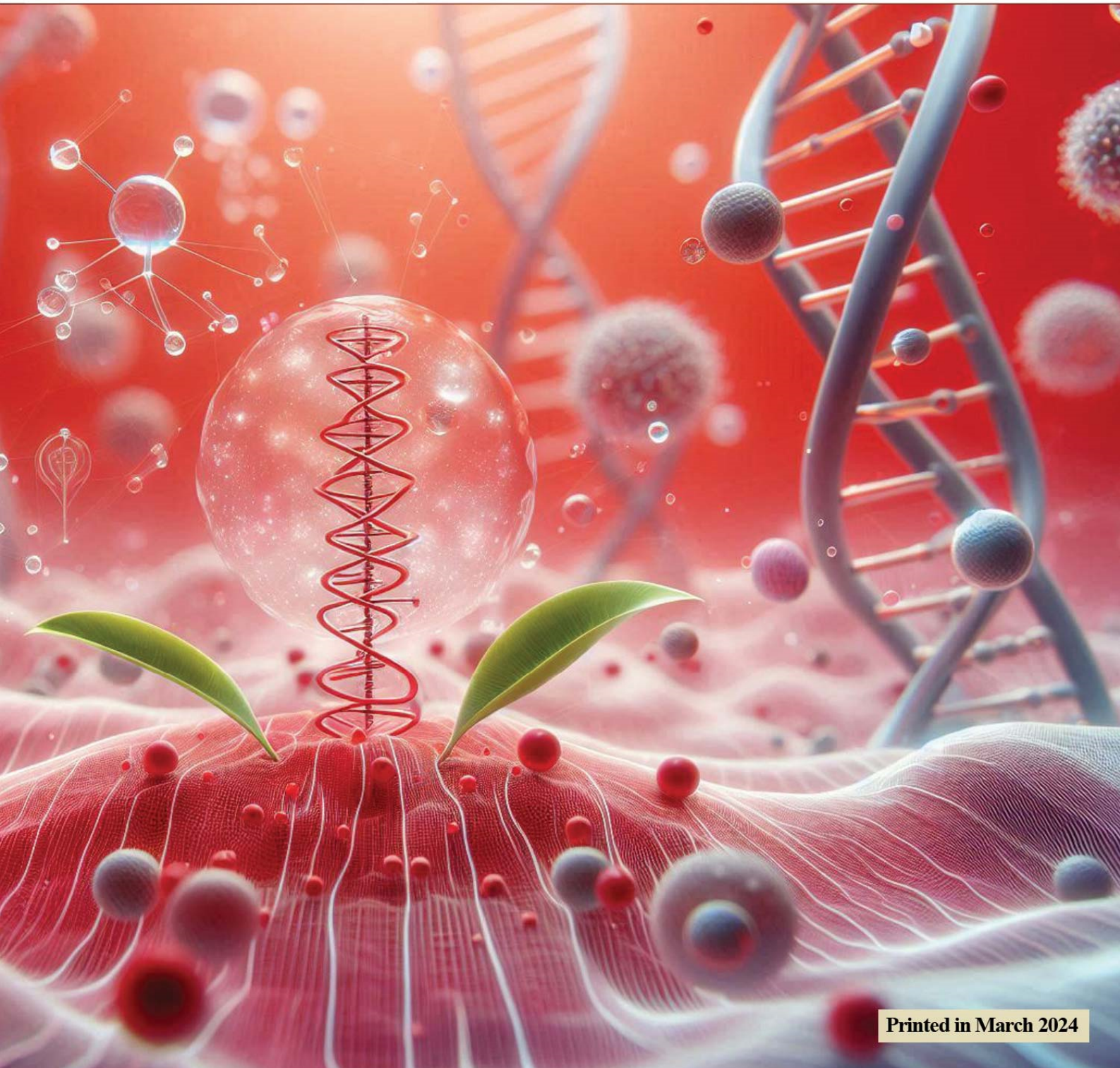
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JPMB

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JPMB is an open-access journal that serves as an advanced forum for research findings in plant molecular genetics and breeding along with other related areas such as plant biology, physiology, taxonomy, stresses, and interactions with other organisms. The journal publishes original research articles, reviews, reports, conference proceedings (peer-reviewed full articles), and short communications. In original research papers, full experimental details must be provided. We also encourage the submission of lab protocols, data management protocols, and analytical procedures in sufficient detail on topics of interest to the plant research community.

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Journal of Plant Molecular Breeding | JPMB is an international, open-access, peer-reviewed, biannual scholarly publication that aims to offer comprehensive coverage of progress in the field of plant molecular breeding. It seeks to present findings to researchers, academics, and students, addressing the growing demand for applied plant improvement technologies, tools, and methodologies.

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Original paper

Ginkgo biloba GbbHLH13 transcription factor regulates flavonoid biosynthesis

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Abstract: *Ginkgo biloba* is the oldest living plant on Earth and one of the most widely used natural medicines worldwide. Flavonoids extracted from *G. biloba* have been shown to have protective effects against cardiovascular and cerebrovascular diseases. The bHLH transcription factors (TFs) are among the most important families of transcription factors in plants, playing a crucial role in regulating plant growth, development, and secondary metabolism. In this study, *GbbHLH13* was isolated and identified. It encodes a protein consisting of 732 amino acids, and transient expression assays in tobacco indicated that *GbbHLH13* is localized in the nucleus. Exogenous hormone treatments revealed that the expression of *GbbHLH13* is affected by methyl jasmonate (MeJA) and abscisic acid (ABA). Transient overexpression experiments further showed that it upregulates the transcription levels of flavonoid metabolism-related genes *PAL*, *CHI*, and *C4H*, suggesting that *GbbHLH13* may be involved in flavonoid biosynthesis.

Keywords: *Ginkgo biloba*, *GbbHLH13*, flavonoids, subcellular localization, RT-qPCR.

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Introduction

In our country, *Ginkgo biloba* resources are widely distributed and abundant, accounting for over 75% of the world's *G. biloba* resources (Wang et al., 2013). They possess unique morphological characteristics, systematic evolution, and taxonomic status. All parts of *G. biloba* have different medicinal values, making it one of the world's most widely used natural medicines. It is commonly used to treat asthma, hypertension, atherosclerosis, and to improve cardiovascular and peripheral vascular diseases (Li et al., 2019). *G. biloba* is especially rich in secondary metabolites (Liu et al., 2021), such as flavonoids, terpenoids, polysaccharides, and phenolic acids, with flavonoids and terpenoids being the main active medicinal components (Liu et al., 2022). Ginkgo flavonoids possess antibacterial (Tagousop et al., 2018), anti-inflammatory (Maleki et al., 2019), and antioxidant activities, as well as neuroprotective effects (Singh et al., 2019) and the ability to regulate the functions of key cell enzymes (Heim et al., 2002). They are essential ingredients in various nutritional supplements, pharmaceuticals, and the cosmetics industry.

Transcription factors are a class of proteins that have specific DNA-binding domains and can bind to cis-regulatory elements to regulate gene expression at the transcriptional level (Biłas et al., 2016). bHLH transcription factors are a type of transcription factor that contains a basic helix-loop-helix (bHLH) structural domain, playing important roles in regulating plant growth and development, stress responses, and signal transduction (Hao et al., 2021). In plants, the first protein reported to have a bHLH structural domain is the product of the *R* gene in *Zea mays* L., which is involved in the regulatory synthesis of flavonoids and anthocyanins (Ludwig et al., 1989). In yeast, bHLH proteins participate in chromosome segregation and metabolic regulation (Robinson and Lopes, 2000). In animals, bHLH proteins are involved in sensing environmental signals, regulating the cell cycle and circadian rhythms, as well as modulating various developmental processes (Stevens et al., 2008). In recent years, a large number of *bHLHs* have been identified in plants such as *Arabidopsis thaliana* (L.) Heynh. (Toledo-Ortiz et al., 2003), carrot (Chen et al., 2015), and *Phyllostachys edulis* (Carrière) J. Houzeau (Cheng et al., 2018), with comprehensive genome-wide characterization conducted. To date, over 160 bHLH transcription factors have been isolated and identified, of which approximately 30% have defined functions (Pires and Dolan, 2010). Among the members of the bHLH transcription factor family in different plants, various bHLH proteins have distinct functions and are involved in different regulatory domains such as plant growth and development and morphogenesis. For example, the Arabidopsis thaliana bHLH proteins HEC2 and HEC3 are involved in regulating the development of the gynoecium (Gremski et al., 2007); additionally, *MdbHLH10* in apples can induce anthocyanin accumulation in both homologous and heterologous systems (Espley et al., 2007). Research has shown that bHLH transcription factors mainly regulate gene expression through two mechanisms. One involves forming MBW complexes by binding to MYB or WD40 transcription factors for transcriptional regulation. The second mechanism involves directly binding to the promoters of structural genes to exert regulatory effects. While there are many studies on the regulatory role of MBW complexes, the direct binding of bHLH factors to promoters requires further investigation. For instance, in apples, *MdbHLH3* can directly bind to the promoters of *MdDFR* and *MdUFGT* to activate their expression (Xie et al., 2012). Additionally, the bHLH transcription factor *VvMYC1* interacts with *MYB5a*, *MYB5b*, *MYBA1/A2*, and *MYBPA1* to induce the synthesis of anthocyanins and proanthocyanidins (Hichri et al., 2010). It is known that most bHLH transcription factors involved in flavonoid synthesis in various plants belong to the IIIf subgroup. Members of the bHLH III(d+e) subfamily can regulate jasmonic acid signaling to enhance the plant defense systems in apples and strawberries, promoting the biosynthesis of flavonoids.

So far, there has been little research on bHLH transcription factors related to ginkgo flavonoids, and the molecular mechanisms by which bHLH transcription factors regulate flavonoid synthesis in *G. biloba* remain unclear. In previous studies, the GbbHLH gene family has been identified (Zhou et al., 2020). In this study, *GbbHLH13* was isolated for the first time. Through exogenous hormone treatment and transient overexpression, it was

found that *GbbHLH13* and flavonoid-related structural genes were significantly upregulated, suggesting that *GbbHLH13* may be associated with flavonoid biosynthesis in *G. biloba*.

Materials and Methods

Cloning and identification of GbbHLH13

The nucleic acid sequence of *GbbHLH13* was obtained from genomic and transcriptomic databases based on previous studies. Using ginkgo leaf cDNA as a template, specific primers (*GbbHLH13*-U/D) were designed with Primer 6 software to amplify *GbbHLH13*, which was then cloned into the pMD19-T vector (Supplementary Table S1). The relative molecular weight and isoelectric point of *GbbHLH13* were predicted using the ExPASy platform. Homologous protein sequences of *GbbHLH13* were downloaded from NCBI, and amino acid sequence alignment was performed using DNAMAN 9.0. The upstream 2000 bp of *GbbHLH13* was extracted as the promoter sequence using TBtools, and cis-regulatory elements in the promoter region were predicted online using PlantCARE, visualization analysis of *GbbHLH13* was conducted using TBtools. Phylogenetic analysis of bHLHs involved in flavonoid regulation across different species was conducted using TBtools, and the results were compared with *GbbHLH13*.

Expression levels of GbbHLH13 in different tissues at various developmental stages

The *G. biloba* materials were sourced from a 37-year-old "Buddha's Hand" *G. biloba* in the ginkgo garden at the West Campus of Yangtze University. Samples from different tissues were collected from March to September 2024. Microstrobilus (M) were collected on March 23; ovulate strobilus (OS) were collected on April 10 and April 17; roots (R) were collected on April 10; stems (S) were collected on April 3, April 17, May 7, May 21, June 3, July 1, and July 15; leaves (L) were collected on March 23, April 3, April 17, May 7, May 21, June 3, July 1, July 15, August 13, and August 26; and fruits (F) were collected on May 7, May 21, June 3, July 1, July 15, August 13, and August 26. The samples were quickly frozen in liquid nitrogen for subsequent experiments.

For qRT-PCR validation, total RNA was extracted from *G. biloba* using the Takara MiniBEST Plant RNA Extraction Kit. The first-strand cDNA was synthesized using the HiScript III First Strand cDNA Synthesis Kit from Vazyme, Nanjing. qRT-PCR was performed according to the protocol of ChamQ Universal SYBR qPCR Master Mix. The primers *DGbbHLH13*-U/D were used to detect the expression of GbbHLH13 in *G. biloba* tissues at different developmental stages. *GbGAPDH* was chosen as the reference gene. Statistical analysis was conducted using IBM SPSS Statistics 26, and relative expression levels were calculated using the 2-ΔΔCt method, presented as mean ± standard error (SE). Duncan's multiple range test was employed to assess significant differences between means, with a significance level set at $P < 0.05$.

Exogenous hormone treatment of G. biloba seedlings

When the one-year-old *G. biloba* seedlings reached 8-10 leaves, plants with consistent growth and no visible disease were selected. The seedlings were treated by spraying both sides of the leaves with 1 mM MeJA, 100 μM ABA, and 1% ethanol solution as a control. Each treatment had three replicates, with eight seedlings per replicate. Treatments were applied at 8:00 AM, and leaves were collected at 0, 3, 6, 12, 24, and 48 hours post-treatment. The samples were quickly frozen in liquid nitrogen, and RNA was extracted and reverse transcribed for subsequent RT-qPCR analysis of *GbbHLH13* expression.

Subcellular localization of GbbHLH13

First, the subcellular localization of the GbbHLH13 protein was predicted using the WoLF PSORT online tool. The *GbbHLH13*, with the stop codon removed, was constructed into the pICH86988 and pICSL50008 fusion vectors. The correctly sequenced recombinant plasmid pICH86988-*GbbHLH13*-pICSL50008 was then introduced into Agrobacterium GV3101 via electroporation. Infection solution (10 mM MES, 10 mM $MgCl_2$, 100 μM acetosyringone, pH = 5.6) was injected into 4-5 week-old tobacco leaves for transient expression. Three days later, fluorescence was observed using a laser confocal microscope (Leica TCS SP8) to detect the localization of *GbbHLH13* in the plant.

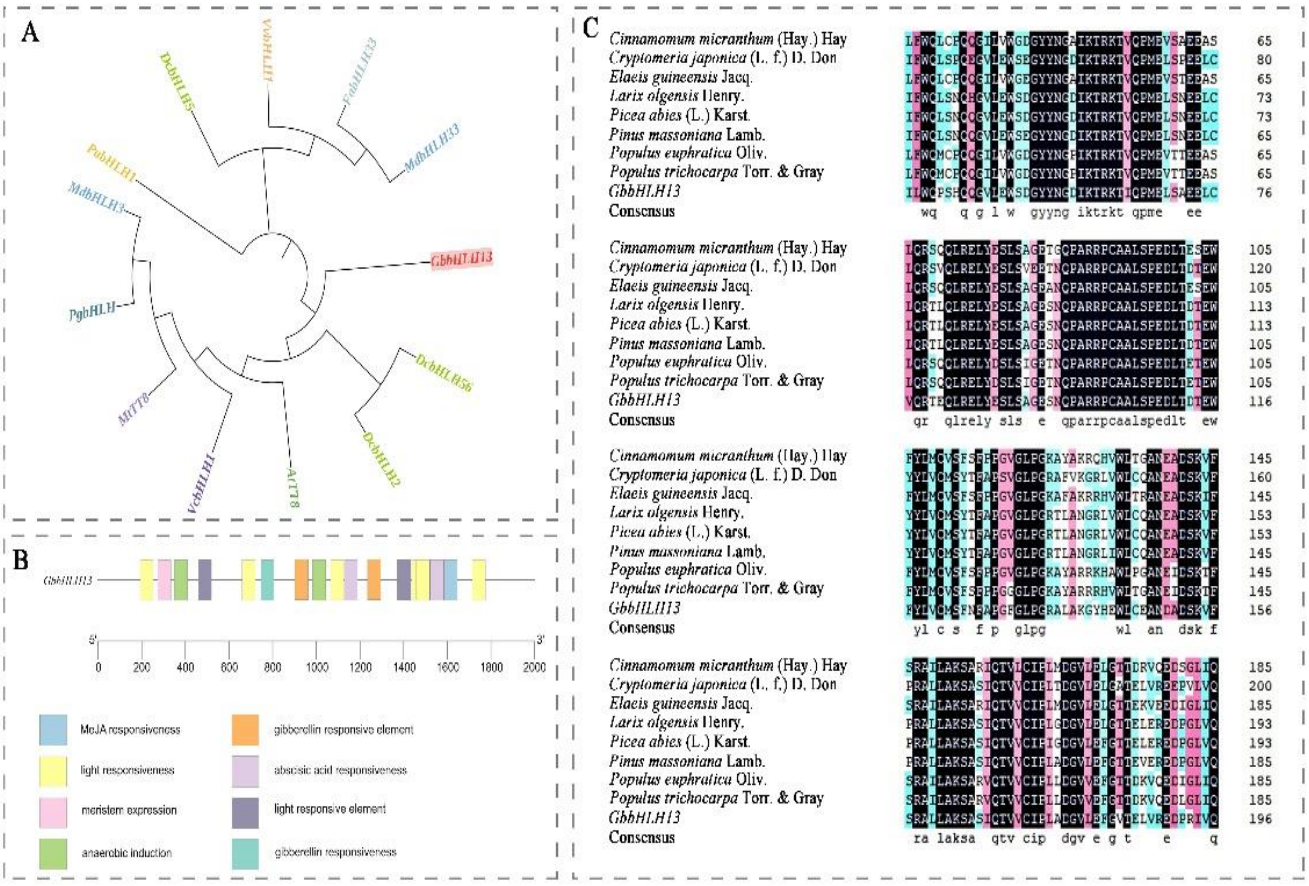


Figure 1. Characterization of *GbbHLH13*. (A) Phylogenetic tree analysis between *GbbHLH13* and verified bHLHs in flavonoid metabolism pathway in other species. Information of gene IDs from other species was shown in Table S1. (B) Promoter analysis for *GbbHLH13*. (C) Multiple sequence alignment for *GbbHLH13* conserved domain and bHLH conserved domains of other species downloaded from NCBI.

Transient overexpression of GbbHLH13 in G. biloba

To investigate the role of *GbbHLH13* in the flavonoid biosynthesis of *G. biloba*, the overexpression vector pCY-H05252 was selected. PCR amplification was conducted using the primers pCY-H05252-*GbbHLH13*-U/D, and the full-length sequence of *GbbHLH13* was subsequently inserted to construct an overexpression recombinant vector. This vector was then transferred into Agrobacterium tumefaciens GV3101 via electroporation and employed for transient overexpression experiments in *G. biloba*. Annual Ginkgo seedlings exhibiting uniform growth and free from disease were selected for these transient expression experiments. The bacterial cultures of pCY-H05252-*GbbHLH13*-GV3101 and pCY-H05252-GV3101 were activated. Once the optical density at 600 nm (OD600) of the bacterial suspension reached approximately 1.0, the culture was centrifuged at 4000 rpm for 5 minutes to collect the bacterial cells, which were then resuspended in an infection solution containing 10 mM MES, 10 mM $MgCl_2$, and 100 μM acetosyringone at pH 5.6. The left leaf of each seedling was injected with the infection solution containing pCY-H05252-*GbbHLH13*, while the right leaf received the control solution. Following a 24-hour incubation period in the dark, the samples

were cultured under normal conditions for one week. Subsequently, samples were rapidly frozen in liquid nitrogen for further experiments.

Results

Isolation and analysis of GbbHLH13

The full length of *GbbHLH13* is 2199 bp, encoding 732 amino acids; the molecular weight is 8.2 kDa, and the isoelectric point is 5.68. Promoter analysis showed that *GbbHLH13* contains a large number of light-responsive elements, gibberellin, methyl jasmonate, and abscisic acid response-related elements; it is also related to anaerobic induction and tissue meristem. (Figure 1B). By comparing bHLH protein sequences with high similarity to *GbbHLH13* using NCBI Blast function, it was found that *GbbHLH13* protein shares a common conserved domain with bHLH proteins of other plants (Fig. 1C). To study the phylogenetic evolution of *GbbHLH13*, this study selected 12 bHLH amino acid sequences involved in flavonoid regulation to construct a phylogenetic tree. The results showed that *GbbHLH13* has a relatively close genetic relationship with dracaena, apple, and grape (Figure 1A).

Expression levels of GbbHLH13 in different tissues at different stages

The expression levels of *GbbHLH13* were analyzed in *G. biloba* microstrobilus (M), ovulate strobilus (OS), roots (R), stems (S), leaves (L), and fruits (F) at different developmental stages. Samples included one period for roots and male flowers, two periods for female flowers, seven periods for stems and fruits, and ten periods for leaves. The results indicated that *GbbHLH13* expression in leaves showed a trend of rising and then falling, peaking at L2 and experiencing a brief increase at L7. The expression was highest in the first period of stems, significantly notable in the first and fourth periods of fruits, and markedly higher in female flowers compared to male flowers and roots (Figure 2). In summary, *GbbHLH13* is expressed in various tissues of *G. biloba* at different stages, with an overall declining trend over time, significant expression in leaves, and almost no expression in roots.

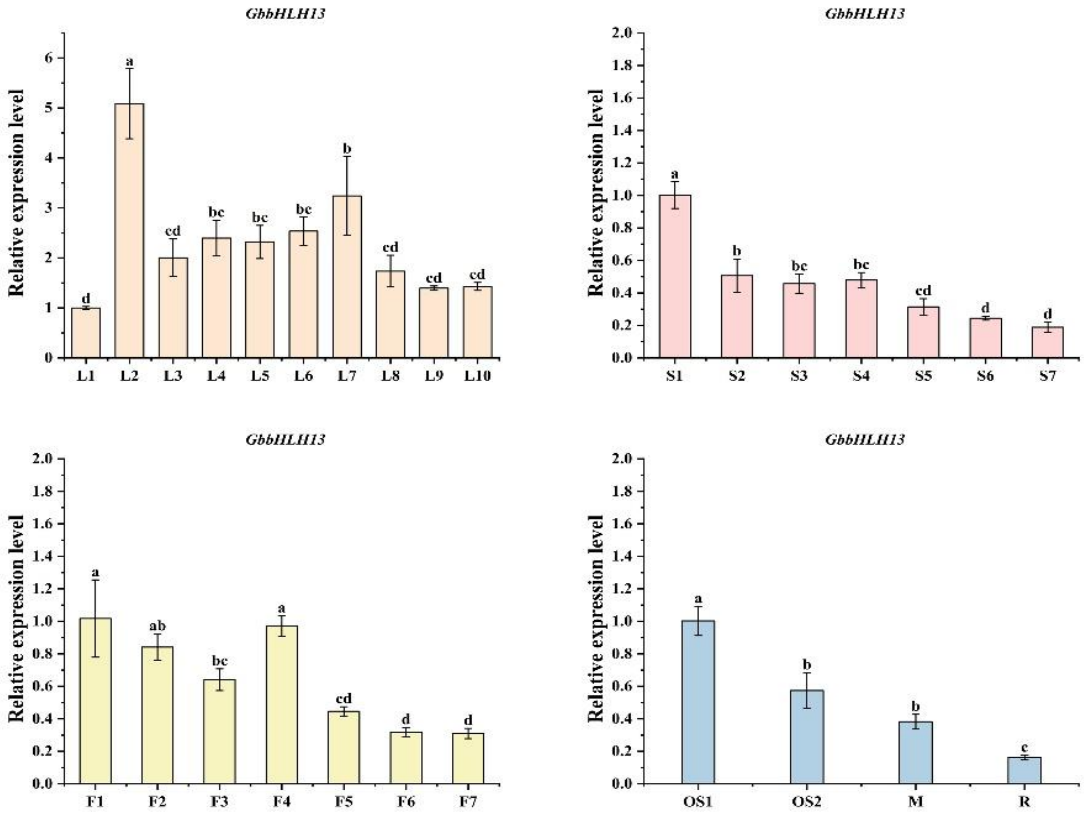


Figure 2. Expression levels of *GbbHLH13* in different tissues at different developmental stages of *Ginkgo biloba*.

