *Ferula assa-foetida* extract exhibits a mixed profile in the PTZ model: it delays seizure onset and improves survival but prolongs motor seizure durations. These findings do not support a classical anticonvulsant effect, suggesting a complex pharmacology requiring further investigation.

Keywords: *Ferula assa-foetida*, Pentylenetetrazole, Seizure, Anticonvulsant, Diazepam.

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Introduction

Epilepsy is one of the most prevalent chronic neurological disorders worldwide, affecting approximately 5.6 per 1000 individuals with active epilepsy and 6.7 per 1000 with lifetime epilepsy (Safeer et al., 2024). The global incidence rate of epilepsy is estimated at 52.5 per 100,000 person-years, with particularly high prevalence observed in West Asian countries (Safeer et al., 2024). In Iran, although comprehensive national epidemiological data are limited, available studies report a prevalence of active epilepsy ranging from 1.2 to 8.3 per 1,000 population depending on the region and study methodology. This condition is characterized by recurrent, unprovoked seizures resulting from excessive and hypersynchronous neuronal discharge in the brain, leading to significant morbidity, cognitive impairment, and reduced quality of life (Jiang et al., 2023). Despite the availability of numerous antiseizure medications (ASMs), 27-30% of patients with epilepsy develop drug-resistant epilepsy (DRE), failing to achieve sustained seizure control with existing therapeutic regimens (Jiang et al., 2023; Alghamdi et al., 2025). Among patients with idiopathic generalized epilepsy, the pooled prevalence of drug resistance has been reported at 27% (95% CI: 0.19-0.36), with psychiatric comorbidities, combined seizure types, and status epilepticus identified as significant risk factors for poor prognosis (Jiang *et al.,* 2023). Furthermore, patients with DRE face increased mortality risks, including sudden unexpected death in epilepsy (SUDEP), which occurs at an estimated incidence of 0.78-1.2 per 1000 patient-years (Wartmann et al., 2024). These limitations underscore the urgent need for novel therapeutic agents with improved efficacy and safety profiles.

The Pentylenetetrazole (PTZ)-induced seizure model remains one of the most widely used and validated preclinical paradigms for screening potential anticonvulsant compounds (Brunal et al., 2021; Samokhina and Samokhin 2018). PTZ, a tetrazole derivative, exerts its epileptogenic effects primarily through non-competitive antagonism of γ-aminobutyric acid type A (GABAA) receptors, thereby reducing inhibitory neurotransmission (Lu et al., 2022). PTZ-induced seizure activity has been detected in the 19-21 Hz beta range, which is positively correlated with generalized clonic seizures (Lu et al., 2022). While lower PTZ doses (40-50 mg/kg) are typically used for threshold tests, the higher dose of 85 mg/kg employed in the present study represents a supramaximal challenge that ensures consistent generalized tonic-clonic seizures in all animals, allowing evaluation of drug effects on severe seizure phases. This approach is particularly useful for detecting potential proconvulsant or paradoxical effects that might be masked at lower doses (Rojas et al., 2014). Additionally, PTZ administration leads to rapid downregulation of connexin 36 protein levels within 30 minutes, contributing to region-specific susceptibility to neuronal hyperactivity (Safeer *et al.,* 2024). The imbalance between inhibitory and excitatory systems results in neuronal hyperexcitability, oxidative stress, neuroinflammation, and generalized tonic-clonic seizures, mimicking key features of human generalized epilepsy (Quintans Júnior et al., 2008). The PTZ model is favored for its simplicity, high reproducibility, cost-effectiveness, and well-characterized behavioral and electroencephalographic correlates, making it an indispensable tool for antiepileptic drug discovery (Samokhina and Samokhin 2018).

In recent years, there has been a resurgence of interest in medicinal plants as alternative or adjunctive therapies for epilepsy, driven by their historical use in traditional medicine systems and potentially lower side effect profiles (Quintans Júnior et al., 2008). Plants of the genus *Ferula* have long been used in traditional medicine to treat various neurological disorders, including seizures, pain, depression, and Alzheimer's disease (Bagheri and Esmailidehaj 2024). *Ferula assa-foetida* L. (family Apiaceae), commonly known as asafoetida or "hing," is a perennial herb native to Central Asia and Iran (Bagheri and Esmailidehaj 2024). The main bioactive compounds of *Ferula* species include coumarins, monoterpenes, sulfide compounds, and polyphenols, which can improve nervous system function through multiple mechanisms (Bagheri and Esmailidehaj 2024). Studies have demonstrated that *Ferula* plants exert protective effects on neuronal cells by reducing pro-inflammatory cytokines such as IL-6, IL-1β, and TNF-α, while also strengthening the antioxidant system and reducing oxidative stress levels in the nervous system (Bagheri and Esmailidehaj 2024; Askari et al., 2020).

Despite the growing body of evidence on the neuropharmacological effects of *Ferula* species, the specific research gap lies in the limited and inconsistent data regarding the hydroalcoholic extract of *Ferula assa-foetida* in acute seizure models. Previous studies have primarily focused on the essential oil, gum, or methanolic extracts of this plant, often using different doses and administration routes. For instance, Kiasalari et al., (2013) investigated the anticonvulsant effect of *Ferula assa-foetida* gum extract (50, 100, and 200 mg/kg, i.p.) in a PTZ-induced kindling model in mice and reported reduced seizure severity (Kiasalari et al., 2013). Bagheri and Esmailidehaj (2024) reviewed the neuroprotective effects of various *Ferula* species, highlighting the use of different extract forms (e.g., hydroalcoholic, methanolic, essential oil) across a wide dose range (25-400 mg/kg) in different neurological conditions (Bagheri and Esmailidehaj 2024). However, most of these studies evaluated seizure latency and mortality without detailed phase-specific seizure duration analysis. Moreover, to the best of our knowledge, no previous study has systematically evaluated the hydroalcoholic extract of *Ferula assa-foetida* aerial parts in the acute PTZ-induced seizure model using a supramaximal PTZ dose (85 mg/kg) with comprehensive assessment of tonic, clonic, tonic-clonic, and total seizure durations alongside Racine scoring. Therefore, to address this research gap, the present study aimed to evaluate the effect of hydroalcoholic extract of *Ferula assa-foetida* (at doses of 50, 100, and 200 mg/kg, i.p.) on PTZ-induced seizures in male Wistar rats, assessing parameters including seizure latency, duration of tonic, clonic, and tonic-clonic phases, total seizure duration, Racine seizure scores, and 24-hour survival rate.

Materials and Methods

Animals and Housing Conditions

In this experimental study, 35 adults male Wistar rats weighing 250–300 g were used. The animals were purchased from the Animal Breeding and Holding Center of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. All rats were housed under standard laboratory conditions at a controlled temperature (22 ± 2°C) and a 12-hour light/dark cycle (lights on from 8:00 AM to 8:00 PM). The animals had free access to standard laboratory rodent chow (containing 22% protein, 4% fat, 55% carbohydrate, and 5% fiber; Behparvar Co., Iran) and tap water ad libitum. Upon arrival, the rats were acclimatized to the animal facility for 7 days before the start of the experiment. During this period, they were housed in polycarbonate cages (four rats per cage) with soft wood chip bedding, which was changed every other day. All experimental procedures were approved by the Ethics Committee of the Islamic Azad University, Izeh Branch (Ethics code: IR.IAU.ID.REC.1403.012) and followed the guidelines for the care and use of laboratory animals (National Research Council, 2011). This reference has been added to the reference list.

Preparation of Hydroalcoholic Extract of *Ferula assa-foetida.*

Ferula assa-foetida L. plants were obtained from a reputable herbal store in Izeh, Iran. The plant material was authenticated by a botanist (Dr. A. Mohammadi, Department of Botany, Islamic Azad University, Izeh Branch), and a voucher specimen (specimen No. IAU-1403-012) was deposited at the university herbarium. The aerial parts of the plant were dried at room temperature (25°C) for 10 days and then powdered using an electric grinder. The powdered material (100 g) was macerated with 800 mL of 80% ethanol (Merck, Germany) for 72 hours at room temperature on an orbital shaker at 120 rpm (Bagheri and Esmailidehaj 2024). The mixture was then filtered through Whatman No. 1 filter paper, and the solvent was evaporated using a rotary evaporator (Heidolph, Germany) at 40°C under reduced pressure. The resulting extract was completely dried in an oven at 40°C for 24 hours and stored at -20°C in airtight dark containers until use (Kiasalari et al., 2013; Jalili et al., 2022). The extraction yield was calculated as (weight of dried extract / weight of dried plant powder) × 100.

Experimental Groups and Drug Administration

Thirty-five rats were randomly divided into five groups of seven animals each (n = 7 per group):

- **Control group:** received normal saline (1 mL/kg, i.p.) + Pentylenetetrazole (PTZ)
- **Three treatment groups:** received 50, 100, and 200 mg/kg of *F. assa-foetida* hydroalcoholic extract (i.p.) 30 minutes before PTZ injection.
- **Positive control group:** received diazepam (1 mg/kg, i.p.) + PTZ.

All injections were performed intraperitoneally using a 1 mL syringe with a 26-gauge needle. The doses of the extract were selected based on previous studies (Mandegary et al., 2012). The extract was freshly dissolved in normal saline on the day of each experiment.

Induction of Seizures

Seizures were induced by a single intraperitoneal injection of Pentylenetetrazole (PTZ, Sigma-Aldrich, USA) at a dose of 85 mg/kg (Lu et al., 2022). This dose was selected as a supramaximal challenge to ensure consistent generalized tonic-clonic seizures in all control animals, allowing evaluation of extract effects on severe seizure phases. Immediately after PTZ injection, each animal was placed individually in a Plexiglas cage (30 × 30 × 40 cm) and observed for 30 minutes for the occurrence of seizure activity (Brunal et al., 2021; Samokhina and Samokhin 2018). Behavioral observations were recorded by two independent observers who were blinded to the treatment groups.

Seizure Parameters Recording

The following seizure parameters were recorded (Rojas et al., 2014):

1. **Latency to seizure onset:** time interval (seconds) between PTZ injection and the first clonic jerk
2. **Duration of tonic phase:** time (seconds) of tonic hindlimb extension
3. **Duration of clonic phase:** time (seconds) of clonic seizures
4. **Duration of tonic-clonic phase:** time (seconds) of tonic-clonic seizures
5. **Total seizure duration:** total time (seconds) from seizure onset to recovery (return to normal locomotor activity)
6. **24-hour survival rate:** percentage of animals surviving 24 hours after PTZ injection

Seizure intensity was scored according to the Racine scale at 5, 10, 15, 20, and 30 minutes after PTZ injection as follows (Rojas *et al.,* 2014):

- **Stage 0:** no seizure activity
- **Stage 1:** mouth and facial jerks
- **Stage 2:** head nodding
- **Stage 3:** forelimb clonus
- **Stage 4:** rearing with forelimb clonus
- **Stage 5:** rearing and falling with generalized tonic-clonic seizures.