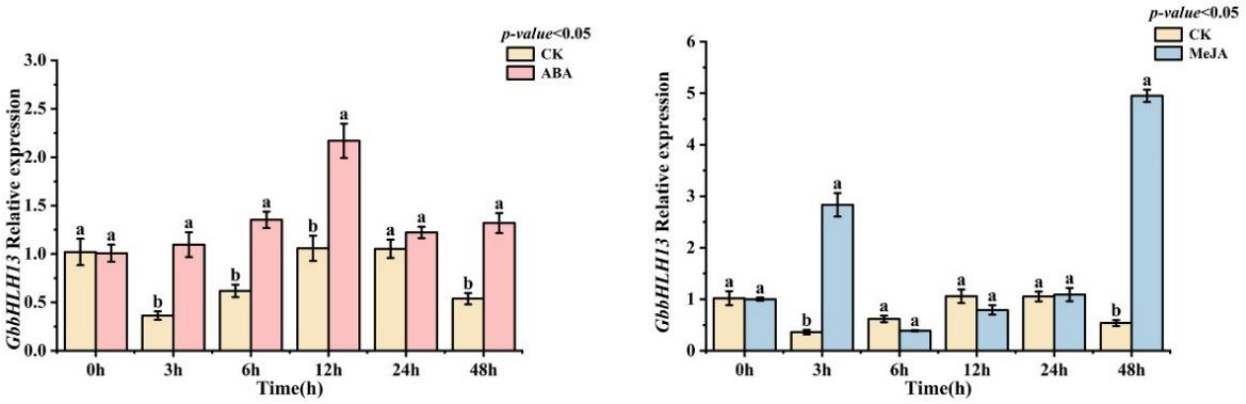


Figure 3. Expression of *GbbHLH13* after exogenous hormone treatment of *Ginkgo biloba.*

Exogenous hormone treatment of G. biloba seedlings

In this study, online prediction of the *GbbHLH13* promoter revealed the presence of response elements for MeJA, ABA, and GA3. Research has shown that treatment with MeJA and ABA can effectively increase flavonoid accumulation. For instance, *MdMYB1* in apple can activate anthocyanin biosynthesis under the influence of ABA, and the isolated *MdbZIP44* from apple can enhance the binding of *MdMYB1* to the promoters of downstream target genes, thus promoting anthocyanin accumulation(An JianPing et al., 2018). Similarly, ABA treatment has been shown to increase the content of flavonoids in pigeon pea (Yang et al., 2021). Additionally, ABA treatment of Fragaria chiloensis can enhance the expression of *FcPAL*, *FcCHS*, and *FcANS*(Mattus-Araya et al., 2022). Studies have found that *Carthamus tinctorius* L. upregulates the expression of *CHSs*, *CHIs*, and *HCTs* while downregulating *ANRs* and *ANSs* through MeJA treatment, thereby promoting flavonoid biosynthesis. In *Camellia vietnamensis* (Chen et al., 2020), MeJA treatment increases the expression of key genes involved in flavonoid synthesis, such as *PAL*, *4CL*, *CHI*, and *FLS*, after 2 hours (Yan et al., 2022). Furthermore, after MeJA treatment, *AmMYB30* interacts with the promoters of *AmCHS*, *AmFLS*, and *AmF3H* to induce flavonoid accumulation (Qi et al., 2024). MeJA treatment also upregulates the expression of key genes like MYC transcription factors in the flavonoid biosynthesis pathway of *Dendrobium officinale* (Jia et al., 2024).

To explore the relationship between *GbbHLH13* and flavonoid content, 1 mM MeJA and 100 μM ABA were applied to the leaves of one-year-old *G. biloba* seedlings. The gene expression patterns of *GbbHLH13* in hormone-treated leaves at different time points were analyzed. The results indicated that after ABA treatment, the expression of *GbbHLH13* first increased and then decreased, peaking at 12 hours. In contrast, after MeJA treatment, *GbbHLH13* expression rose sharply, then fell, and subsequently increased again to reach a peak (Figure 3). These findings suggest that *GbbHLH13* may be involved in the regulation of flavonoids in *G. biloba*.

Subcellular localization of GbbHLH13

Online prediction indicates that *GbbHLH13* is localized in the nucleus. By constructing the fusion expression vector pICH86988-*GbbHLH13*-pICSL50008, using pICH86988-pICSL50008 as a control, we employed laser confocal microscopy to detect the subcellular localization of *GbbHLH13* fused with green fluorescent protein. The experimental results showed that the control group displayed fluorescence at both the cell membrane and nucleus. In contrast, when expressing the pICH86988-*GbbHLH13*-pICSL50008 fusion, strong fluorescence was only observed in the nucleus, which is consistent with the expected results (Figure 4).

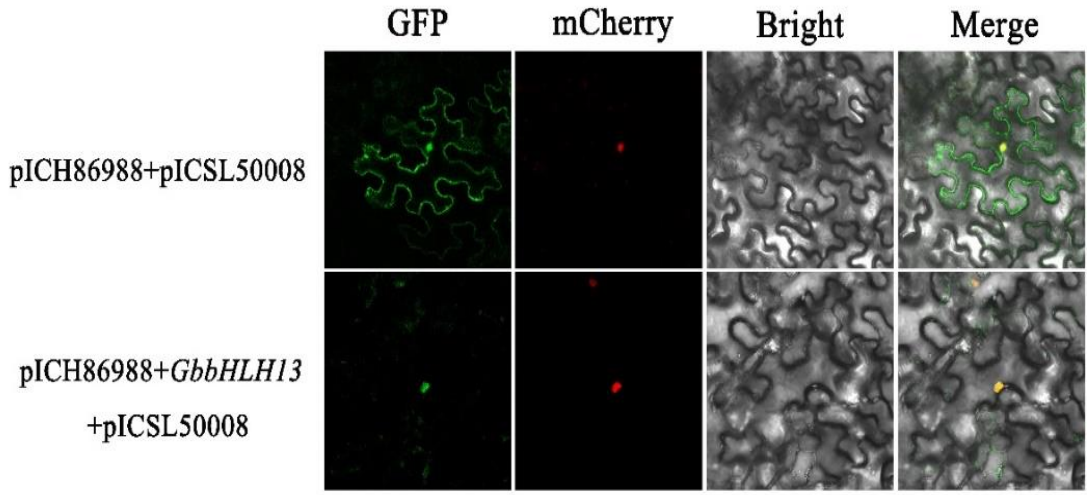


Figure 4. Subcellular localization of *GbbHLH13*.

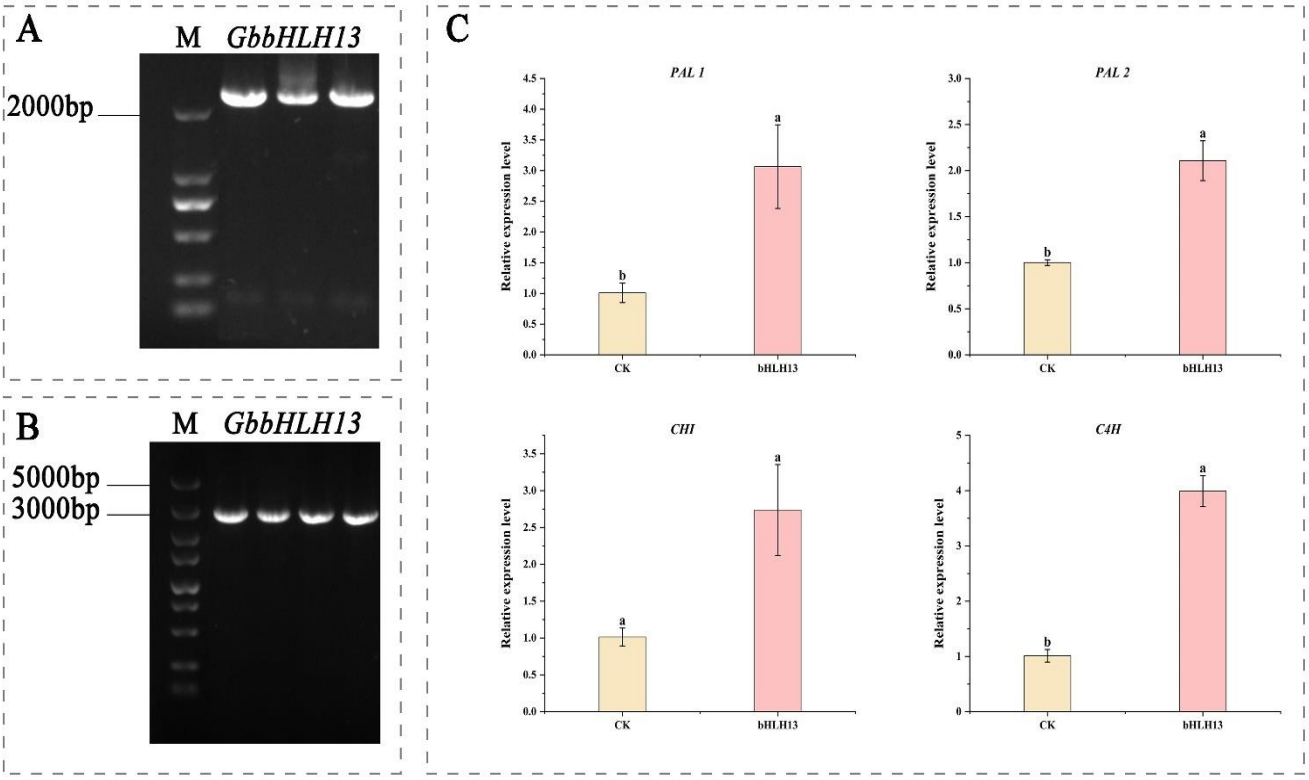


Figure 5. (A) *GbbHLH13* clone product electrophoresis pattern. (B) pCY-H05252-*GbbHLH13* electrophoresis pattern. (C) *GbbHLH13* regulates expression changes of structural genes.

Transient overexpression of GbbHLH13 in G. biloba

Since a stable genetic transformation system has not yet been established for ginkgo, this study chose to use a transient overexpression method by injecting ginkgo leaves to predict the function of *GbbHLH13*. After cloning *GbbHLH13* (Figure 5A), the pCY-H05252-*GbbHLH13* vector was constructed (Figure 5B). The pCY-H05252-*GbbHLH13* vector was used as the experimental group, while pCY-H05252 empty vector served as the control group. Four structural genes involved in the flavonoid biosynthesis pathway in *G. biloba PAL 1*, *PAL 2*, *C4H*, and *CHI* were selected as targets. RT-qPCR experiments were conducted to investigate the expression changes of these four structural genes in the experimental and control groups. The results showed that the expression levels of *PAL 1*, *PAL 2*, and *C4H* significantly increased in a short time (Figure 5C), suggesting that *GbbHLH13* may be involved in the regulation of flavonoids in *G. biloba*.

Discussion

This study provides the first analysis and identification of *GbbHLH13*, which is located in the nucleus. Its nucleotide sequence is 2199 bp, encoding 732 amino acids. It was found that *GbbHLH13* shares common conserved domains with bHLH proteins from various plants, including *Cryptomeria japonica* (Thunb. ex L. f.) D. Don (XP_057844388.1), *Larix gmelinii* (Rupr.) Kuzen. (UNP37115.1), *Pinus massoniana* Lamb. (UGN74583.1), *Picea abies* (L.) H. Karst. (ANB66421.1), *Elaeis guineensis* Jacq. (XP_010906882.1), *Populus trichocarpa* Torr. & Gray (XP_006383662.2), *Cinnamomum micranthum* (Hay.) Hay (RWR95376.1), and *Populus euphratica* Oliv. (XP_011032729.1), (Figure 1C). The phylogenetic tree indicates that *GbbHLH13* is phylogenetically related to the identified bHLH transcription factors involved in flavonoid regulation. Previous studies have shown that *DcbHLH2*, *DcbHLH5*, and *DcbHLH56* regulate flavonoid biosynthesis by binding to promoters(Zhu et al., 2020). Heterologous expression of *PgMYB5*-like and *PgbHLH* in pomegranate induced the accumulation of dihydroflavonols(Arlotta et al., 2020). Research has indicated that the expression of *TT8* is controlled by different combinations of MYB and bHLH transcription factors in plants, specifically inducing the biosynthesis of proanthocyanidins(Baudry et al., 2006). Overexpression of *VvbHLH1* significantly increased the accumulation of flavonoids in transgenic Arabidopsis plants, while enhancing salt and drought tolerance(Wang et al., 2016). *MtTT8*, *MtWD40-1*, and MYB transcription factors interact with *MtPAR* or *MtLAP1* to activate the promoters of flavonoid reductase and anthocyanin synthase, thereby regulating anthocyanin biosynthesis(Li et al., 2016). Differential expression gene (DEG) analysis revealed that the expression pattern of MYB-bHLH-WD40 genes positively correlates with anthocyanin accumulation in blueberry fruits(Zhao et al., 2019). In summary, *GbbHLH13* may play a regulatory role in the flavonoid synthesis pathway. Promoter analysis can help reveal the potential response mechanisms of genes to multiple stresses. Binding sites related to meristem formation, organogenesis, and anaerobic induction were identified in the *GbbHLH13* promoter, suggesting that *GbbHLH13* is closely associated with plant growth and development. When plants are subjected to specific environmental influences, plant hormone signaling pathways are activated to regulate various downstream biological processes. Exogenous hormone treatments showed that the expression of *GbbHLH13* is influenced by MeJA and ABA, both of which have been shown to affect flavonoid synthesis in various plants. Therefore, it can be inferred that *GbbHLH13* may regulate flavonoid synthesis. Additionally, transient overexpression in ginkgo leaves indicated that *GbbHLH13* also upregulated the transcription levels of genes related to flavonoid metabolic pathways, including *PAL*, *CHI*, and *C4H*.

Conclusion

In this study, GbbHLH13, located in the nucleus, was cloned for the first time, and its function was analyzed and identified. It is speculated that GbbHLH13 is involved in the regulation of flavonoid synthesis in Ginkgo through hormone treatment. Furthermore, the transient expression of GbbHLH13 in Ginkgo can upregulate the transcription levels of genes related to the flavonoid metabolism pathway, specifically PAL, C4H, and CHI. Additionally, the transcription factor (TF)

protein that interacts with GbbHLH13 should be further identified and analyzed.

This study suggests that *GbbHLH13* may be involved in the flavonoid metabolic pathway, providing valuable insights for expanding the control network of flavonoid metabolism. Future research is needed to explore the downstream target genes of *GbbHLH13* and how they participate in the regulation of flavonoids in *G. biloba*.

Supplementary Materials

The supplementary material for this article can be found online at: https://www.jpmb-gabit.ir/article_717860.html.

Supplementary Table S1. *GbbHLH13* genes and primers information.

Author Contributions

Conceptualization, formal analysis, visualization, writing- original draft, validation, and investigation; Y. L.

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Conflict of Interest Statement

The author declares no conflict of interest.

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مقاله پژوهشی

نقش فاکتور رونویسی *GbbHLH13* در تنظیم بیوسنتز فلاونوئید در گیاه جینکو بیلوبا

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چکیده: جینکو بیلوبا از قدیمی‌ترین گیاهان زنده روی زمین و یکی از پرمصرف ترین داروهای طبیعی در سراسر جهان می‌باشد. بررسی‌ها نشان داده است که فلاونوئیدهای استخراج شده از *G. biloba*، دارای اثرات محافظتی در برابر بیماری‌های قلبی عروقی و عروقی مغز می‌باشند. فاکتورهای رونویسی bHLH (TFs) یکی از مهم‌ترین خانواده‌های فاکتورهای رونویسی در گیاهان بوده که نقش مهمی در تنظیم رشد، نمو و متابولیسم ثانویه گیاه دارند. در این مطالعه جداسازی و شناسایی ژن *GbbHLH13* مدنظر قرار گرفت. توالی این ژن، پروتئینی متشکل از ۷۳۲ اسید آمینه را کد می‌نماید. سنجش بیان موقت این ژن در تنباکو نشان داد که بیان ژن *GbbHLH13* در هسته می‌باشد. تیمارهای هورمونی اگزوژن نشان داد که بیان *GbbHLH13* تحت تأثیر دو هورمون اسید آبسیزیک (ABA) و متیل جاسمونات (MeJA) قرار دارد. آزمایش‌های بیش‌بیان موقت این ژن نشان داد که سطح رونویسی ژن‌های مرتبط با متابولیسم فلاونوئید شامل *PAL* ، *CHI*، و *C4H* افزایش یافته که احتمالا می‌تواند حاکی از نقش *GbbHLH13* در بیوسنتز فلاونوئید باشد.

کلمات کلیدی: جینکو بیلوبا، GbbHLH13، فلاونوئیدها، مکان‌یابی درون سلولی، RT-qPCR.

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Transposable elements: mechanistic insights into a dynamic regulatory landscape of plant stress response with agricultural implications

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Review paper

Abstract: Transposable genetic elements (TEs) are dynamic DNA sequences that significantly impact plant gene expression, enabling adaptation to environmental stresses. This review explores the role of TEs in plant adaptation, focusing on the mechanisms of TE activation and suppression, including chromatin remodeling, DNA modifications, and small interfering RNAs (siRNAs). Stress conditions trigger TE activation through interactions between stress-induced transcription factors and TE promoters, as seen with retrotransposon families like COPIA93 and ONSEN in modulation of stress-responsive genes. Understanding these mechanisms provides valuable insights for agriculture, particularly in developing crops that are resilient to climate change. Leveraging TE-mediated gene regulation presents innovative strategies for enhancing plant adaptation, highlighting the potential of TEs in genetic manipulation for plant improvement.

Keywords: Transposable elements, epigenetic regulation, environmental adaptation, noncoding RNAs, plant stress.

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Introduction

Epigenetic research has demonstrated that heritable modifications in gene function, which do not include any changes to the DNA sequence, play a crucial role in plants' ability to adapt to environmental stress (Kakoulidou *et al.*, 2021; Hoseini and Arzani, 2023; Hoseini *et al.*, 2024). Epigenetics refers to these heritable changes in gene activity that do not involve changes to the underlying DNA sequence. The alterations mentioned above, which are facilitated by several processes including DNA methylation, histone modification, and RNA interference, highlight the inherent adaptability of plant genomes in reaction to external stimuli (Akhter *et al.*, 2021). Transposable elements (TEs) are dynamic DNA sequences found in both eukaryotic and prokaryotic genomes. TEs have the ability to move within the genome, introducing genetic variations that significantly impact gene expression and contribute to plant adaptability (Fambrini *et al.*, 2020; Ramakrishnan *et al.*, 2022). These variations help plants to cope with both biotic and abiotic stressors. Abiotic stress refers to the negative impact of non-living factors, such as drought, salinity, and extreme temperatures, on plants (Hoque *et al.*, 2020). TEs were historically considered genomic parasites; however, recent research has highlighted their crucial role in genome evolution and stress adaptation (Pandita and Pandita, 2023). The responsibilities of TEs go beyond their function as genetic disruptors. They actively engage in epigenetic regulation, which enables plants to respond to environmental challenges by modifying chromatin, integrating regulatory sequences, and producing noncoding RNA (Mozgova *et al.*, 2019; Klein and Anderson, 2022). Recent advancements in cutting-edge technologies, such as CRISPR-based tools, have significantly advanced TE research by allowing precise manipulation and investigation of TEs, further uncovering their roles in plant resilience (Zhang *et al.*, 2022).

This review emphasizes the evolutionary importance of TEs and specifically examines their influence on gene expression under abiotic stress conditions. This study also explores recent research that highlights the significance of TEs, which were previously regarded as genomic parasites, in relation to their crucial role in enhancing plant adaptability and survival across many settings. The narrative commences by acknowledging Barbara McClintock's groundbreaking research (McClintock, 1950), which revealed the dynamic characteristics of TEs, establishing the foundation for our present comprehension of their essential function in the evolution and functioning of plant genomes, specifically under stress conditions (Pulido and Casacuberta, 2023).

TEs have been extensively studied and found to play a significant role in the development and operation of the host genome, specifically in how plants respond to environmental challenges (Ramakrishnan *et al.*, 2022). In general, TEs have a role in regulating genes and modifying gene responses to their surroundings, hence influencing the dynamics of the genome (Schrader and Schmitz, 2019; Diehl *et al.*, 2020). Studies have demonstrated that TEs occupy several functions within plant genomes. The mechanisms above encompass the facilitation of gene duplication or deletion, the modulation of gene expression or activity, and the amalgamation of genes from disparate sites into innovative combinations (Gill *et al.*, 2021). TEs interfere with the functioning of genes by infiltrating genes or gene regulatory elements. They can induce chromosomal rearrangements by undergoing aberrant recombination between numerous TE copies (Almojil *et al.*, 2021).

To quickly and consistently adapt to environmental changes, plants need a high degree of growth flexibility, which enables them to flourish diverse and unfamiliar conditions (Majid *et al.*, 2017). The presence of genetic variation across plant populations has been found to augment their ability to withstand both biotic and abiotic challenges (Ghasemi-Soloklui *et al.*, 2023). According to the selfish DNA theory, TEs have been conserved on different chromosomes throughout the evolutionary history of plants, exhibiting significant variations in terms of quantity, variety, and positioning (Kent, 2023). The content of TEs in plant genomes varies greatly, ranging from a minimum of 3% in aquatic plants such as *Utricularia gibba* to more than 85% in complex polyploids like wheat (Pegler *et al.*, 2023). Our objective is to gain insight into the delicate equilibrium between genomic stability and flexibility, which is crucial for plant resilience in

response to shifting environmental conditions. This study presents a synthesis of recent research findings regarding the roles of TEs in facilitating changes in gene expression during periods of stress, and discusses their implications for future agricultural applications. As a result, it contributes to the wider academic conversation surrounding plant adaptation techniques.