The maximum observed score for each animal during the 30-minute observation period was recorded for analysis. All behavioral assessments were performed by an observer blinded to the experimental groups.

Statistical Analysis

Data was analyzed using SPSS software version 26 (IBM, USA). Normality of data distribution was confirmed using the Kolmogorov-Smirnov test and further verified with the Shapiro-Wilk test (recommended for sample sizes $n < 50$). Homogeneity of variances was assessed using Levene's test. For all dependent variables, homogeneity of variances was confirmed ($p > 0.05$). One-way analysis of variance (ANOVA) was used for comparisons between groups, followed by the LSD (Least Significant Difference) post-hoc test for pairwise comparisons when ANOVA was significant. The LSD post-hoc test was chosen due to its appropriate sensitivity for a limited number of pairwise comparisons (five groups) and because homogeneity of variances was confirmed. Effect sizes were calculated as eta squared (η^2), with values of 0.01, 0.06, and 0.14 considered small, medium, and large effects, respectively.

All data are expressed as mean ± standard error of the mean (SEM). Exact p-values are reported where appropriate (e.g., $p = 0.003$ rather than only $p < 0.01$). Differences were considered statistically significant at $p < 0.05$.

Results

Effect of *Ferula assa-foetida* extract on seizure latency.

As shown in Figure 1, administration of 100 and 200 mg/kg of *F. assa-foetida* hydroalcoholic extract significantly increased the latency to seizure onset compared to the control group ($p < 0.001$, $\eta^2 = 0.42$). The 50 mg/kg dose did not show a significant difference in seizure latency compared to the control group ($p > 0.05$). Although the 100 and 200 mg/kg doses increased seizure latency compared to saline ($p < 0.001$), they showed significantly lower latency compared to the diazepam-treated group ($p < 0.001$). These data indicate that the extract delays seizure onset in a dose-dependent manner.

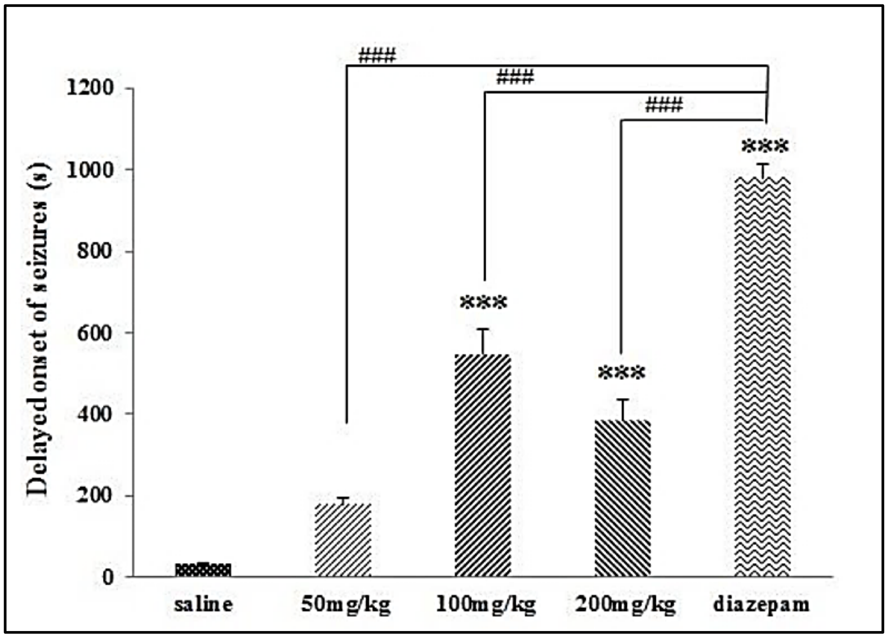

Figure 1. Comparison of seizure latency (seconds) in the control (saline) group, groups treated with different doses of Ferula assa-foetida hydroalcoholic extract (50, 100, and 200 mg/kg, i.p.), and the diazepam-treated group (1 mg/kg, i.p.) as positive control ($n = 7$). The extract at 100 and 200 mg/kg significantly increased seizure latency compared to control. Data are presented as mean ± SEM. One-way ANOVA followed by LSD post-hoc test. $^{***}p < 0.001$ when compared to the control group; $^{###}p < 0.001$ when compared to the diazepam group. Effect size (η^2) for treatment on latency: 0.42 (large effect).

Effect of *Ferula assa-foetida* extract on tonic phase duration.

Figure 2 demonstrates that all treatment groups receiving different doses of *F. assa-foetida* extract, as well as the diazepam-treated group, showed a significant increase in tonic phase duration compared to the control group ($p < 0.01$ and $p < 0.001$; $\eta^2 = 0.38$). Furthermore, the groups treated with 50 and 200 mg/kg exhibited a significant increase in tonic phase duration compared to the diazepam-treated group ($p < 0.001$ and $p < 0.05$, respectively). Thus, contrary to a classical anticonvulsant effect, the extract prolonged the tonic phase of PTZ-induced seizures.