Transposable elements: An epigenetic perspective

TEs play a critical role in the epigenetic regulation of plant genomes, offering a unique lens to understand how plants adapt to stress (Figure 1). Classified broadly into 'copy and paste' (Class I, or Retrotransposons) and 'cut and paste' (Class II, DNA transposons), these elements contribute to genomic diversity and flexibility (Wells and Feschotte, 2020). Class I TEs replicate through an RNA intermediary, a process involving transcription to RNA followed by reverse transcription back into DNA, facilitated by reverse transcriptase enzymes. These are prevalent in large plant genomes and include subtypes with long terminal repeats (LTRs) and those without, impacting genome structure and function differently (Kojima, 2019).

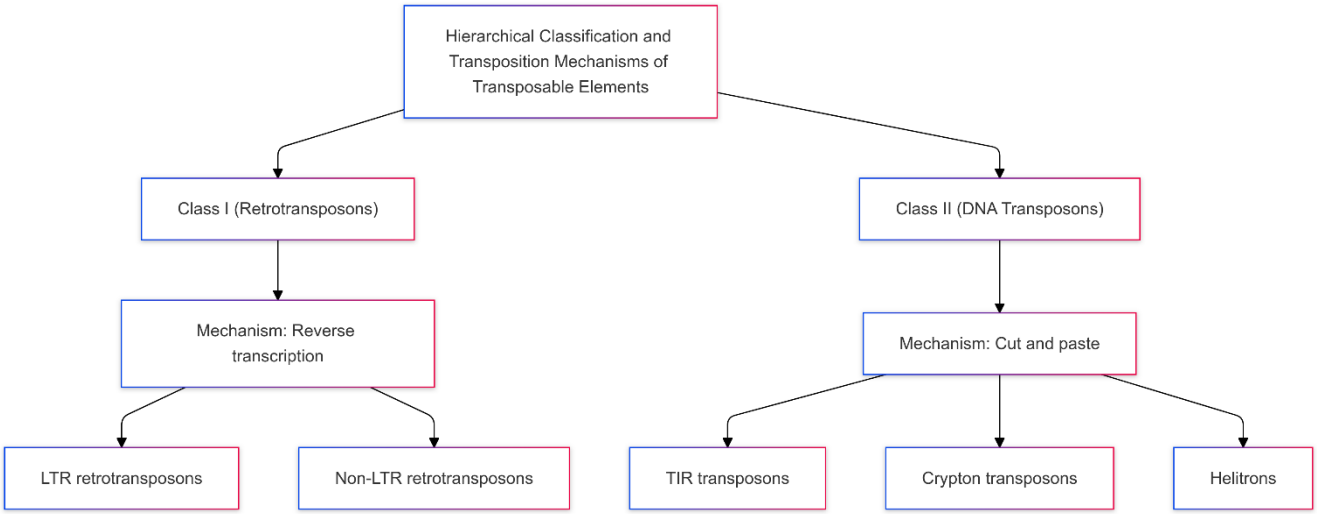


Figure 1. Classification of transposable elements in eukaryotes. This figure provides an overview of the different classes and subclasses of transposable elements, helping to establish a foundation for understanding their diverse roles and mechanisms within eukaryotic genomes.

On the other hand, Class II TEs, found across eukaryotes, maneuver through a direct 'cut and paste' mechanism, enabling them to move autonomously within the genome. This movement, governed by the transposase enzyme, adds to the genetic diversity essential for plants' stress adaptation mechanisms (Schrader and Schmitz, 2019; Bhat *et al.*, 2022).

Both autonomous and non-autonomous TEs, with the former capable of independent relocation and the latter requiring assistance from autonomous elements, enrich the plant's response to environmental changes. The variety in TE size and encoded genes underscores their complexity and influence on plant genome architecture. For example, TEs make up significant portions of the genome in many plants, such as around 85% in maize, with varying impacts on gene expression and regulation (Oliver *et al.*, 2013; Jouffroy *et al.*, 2016; Quesneville, 2020; Ramakrishnan *et al.*, 2021). In sum, TEs are not just genomic relics but active participants in shaping gene expression and plant adaptation to stress through epigenetic mechanisms.

Mechanisms of transposable element activation and silencing

TEs undergo strict regulation to balance their contributions to genetic diversity against the risk of genomic instability (Kremer *et al.*, 2020). Activation of TEs is often triggered by environmental stressors, developmental cues, or certain phases of the life cycle, enhancing genetic variability crucial for plant adaptation (Figure 2) (Mustafin and Khusnutdinova, 2019). For example, under drought stress, the frequency of activated TEs in maize was found to increase significantly, highlighting the role of environmental factors in triggering TE activity (Makarevitch *et al.*, 2015). However, unregulated TE activity can threaten genomic integrity, necessitating robust mechanisms for their repression (Almojil *et al.*, 2021).

Activation mechanisms

External stresses—such as temperature fluctuations, drought, and pathogen invasion—can prompt TE activation by altering the cellular environment and epigenetic status, initiating transcription. For instance, the ONSEN retrotransposon in *Arabidopsis* becomes active under heat stress, potentially facilitating adaptation (Nozawa *et al.*, 2022). Quantitative data also indicate that the frequency of ONSEN copies increases significantly—up to tenfold—under repeated heat stress conditions, demonstrating transgenerational effects (Ito *et al.*, 2016). Developmental signals, particularly during gametogenesis and embryogenesis, can also increase TE activity, enriching genetic diversity in progeny (Maupetit-Mehouas and Vaury, 2020).

Silencing mechanisms

DNA methylation, histone modifications, and RNA interference (RNAi) are epigenetic controls that rely heavily on TE suppression (Choi and Lee, 2020). Different researchers found that DNA methyltransferases are essential for DNA methylation at TE promoters, which inhibits TE transcription (Deniz *et al.*, 2019; Domb *et al.*, 2020)..

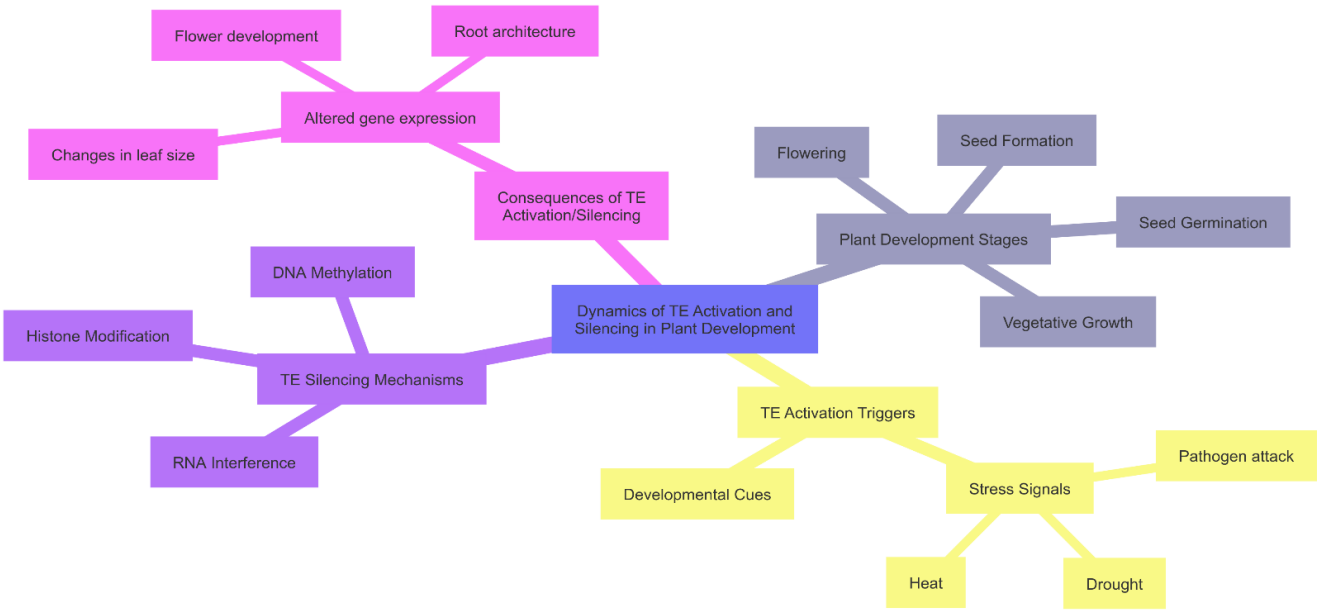


Figure 2. Dynamics of TE activation and silencing in plant development. This figure illustrates the processes of TE activation and silencing, showing how these elements are regulated during different stages of plant development and in response to environmental cues. It supports the discussion on the balance between TE activity and repression, contributing to plant adaptability.

Quantitative analysis has shown that in stressed versus unstressed conditions, levels of DNA methylation at TE sites can decrease significantly, corresponding to increased TE activity during stress (Secco *et al.*, 2015). Heterochromatin formation and TE silencing are both impacted by histone modifications, such as H3 lysine 9 dimethylation (H3K9me2) (Underwood and Choi, 2019). The RNA-directed DNA methylation (RdDM) pathway is essential for maintaining TE suppression by specifically methylating TE sequences; RNA interference (RNAi) mechanisms also produce small noncoding RNAs that target TEs for silencing (Erdmann and Picard, 2020).

Dynamic balance

The equilibrium between TE activation and suppression underscores the plant genome's adaptability and stability. While activation under stress can introduce beneficial traits for stress tolerance, robust silencing mechanisms ensure genomic stability is restored post-stress (Liu *et al.*, 2022). For example, studies have shown that after stress conditions subside, TE silencing is reestablished within a few cell cycles, reducing the frequency of TE transcription events compared to peak activation levels (Naito *et al.*, 2009). This dynamic interplay between activation and silencing mechanisms allows plants to adapt to changing environments without compromising genomic integrity

Transposable elements as epigenetic regulators

TEs extend beyond their mobility within the genome, playing pivotal roles in epigenetic regulation (Figures 3 and 4). These elements significantly influence genome responsiveness to environmental stimuli, underpinning adaptability and evolutionary processes (Nishihara, 2019; Colonna Romano and Fanti, 2022). TEs modulate epigenetic landscapes through several mechanisms (Figure 4): they alter DNA methylation patterns, reshape histone configurations, and generate noncoding RNAs, each contributing to the complexity of gene regulation (Ali *et al.*, 2021). Notably, similar regulatory roles of TEs have been observed in other eukaryotes, including animals and fungi, where TEs contribute to chromatin modifications and influence stress responses (Slotkin and Martienssen, 2007; Rebollo *et al.*, 2010).

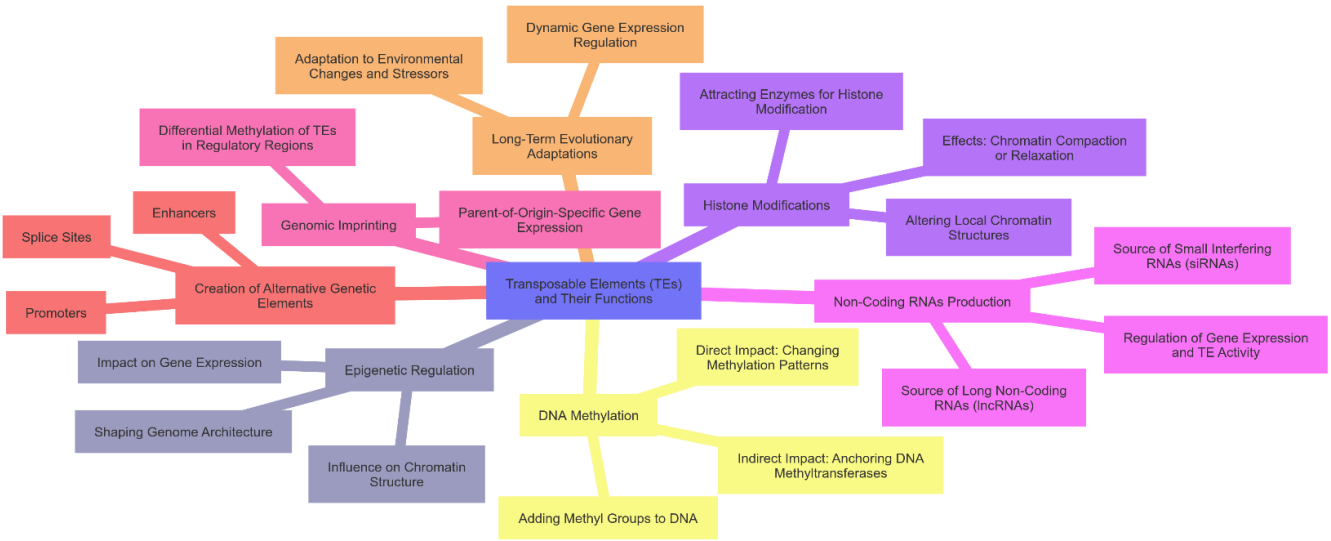


Figure 3. The mind map diagram illustrating the roles and functions of Transposable Elements (TEs). This diagram offers a comprehensive overview of the multiple roles that TEs play in genome evolution, gene regulation, and stress response, reinforcing the detailed discussion provided in the text.

DNA methylation

TEs directly influence the DNA methylation landscape, with their integration altering methylation patterns near or within genes, potentially leading to changes in gene activity. They also serve as platforms for DNA methyltransferases, indirectly modulating genome-wide methylation and affecting gene expression, particularly in stress responses and development (Deniz *et al.*, 2019; Kumar and Rani, 2023).

Histone modifications

A change in local chromatin architecture can occur as a result of TE integration into the genome, which attracts enzymes that modify histones. Their ability to methylate, acetylate, and ubiquitinate histones impacts the transcription machinery's ability to access DNA. For example, under drought stress, TEs have been shown to promote H3K9me2 (histone H3 lysine 9 dimethylation), leading to the formation of heterochromatin and suppression of nearby genes (Bej and Basak, 2017). Conversely, heat stress can induce H3K27ac (histone H3 lysine 27 acetylation), resulting in the activation of TEs and adjacent genes, aiding in plant adaptation (Korotko *et al.*, 2021). According to (Drongitis *et al.*, 2019; Bousios *et al.*, 2020), the presence of TEs can induce either a chromatin state that silences genes or an activating one.

Noncoding RNAs

Long noncoding RNAs (lncRNAs) and small interfering RNAs (siRNAs) also originate from TEs. These molecules are essential for gene regulation; lncRNAs may influence gene expression close to TEs, and siRNAs silence particular genes or TEs. This ability to regulate adds a new layer to epigenetic regulation, which affects mechanisms of stress response and development (Ariel and Manavella, 2021; Gebrie, 2023).

Genomic imprinting and regulatory element formation

Beyond these mechanisms, TEs contribute to genomic imprinting by differentially methylating regulatory regions of imprinted genes. They also foster genomic diversity by creating alternative promoters, enhancers, and splice sites, enhancing the functional repertoire of the genome (Batista and Köhler, 2020; Gill *et al.*, 2021).

TEs' role as epigenetic regulators is central not only to immediate environmental response but also to long-term evolutionary adaptation. By influencing epigenetic modifications, TEs enable dynamic regulation of gene expression, facilitating organismal adaptation to changing environments and stressors. This underscores the significance of TEs not only in plants but also across other eukaryotic organisms, where they offer mechanisms for rapid genomic adaptation to new challenges (Slotkin and Martienssen, 2007; Klein and Anderson, 2022). This underscores TEs' integral role in the evolutionary narrative, offering mechanisms for rapid genomic adaptation to new challenges (Baduel and Quadrana, 2021; Klein and Anderson, 2022).

Evolutionary perspectives of transposable elements

The presence of TEs has played a crucial role in influencing the evolutionary path of plant genomes (Colonna Romano and Fanti, 2022). TEs play a crucial role in genomic innovation, promoting diversity and complexity throughout the plant kingdom, rather than being only genetic curiosities (Pandita and Pandita, 2023). The relevance of their involvement in evolution is underscored by their ability to introduce new genetic material, facilitate gene and genome duplications, and contribute to the formation of novel regulatory networks (Bhat *et al.*, 2022).

TEs are dynamic elements of the genome that can generate new genomic arrangements (Stitzer *et al.*, 2021). The capacity to relocate and integrate into diverse genomic sites facilitates the incorporation of novel genetic material. The ability to move has made it easier for genes with new functions to develop and for gene families to grow, which has improved the ability of plants to adapt (Ramakrishnan *et al.*, 2021). For example, in wheat (*Triticum aestivum*), a hexaploid species, TEs have played a key role in polyploid genome evolution. The rapid amplification of TEs contributed to genome expansion and facilitated adaptive traits such as increased resilience to stress conditions, including cold and drought (Wicker, 2018).

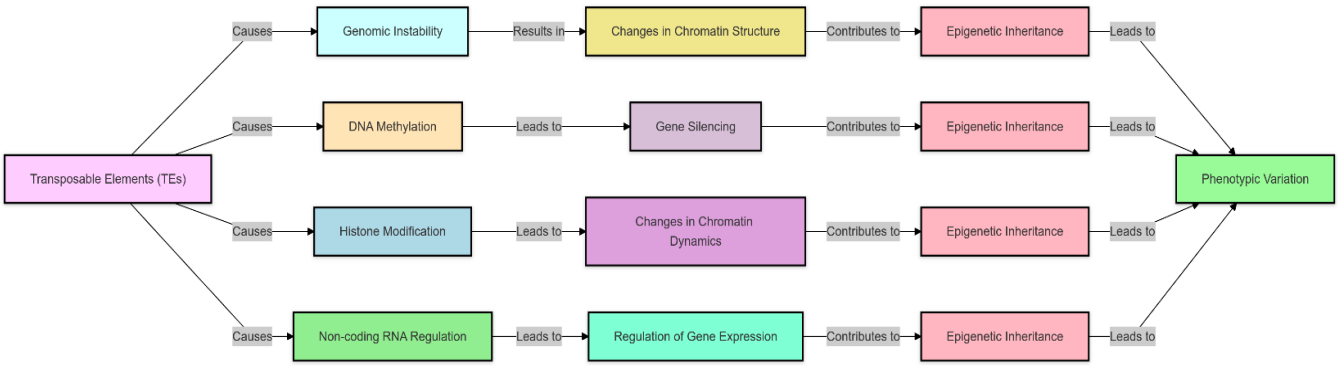


Figure 4. A flowchart diagram illustrating the epigenetic modifications mediated by Transposable Elements (TEs). This figure explains how TEs influence epigenetic modifications, such as DNA methylation and histone changes, emphasizing their role in gene regulation and chromatin structure.

The correlation between the abundance of TEs in the maize genome and its notable genetic variability has been shown, hence playing a role in the development of traits such as stress tolerance and nutrient utilization efficiency (Stitzer *et al.*, 2021).

Gene duplication events, frequently facilitated by transposable elements (TEs), serve as a fundamental origin of novel genetic material inside plant genomes. Transposable elements (TEs) can promote these duplications by interfering with each other or by acting as templates for the replication of neighboring genomic segments (Gill *et al.*, 2021). Polyploidy, also known as genome duplication, is a pivotal evolutionary occurrence in plants, serving as a fundamental catalyst for evolutionary advancements (Wang *et al.*, 2021). In wheat, polyploidy is associated with extensive TE activity, which has been linked to both gene loss (nonfunctionalization) and the evolution of new gene functions (neofunctionalization) that enhance adaptability to diverse environments (Wicker, 2018). TEs have been associated with the structural changes that occur during polyploidy, allowing for both the nonfunctionalization and long-term neofunctionalization and subfunctionalization of duplicate genes (Fambrini *et al.*, 2020).

The introduction of new promoters, enhancers, and silencers by TEs has been crucial in the evolution of gene regulation networks (Palazzo and Marsano, 2021). These factors can modify the way genes are expressed, resulting in the expansion of regulatory pathways and the creation of intricate developmental processes (Kakoulidou *et al.*, 2021). The enduring influence of TE-derived regulatory sequences on gene regulation is demonstrated by their presence in plant promoters (Hannan Parker *et al.*, 2022).

The adaptive flexibility given by TEs contributes to the evolutionary success of plants. Through the production of genetic diversity, TEs facilitate the ability of plants to swiftly adjust to shifting environmental circumstances. In maize (*Zea mays*), specific TEs have been linked to the adaptation of plants to different climatic conditions by modulating gene expression related to drought and nutrient stress (Makarevitch *et al.*, 2015). The ability to adapt is essential for the survival and diversification of plants, enabling them to establish themselves in new ecological areas and develop into unique species (Baduel and Quadrana, 2021). The importance of TEs on plant diversity is underscored by their involvement in generating sympatric speciation, which refers to the emergence of new species from a single ancestral species while residing in the same geographic region.

Molecular tools and techniques

TEs exert epigenetic influences, and recent developments in molecular methodologies have revolutionized our understanding of their role in genomic regulation and adaptation (Baduel *et al.*, 2021). These advancements include the novel CRISPR-Cas system for targeted epigenetic modifications, sophisticated sequencing

technologies, and a set of methods for analyzing TE-driven epigenetic changes (Mohan *et al.*, 2022).

Techniques for studying epigenetic effects of transposable elements

In-depth molecular analysis techniques and advanced methodologies are necessary to analyze the epigenetic roles of TEs. One essential method for studying DNA methylation is bisulfite sequencing (Chen *et al.*, 2022). This process involves treating DNA with sodium bisulfite, which turns unmethylated cytosines into uracil while protecting methylated cytosines. The resulting sequencing data can shed light on epigenetic modifications brought about by TEs by providing information on the methylation status throughout the entire genome (Urich *et al.*, 2015). Chromatin immunoprecipitation followed by sequencing (ChIP-seq) is another essential method that is used to examine histone modifications close to translocations (TEs). This technique highlights the impact of TEs on chromatin architecture and gene regulation by isolating and sequencing genomic regions linked to different histone marks using particular antibodies that target modified histones (Lerat *et al.*, 2019).

Clarifying how TEs impact gene expression has also been made possible by RNA sequencing, or RNA-seq (Wang *et al.*, 2017). RNA-seq offers a comprehensive view of the transcriptome by measuring and sequencing RNA transcripts. It also demonstrates how TEs may impact alterations in gene expression through epigenetic mechanisms (Stuart, 2018). Furthermore, the increasing use of ATAC-seq (Assay for Transposase-Accessible Chromatin with high-throughput sequencing) to assess chromatin accessibility sheds more light on the role of TEs in altering the chromatin environment (Bourguet *et al.*, 2021).

CRISPR-Cas system in epigenetic editing

Initially identified as a bacterial defense strategy, the CRISPR-Cas system has been repurposed into a potent genome and epigenome editing tool (Jogam *et al.*, 2022). This technology facilitates precise DNA sequence modifications, including areas around TEs (Merkulov *et al.*, 2023). By leveraging this system, researchers can intentionally modify epigenetic marks at specific TE sites, enabling them to probe their influence on gene regulation and chromatin structure (Liu *et al.*, 2022). The CRISPR-Cas system is adept at directing epigenetic modifiers to particular genomic regions, allowing the investigation of the direct and reversible impacts of epigenetic alterations (Selma and Orzáez, 2021). This precision editing paves the way for unraveling the interplay between TE activities, epigenetic changes, and resultant phenotypic effects (Kirov, 2023).

CRISPR-Cas has also evolved to enable epigenetic modifications without changing the DNA sequence . By inactivating the nuclease function of Cas9 (dCas9) and fusing it with enzymes that alter DNA methylation or histones, researchers can target specific epigenetic changes to TE regions. This innovative use of CRISPR-dCas9 offers unparalleled control over the epigenetic state of TEs, facilitating in-depth analyses of their role in gene expression regulation and genomic variation (Shin *et al.*, 2022).

Advances in epigenomic sequencing technologies

The field of epigenomics has advanced significantly as a result of the development of high-throughput sequencing technologies (Gogolev *et al.*, 2021). The whole genome's epigenetic modifications can now be comprehensively mapped thanks to these technologies (Figure 5). The application of next-generation sequencing (NGS) platforms has led to significant cost savings and improvements in sequencing productivity, which has made extensive studies of epigenetics possible (Kulski, 2016). With methods like whole-genome bisulfite sequencing (WGBS), comprehensive maps of DNA methylation can be obtained throughout the entire genome, including TE regions. By applying this methodology, researchers can link methylation patterns to phenotypic traits and gene expression (Omony *et al.*, 2020).

Additionally, the field of epigenetic research is expanding with the use of third-generation sequencing technologies like single-molecule real-time (SMRT) and nanopore sequencing. These technologies' longer read lengths allow for the simultaneous sequencing of TEs and the areas around them (Usha *et al.*, 2022). This feature is useful for studying TEs, which often show repetitive patterns and are challenging to analyze with short-read sequencing methods. The

application of long-read sequencing methods advances our knowledge of the intricate genomic terrain sculpted by transposons and the corresponding epigenetic changes (Shahid and Slotkin, 2020).

In parallel with these sequencing advancements, developments in computational biology have been essential in interpreting the extensive data generated. Bioinformatics tools and algorithms are continually refined to analyze epigenetic data, offering insights into the dynamic interactions between TEs and the epigenome (Pandita and Pandita, 2023). These tools facilitate the integration of diverse data types, such as DNA methylation, histone modification, and RNA expression, creating a holistic view of the epigenetic influences exerted by TEs (Ramakrishnan *et al.*, 2022).

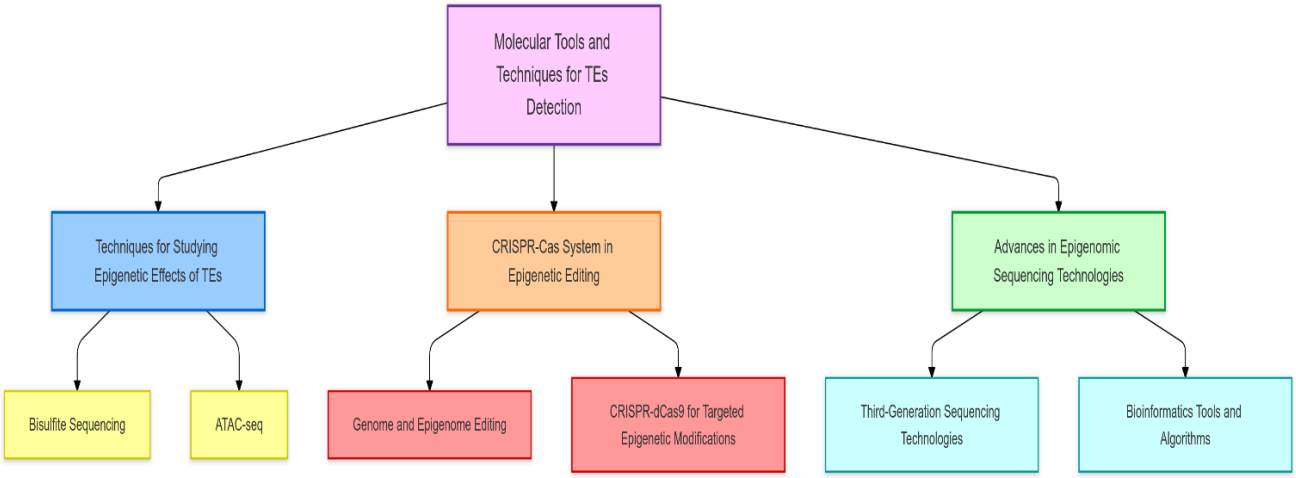


Figure 5. A flowchart diagram illustrating the molecular tools and techniques for Transposable Elements (TEs) detection. This figure shows the various tools and techniques used to detect and study TEs, including PCR-based methods, sequencing technologies, and CRISPR tools. It is closely related to the discussion on advancements in TE research.

Transposable elements in plants: regulatory dynamics and impact on gene expression

Transposable elements (TEs) significantly impact gene expression patterns and genomic integrity, acting as key players in gene regulation and adaptation processes (Galindo-González *et al.*, 2018). Research has shown that TEs can modify the expression of nearby genes by acting as stimulators, silencers, enhancers, or targets of epigenetic modification. This modulation can lead to the creation of mutations, which are often crucial for adaptation to environmental changes. For instance, TEs like the Ac/Ds in peas can result in distinct phenotypic changes, such as the shriveled phenotype, by inserting into genes like the starch branching enzyme SBEI (Fambrini *et al.*, 2020).

Beyond causing mutations, TEs can influence gene expression by disrupting regulatory regions of host genes, both positively and negatively (Hirsch and Springer, 2017). This is evident in various plant species where changes in flowering time and other developmental processes have been linked to TE insertions (Lisch, 2013). For example, inserting MITEs in maize and retrotransposons in the *BrFT2* gene illustrates how TEs can disrupt gene expression (Negi *et al.*, 2016). Similarly, a retrotransposon near the maize tb1 locus leads to increased apical dominance, showcasing the role of TEs in morphological traits (Stitzer *et al.*, 2021).

Maintaining a delicate balance between TE expression and repression is crucial for plant survival and evolution (Galindo-González *et al.*, 2018). This balance ensures that TEs' expression is neither detrimentally high nor insufficiently low (Mhiri *et al.*, 2022). Retrotransposons, which constitute a significant part of many organisms' genomes, modulate this balance by influencing the

expression and repression of essential genes (Negi *et al.*, 2016). Interestingly, TEs are not randomly distributed throughout the genome; their insertion locations are often biased, suggesting a strategic placement that aids in maintaining the balance of expression and suppression (Sahebi *et al.*, 2018).

This balance is further maintained by the host's use of various factors, including chromatin structure, DNA modification pathways, siRNA, and sequence-specific repressors like KRAB-ZFPs (Giménez-Orenga and Oltra, 2023). In addition, self-regulatory mechanisms in elements like Mariner-like elements (MLEs) control their amplification and copy number through overproduction inhibition (Ramakrishnan *et al.*, 2021). In reproduction, plants have developed strategies where TEs are only activated in germlines, not somatic cells, indicating a sophisticated regulatory system (Pachamuthu and Borges, 2023).

Furthermore, TE repression is relieved in a homologous cell produced during meiosis in flowering plant species, which does not pass genetic information to the next generation (Vigneau and Borg, 2021). This strategy exemplifies the intricate mechanisms plants have evolved to regulate TE activity, ensuring genomic stability while allowing for necessary genetic variability.

Implications for agriculture

The understanding of transposable element (TE) mechanisms offers significant potential for agricultural improvement. For instance, the manipulation of TEs in rice (*Oryza sativa*) has been used to enhance tolerance to submergence by activating the *Sub1A* gene, a key regulator in the plant's response to flooding (Xu *et al.*, 2006). In maize (*Zea mays*), TEs have been harnessed to improve drought tolerance by modulating genes involved in water use efficiency and root architecture (Makarevitch *et al.*, 2015). Additionally, CRISPR-based targeting of TEs has been employed to alter their activity, leading to changes in gene expression that improve grain yield under stress conditions (Rodríguez-Leal *et al.*, 2017). These examples illustrate how leveraging TE activity can be instrumental in developing crops with enhanced resilience and productivity, which is crucial in the face of climate change and global food security challenges.

Implications for plant growth and development

The function of transposable elements in developmental control

Transposable elements (TEs) play a pivotal role in the regulation of plant development, influencing gene activity and contributing to developmental flexibility and diversity. Their presence and activity within the genome are intricately linked to the developmental stages of plants, acting as key regulatory elements that modulate gene expression in response to developmental cues (Gebrie, 2023; Zhang *et al.*, 2023). Activation of specific TEs during certain growth phases can alter the expression of neighboring genes, leading to variations in plant morphology such as leaf size, flower structure, and root system architecture, which, in turn, affects the plant's ability to adapt to its environment (Rymen *et al.*, 2020).

TEs induce epigenetic modifications, including changes in DNA methylation and histone configurations, that influence gene expression throughout plant development. These epigenetic alterations can be stable and heritable, playing a crucial role in shaping plant growth patterns and traits (Ariel and Manavella, 2021). By introducing new promoters, enhancers, or alternative splicing sites, TEs contribute to the formation of novel gene regulatory networks, facilitating the emergence of new phenotypes and aiding in species diversification (Deneweth *et al.*, 2022).

The insertion of TEs into or near genes can disrupt existing functions or create new ones, enhancing genetic diversity within plant populations. This diversity is vital for plant evolution and adaptation, enabling species to navigate and thrive in changing environmental conditions (Schrader and Schmitz, 2019). Furthermore, TEs can cause genomic rearrangements, leading to the creation of new genes or gene variants—a process that is particularly advantageous in dynamic environments where novel traits may confer a survival benefit (Ramakrishnan *et al.*, 2022).

Stress memory and transgenerational epigenetic inheritance in plants

Transposable elements (TEs), such as the ONSEN retrotransposon in *Arabidopsis* (Roquis *et al.*, 2021) and the Suppressor-mutator (Spm) element in

maize (Baduel and Colot, 2021), are central to stress memory and transgenerational epigenetic inheritance in plants. This area is drawing more attention due to its relevance in plant adaptation and evolution (Ramakrishnan *et al.*, 2022). Activated by environmental stressors like heat, drought, or salinity, these elements can cause significant epigenetic changes. These changes impact the stress response of the current generation of plants and can also be inherited by future generations, enhancing their stress resilience (Chang *et al.*, 2020).

The notion of stress memory suggests that a plant's initial exposure to stress leads to genetic and epigenetic alterations, preparing it for more effective responses to similar stress (Sun *et al.*, 2021). An example is the ONSEN retrotransposon in *Arabidopsis* (Nozawa *et al.*, 2022). Upon heat stress exposure, ONSEN becomes active, producing small RNAs targeting genes related to heat stress response (Matsunaga *et al.*, 2015). The resulting changes in gene expression create stable epigenetic marks, which are inherited by the next generation, thereby enhancing their heat tolerance (Roquis *et al.*, 2021). In maize, the Spm element is similarly activated under stress conditions, causing genome-wide changes in DNA methylation patterns and altering the expression of stress-responsive genes (Gill *et al.*, 2021). This TE-driven epigenetic modification under stress is vital for developing stress memory, helping plants respond more efficiently to future stressors (Marin *et al.*, 2020).

Transgenerational epigenetic inheritance refers to the passage of these stress-induced epigenetic changes from generation to generation (Shanker *et al.*, 2020). In various plant species, such as rice with the Tos17 retrotransposon, offspring of stressed plants exhibit altered stress responses without direct exposure to the stressor . This indicates that the stress experiences of parent plants directly affect their offspring's adaptability and survival. The underlying molecular mechanisms of stress memory and transgenerational epigenetic inheritance involve complex interactions between TEs, epigenetic processes, and environmental signals (Gallusci *et al.*, 2023). TEs can trigger epigenetic changes through DNA methylation, histone modification, and the production of noncoding RNAs, altering gene expression to better handle stress . These changes can be stable enough to pass through the germline to offspring. Furthermore, TE activation under stress can lead to genomic rearrangements and the creation of new gene variants, increasing genetic diversity within plant populations . This TE-induced variability is crucial for plant evolution, aiding adaptation to environmental changes.

Ongoing research into TEs' role in stress memory and transgenerational epigenetic inheritance reveals how plants adapt to their environment (Shanker *et al.*, 2020; Baduel and Colot, 2021). It shows that TEs are more than just genomic elements; they actively contribute to plant survival and evolution (Mhiri *et al.*, 2022). They enable rapid adaptation to environmental changes, not just through immediate responses but also by conveying these adaptations to subsequent generations.

Interaction between epigenetic and genetic elements in plants

The complex interaction between epigenetic and genetic elements, driven by transposable elements (TEs) like maize's Activator/Dissociator (Ac/Ds) system , barley's BARE-1 , and rice's Tos17 , plays a crucial role in defining plant genomes and their reaction to environmental factors. Once regarded as mere 'junk DNA', TEs are now recognized for their substantial impact on genetic and epigenetic processes, which are crucial for plant adaptation and evolution . These elements are dynamic within the genome, capable of causing both structural and functional modifications.

TEs such as Ac/Ds in maize and BARE-1 in barley are notable for their genome mobility, leading to genetic variation. Their transposition can cause mutations by disrupting functional genes or changing gene regulatory networks . For example, the Ac/Ds system in maize is well-studied for its mutation-inducing ability, contributing to genetic diversity. These mutations can be advantageous, serving as a basis for natural selection and enabling plants to adapt to environmental shifts. Similarly, BARE-1 in barley is linked to generating genomic diversity, which is essential for plant survival and adaptation.

Beyond their role in genetic diversity, TEs like Tos17 in rice also engage in epigenetic modifications (Cui and Cao, 2014). They can reshape the epigenetic

landscape by altering DNA methylation patterns and histone modifications (Galindo-González *et al.*, 2018). The activation or silencing of Tos17 under various stress conditions can lead to DNA methylation changes, influencing gene expression nearby. This epigenetic regulation is vital for plant development and stress response, offering a flexible and reversible method to control gene expression (Ramakrishnan *et al.*, 2021).

The synergy between TE-induced genetic changes and epigenetic modifications forms a complex regulatory network (Mirouze and Vitte, 2014). For example, a TE insertion into a gene or its regulatory area can disrupt its function and attract epigenetic modifications, further affecting gene expression. This interplay is crucial for plant development, integrating environmental signals into the genomic framework and leading to adaptive gene expression changes.

The combined genetic and epigenetic influences of TEs are pivotal in plant development and adaptation (Galindo-González *et al.*, 2018). They allow plants to respond rapidly to environmental alterations, either through new genetic variants via mutations or by modulating gene expression through epigenetic adjustments (Rey *et al.*, 2016). This adaptability is critical for plant survival, enabling them to withstand various stressors like drought, salinity, and temperature changes . TEs also contribute significantly to the long-term evolution of plant species. By inducing genetic and epigenetic variability, they provide material for natural selection, potentially leading to new traits and adaptation to novel environments (Bennetzen and Wang, 2014). The dynamic nature of TEs, along with their responsiveness to environmental cues, positions them as key contributors to the evolutionary narrative of plants (Bousios and Gaut, 2016).

Impact of TEs on plant genome architecture

Transposable elements (TEs) are dynamic entities that modify the dimensions, composition, and capabilities of the plant genome. These are more than simply inactive DNA segments found in the genomes (Baduel *et al.*, 2021). Their activities result in a range of genomic alterations, from small tweaks to large reorganizations, all of which are necessary for plant adaptation and the processes of evolution (Colonna Romano and Fanti, 2022).

One of the most evident effects of TEs on plant genomes is variation in genome size. TEs can make up a significant portion of plant genomic DNA, and as TEs multiply, the genome gets bigger (Stuart, 2018). For instance, TEs play a major role in the genomes of species like maize and wheat, which helps explain the latter's enormous size (Fambrini *et al.*, 2020). The correlation between the genome's size and TE content emphasizes the importance of TEs in the process of genomic expansion by serving as a genomic buffer that may facilitate evolutionary innovations and adaptations (Giménez-Orenga and Oltra, 2023).

Furthermore, TEs are the source of chromosomal rearrangements like translocations, inversions, deletions, and duplications. These rearrangements can lead to phenotypic variability, which can impact gene expression and function (Kremer *et al.*, 2020). For example, transposon migration (TEs) can alter gene sequences or gene regulatory regions, which can impact patterns of gene expression. These rearrangements have been connected to the evolution of novel traits in plants and can help them quickly adapt to changing environmental conditions (Fambrini *et al.*, 2020).

Role of transposable elements in plant stress responses

Systematic analyses across various plant systems have revealed that Transposable Elements (TEs) significantly regulate host gene expression and genomic rearrangement. This involvement of TEs in gene expression control is evident under different environmental conditions, as outlined in studies by Lisch (2013). TEs contribute to the release of cis-regulatory elements, enabling the host genome to manage its genes effectively. This gene regulation manifests in two primary forms: short-term adjustments, adapting to immediate environmental changes, and long-term adjustments, contributing to evolutionary changes, as discussed by Ramakrishnan *et al.* (2021).

The transposition of TEs at the genome level can lead to mutations that create new functional protein-coding sequences, a concept explored by (Chuong *et al.*, 2017; Pisoschi *et al.*, 2021). These mutations often result in environmental adaptation.

For instance, in plants, TEs-induced mutations have been observed to foster adaptation to local environments. In Arabidopsis, this is exemplified through the expression of photoregulation genes associated with FAR1 and FHY3, derived from a mutator-like transposase. The increased gene expression through this protein's direct binding to target genes' promoters suggests Arabidopsis's adaptation to changing light environments, as noted by Lin *et al.* (2007).

In soybeans, phenotypic experiments and allelic distribution analysis demonstrated adaptation to higher latitudes due to the TE insertion in GmphyA2, a paralog of encoding phytochrome A. This was explored by (Liu *et al.*, 2008; Kanazawa *et al.*, 2009). Similarly, various mutations in vernalization genes, essential for changes in growth habits, were induced by TEs in wheat. These mutations have contributed to wheat adaptation to a wide range of environments, as observed by (Yan *et al.*, 2006; Chu *et al.*, 2011).

These findings underscore the pivotal role of TEs in plant stress responses. By inducing mutations and affecting gene expression, TEs enable plants to adapt to their immediate surroundings and evolve, facing various environmental challenges. This dynamic interplay between TEs and plant genomes highlights the complexity and adaptability of plant life, showcasing how genetic mechanisms contribute to the survival and evolution of plant species in diverse ecosystems.

TEs - activation cues under stress

Plants respond to various stress levels, including molecular, tissue, anatomical, and morphological changes. These responses involve alterations in membrane regulation, cell wall structure, transcription, and metabolic processes (Shinozaki *et al.*, 2003; Hirayama and Shinozaki, 2010). Research indicates that under biotic and abiotic stress, Transposable Elements (TEs) are expressed and activated through translocation. Stress often activates TEs by reducing their inactive epigenetic state or through Transcription Factors (TFs) (Sun *et al.*, 2020). Activated TEs under stress can alter gene expression near their insertion sites, leading to phenotypic adaptability (Negi *et al.*, 2016; Chuong *et al.*, 2017; Dubin *et al.*, 2018). However, some studies also report TE suppression or harmful effects of TE activation under stress, highlighting a complex TE-stress relationship (Sun *et al.*, 2020). Specific TE sequences linked to stress response, such as LTR retrotransposons with cis-regulatory elements in their 5' LTR, have been identified, triggering transposon activation under stress (Kumar and Bennetzen, 1999).

Transposon transcription under environmental stress involves two mechanisms. The first involves specific TFs interacting with the transposon promoter sequence. In plant retrotransposons, DNA elements related to stress response signals, like plant hormones, are found in the 5'LTR (Casacuberta and González, 2013). These elements act as crucial motifs for gene activation under stress. For example, in Arabidopsis, the ONSEN LTR retrotransposon is activated by heat stress factors recognizing a sequence in its promoter (Ito *et al.*, 2011; Cavrak *et al.*, 2014). ONSEN transcription is triggered at 37°C (Ito *et al.*, 2011), with the heat shock factor binding to the heat shock element in the LTR 5' ONSEN (Cavrak *et al.*, 2014). Another study on Arabidopsis showed ONSEN's insertion site preference related to specific chromatin states, particularly those rich in histone H2A.Z and H3K27me3 (Roquis *et al.*, 2021). In rdd-regulated stress response promoters, short TE sequences and their demethylation confer resistance against *Fusarium oxysporum* (Le *et al.*, 2014). In *A. lyrata* and *A. thaliana*, heat-responsive TEs like ONSEN, COPIA37, TERESTRA, and ROMANIAT5 were identified, with COPIA families in seven Brassicaceae species showing heat-responsiveness due to HSF-binding HREs in their long terminal repeats (Pietzenuk *et al.*, 2016). In tomatoes, the Rider Copia retrotransposon is expressed under drought stress and abscisic acid signaling, containing dehydration-responsive elements in its promoter (Benoit *et al.*, 2019).

TE insertion in regulatory regions can induce stress-related gene expression. In corn, MITE 82 insertion in the ZmNAC111 promoter led to gene expression repression, with ZmNAC111 overexpression enhancing drought stress tolerance and water use efficiency through H3K9 demethylation (Mao *et al.*, 2015). In rice, the GSTL2 promoter contains two transposons, affecting expression while being balanced by enhancer elements (Hu et al., 2011).

Another mechanism involves reactivating suppressed transposon sequences under stress, as environmental stress alters epigenetic modifications and gene expression patterns, including transposons (Kumar *et al.*, 2013). In rice, many LTR-retrotransposons were up-regulated under iron stress, with 156 out of 369 families expressed (Finatto *et al.*, 2015). Maize, with its diverse TE types, shows TE-regulated gene expression under various stresses (Makarevitch *et al.*, 2015). In *Antirrhinum majus*, the Tam3 DNA transposon, inactive at high temperatures, activates at low temperatures, and is linked to DNA methylation changes (Kitamura 2001). Arabidopsis experiences reduced chromosomal interactions under heat stress, correlating with TE activation (Sun *et al.*, 2020). In rice, new mPing MITE insertions up-regulate adjacent genes under low temperature or salinity, with no expression change in control conditions (Naito *et al.*, 2009; Yasuda *et al.*, 2013). Figure 6 presents examples of TE activation under stress.