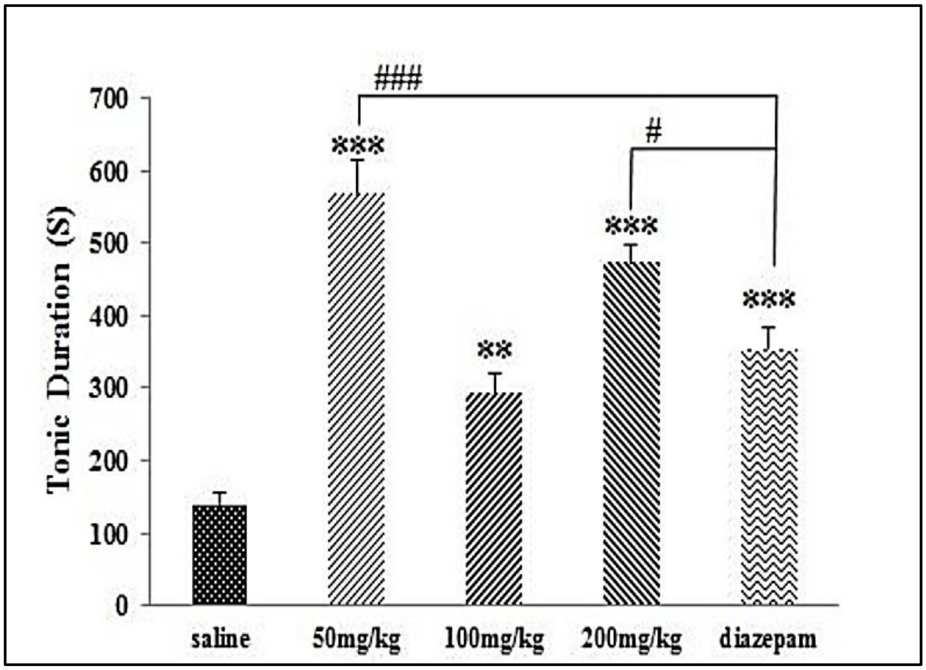

Figure 2. Comparison of tonic phase duration (seconds) in the control (saline) group, groups treated with different doses of *Ferula assa-foetida* hydroalcoholic extract (50, 100, and 200 mg/kg, i.p.), and the diazepam-treated group (1 mg/kg, i.p.) (n = 7 per group). All doses of the extract significantly increased tonic phase duration compared to the control group. Data are presented as mean ± SEM. One-way ANOVA followed by LSD post-hoc test. **$p < 0.01$, ***$p < 0.001$ when compared to the control group; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ when compared to the diazepam group. Effect size (η^2) = 0.38 (large effect). Note: Increased tonic duration indicates that the extract did not produce a classical anticonvulsant effect on this parameter.

Effect of *Ferula assa-foetida* extract on clonic phase duration.

Treatment with 50 mg/kg of *F. assa-foetida* extract significantly increased clonic phase duration compared to the control group ($p < 0.001$; $\eta^2 = 0.31$). Additionally, the groups treated with 50 and 100 mg/kg of the extract showed a significant increase in clonic phase duration compared to the diazepam-treated group ($p < 0.001$ and $p < 0.05$, respectively). The 200 mg/kg dose did not significantly differ from the control group ($p > 0.05$). These findings indicate that lower doses of the extract paradoxically prolonged clonic seizure activity.

Effect of *Ferula assa-foetida* extract on tonic-clonic phase duration.

While the diazepam-treated group showed a significant decrease in tonic-clonic phase duration compared to the control group ($p < 0.01$; $\eta^2 = 0.44$), all groups treated with different doses of *F. assa-foetida* extract exhibited a significant increase in tonic-clonic phase duration compared to the diazepam-treated group ($p < 0.05$ and $p < 0.01$). Compared to the control group, the extract-treated groups showed no significant reduction in tonic-clonic duration. This further supports that the extract does not produce a classical anticonvulsant effect.