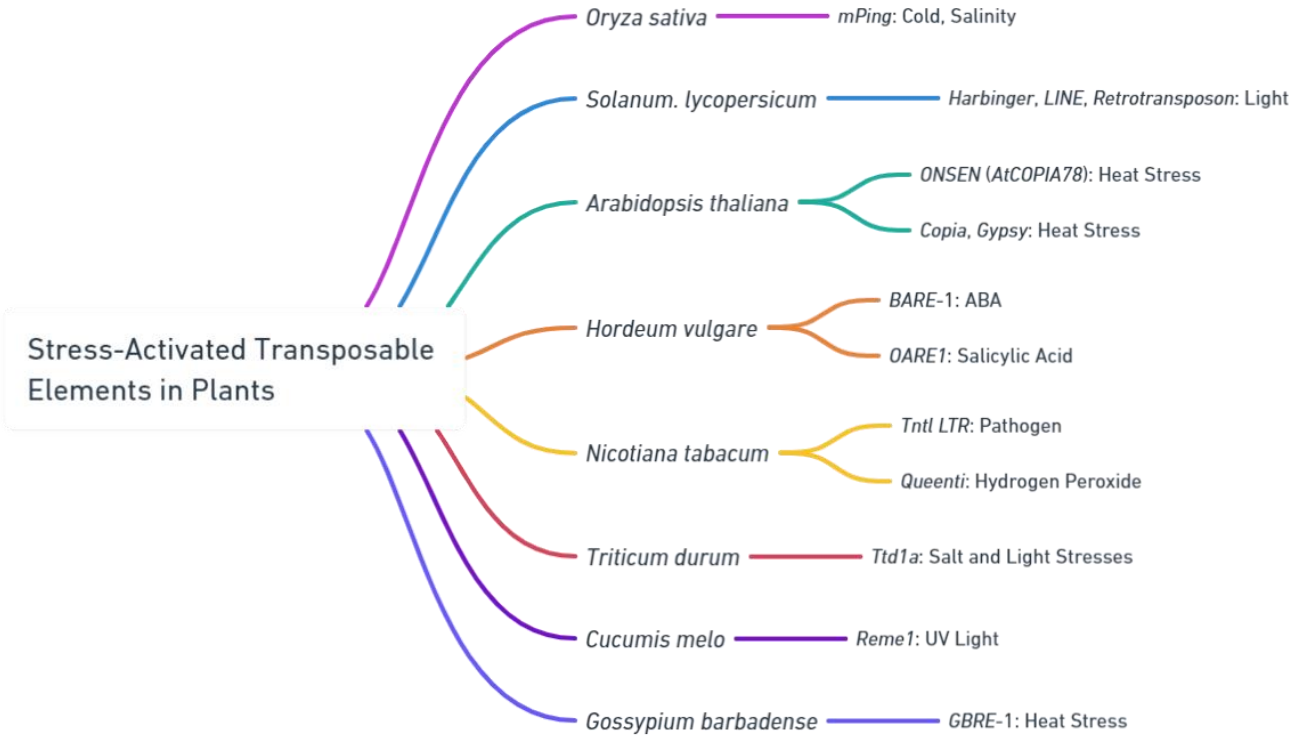


Figure 6. Examples of stress-activated TEs in some plants. This figure presents specific examples of TEs that are activated under stress conditions in different plant species, providing quantitative data to support the discussion on stress-induced TE activation and its adaptive significance.

According to the studies that have been conducted, it has been stated that TEs in plants may induce the reaction of adjacent genes via providing stress-responsive enhancer functions and activation of TEs under stress conditions affiliated with factors such as stress type, TEs type, its genomic location, and the genetic background of the host (Figure 7) (Deneweth *et al.*, 2022).

Activation of TEs does not always occur under stress conditions, but stress activates and suppresses TEs depending on the context. For example, transposable elements' response to high temperature was investigated in several ecotypes of *Arabidopsis thaliana*, the results showed that in 7 ecotypes, most TEs had a higher expression under stress. In comparison, in the other 3 ecotypes, most transposable elements had lower expression levels under stress conditions (Barah *et al.*, 2013; Horváth *et al.*, 2017), which indicates that the suppression of TEs may also occur in the stress condition. Excessive

iron stress altered the transcription of 37% of LTR-retrotransposon, suggesting that the expression level of 95.4% increased and about 4.6% showed a decrease in expression (Finatto *et al.*, 2015).

Stress regulation by Te-lncRNAs

Long noncoding RNAs (lncRNAs), a subgroup of RNAs, play a crucial role in regulating gene expression and key cellular activities, including reactions to phosphate and sulfur deprivation, as well as salt stress. These RNAs, characterized by lengths exceeding 200 nucleotides, cannot code for proteins. In Arabidopsis, numerous lncRNAs exhibit significant reactions to stressors like drought, cold, high salt levels, and abscisic acid (Di *et al.*, 2014; Qin *et al.*, 2017; Zhao *et al.*, 2018). *Arabidopsis thaliana* has a notably higher count of TE-containing lincRNAs (Te-lincRNAs) than *Zea mays* and *Oryza sativa*, correlating with the number of transposable elements (TEs) in their genomes. Certain Te-lincRNAs play a role in stress response. Research on Arabidopsis revealed that lincRNAs are crucial in abiotic stress response, with Arabidopsis thaliana mutants in Te-lincRNA11195 displaying enhanced resistance to ABA treatment compared to wild types (Wang *et al.*, 2017). In maize, 65% of 23,309 identified lncRNAs showed sequence similarity to transposable elements, predominantly retrotransposons with long terminal repeats from the *Copia* and *Gypsy* superfamilies, with over 1077 reacting to abiotic stress (Lv *et al.*, 2019). Studies on *Solanum lycopersicum* and *S. pennellii* showed that Lycopersicon-specific lncRNA sites originate from TEs (Wang *et al.*, 2016).

Stress regulation by Te-siRNA

Epigenetic mechanisms play a crucial role in silencing and deactivating most transposable elements (TEs) in plant genomes, with the epigenetic activity of TEs influencing the host plant's stress response (Lippman *et al.*, 2003; McCue *et al.*, 2012). DNA methylation is a critical factor in controlling transposon expression. In Arabidopsis, Attila LTR retrotransposons, which form the largest TE family, are transcriptionally silenced through symmetric CG DNA methylation. This silencing can be reversed by reducing or removing DNA methylation, leading to transcriptional activation (Wright and Voytas, 1998; Steimer *et al.*, 2000). In DNA hypomethylation mutants, activation of TEs is observed (Mirouze *et al.*, 2009; Tsukahara *et al.*, 2009). Methylation acts as a defense mechanism, preventing excessive TE activation and protecting against abnormal transposon activity. RNA-directed DNA methylation (RdDM) is another method for regulating TEs, using siRNA-mediated DNA methylation to control them (Wierzbicki *et al.*, 2008; Gao *et al.*, 2010).

Gene expression is also regulated by suppressing TE activity through small RNAs. Research on *Arabidopsis thaliana* indicates that the production of TE-associated endogenous small interfering RNAs (Te-siRNAs) differs from other gene-regulatory small RNAs (sRNAs), such as microRNAs or trans-acting siRNAs (McCue *et al.*, 2012).

TEs are known to activate under biotic and abiotic stresses, impacting the expression of stress-related genes. A study on the *Ty1/copia* retrotransposon ONSEN, activated by heat stress in Arabidopsis, revealed that siRNA pathways regulate ONSEN's transcriptional activation (Matsunaga *et al.*, 2015). DNA demethylases like ROS1, DML2, and DML3 play roles in fungal disease resistance in Arabidopsis by maintaining or enhancing the expression of stress response genes necessary for combating *Fusarium oxysporum*. An rdd triple mutant of DNA demethylase (ros1 dml2 dml3) showed increased susceptibility to this pathogen (Le *et al.*, 2014). Furthermore, a study on *A. thaliana* under phosphate starvation showed that a cluster of sRNAs (smRPi1LTR) derived from the Copia95 LTR retrotransposon indicates TE-mediated regulation of plant stress response gene expression through small noncoding RNAs derived from TEs during stress (Hsieh et al., 2009).

Future directions and challenges in TE research

The field of transposable element (TE) research stands at a dynamic juncture, propelled by technological advancements and deepening theoretical insights. However, it also faces significant challenges that must be addressed to unravel the complexities of TEs and their roles in genomes (Figure 8). Recent years have seen remarkable progress in sequencing technologies and bioinformatics tools, offering unprecedented opportunities to study TEs (Omony *et al.*, 2020). Long-read sequencing technologies, such as

nanopore and single-molecule real-time (SMRT) sequencing, have begun to overcome the limitations of short-read sequencing in resolving repetitive TE sequences (Kulski, 2016; Usha *et al.*, 2022). Furthermore, the application of CRISPR-Cas systems for precise editing and epigenetic modification of TEs opens new avenues for functional studies (Saika *et al.*, 2019; Merkulov *et al.*, 2023). Looking forward, the integration of these technologies with advanced computational models and machine learning algorithms promises to enhance our ability to identify, annotate, and understand the functional dynamics of TEs in various genomes.

The conceptual frameworks guiding TE research are evolving. There is a growing recognition of the need to view TEs not just as isolated genetic elements but as integral components of complex regulatory networks within the genome (Bennetzen and Wang, 2014). This perspective necessitates a holistic approach to studying TEs, combining genomics, epigenetics, systems biology, and evolutionary biology (Galindo-González *et al.*, 2018). Future research will likely focus on elucidating the systemic effects of TEs, including their impact on gene regulatory networks, chromatin architecture, and genome evolution.

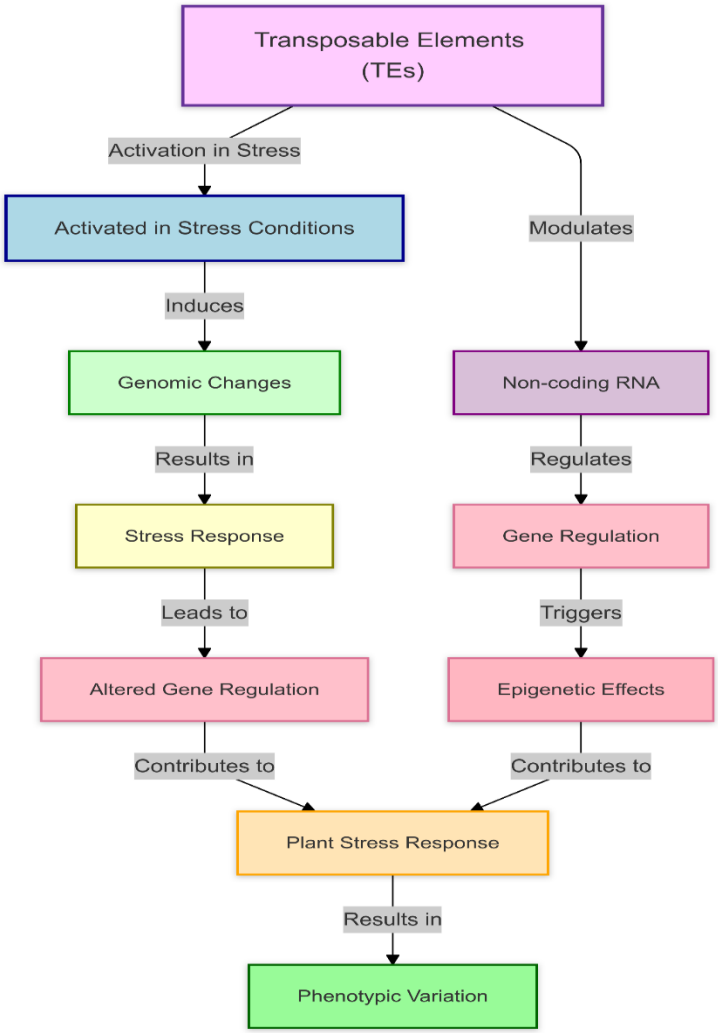


Figure 7 A flowchart diagram illustrating the role of Transposable Elements (TEs) in plant stress response. This figure shows the interplay between TE activation and silencing under stress conditions, highlighting their contribution to the plant's ability to adapt to environmental challenges.

Despite these advancements, TE research faces several challenges. One of the primary obstacles is the accurate annotation and characterization of TEs in genome sequences. The repetitive and diverse nature of TEs makes them difficult to identify accurately, requiring the development of more sophisticated computational tools and algorithms (Platt *et al.*, 2016). Another challenge lies in deciphering the complex regulatory roles of TEs, particularly their interactions with host genomes and their influence on gene expression and epigenetic states. Additionally, understanding the evolutionary dynamics of TEs and their contributions to genetic diversity and adaptation remains a daunting task (Viviani *et al.*, 2021).

Addressing these challenges will require concerted efforts across multiple disciplines. Improvements in sequencing technology and bioinformatics are essential for better TE annotation and characterization. Functional studies of TEs will benefit from innovative genetic and epigenetic editing techniques, allowing for the dissection of their regulatory roles. Moreover, collaborative research initiatives that bring together experts from genetics, epigenetics, computational biology, and evolutionary biology can accelerate the generation of comprehensive models of TE activity and function.

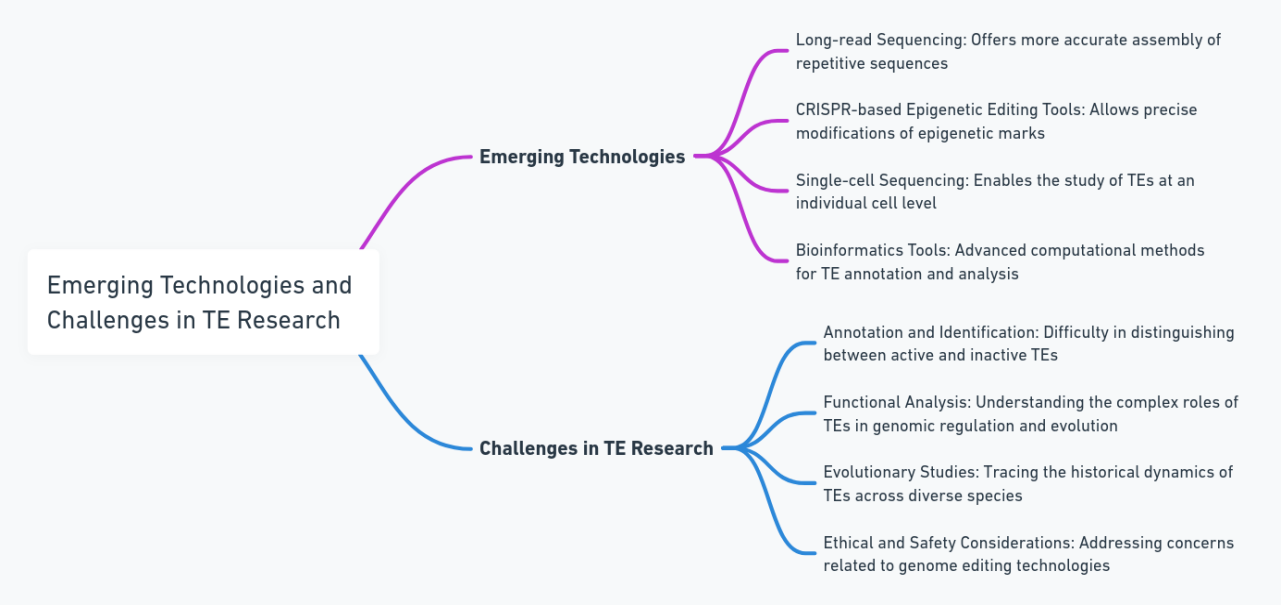


Figure 8. Emerging technologies and challenges in TE research. This figure summarizes the latest technological advancements in TE research, such as CRISPR-based tools, and discusses the challenges that researchers face. It aligns with the discussion on the future directions of TE studies and their implications for plant breeding.

Ethical and ecological considerations

While the application of TE-based biotechnologies holds immense promise, it also raises several ethical and ecological concerns. Manipulating TEs could lead to unintended off-target effects, potentially disrupting non-target genes and causing deleterious traits. Ecologically, the activation of TEs might introduce novel genetic variations that could impact biodiversity, especially if engineered plants crossbreed with wild relatives. Furthermore, the long-term consequences of altering TE dynamics on genome stability are not fully understood, necessitating careful assessment and regulation. Ethical considerations include public acceptance of genetic modifications, especially those involving elements with potential mobility, which may evoke concerns about the safety of such technologies. Therefore, responsible research, rigorous regulatory frameworks, and transparent public communication are essential for the sustainable

application of TE-based innovations in agriculture (Howell *et al.*, 2018).

Conclusion

Transposable elements (TEs), once overlooked as 'junk DNA,' have emerged as fundamental components of plant genomes, playing a pivotal role in their adaptation to environmental stresses. Particularly, retrotransposons, residing within both heterochromatin and euchromatin regions of chromosomes, underscore their functional significance in genomic structure and activity. Their strategic distribution across the genome facilitates vital processes such as gene regulation, creation of new genes, and genetic recombination, contributing to plant diversity and evolutionary adaptability.Our review underscored the dynamic role of TEs, especially retrotransposons, in modulating gene expression in response to environmental changes. By either up-regulating or down-regulating adjacent genes, TEs serve as key regulators of plant stress responses, enabling resilience against various abiotic and biotic stressors. The production of transposon-derived small interfering RNAs (Te-siRNAs) and long intergenic noncoding RNAs (TE-lincRNAs) further exemplifies their intricate role in gene expression regulation and epigenetic silencing, highlighting the complex interplay between TEs and plant stress mechanisms.

Despite significant advancements in understanding the relationship between TEs and plant stress responses, several areas warrant further exploration. Future research should aim to delineate the precise molecular pathways through which TEs influence neighboring gene expression under stress conditions. Investigating the role of TEs in a broader spectrum of plant species will enrich our comprehension of their universal and species-specific impacts on stress adaptation.

The practical applications of our expanding knowledge of TEs are vast, especially in the fields of agriculture and biotechnology. Understanding the mechanisms by which TEs contribute to stress resilience offers promising avenues for the development of climate-resilient crop varieties. By harnessing the natural genetic diversity and regulatory capabilities of TEs, we can engineer crops that are better equipped to withstand environmental challenges, thus ensuring food security in the face of changing global climates.

In conclusion, TEs are not mere genomic relics but are dynamic and integral to plant stress responses and evolution. Their study not only reveals the complexity of plant genomes but also opens up new frontiers in agriculture and biotechnology, where their potential can be harnessed for sustainable and resilient crop production. Looking ahead, advancements in sequencing technologies, coupled with the development of precise genome-editing tools such as CRISPR, hold promise for uncovering novel functions of TEs and their regulatory networks. Future breakthroughs may involve the targeted activation or silencing of specific TEs to enhance desirable traits in crops, such as increased tolerance to extreme temperatures or improved nutrient efficiency. Moreover, addressing the ecological and evolutionary implications of manipulating TEs will be crucial for safely implementing these technologies. The integration of interdisciplinary approaches will be key to unraveling the full potential of TEs in plant adaptation, paving the way for innovative solutions to agricultural challenges in the face of climate change.

Supplementary Materials

No supplementary material is available for this article.

Author Contributions

MN, MK and AKGS developed the concept, performed the database search, partially collected the data, analyzed, interpreted the results and finalized the draft. PS revised the manuscript. MK and PS supervised this study. All authors approved the final version of the manuscript.

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Conflict of Interest Statement

The author declares no conflict of interest.

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مقاله مروری

عناصر جابه‌جاشونده: بینشی مکانیکی به چشم‌انداز تنظیم‌کننده پویا از پاسخ به تنش‌ها در گیاهان (با تکیه بر مفاهیم کشاورزی)

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چکیده: عناصر ژنتیکی جابه‌جاشونده (TEs) توالی‌های DNA پویایی هستند که به‌طور قابل‌توجه بر بیان ژن در گیاهان تأثیر می‌گذارند و امکان سازگاری با تنش‌های محیطی را فراهم می‌کنند. این بررسی نقش TEها را در سازگاری گیاه، با تمرکز بر مکانیسم‌های فعال‌سازی و سرکوب TE، از جمله بازسازی کروماتین، تغییرات DNA، و RNAهای مداخله‌کننده کوچک (siRNA) بررسی می‌کند. شرایط تنش‌زا از طریق تعامل بین فاکتورهای رونویسی ناشی از تنش و محرک های TE باعث فعال شدن TE می‌شود، همانطور که در خانواده‌های رتروترانسپوزون مانند COPIA93 و ONSEN در تعدیل ژن‌های پاسخگو به تنش دیده می‌شود. درک این مکانیسم‌ها بینش ارزشمندی را برای کشاورزی، به‌ویژه در توسعه گیاهانی که در برابر تغییرات آب و هوایی مقاوم هستند، ارائه می دهد. استفاده از تنظیم ژن با واسطه TE، استراتژی‌های نوآورانه‌ای را برای افزایش سازگاری گیاه ارائه می‌کند و پتانسیل TEs را در دستکاری ژنتیکی برای به‌نژادی گیاهی برجسته می‌کند.

کلمات کلیدی: عناصر جابه‌جاشونده، تنظیم اپی‌ژنتیکی، سازگاری محیطی، RNAهای غیرکدکننده، تنش گیاهی.

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Phytohormones and plant defense

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Review paper

Abstract: Phytohormones, also known as plant growth regulators, regulate various physiological processes in plants, including germination, growth, and response to biotic and abiotic stresses. Plant diseases, caused by pathogens such as fungi, bacteria, and viruses, often alter hormonal pathways, leading to the simultaneous induction of antagonistic and synergistic hormones in plants. In resistant varieties, however, the hormonal responses follow a more sequential pattern. Plant hormone signaling pathways are primarily polarized along two antagonistic axes: the salicylic acid (SA) and jasmonic acid (JA) pathways on one side, and the ethylene pathway on the other. In addition to SA, JA, and ethylene, other growth regulators, such as auxins, brassinosteroids, cytokinins, and abscisic acid (ABA), also play significant roles in plant responses to biotic stress and are increasingly recognized for their importance in plant-pathogen interactions. Pathogens can modulate hormone biosynthesis and signaling to suppress plant defenses and alter the cellular environment, promoting their infection and proliferation. In this article, we will review the latest advances in understanding the function and regulation of plant hormones, the modulation of plant defense responses, and their synergistic and the crosstalk between phytohormones and defense pathways.

Keywords: Biotic stress, crosstalk, hormonal regulation, plant defense.

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Introduction

In nature, plants encounter a wide range of biological factors including fungi, bacteria, viruses, nematodes and other pathogens and pests that lead to various biotic stress responses.These factors, can cause diseases that lead to severe loss of crops productivity and quality. Their global reduction of agricultural productivity and economic losses threaten global food security and natural resources (Nawaz et al., 2023).