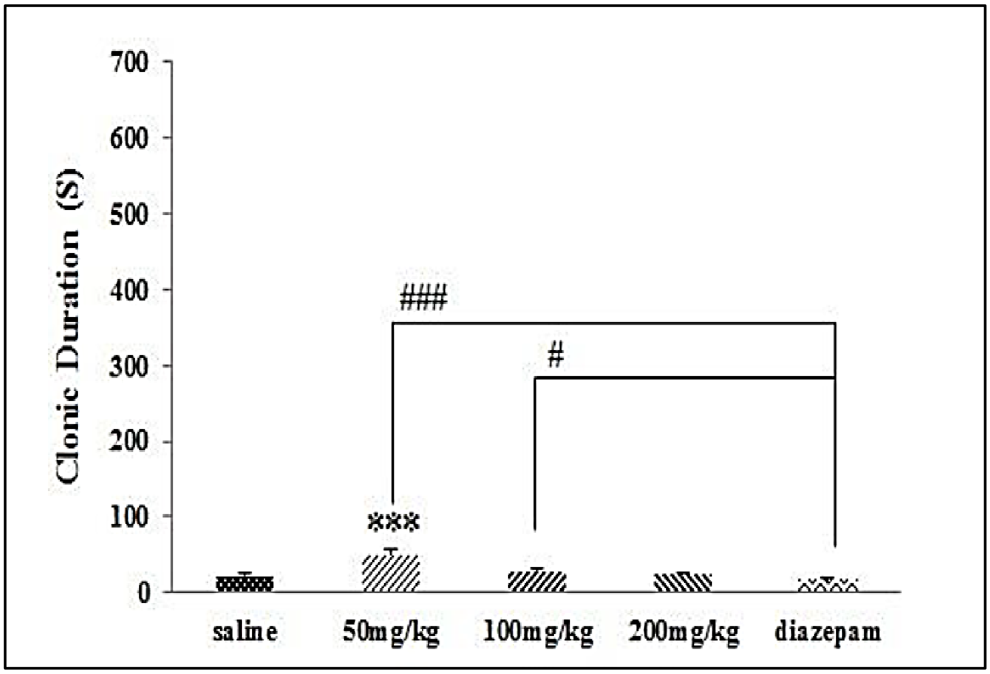

Figure 3. Comparison of clonic phase duration (seconds) in the control (saline) group, groups treated with different doses of *Ferula assa-foetida* hydroalcoholic extract (50, 100, and 200 mg/kg, i.p.), and the diazepam-treated group (1 mg/kg, i.p.) (n=7 per group). The 50 mg/kg dose of the extract significantly increased clonic phase duration compared to the control group. Data are presented as mean ± SEM. One-way ANOVA followed by LSD post-hoc test. ***$p < 0.001$ when compared to the control group; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ when compared to the diazepam group. Effect size (η^2) = 0.31 (large effect).

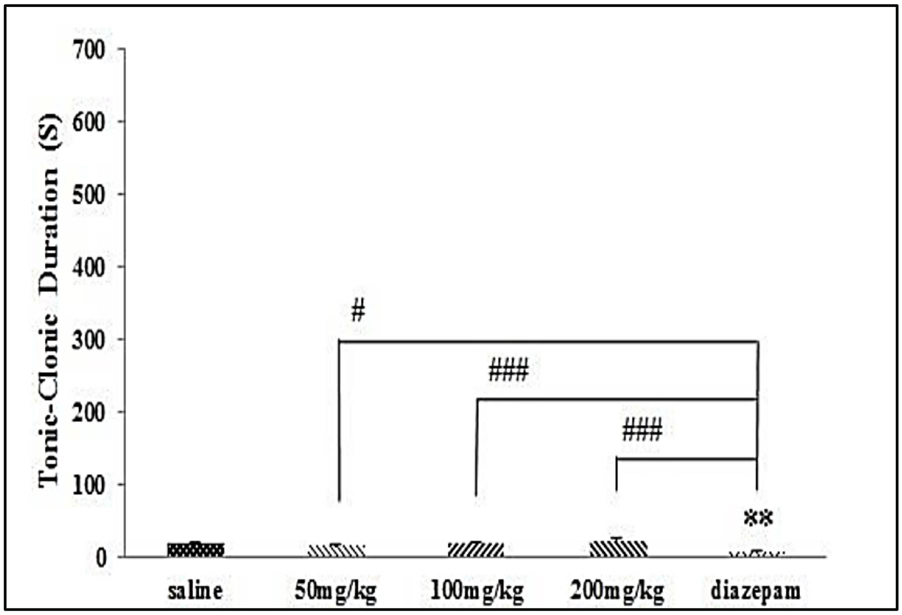

Figure 4. Comparison of tonic-clonic phase duration (seconds) in the control (saline) group, groups treated with different doses of *Ferula assa-foetida* hydroalcoholic extract (50, 100, and 200 mg/kg, i.p.), and the diazepam-treated group (1 mg/kg, i.p.) (n=7 per group). Diazepam significantly reduced tonic-clonic duration compared to control, whereas all doses of the extract increased tonic-clonic duration compared to diazepam, with no significant reduction compared to control. Data are presented as mean ± SEM. One-way ANOVA followed

by LSD post-hoc test. $^{**}p < 0.01$ when compared to the control group; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ when compared to the diazepam group. Effect size (η^2) = 0.44 (large effect).

Effect of *Ferula assa-foetida* extract on total seizure duration.

As illustrated in Figure 5, the groups treated with 100 and 200 mg/kg doses of *F. assa-foetida* extract, as well as the diazepam-treated group, showed a significant increase in total seizure duration compared to the control group ($p < 0.01$; $\eta^2 = 0.36$). This indicates that the extract, rather than abbreviating seizure activity, prolonged the overall seizure episode.