Therefore, understanding the natural defense mechanisms in plants to cope with stresses and controlling plant diseases is important for sustainable and eco-friendly exploitation of natural resources.The induction of defense responses to biotic and abiotic stresses through morphological, physiological, and biochemical mechanisms is regulated by plant hormones (Husen, 2021). Plants synthesize various hormones, including abscisic acid (ABA), gibberellic acid (GA), auxin, cytokinin (CK), salicylic acid (SA), jasmonic acid (JA), ethylene (ET), strigolactones (SL), and brassinosteroids (BR). Plant growth regulators, or phytohormones, are natural organic signaling molecules with low molecular weights (Husen, 2021). Phytohormones play a crucial role in regulating various growth and developmental processes, as well as controlling physiological responses, including defense mechanisms against stress in plants (Sutaoney et al., 2023).

The immune response activated by pathogen or insect attacks is regulated by the induced production of specific plant hormones. Salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and abscisic acid (ABA) are important regulators of inducible defense mechanisms (Dehkordi et al., 2018). The SA pathway is mainly induced by biotrophic pathogens and the JA pathway is mainly induced by necrotrophic pathogens and insect herbivores (Vos et al., 2013).

Some studies have highlighted the roles of abscisic acid (ABA), auxin, gibberellic acid (GA), cytokinin (CK), brassinosteroids (BR), and peptide hormones in plant defense signaling pathways (Bari and Jones, 2009; Nawaz et al., 2023).For an effective response to biological stresses, each hormone initiates a specific molecular pathway. These signaling pathways are integrated into a complex network of synergistic, antagonistic, and additive interactions, collectively referred to as hormonal crosstalk (Aerts et al., 2021). While the role of hormones in plant growth and their biosynthesis mechanisms is well established, this article aims to review recent advances in understanding the functions and regulation of plant hormones, their role in modulating plant defense responses, and the impacts of their synergistic and antagonistic interactions in various plant diseases.

Significance of Plant Defense Mechanisms

Plant health is a critical aspect of food security, yet it is constantly threatened by plant pathogens, which cause up to 40% of global crop production losses (Butt et al., 2020). The defense mechanisms of plants against pathogens are highly complex, relying on both innate immune systems and preformed defense structures.

The first line of defense in plants is passive and includes physical barriers such as the cuticle, wax, and trichomes, which serve to prevent the entry of pathogens and insects. Beyond these barriers, plants activate innate immune responses involving two levels of pathogen recognition (Checker et al., 2018). The first level involves pattern recognition receptors (PRRs) located on the plant cell surface. These receptors identify pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs) (Saberi Riseh et al., 2022). This recognition triggers pattern-triggered immunity (PTI), which inhibits pathogen growth and colonization. However, some pathogens have evolved proteins capable of suppressing PTI, enabling further infection (Fu and Dong, 2013).

The second level of recognition relies on intracellular resistance (R) proteins, which can directly or indirectly detect specific pathogen effectors. This induces a more specialized and robust defense response known as effector-triggered immunity (ETI) (Saberi Riseh et al., 2022). ETI responses are faster and more potent than PTI and often lead to the formation of necrotic lesions at infection sites. These lesions restrict the pathogen's movement, effectively transforming a localized infection into a systemic defense response (Fu and Dong, 2013).

In general, both types of plant immune responses, PTI and ETI, activate similar defense mechanisms.

These include the production of reactive oxygen species (ROS), an increase in intracellular Ca^{2+} concentrations, activation of mitogen-activated protein kinases (MAPKs), upregulation of defense-related genes, and the production of antimicrobial compounds in damaged tissues (Checker et al., 2018).

Finally, the involvement of phytohormones in the regulation of plant biotic defense responses should not be ignored. ETI and PTI cause specific downstream signaling pathways, in which three phytohormones, SA, JA, and ET, are vital. SA pathways respond to biotrophic and hemibiotrophic pathogens and JA and ET pathways respond to necrotrophic agents and pests (De Vleesschauwer et al., 2014). SA provoke the SAR pathway that promotes the expression of PR genes, which confers tolerance to a wide range of pathogens (Dehkordi et al., 2018).

Phytohormones: Types and Functions

Phytohormones are biochemical compounds that exist in plants in very low concentrations. They play important regulatory roles in physiological processes, growth, plant development and plant response to biotic (Nawaz et al., 2023) and abiotic stresses (Haghpanah et al., 2024).

Phytohormones are traditionally divided into two groups: growth-stimulating and stress-related hormones. Growth stimulating hormones included gibberellins, cytokinins, auxins, brassinosteroids and strigolactones and stress/defense- related hormones included salicylic acid (SA), jasmonic acid (JA), ethylene (ET) and abscisic acid (ABA). However, in recent years, these divisions have become increasingly unclear, as more data become available indicating that the crosstalk between plant hormones during growth and stress responses is extensive, and that all hormones, regardless of their current name, play a role in defense of Plants (Gilroy and Breen, 2022).

Plant hormones also vary in their chemical nature and biological functions. In terms of chemical nature, they are derived from amino acids (IAA, ethylene, salicylic acid), lipids (jasmonic acid), isoprenoid (cytokinin, gibberellin, abscisic acid, brassinosteroids) (Madani et al., 2019) (Figure. 1).

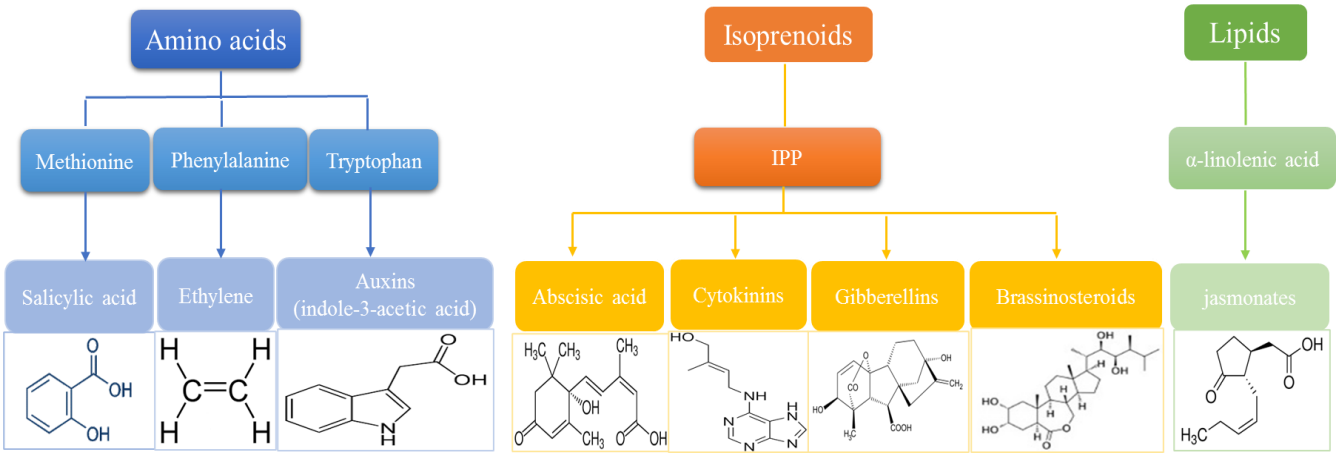


Figure 1. Types of phytohormones based on chemical structure and precursors of their biosynthesis (Base on Madani et al. 2019; Haghpanah et al. 2024 with some modifications).

Regulators of Plant Defense

Salicylic Acid (SA)

Salicylic acid (SA) is a crucial phytohormone involved in the regulation of plant immune responses, such as PTI, ETI, and systemic acquired resistance (SAR) (Kim et al., 2022; Li et al., 2022a). Upon pathogen attack, plants synthesize SA, which plays a central role in activating both local and systemic defense mechanisms. These defense responses include the synthesis of pathogenesis-related (PR) proteins, the accumulation of callose

deposits, enzymatic and non-enzymatic antioxidants, and localized defense response (hypersensivity) that involves rapid, programmed cell death(Gomez‐Munoz et al., 2017).

SA's involvement in disease resistance is multifaceted. It regulates small interfering RNAs (siRNAs) and interacts with RNA silencing pathways to suppress viral replication, as seen in the resistance of sour orange to Citrus tristeza virus (Gomez‐Munoz et al., 2017). Additionally, mutations in the SA biosynthesis or signaling pathways can render plants more susceptible to viral infections, even in the presence of resistance (R) genes. On the other hand, overexpression of SA synthesis genes or exogenous SA application can enhance basal immunity by delaying viral infections (Li et al., 2022a).

SA's action is largely mediated by the NONEXPRESSOR OF PATHOGENESIS-RELATED GENES (NPR) family, with NPR1 being the key mediator of SA perception. Upon SA binding, NPR1 undergoes a conformational change, transitioning from an inactive oligomeric to an active monomeric form, which then translocates to the nucleus. There, NPR1 interacts with TGA transcription factors, activating defense-related genes, including those encoding PR proteins and WRKY transcription factors (Zhang and Li, 2019). Moreover, the regulation of SA signaling involves post-translational modifications, such as methylation and glycosylation, which can modulate its effects on plant immunity and growth (Vora et al., 2023).

Exogenous treatment with salicylic acid (SA) has been shown to enhance disease resistance by activating defense enzymes such as polyphenol oxidase, chitinase, and phenylalanine ammonia-lyase, while also boosting the expression of pathogenesis-related (PR) proteins. For instance, the application of SA in plants like tobacco has been shown to increase resistance to pathogens such as the tobacco mosaic virus (TMV) (Li et al., 2022a). However, the effect of SA on plant growth is concentration-dependent: lower concentrations promote growth, while higher concentrations inhibit it (Vora et al., 2023).

In summary, Salicylic acid (SA) is a key phytohormone in regulating plant immunity, playing an essential role in both local and systemic defense mechanisms. Upon pathogen attack, SA triggers the activation of immune responses, including the production of pathogenesis-related (PR) proteins, callose deposition, and hypersensitive responses, which restrict pathogen spread. SA mediates its effects through complex signaling pathways, primarily involving NPR1, TGA transcription factors, and siRNA pathways. Moreover, exogenous SA application has been shown to enhance disease resistance in plants such as tobacco, improving defense against pathogens like the *Tobacco mosaic virus* (TMV). However, its effects on plant growth are concentration-dependent, with lower concentrations promoting growth and higher concentrations inhibiting it. Understanding SA's role in plant immunity is crucial for developing strategies to improve crop protection and resistance against various pathogens (Gomez‐Munoz et al., 2017; Li et al., 2022a; Vora et al., 2023).

Jasmonic acid (JA):

Jasmonic acid (JA) is a key regulator in plant defense, particularly in response to biotic stressors such as herbivory and infections by necrotrophic pathogens. Upon wounding or insect attack, JA is synthesized and triggers various defense mechanisms, including the production of antimicrobial compounds. JA is known to function in antagonism to salicylic acid (SA)-mediated pathways, which are primarily involved in responses to biotrophic pathogens. This interaction between JA and SA helps plants to fine-tune their immune responses, ensuring an appropriate defense mechanism is activated depending on the nature of the pathogen or stress. Recent studies have highlighted the critical role of JA in modulating plant defenses and its crosstalk with other phytohormones, including SA, to enhance resistance to a broad range of threats (Vora et al., 2023).

For example, the response to wounding in tomato plants activates JA biosynthetic enzymes, leading to the expression of defense genes like proteinase inhibitors. This function is critical in establishing systemic induced resistance (SIR). JA promotes the synthesis of bioactive forms, such as jasmonyl-lisoleucine (JA-Ile), in both the damaged and healthy tissues of plants (Sutaoney et al., 2023; Vora et al., 2023). These hormones modulate the

expression of resistance-related genes, initiating defense mechanisms through signaling pathways. The activation of JA responses is facilitated by proteins such as CORONATINE INSENSITIVE1 (COI1), JASMONATE ZIMDOMAIN (JAZ), and MYC2, which interact to regulate gene expression and activate defense systems by degrading JAZ repressors through the 26S proteasome pathway (Rubio et al., 2021; Li et al., 2022b).

Furthermore, jasmonic acid (JA) plays a key role in virus defense; its application during the early stages of viral infections, such as Potato virus Y (PVY) and Potato virus X (PVX), enhances resistance. However, in later stages of infection, JA may increase susceptibility, suggesting that the timing of JA application influences its effectiveness (García-Marcos et al., 2013). Additionally, research indicates that JA modulates the balance between its signaling and salicylic acid (SA) responses. Pretreatment with JA, followed by SA application, has been shown to enhance resistance to Tobacco mosaic virus (TMV) in Nicotiana benthamiana, although other studies suggest JA's indirect role in viral resistance, possibly by modulating SA-mediated resistance. This balance between JA and SA levels is crucial for determining the plant's resistance to various pathogens (García-Marcos et al., 2013; Zhu et al., 2014).

Furthermore, manipulation of JA signaling has been shown to influence pathogen dissemination in susceptible plant genotypes, highlighting the hormone's pivotal role in regulating plant defense mechanisms against both biotic and abiotic stresses.

Ethylene (ET):

Ethylene (ET) plays a critical role in plant immunity, particularly in the defense response to necrotrophic pathogens (Bürger and Chory, 2019). It is a central regulator of the plant immune signaling network, contributing to pattern-triggered immunity against biotrophic infections. ET also interacts with other hormones like auxin and jasmonic acid (JA), working together with JA to enhance defense responses against pathogens and herbivores (Ning et al., 2019). ET is perceived by several receptors, including ethylene response 1 (ETR1), ethylene response sensor 1 (ERS1), and ethylene insensitive 4 (EIN4), which are located in the endoplasmic reticulum (Vora et al., 2023). These receptors are histidine kinases that, upon activation, inhibit the downstream serine/threonine kinase, CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1), and regulate ET signaling (Chen et al., 2021). Mutations in these receptors result in ET insensitivity and increased susceptibility to necrotrophic pathogens, highlighting the importance of ET in pathogen resistance.

Mutations in these receptors cause ET insensitivity and increase sensitivity to necrotrophic pathogens (Vora et al., 2023).

Ethylene influences plant defense mechanisms against various pathogens through both endogenous and exogenous pathways. During plant-pathogen interactions, a significant increase in endogenous ethylene synthesis occurs, activating defense-related genes such as L-PAL, 4-coumarate CoA ligase (4-CL), chalcone synthase (CHS), and hydroxyproline-rich glycoproteins (HRGP). Exogenous application of ET and its precursors has demonstrated a direct link between this volatile plant hormone and the activation of defense responses against pathogens (Sutaoney et al., 2023; Vora et al., 2023).

Modulators of Plant Defense

Abscisic acid (ABA):

ABA plays distinct roles in plant immunity depending on the infection stage and the pathogen involved. In the early stages of infection, ABA helps regulate plant defense by causing stomatal closure and inducing callose deposition. However, when activated at later stages, ABA can suppress ROS induction and inhibit SA or JA signaling pathways, thereby negatively affecting plant immunity (Bharath et al., 2021). ABA modulates plant immune responses and defense mechanisms depending on the type and lifestyle of the pathogen. For example, in *Arabidopsis thaliana*, ABA enhances resistance to the necrotrophic fungus *Alternaria brassicicola* but suppresses resistance to *Pseudomonas syringae* (Mohr and Cahill, 2003; García - Andrade et al., 2011). Additionally, ABA increased resistance to *Cochliobolus miyabeanus* and enhanced susceptibility to *Magnaporthe oryzae* in Arabidopsis thaliana (Jiang et al., 2010). Studies have shown that interactions between *Tobacco mosaic virus* (TMV) and ABA affect ABA accumulation(Chen et al., 2021). Dehkordi et al. (2018) reported that ABA negatively influences

the immune response in plants with resistance genes against viruses.

Cytokinin (CK):

Cytokinins play a significant role in enhancing resistance against biotrophic pathogens, often working in tandem with salicylic acid (SA) in its signaling pathway. The anti-biotrophic effect of cytokinins is reported to be both SA-dependent and dose-dependent. Various studies have highlighted the role of cytokinins in providing resistance against *Pseudomonas syringae* in Arabidopsis and tobacco (Choi et al., 2010). In contrast, research by Ma and Ma (2016) suggested that modulating the cytokinin pathway may actually suppress plant defense responses. Cytokinins are thus recognized as essential regulators in the complex interactions among plants, microbes, and insects, affecting both plant growth and defense mechanisms. Dehkordi et al. (2018) described, SA signaling is expected to activate the cytokinin response, potentially inducing resistance to *Plum pox* virus. The application of exogenous CK promoted the resistance against biotrophic and hemibiotrophic pathogens in rice and tobacco (Jiang et al., 2013).

Indole-3-acetic acid/Auxin

By interacting with other plant hormones, auxins play an important role in mediating responses to both biotic and abiotic stresses. While auxins generally promote plant growth, they have also been shown to weaken defense responses in certain situations. Studies indicate that exogenous application of auxins can exacerbate the severity of some plant diseases (Checker et al., 2018).

Auxin signaling interacts with various other phytohormone pathways, such as those of salicylic acid , jasmonic acid, and ethylene. Auxin and SA act in a mutually antagonistic manner, while auxin shares common functions with JA in plant defense mechanisms (Checker et al., 2018). Exogenous auxin treatments are known to increase pathogen severity and worsen disease outcomes. In the case of biotrophic pathogen infections, auxin signaling is typically suppressed, with the Aux/IAA repressor being stabilized by SA. This suppression is likely due to the synergistic role of auxin in enhancing JA-mediated resistance against necrotrophic pathogens (Vora et al., 2023).

Recent data highlight auxin's crucial role in the hormonal signaling network that governs plant defense against both biotrophic and necrotrophic infections. Auxin regulates the generation, degradation, and expression of genes involved in other hormone signaling pathways, in addition to developmental and defense responses (Gomes and Scortecci, 2021; Sutaoney et al., 2023). Interestingly, some pathogens themselves produce auxin or enhance its biosynthesis and accumulation during infection, influencing both plant defense and growth mechanisms (Checker et al., 2018).

Brassinosteroids (BRs):

Brassinosteroids play a role in controlling the plant's defense response against a wide range of microorganisms such as bacterial, viral and fungal diseases. BRs have long been known as a favorable modulator in plant resistance to disease, however, recent studies have shown different functions of BR in response to disease (Sutaoney et al., 2023). The use of these hormones in agriculture can increase productivity, improve resistance to plant diseases and reduce environmental stress.

BRs further regulate the antioxidant system by stimulating apoplastic H2O2 accumulation, thereby inducing stress tolerance in plants (Jiang et al., 2013). Application BR reduced the level of TMV and other biotrophs in tobacco plants (Hayat et al., 2011). Several pattern recognition receptors (PRRs) interact with receptor BRI1-associated kinase 1 (BAK1) in a ligand-dependent manner. BAK1 is known as a general regulator of plant immunity against some biotrophs and hemibiotrophs (Alazem and Lin, 2015). This group of hormones interacts with other phytohormones, especially auxin. A close relationship between BRs and auxin signaling has been seen. There is a strong synergy between auxins and BRs In addition, BRs are involved in the modulation of JA signaling and in JA-dependent plant defense response (Chaiwanon and Wang, 2015)

Crosstalk Between Phytohormones in Defense Pathways

When plants are simultaneously exposed to multiple pathogen stresses, hormone crosstalk becomes increasingly important. In recent decades, accumulating evidence has defined the role of

various plant hormones in immunity and how they cooperate to coordinate the activation of the immune system through hormonal crosstalk. Hormone crosstalk is regulated at multiple levels, including protein stability, gene expression, transcriptional modification, protein modification, and hormone homeostasis (Ning et al., 2019; Aerts et al., 2021; Neil et al., 2021).

Phytohormones such as auxins, cytokinins, gibberellins, salicylic acid, jasmonic acid, ethylene , abscisic acid and brassinosteroids respond to stresses through crosstalk with signaling pathways (Figure. 2) (Checker et al., 2018; Aerts et al., 2021).

As the backbone of plant immune responses, plant signaling molecules SA, JA, and ET play a dominant role in inducible defense against microbial pathogens and herbivorous insects in a network of interconnected signaling pathways (Ning et al., 2019). While ABA, auxin, BR, GA, CK, and SL are involved in plant immune response through modulation.

Antagonistic interaction between the SA and JA pathways is the most common form and is studied, although large-scale additive and synergistic interactions have also been described (Hickman et al., 2019).

JA and SA signaling intersect negatively against necrotrophic pathogens and herbivores. NPR1 is a key intersecting in antagonistic crosstalk between SA and JA. SA and its functional analogs INA and BTH repress the expression of JA-responsive genes such as lipoxygenase 2 (LOX2), plant defensin 1.2, and vegetative storage protein (VSP), perhaps through inhibition of JA biosynthesis and action. The WRKY 70 transcription factor family also plays a prominent role in the antagonistic interactions of SA and JA. (Checker et al., 2018). Overexpression of the SA pathway, conferring resistance to a wide range of microbial pathogens can have deleterious effects on the JA/ET pathway. JA and ET have synergistic interactions and regulate the synthesis of genes encoding defense proteins, such as proteinase inhibitors and plant defensins.

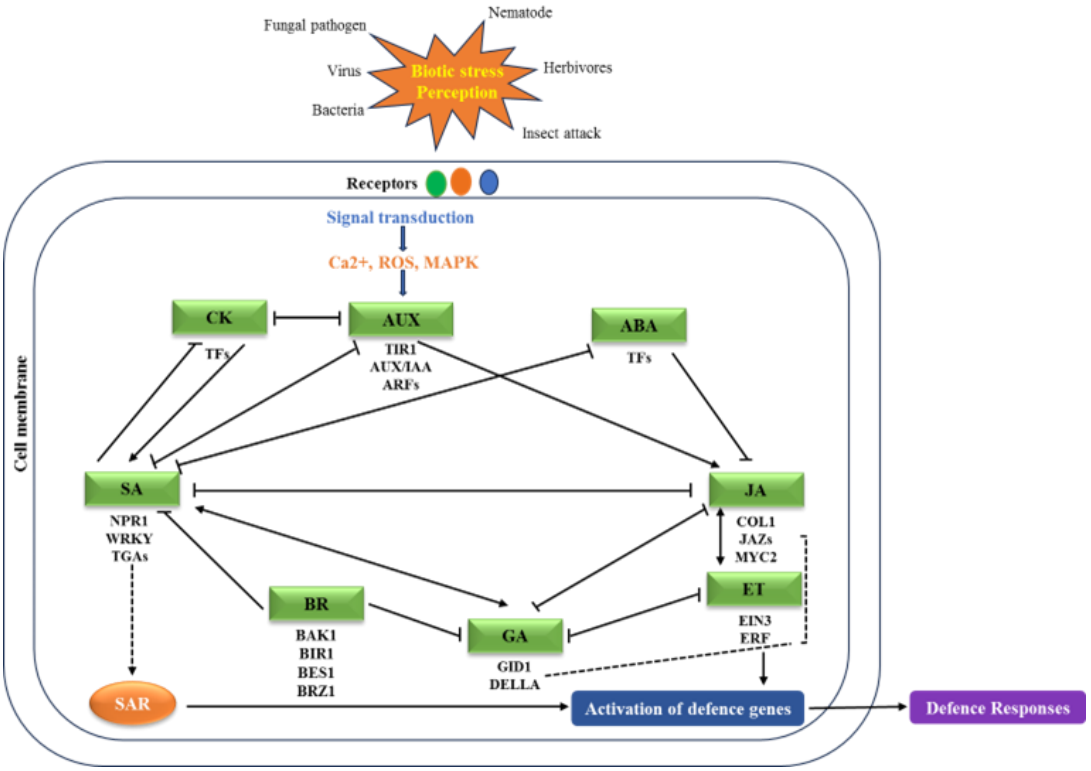


Figure 2. Scheme for plant hormone signaling networks and their crosstalk in stress responses (Shigenaga et al. 2017; Dehkordi-Nikbakht et al.2018).

In some cases, their cooperative action stimulates SA action, leading to increased PR gene (Checker et al., 2018). Studies have shown the key role of cytokinins in response to biotic stress. CK and SA signaling pathways converge to regulate plant defense, and SA defense responses are promoted by cytokinin-activated transcription factor (Reusche et al., 2013).

GA and JA also interact as DELLA proteins with key repressors of JA signaling, JAZ1, thereby preventing JA-mediated transcriptional repression. JA also regulates the expression of the GA1-3 (RGL3) suppressor, that positively regulates the JA-mediated response against necrotrophs by competing with MYC2 (Checker et al., 2018).

The crosstalk between pathways provides great regulatory potential to activate multiple resistance mechanisms in different combinations and may help the plant to prioritize the activation of a particular defense pathway over another. The effects of crosstalk between pathways may have a large impact on crop plants, providing better resistance against pathogens and insects in plants that are manipulated through genetic engineering of defense pathways or through the use of chemical plant protection agents that mimic the function of specific defense signaling molecules.Table 1 shows some of the antagonistic or synergistic interactions between plant hormones that contribute to plant immunity.

Table 1. Interactions between plant hormones that contribute to plant immunity.

Hormone Crosstalk	Interaction	Species	Reference
SA -JA/ET	Antagonistic	Various monocot and eudicot species	Thaler et al. 2012
SA-JA	Antagonistic	*Arabidopsis thaliana* wheat Maize *Oryza sativa*	Ding et al. 2016; Gorman et al. 2020, Huang et al. 2023; An et al. 2022
SA - CK	Synergistic	*Oryza sativa*	Jiang et al. 2013
ET- CK	Antagonistic	wheat	Veselova et al. 2021
JA-CK	Synergistic	*Oryza sativa*	Zhang et al. 2022
ABA -SA	Antagonistic	*Oryza sativa*	Xu et al. 2013
GA -JA/ET	Antagonistic	*Arabidopsis thaliana*	Hou et al. 2010
CK-AUX	Antagonistic	*Arabidopsis thaliana*	Naseem et al. 2014
BR-SA	Synergistic	*Arabidopsis*	Kim et al. 2022
BR-JA	Antagonistic	*Arabidopsis thaliana*	Liao et al. 2020
BR-ET	Synergistic	*Nicotiana benthamiana*	Xiong et al. 2020

Conclusion

Although chemical control agents can effectively combat plant pathogens, they pose severe environmental risks. Therefore, the use of efficient and effective solutions compatible with the environment and human health are promising alternatives to fight pathogens. One of the promising strategies for pathogen control is the use of plant hormones. The exogenous application of phytohormones significantly reduces the damage caused by various pathogens, both directly and through the activation of defense and antioxidant responses in different plant organs.

Over the past years, the understanding of the importance of hormonal regulation in plants under stress and hormonal signaling pathways has improved dramatically. Also, many efforts have been made to develop crops resistant to pathogenic agents and to understand different mechanisms of tolerance to these agents. However, more research is needed to elucidate the effect of different plant hormones on different crops and to better understand the complex molecular processes that control hormonal signaling and phytohormone-

mediated defense responses to develop successful crop engineering programs for resistance against diseases and pests in the future tense. Due to their high level of complexity, many of the underlying molecular mechanisms by which phytohormones induce stress tolerance in crops are poorly understood and require further study. In addition, the crosstalk among the phytohormones and the basic mechanisms of the role of molecular components in hormonal crosstalk against stress are not yet fully understood due to their complexity and need further investigation. Since the crosstalk among phytohormones regulates the balance between plant growth and defense resistance, further molecular and genetic studies will expand our understanding of the underlying mechanisms of hormonal crosstalk in modulating plant growth and development under stress conditions.

In recent years, scientists have been trying to understand the mechanisms of resistance to pathogenic agents in plants through the exogenous application of phytohormones and genetic manipulation. Despite these important advances, some problems still need to be addressed, such as plant growth stage and cell metabolism during development, which are directly related to plant stress response under unfavorable conditions. Therefore, it is essential to investigate the crosstalk between plant growth stages and response to pathogen in hormonal biosynthesis and hormone signaling networks under stress conditions.

Supplementary Materials

No supplementary material is available for this article.

Author Contributions

Conceptualization; A.N.D and P.M.G., writing original draft, investigation, reviewing and editing; A.N.D, supervision; A.N.D All authors listed have made substantial, direct, and intellectual contributions to the work and have approved it for publication. All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

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Conflict of Interest Statement

The authors declare no conflict of interest.

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مقاله مروری

فیتوهورمون‌ها و دفاع گیاهی

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چکیده: هورمون‌های گیاهی فرآیندهای فیزیولوژیکی مختلفی را در گیاهان تنظیم می‌کنند، از جمله جوانه‌زنی، رشد و واکنش‌ها به تنش‌های زیستی و غیر زیستی. بیماری‌های گیاهی که توسط پاتوژن‌هایی همچون قارچ‌ها، باکتری‌ها و ویروس‌ها ایجاد می‌شوند، اغلب مسیرهای هورمونی گیاهان را تغییر داده و منجر به مهار و یا تقویت همزمان هورمون‌ها در گیاهان می‌شوند. در واریته‌های مقاوم، با این حال، واکنش‌های هورمونی الگوی با آرایش‌تری دارند. مسیرهای سیگنال‌دهی هورمون‌های گیاهی عمدتاً در دو سوی متفاوت برنامه ریزی شده‌اند: مسیرهای اسید سالیسیلیک (SA) و اسید جاسمونیک (JA) از یک طرف و مسیر اتیلن از طرف دیگر. علاوه بر سالیسیلیک، جاسمونیک و اتیلن، هورمون‌های دیگری همچون اکسین‌ها، براسینوستروئیدها، سیتوکینین‌ها و اسید آبسیزیک (ABA) نیز نقش‌های مهمی در واکنش‌های گیاه به تنش‌های زیستی ایفا می‌کنند و به طور فزاینده‌ای به دلیل اهمیت‌شان در تعاملات گیاه–پاتوژن شناخته شده‌اند. پاتوژن‌ها می‌توانند این مسیرهای هورمونی را دستکاری کرده تا دفاع‌های گیاهی را سرکوب کرده که این امر موجب تسهیل آلودگی و گسترش آن می‌شود. در این مقاله، آخرین پیشرفت‌ها در درک عملکرد و تنظیم هورمون‌های گیاهی، پاسخ‌های دفاعی گیاهان و آثار متقابل بین هورمون‌های گیاهی در مسیرهای دفاعی بررسی خواهد شد.

کلمات کلیدی: اثر متقابل، تنش زیستی، تنظیم هورمونی، دفاع گیاه.

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Genetic diversity of conserved West African plantain (*Musa* spp.) using simple sequence repeat markers

Original paper

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Abstract: Assessment of the genetic diversity of germplasm is essential for sound germplasm management and its successful utilization in breeding programs. This study aimed to estimate genetic diversity among plantain accessions and establish relationships among the genotypes using simple sequence repeat (SSR) markers. SSR markers amplified 21 alleles, 3.50 alleles per locus, and major allele frequency (mean ± SD, 0.80 ± 0.34) across the 20 plantain accessions. The polymorphic information content (PIC) and Shannon's diversity index ranged from 0.054 to 0.919 and 0.000 to 1.864, respectively. Analysis of molecular variance (AMOVA) showed that 88% genetic variation occurred among genotypes within populations, with minimal variation observed between populations. This resulted in Nei genetic distance and FST values being negligible when distinguishing the populations. The gene flow rate unequivocally demonstrated the efficacy of employing co-dominant markers, as evidenced by both the Principal Coordinates Analysis (PCoA) and the dendrogram. This study revealed a clear-cut genetic variation among the 20 plantain accessions across plantain populations and established new cluster groups, providing valuable insights for future use in breeding programs.

Keywords: *Musa* spp., SSR markers, genetic diversity, genetic relationship, gene flow rate, heterozygosity.

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Introduction

Plantains, a staple crop for many Nigerians, are a significant food source in tropical regions, particularly in Africa, Latin America, and the Caribbean (Lescot, 2004; Ekunwe and Ajayi, 2010). They are a good source of nutritional energy and nutrients and can be cooked in various ways, such as boiling, frying, grilling, or roasting (Elayabalan et al., 2017). In 2020, plantains were grown in 54 countries, with global production exceeding 43 million metric tons (Data, 2020). This crop is critical for food security and revenue generation for small-scale farmers in tropical countries (Kahane et al., 2013). In Nigeria, plantain output is projected to reach 2.4 million metric tons, with the most production coming from the country's southern states (Folayan and Bifarin, 2011). However, plantains face various challenges that may reduce productivity and availability. Pests and diseases, such as plant-parasitic nematodes, can reduce nutrient and water transport to the main plant system, causing plant toppling and yield loss (Gowen et al., 2005; Godefroid et al., 2017). Synthetic pesticides are commonly used to control or manage these pests, but they have harmful effects on other beneficial soil-living species (Lafont et al., 2007; Carrascosa et al., 2015). Efforts to address these flaws have resulted in the creation of new hybrid types through breeding programs to establish lines with resistance or tolerance (Quain et al., 2018). However, over the years, their resistance or tolerance to pests and diseases has been genetically compromised by locals' activities in mixed agriculture systems involving hybrids, landraces, and other aspects of farming practices (Quain et al., 2018). Therefore, understanding the current alignment among plantain genotypes in the population would provide the initial knowledge needed to develop better-adapted varieties for sustainable agriculture in plantain production.

Simple sequence repeats (SSRs), also known as microsatellites, are suitable targets and methods for analyzing genetic variation and genotype relationships due to their genome-wide coverage, robustness, excellent reproducibility, co-dominant inheritance, high polymorphism with multiple alleles per locus, transferability between species, and low requirements for knowledge and instruments (Park et al., 2009; Foster et al., 2020; Bhattarai et al., 2021; Cusaro et al., 2021). SSR markers are comparatively low-cost for plant genotyping and can be employed in small laboratories. Fingerprinting, genetic diversity research, population structure analysis, association mapping, and linkage mapping have all used SSR markers, contributing to the expansion of fundamental genetic research and plant breeding efforts (Bhattarai et al., 2021). Several crops and plant diseases are being targeted with SSR markers, including genetic diversity studies based on genetic distance, gene flow and crossing over rate, evolutionary studies, building linkage maps, mapping loci involved in quantitative traits (QTL), marker-assisted selection, and defining cultivar DNA fingerprints (Jonah et al., 2011; Kalia et al., 2011). Several studies have shown the extent to which SSR markers have been utilized, such as those by (Tenkouano, 2008; Wang, 2016; Yamasaki, 2016; Leal, 2018; Wu, 2018; Jiang, 2020). These studies have shown that SSR markers are an effective technique for identifying genomic regions linked with significant traits or diseases in plants and have been used in genome-wide association studies (GWAS). Therefore the purpose of the study was to assess the genetic diversity among plantain accessions in West Africa and establish relationships among the genotypes using SSR markers.

Materials and Methods

Experimental site

This experiment was conducted at the Genetic Resources Center and Bioscience Center located at the International Institute of Tropical Agriculture (IITA), Ibadan, Oyo State, Nigeria.

Plant material, experimental design, and treatment

Twenty (20) plantain accessions from the Genetic Resources Center (GRC) of the International Institute of Tropical Agriculture (IITA) were used in this study. Table 1 shows the plantain accessions originating in three West African countries: Nigeria, Côte d'Ivoire, and Ghana. The experiment was laid out in a Completely Randomized Design (CRD), utilizing a factorial experiment with three replicates. The twenty (20) plantain accessions

(Table 1) and six SSR markers listed in Table 2 [DN239472 (locus_1), DN240063 (locus_2), Ma513026332 (locus_3), Ma513019043 (locus_4), Ma513036168 (locus_5), and Ma513035997 (locus_6)] were prepared by Inqaba Biotec, South Africa, served as the treatment.

Genomic DNA extraction of the biological sample and SSR primer sequences

Biological samples from 20 plantain accessions were taken from the young leaves of 8-week-old vegetatively propagated plantain seedlings maintained in the greenhouse at IITA, Ibadan. The collected fresh leaf samples (100–110 mg each) of the twenty plantain accessions were further shared in three replicates and placed in 2 ml autoclaved and labeled Eppendorf tubes and frozen for the next 24 h at -20°C. After 24 h, the biological samples were further dried in a freeze dryer for another 24 h and then ground with a Geno Grinder (MM-200, Retsch) for 3 minutes. Genomic DNA was extracted in three replications using the Doyle and Doyle (1987) plant tissue DNA extraction protocol.

DNA quantity and quality were assessed by a Nanodrop 2000 spectrophotometer (Thermo-Scientific, USA) and 1% agarose gel. Samples with high band intensity, purity, and lesser smear were selected and normalized to 50 ng/µl for further PCR analyses.

Six (6) Expressed Sequence Tag–Simple Sequence Repeat (EST–SSR) markers, created by Mbanjo et al. (2012), were used to assess twenty (20) plantain accessions. The SSR patterns of each subject were examined according to the procedure established by Quain et al. (2018), using the automated infrared fluorescence technology of a Li-COR 4300 sequencer (Li-COR, USA). Six SSR loci were amplified using particular primers modified with an extension M13 sequence at the 5'-end (5'-CACGACGTTGTAAAAC GAC-3') of the forward primer shown in Table 2 (Quain et al., 2018).

Table 1. List of plantain accessions sourced from the Genetic Resources Center, IITA.

S/N	Accession No.	Cultivar name	Collection	Origin
1	TMp-20	Apem onniaba	*Musa* collection	Pop_1 – Ghana
2	TMp-39	Apem pa	*Musa* collection	Pop_1 – Ghana
3	TMp-52	Abomienu	*Musa* collection	Pop_1 – Ghana
4	TMp-10	Nselouka	*Musa* collection	Pop_2 - Cote d'Ivoire
5	TMp-46	Diby 2 off-type	*Musa* collection	Pop_2 - Cote d'Ivoire
6	TMp-54	Didiede	*Musa* collection	Pop_2 - Cote d'Ivoire
7	TMp-55	Niangafelo	*Musa* collection	Pop_2 - Cote d'Ivoire
8	TMp-81	Poupoulu	*Musa* collection	Pop_2 - Cote d'Ivoire
9	TMp-5	Pome	*Musa* collection	Pop_3 – Nigeria
10	TMp-7	Mbi egome 1	*Musa* collection	Pop_3 – Nigeria
11	TMp-22	Akpakpak	*Musa* collection	Pop_3 – Nigeria
12	TMp-24	Egjoga	*Musa* collection	Pop_3 – Nigeria
13	TMp-25	Mbi egome 3	*Musa* collection	Pop_3 – Nigeria
14	TMp-26	Ntanga 3	*Musa* collection	Pop_3 – Nigeria
15	TMp-28	26285.0	*Musa* collection	Pop_3 – Nigeria
16	TMp-29	11669.0	*Musa* collection	Pop_3 – Nigeria
17	TMp-30	1199-1	*Musa* collection	Pop_3 – Nigeria
18	TMp-31	1199-6	*Musa* collection	Pop_3 – Nigeria
19	TMp-62	Eberedia ukom	*Musa* collection	Pop_3 – Nigeria
20	TMp-69	Okoyo ukom	*Musa* collection	Pop_3 – Nigeria

TMp – Tropical *Musa* plantain, Pop_1/2/3 – Individual population

Table 2. List of the SSR loci used in this study.

Locus	Primer sequences (5' - 3')	A.S.R.O.	T_a (°C)
DN239472	F : CACGACGTTGTAAAACGACCCGTGTTACAGATTGTATTCCCTTGT	105-126	58
	R : CGGCTGCGTCAACAAGCC		
DN240063	F : CACGACGTTGTAAAACGACCGCGGATGATTCATCGTGGACCG	170-174	58
	R : ACAGCAGCAACAATCTCGTCGT		
Ma513026332	F : CACGACGTTGTAAAACGACCCAACTTTCTCCAAGATCAG	164-170	58
	R : TCCAACAAGCAGCCCGT		
Ma513019043	F : CACGACGTTGTAAAACGACGTTAACGGCCACCTGCATGG	174-180	58
	R : GCCTCTTCACTGTGTTAAGTGCACAA		
Ma513036168	F : CACGACGTTGTAAAACGACCGCAGTAGCAGCAGGCAG	158-169	58
	R : GCCACAGCAGGATCCACC		
Ma513035997	F : CACGACGTTGTAAAACGACGAGGACCAATCTGCGTTCGC	114-124	58
	R : ACGCAGCACAAGTCGTCCA		

F – Forward, R – Reverse, A.S.R.O. – Allele size range observed, Ta (°C) – Annealing temperature

Polymerase chain reaction (PCR) product followed by gel electrophoresis

The six SSR markers, or loci, were used to estimate genetic variation and relationships among plantain accessions. The Gene-Amp® PCR System 9700 thermal cycler was used to conduct the PCR analysis in a total volume of 12.5 µl. Briefly, the recipe contains 6.25 µl of 1x [one *Taq* 2x Master Mix (M04821), New England Bio-Labs USA, with standard buffer, which contains all PCR components: 3 mM $MgCl_2$, 10x PCR buffer, 2.5 mM dNTPs, and 1 U *Taq* DNA polymerase (Fermentas)], 0.5 µl of 10 µM forward primer, 0.5 µl of 10 µM reverse primer, 0.25 µl of 3% dimethyl sulfoxide (DMSO, v/v), 0.1 µM of M13 primer-fluorescent dye IR700 or IR800 (Biosynthesis, Lewisville, Texas, USA), 2 µl of 20 ng genomic DNA, and 3 µl nuclease-free water. The PCR was programmed with an initial denaturation phase of 3 min at 94°C, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 58°C for 1 min, and extension at 68°C for 1 min, with a final extension at 68°C for 20 min, and a holding step at 4°C. The PCR products were loaded on a 1% agarose gel (w/v) using a 6x loading dye that contained GelRed. Electrophoresis was carried out in 1x Tris-acetate-EDTA (TAE) buffer for 3 h at 100 constant volts. The gel was stained with GelRed, and a Bio Doc-IT Imaging device (Cambridge, UK) was used to view the gel image under UV light. A 50–1500 bp AMPIGENE DNA ladder was used as a molecular ruler to compare DNA bands and estimate the sizes of the DNA fragments.

Data analysis

In this study, the following variables were computed to analyze the genetic diversity within and among plantain accessions. For each locus, the number of alleles (Na), average number of alleles per locus (A), observed heterozygosity (Ho), expected heterozygosity (He), Shannon's information index (I), and inbreeding coefficient (FIS) were calculated by GenAlEx software 6.5v (Peakall and Smouse, 2006). Then, polymorphic information content (PIC) was calculated to evaluate the informativeness of each marker using Power Marker software 3.25v (Liu and Muse, 2005). An analysis of molecular variance (AMOVA) was conducted to examine the genetic structure of several plantain accessions by comparing within and among population variations. The importance of AMOVA was evaluated using a non-parametric permutation method using 999 permutations. The genetic differentiation index was assessed using FST values among subpopulations. Pairwise genetic distances were calculated using unbiased genetic distance (Nei, 1978). Principal coordinate analysis (PCoA), implemented in GenAlEx software version 6.5, was used to elucidate the genetic structure, relying on the pairwise distance matrix. Additionally, to enhance the examination of genetic structure, a dendrogram was generated using the unweighted neighbor-joining approach, utilizing genetic dissimilarity among the 20 plantain accessions via DARwin software version 6.0.12 (Perrier, 2006).

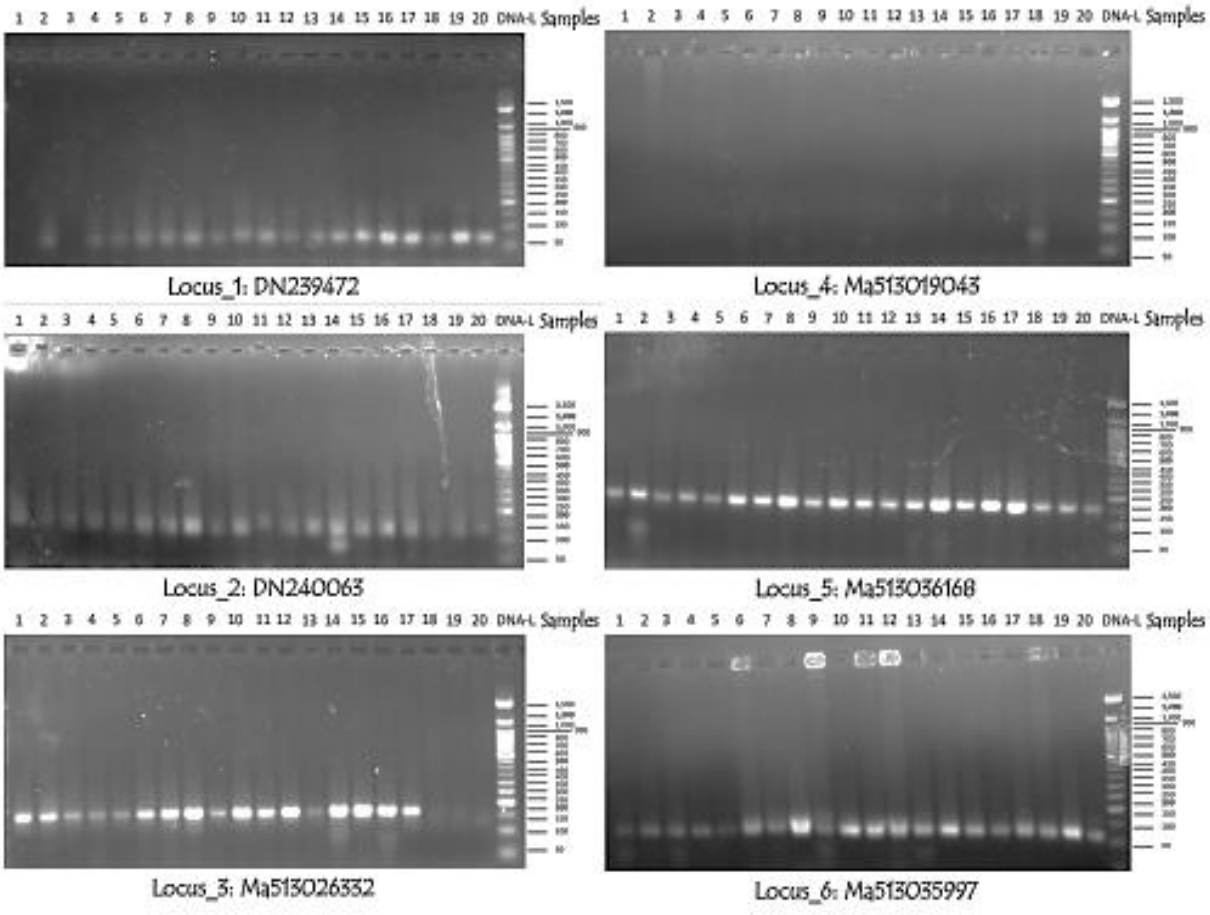


Figure 1. Polymorphisms observed from 20 plantain accessions across the six SSR loci.