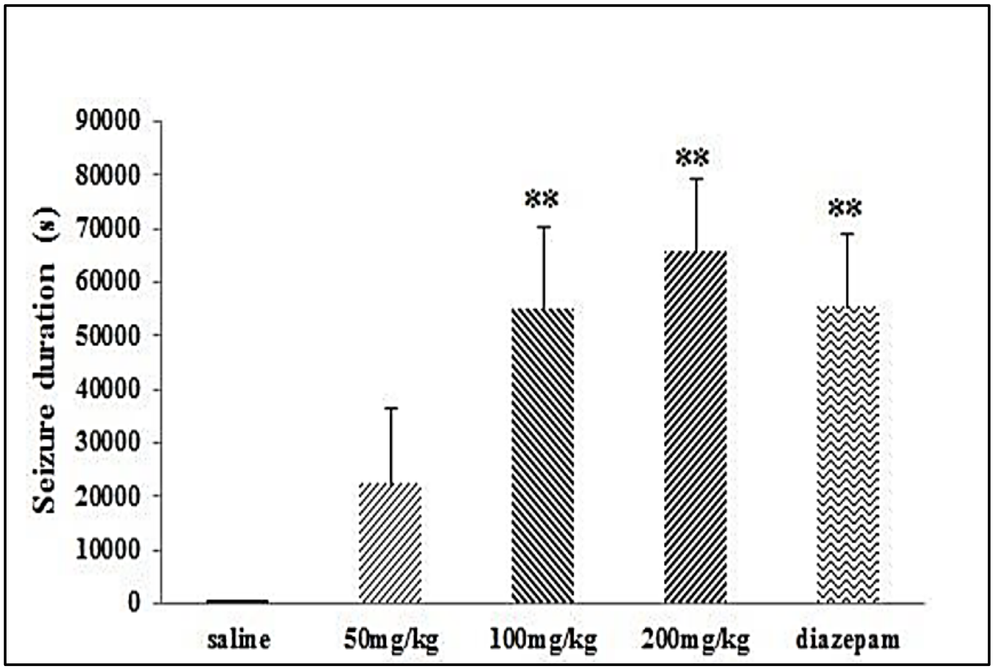

Figure 5. Comparison of total seizure duration (seconds) in the control (saline) group, groups treated with different doses of *Ferula assa-foetida* hydroalcoholic extract (50, 100, and 200 mg/kg, i.p.), and the diazepam-treated group (1 mg/kg, i.p.) (n=7 per group). The extract at 100 and 200 mg/kg significantly increased total seizure duration compared to the control group. Data are presented as mean ± SEM. One-way ANOVA followed by LSD post-hoc test. $^{**}p < 0.01$ when compared to the control group. Effect size (η^2) = 0.36 (large effect). Note: Prolongation of total seizure duration is opposite to the effect expected from a classical anticonvulsant.

Effect of *Ferula assa-foetida* extract on 24-hour survival rate.

Figure 6 shows that the 24-hour survival rate in groups treated with 100 and 200 mg/kg doses of *F. assa-foetida* extract, as well as the diazepam-treated group, significantly increased compared to the control group in a dose-dependent manner ($p < 0.01$; $\eta^2 = 0.29$). This survival benefit occurred despite the prolongation of seizure durations, suggesting that the extract may protect against seizure-induced mortality through mechanisms independent of seizure duration.

Effect of *Ferula assa-foetida* extract on seizure intensity (Racine scale)

Racine scores were recorded at 5, 10, 15, 20, and 30 minutes post-PTZ injection (Figure 7). The control group reached a mean maximum Racine score of 4.8 ± 0.2. The extract at 50, 100, and 200 mg/kg produced mean maximum Racine scores of 4.6 ± 0.3, 4.4 ± 0.3, and 4.1 ± 0.4, respectively. None of these differences reached statistical significance compared to control ($p > 0.05$ for all comparisons; $\eta^2 = 0.11$, small effect). The diazepam group showed a significantly lower Racine score (2.1 ±

0.3, $p < 0.001$ vs. control; $\eta^2 = 0.51$, large effect). Thus, the extract did not significantly reduce seizure intensity as measured by the Racine scale.

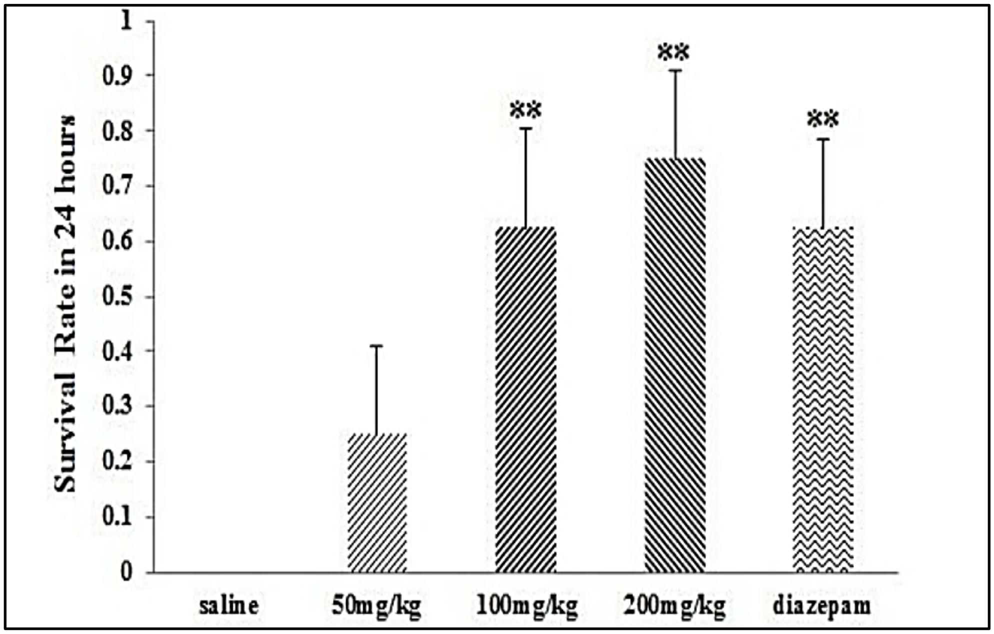

Figure 6. Comparison of 24-hour survival rate (%) in the control (saline) group, groups treated with different doses of *Ferula assa-foetida* hydroalcoholic extract (50, 100, and 200 mg/kg, i.p.), and the diazepam-treated group (1 mg/kg, i.p.) (n=7 per group). The extract at 100 and 200 mg/kg significantly increased survival rate in a dose-dependent manner. Data are presented as mean ± SEM. One-way ANOVA followed by LSD post-hoc test. $^{**}p < 0.01$ when compared to the control group. Effect size (η^2) = 0.29 (large effect).

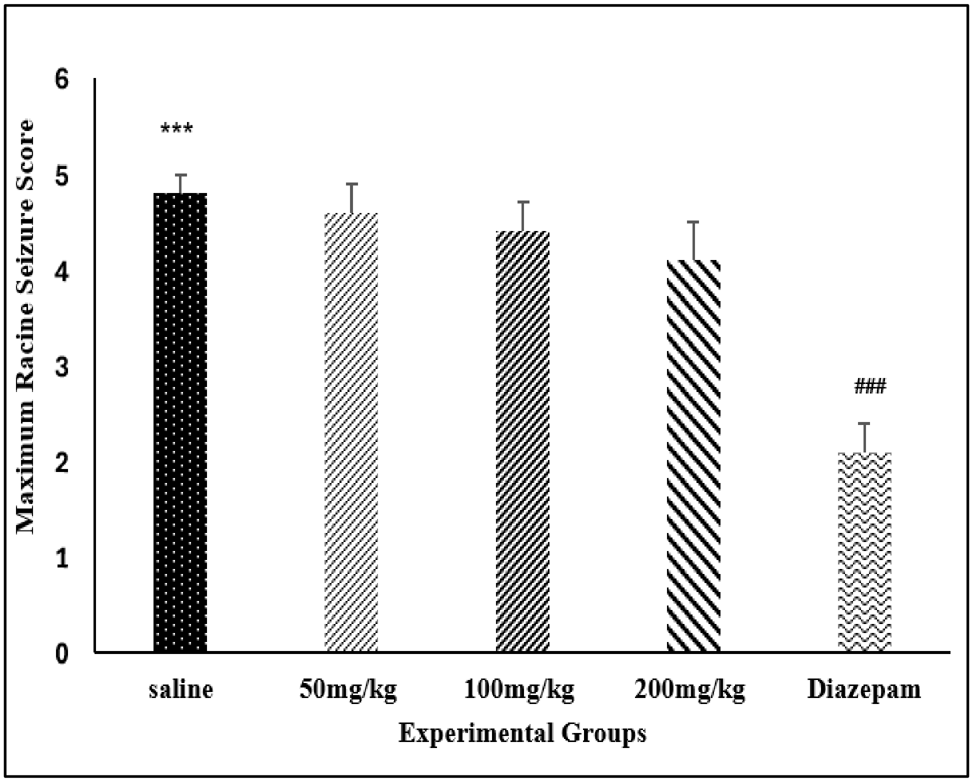

Figure 7. Comparison of maximum Racine seizure scores in the control (saline) group, groups treated with different doses of *Ferula assa-foetida* hydroalcoholic extract (50, 100, and 200 mg/kg, i.p.), and the diazepam-treated group (1 mg/kg, i.p.) (n = 7 per group). Scores were recorded at 5, 10, 15, 20, and 30 minutes after PTZ injection, and the maximum score for each animal is presented. The extract did not significantly reduce Racine scores at any dose. Only diazepam produced a significant reduction. Data are presented as mean ± SEM. One-way ANOVA followed by LSD post-hoc test. $^{***}p < 0.001$ when compared to the control group; $^{\#\#\#}p < 0.001$ when compared to the diazepam group. Effect size (η^2) = 0.51 (large effect for diazepam, non-significant for extract).

Phytochemical characterization of the extract (limitation statement)

As acknowledged in the Discussion section, no phytochemical analysis (e.g., total phenolic content, total flavonoid content, HPLC, or GC-MS) was performed on the specific batch of extract used in this study. This limitation is fully described in the Discussion.

Discussion

The findings of this study demonstrated that intraperitoneal administration of PTZ (85 mg/kg) successfully induced generalized seizures in adult male Wistar rats, which is consistent with previous reports (Esmaeili et al., 2017; Güneş et al., 2014). The present study evaluated the effects of different doses (50, 100, and 200 mg/kg) of *Ferula assa-foetida* hydroalcoholic extract on PTZ-induced seizures and revealed a complex and mixed pharmacological profile (Alimohammadi et al., 2014). Specifically, the extract significantly increased the latency to seizure onset and improved 24-hour survival in a dose-dependent manner (Figure 1 and Figure 6). However, contrary to classical anticonvulsant agents such as diazepam, the extract significantly increased the duration of tonic, clonic, and tonic-clonic phases, as well as total seizure duration (Figures 2-5). Racine seizure scores were not significantly reduced (Figure 7). This pattern – delayed onset but prolonged motor seizures – is unusual and warrants careful interpretation. A true anticonvulsant should shorten seizure duration; therefore, the present extract does not meet the criteria for a classical anticonvulsant agent (Stafstrom and Carmant, 2015).

The effects observed in this study are partially consistent with and partially contradictory to previous reports on *Ferula* species. Several studies have reported anticonvulsant or neuroprotective effects for different *Ferula* species. For example, Bagheri and Esmailidehaj (2024) reviewed the neuroprotective effects of the *Ferula* genus and reported anticonvulsant activity for some species, including *Ferula gummosa* and *Ferula sinkiangensis*, in various seizure models (Bagheri and Esmailidehaj, 2024). However, those studies primarily evaluated seizure latency and mortality, not detailed phase-specific seizure durations (Bagheri and Esmailidehaj, 2024). In agreement with our finding of increased seizure latency, previous studies have also reported that *Ferula* species can delay seizure onset, likely through GABAergic modulation (Ghanbari et al., 2021; Quintans Júnior et al., 2008).

In contrast, our finding of prolonged seizure durations contradicts some previous reports. Kiasalari et al., (2013) reported that *Ferula assa-foetida* gum extract reduced seizure severity in a PTZ-induced kindling model in mice, which appears inconsistent with our finding of prolonged seizure durations (Kiasalari et al., 2013). This discrepancy may be explained by differences in the extraction method (gum vs. aerial parts), animal species (mouse vs. rat), seizure model (kindling vs. acute), or the PTZ dose used (lower doses in kindling vs. supramaximal 85 mg/kg in the present study) (Rojas et al., 2014). Similarly, some studies on other medicinal plants have reported paradoxical proconvulsant effects at certain doses, which has been attributed to the presence of multiple bioactive compounds with opposing actions (Quintans Júnior et al., 2008). To our knowledge, no previous study has reported a paradoxical prolongation of motor seizure phases by *Ferula assa-foetida* extract, making our finding novel but also highlighting the need for replication.

The present study was not designed to elucidate the molecular mechanisms underlying the observed effects. However, based on existing literature on *Ferula* species, several hypotheses can be proposed for future investigation. Phytochemical analyses have identified numerous bioactive constituents in *Ferula* species, including sesquiterpene coumarins (farnesiferol A, ferocolicin), flavonoids (luteolin, quercetin, kaempferol), volatile sulfur compounds, and phenolic acids (Kartal et al., 2006). Flavonoids have been shown to exert benzodiazepine-like effects by binding to GABAA receptors, enhancing chloride channel opening, and promoting neuronal hyperpolarization (Ghanbari et al., 2021). Studies have demonstrated that flavonoids can increase the seizure threshold and reduce seizure severity through GABAergic mechanisms (Okoye, Akah, and Omeke, 2010). The structural similarity between certain flavonoids and benzodiazepines supports their potential as natural anticonvulsant agents (Quintans Júnior et al., 2008). These mechanisms could potentially explain the observed delay in seizure onset in our study.

The present study revealed that the hydroalcoholic extract of *Ferula assa-foetida* exerts a mixed pharmacological profile in the PTZ-induced seizure model. Although the extract significantly delayed seizure onset and improved survival, it prolonged motor seizure durations and did not reduce Racine scores, indicating that it does not possess a classical anticonvulsant effect comparable to diazepam. The paradoxical prolongation of seizure phases might be attributed to sulfur-containing compounds or biphasic effects of certain phytochemicals at higher doses, though these remain speculative hypotheses requiring experimental verification (Bagheri and Esmailidehaj, 2024; Quintans Júnior et al., 2008).

The study probably suggests that lower PTZ doses (40-50 mg/kg) might be required to detect potential anticonvulsant effects masked by the supramaximal dose (85 mg/kg) used here (Rojas et al., 2014; Samokhina and Samokhin, 2018). Overall, it is imperative to conduct additional research, specifically examining phytochemical characterization of the extract, adverse effects (motor coordination, sedation, behavioral changes), sex differences, plasma/brain concentrations of bioactive compounds, and antagonist studies (e.g., flumazenil for GABAA receptors) (Bagheri and Esmailidehaj, 2024; Kartal et al., 2006; Stafstrom and Carmant, 2015; Ghasemi and Zahediasl, 2012; Nassiri-Asl, Shariati-Rad, and Zamansoltani, 2008; Ghanbari et al., 2021). Understanding the role of these factors can provide valuable insights into the mechanisms underlying the paradoxical profile of *Ferula assa-foetida* extract and facilitate the development of potential therapeutic interventions.

Conclusion

The results of this study demonstrate that the hydroalcoholic extract of *Ferula assa-foetida* has a mixed effect in the PTZ-induced seizure model in male Wistar rats. While it significantly increased seizure latency and reduced seizure-induced mortality in a dose-dependent manner, it prolonged the duration of tonic, clonic, tonic-clonic, and total seizure phases without significantly reducing Racine scores. Therefore, the extract does not exhibit a classical anticonvulsant profile comparable to diazepam. Instead, its effects suggest a complex pharmacological action that may involve simultaneous modulation of multiple targets. These findings do not support the use of *F. assa-foetida* as a standalone anticonvulsant based on this study alone. Further studies – including EEG monitoring, receptor binding assays, toxicity assessments, fractionation to identify individual active compounds, and testing across a range of PTZ doses – are essential to clarify the mechanisms underlying this paradoxical profile and to assess whether any therapeutic potential exists.

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Conflicts of interest

There are no conflicts of interest.

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