Results

Allelic polymorphisms of SSR loci

Six (6) SSR markers were employed to assess the genetic diversity and relationships of twenty (20) plantain accessions available within the locality (Figure 1). The results presented in Table 3 revealed polymorphic profiles for the six SSR markers. A total of 21 alleles were identified across the six loci, resulting in an average of 3.50 alleles per locus. These loci (DN239472, DN240063, Ma513026332, Ma513036168, and Ma513035997) displayed appreciable levels of polymorphisms needed for genetic diversity assessment, with 3, 4, 5, 4, and 4 alleles, respectively. This cascaded on the major allele frequency (mean ± SD, 0.80 ± 0.34), where the following loci (DN240063, Ma513036168, and Ma513035997) assumed full availability (1 each) across the selected twenty (20) plantain accessions. The discriminatory power of each locus was evaluated by polymorphic information content (PIC). The average PIC value was relatively high 0.733 ± 0.307, with a maximum value of 0.919 recorded for the Ma513026332 locus and a minimum value of 0.054 observed for the Ma513019043 locus; the latter showed the most undesirable traits across the measured variables.

SSR markers and genetic diversity analyses

Genetic diversity traits are variables used by co-dominant markers (SSRs, SNPs, etc.) to assess the variation within and among populations. The results revealed the average number of alleles (Na),

as explained in Table 3. The number of effective alleles (Ne) mean values ranged from 1 to 6 (Supplementary Table 1). The results showed that locus_4 (Ma513019043) received the lowest value (mean ± SD) of 1.000 ± 1.523 Ne across the populations, while locus_3 (Ma513026332) had the highest mean value of 6 ± 1.523 Ne present in population_3 (Pop_3). Shannon`s information index (I) illustrated the values range from 0 to 1.864 I, with locus_4 (Ma513019043) was found with the lowest mean value of 0 ± 0.568 I over at least two populations (Pop_1 and Pop_2). However, the highest mean value was recorded for locus_3 (Ma513026332) with 1.864 ± 0.568 I in Pop_3. Observed heterozygosity (Ho) values ranged from 0 to 0.667. The maximum mean value resulting from locus_6 (Ma513035997), with an average of 0.667 Ho. On the other hand, the average expected heterozygosity (He), which reflects genetic diversity ranged from 0 to 0.833 (Supplementary Table 1). Here, locus_4 (Ma513019043) had the lowest mean value (0 ± 0.262, He), and locus_3 (Ma513026332) had the most admirable mean value (0.833 ± 0.262, He).

Table 3. Mean performance of allelic polymorphisms at the studied SSR loci.

S/N	Locus	Number of alleles	Major allele frequency	PIC
1	DN239472	3.00	0.90	0.799
2	DN240063	4.00	1.00	0.890
3	Ma513026332	5.00	0.85	0.919
4	Ma513019043	1.00	0.05	0.054
5	Ma513036168	4.00	1.00	0.906
6	Ma513035997	4.00	1.00	0.832
	Total	21.00	-	-
	Mean	3.50	0.80	0.733
	Standard Deviation	1.26	0.34	0.307

PIC – Polymorphism information content

This trend was seen on the unbiased expected heterozygosity (uHe), which ranged from 0 to 0.889. Same locus_4 (Ma513019043) had the least mean value (0 ± 0.284, uHe), while locus_3 (Ma513026332) showed the highest value across the populations with the highest mean value of 0.889 ± 0.284, uHe. On the fixation index (F), also known as the inbreeding coefficient, negative values denote excess in the heterozygote individuals within a given population, whereas positive values are for homozygotes. Hence, the inbreeding coefficient values ranged from -0.091 to 1. Locus_6 (Ma513035997) was found to have an appreciable mean value for the expected heterozygosity only in Pop_1 (-0.091 ± 0.386, F). On the contrary, loci (DN239472, 1.000 ± 0.386; DN240063, 1.000 ± 0.386; Ma513026332, 1 ± 0.386; Ma513019043, 1.000 ± 0.386; Ma513036168, 1 ± 0.386) had positive mean values for the inbreeding coefficient (F).

Genetic diversity expressed by populations of plantain accessions across the SSR loci

The results presented in Table 4 revealed the gene diversity information over the three populations of plantain accessions at the six SSR loci. The number of individuals per population (N) ranged from 3 to 12, with a grand mean of 6.667 ± 0.936. The number of alleles (Na) ranged from 2.500 ± 0.342 to 5.667 ± 0.882, with a mean value of 3.889 ± 0.471. Similarly, the number of effective alleles (Ne) showed the same trend, with values ranging from 2.324 ± 0.320 to 4.419 ± 0.749 on a grand mean of 3.331 ± 0.369. The

Shannon`s information index (I) ranged from 0.809 ± 0.176 to 1.471 ± 0.253 with a grand mean value of 1.130 ± 0.138. On the contrary, the pattern changed as observed heterozygosity (Ho) had values ranging from 0 to 0.167 ± 0.114 on a general mean value of 0.069 ± 0.041. In contrast, expected heterozygosity (He) recorded values ranged from 0.500 ± 0.105 to 0.688 ± 0.109 on a grand mean value of 0.596 ± 0.064 (Table 4). The results maintained the trend on unbiased expected heterozygosity (uHe), which ranged from 0.600 ± 0.126 to 0.717 ± 0.113 over the general mean value of 0.661 ± 0.069. The fixation index (inbreeding coefficient, F) reported positive values across the populations, indicating fewer heterozygote individuals among the studied populations. The inbreeding coefficient values ranged from 0.673 ± 0.199 to 1, with a grand mean value of 0.878 ± 0.070. Altogether, the findings on percentage polymorphic loci (% P) indicated the levels of genetic diversity or similarity expressed by the 20 plantain accessions. The grand mean value was notably high (88.89% ± 5.56). The lowest mean value among the populations was even on the high side at 83.33%, shared between Pop_1 and Pop_2. The highest recorded percent polymorphism of the six SSR loci studied was found in Pop_3, with a mean value of 100.00%.

Table 4. The mean performance of the three plantain populations on genetic diversity indexes across the SSR markers.

Population	N	Na	Ne	I	Ho	He	uHe	F	%P
Pop_1	3.000 (0.000)	2.500 (0.342)	2.324 (0.320)	0.809 (0.176)	0.167 (0.114)	0.500 (0.105)	0.600 (0.126)	0.673 (0.199)	83.33%
Pop_2	5.000 (0.000)	3.500 (0.563)	3.249 (0.537)	1.110 (0.233)	0.000 (0.000)	0.600 (0.122)	0.667 (0.135)	1.000 (0.000)	83.33%
Pop_3	12.000 (0.000)	5.667 (0.882)	4.419 (0.749)	1.471 (0.253)	0.042 (0.028)	0.688 (0.109)	0.717 (0.113)	0.948 (0.036)	100.00%
Grand M	6.667	3.889	3.331	1.130	0.069	0.596	0.661	0.878	88.89%
SE	(0.936)	(0.471)	(0.369)	(0.138)	(0.041)	(0.064)	(0.069)	(0.070)	(5.56)

Pop_1/2/3 – Individual population, N – Number of individuals per population, Na – Number of different alleles, Ne – Number of effective alleles, I – Shannon`s information index, Ho – Observed heterozygosity, He – Expected heterozygosity, uHe – Unbiased expected heterozygosity, F – Fixation index (inbreeding coefficient), %P – Percent polymorphic loci, GM – Grand mean, SE – Standard error.

Wright's F-statistics for 20 plantain accessions based on the SSR markers

Table 5 presents Wright's F-statistics for 20 plantain accessions across the six SSR markers. Wright's F-statistics include the inbreeding coefficient (FIS), global genetic fixation index (FIT), genetic differentiation index (FST), and estimated gene flow rate (Nm). Regarding the inbreeding coefficient (FIS), the results showed values ranging from 0.607 to 1, with a mean of 0.902 ± 0.064. The global genetic fixation index (FIT), or, in other words, total genetic diversity, revealed high positive values ranging from 0.666 to 1 with a mean value of 0.919 ± 0.054. On the contrary, the genetic differentiation index (FST) among the three plantain populations based on the SSR loci was very low, indicating unity among the populations, with a general mean of 0.168 ± 0.027. The FST values ranged from 0.057 to 0.252. The overall findings indicated that the estimated gene flow rate (Nm) was significant, with values ranging from 0.741 to 4.125 and a mean of 1.614 ± 0.511. Therefore, these results suggest that for every generation among the populations, the highest percent of gene crossing over between populations was approximately 4.13%, it was argued that it is still very low to influence the genetic structure of any population but enough to seek precautions in maintaining pure breeds or lines among the populations.

Genetic differentiation indexes (AMOVA, F_{ST} values, and Nei genetic distance) based on SSR markers for 20 accessions of Musa spp.

The analysis of molecular variance (AMOVA) results indicates that there is little or no genetic variation among populations (6% var.), as shown in Supplementary Table 2. The majority of genetic variance (88% var.) can be attributed to variation among genotypes or individuals across the three populations. However, the percentage variation resulting from within individuals of respective populations was very low, accounting for 6%. This finding suggests a lack of significant population structure destruction, which can be linked to the observed low genetic distances that would amount to a full breakdown of desired traits among the populations. The pairwise population matrix FST values revealed minimal levels of differentiation among the populations, as indicated in Supplementary Table 3 (below the diagonal), ranging from 0.076 to 0.193. Furthermore, the pairwise population matrix based on Nei genetic distance in Supplementary Table 3 (above the diagonal) demonstrated a close genetic relationship within plantain accessions from 0.398 to 0.862. Conversely, Nei genetic distance results revealed wider distances between the populations, which represented the percentage variation found among individuals across the three plantain populations in the AMOVA Table.

Table 5. Wright's F-statistics for 20 plantain accessions based on SSR markers.

Locus	FIS	FIT	FST	Nm
DN239472	1.000	1.000	0.252	0.741
DN240063	0.962	0.969	0.172	1.200
Ma513026332	1.000	1.000	0.165	1.262
Ma513019043	1.000	1.000	0.057	4.125
Ma513036168	0.845	0.877	0.208	0.949
Ma513035997	0.607	0.666	0.151	1.406
Mean ± SE	0.902 ± 0.064	0.919 ± 0.054	0.168 ± 0.027	1.614 ± 0.511

FIS – Inbreeding coefficient, FIT – Global genetic fixation index, FST – Genetic differentiation index, Nm – Estimated gene flow rate, SE – Standard error

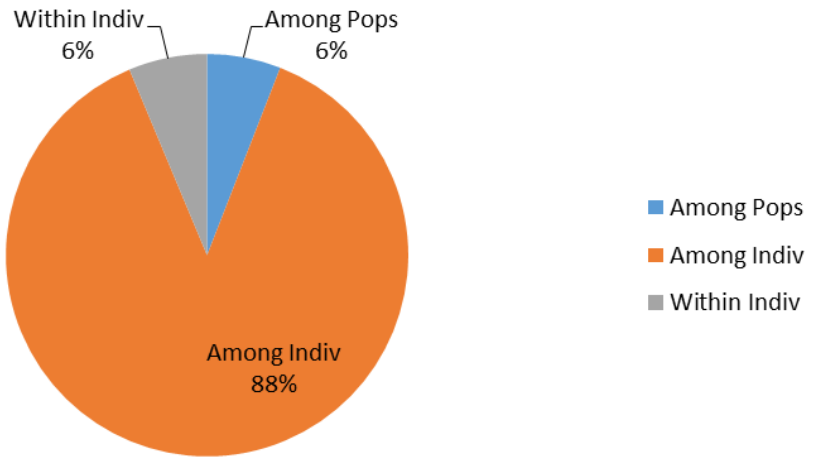


Figure 2. AMOVA for the genetic differentiation indexes on 20 accessions of Musa spp.

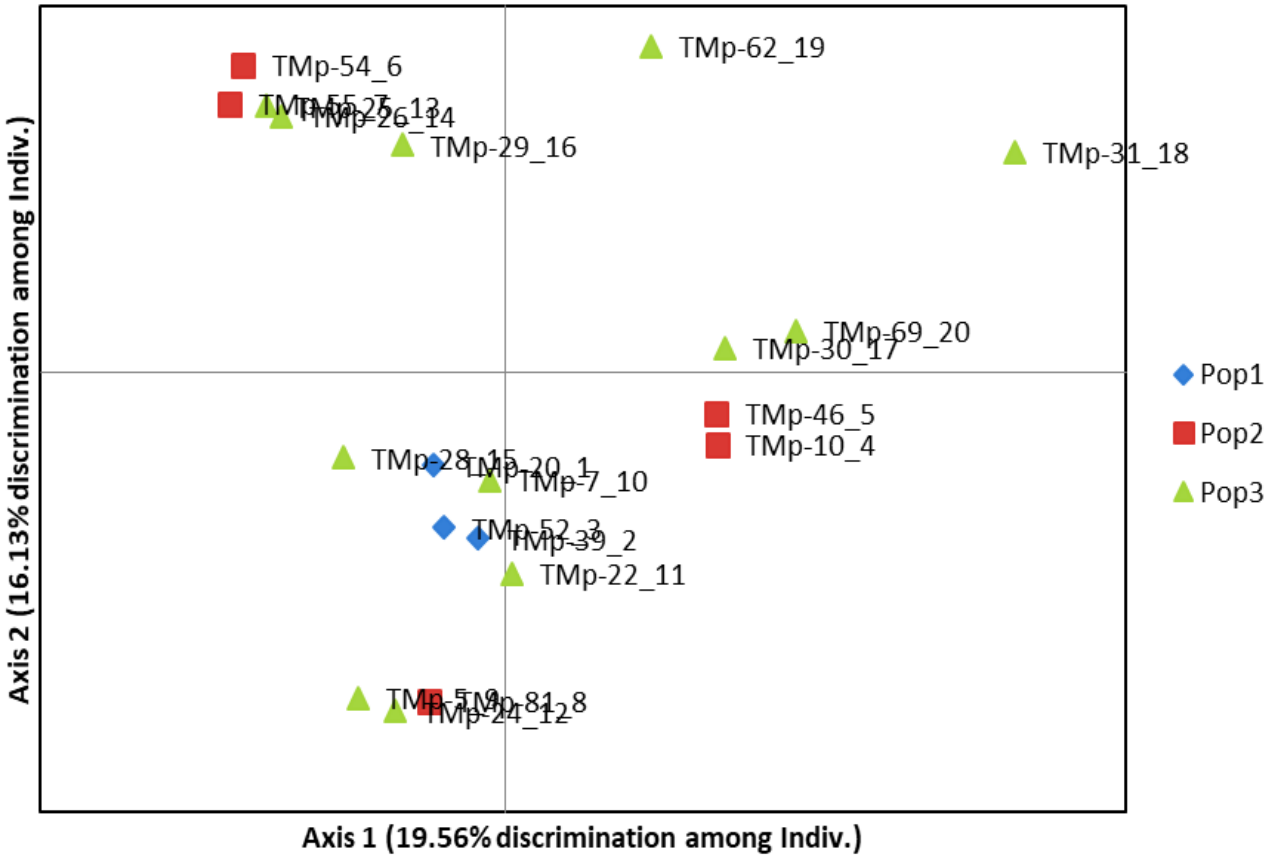


Figure 3. In a Principal Coordinates Analysis (PCoA) scatter plot of 20 plantain accessions based on the genetic distance matrix, the plane of the first, second, and third main PCoA axes accounted for 48.05% of the total variation. The first axis = 19.56% of total information; the second axis = 16.13%.

Genetic structure analysis

The principal coordinates analysis plot presents the genetic relationships among the 20 plantain accessions (Figure 3). The first three axes were the most informative PCoA, accounting for 48.05% of the total variation. The graphic representation of the PCoA showed proficient differentiation among the individuals or genotypes across the three populations based on the Nei genetic distance matrix. However, all three plane axes still did not greatly separate the plantain populations because the total variation was below average.

Though the unweighted neighbor-joining method was employed to justify the dissimilarities among the plantain populations, a dendrogram based on a dissimilarity matrix was generated, which confirmed the relationships and dissimilarities among the genotypes of plantain populations (Figure 4). The bootstrap values of all the nodes were depicted as numbers at the forks of the consensus tree diagram. To this end, three cluster groups were formed (rearranged due to gene flow or crossover effects).

Discussion

The evaluation of genetic diversity in agricultural germplasm is critical for defining a strategic approach to germplasm management and its use in breeding operations. Many studies in West African countries have focused on assessing a range of varieties, from known to unknown, such as the morphological diversity of plantain genotypes, and now using molecular tests. These physical characteristic investigations have usually found extremely minor differences in plantain traits across

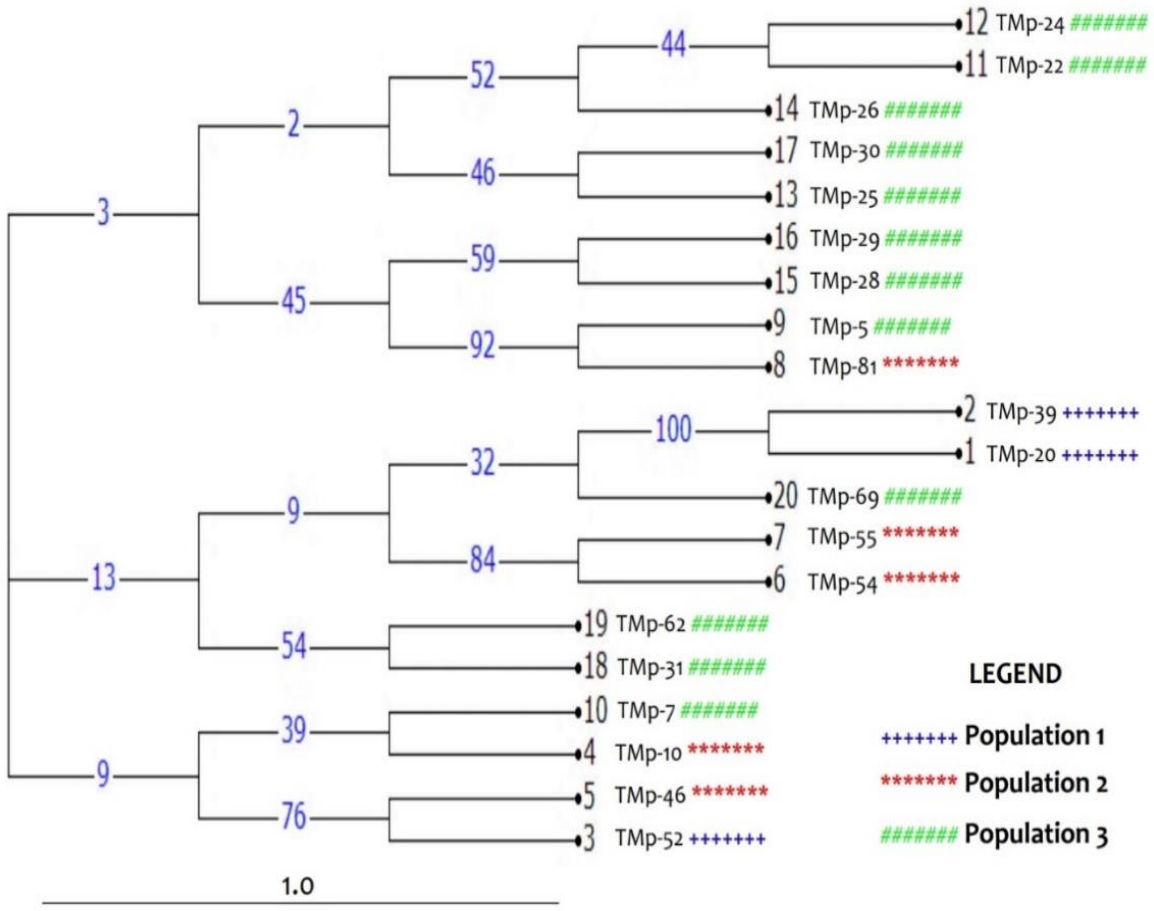


Figure 4. Unweighted neighbor-joining dendrogram depicting genetic relationships and dissimilarities among 20 plantain accessions; numbers along branches denote bootstrap support (shown for all values from 1000 bootstrap replicates).

the region, highlighting the significance of understanding and protecting *Musa* spp. genetic diversity. (Selatsa et al., 2009), for example, carried out a successful study in Cameroon that studied the morphological variety of plantain cultivars collected from several parts of the country. They discovered significant differences in plant height, leaf form, bunch size, and fruit characteristics among the studied genotypes, showing the considerable genetic diversity of plantains in Cameroon. Yet, many phenotypic features are strongly influenced by environmental influences, resulting in continuous variation (Creste et al., 2004). As a consequence, we decided to use molecular method (engaging co-dominant markers, SSRs) to evade the influence of the environment. Therefore, we set out to estimate genetic variation within and among the selected plantain accessions and to establish genetic relationships among the plantain accessions using co-dominant markers, SSRs. In all 20 plantain genotypes, at least 83.33% of the primers successfully produced scoreable amplicons. The microsatellite markers were obtained from diploid genotypes of East African highland banana cultivars, which are known to have substantial genetic variation (Mbanjo et al., 2012). The total number of alleles, average alleles per locus, major alleles frequency, and PIC values were all reported. The six primers amplified a total of 21 alleles, with an average of 3.50 alleles per locus, and expressed a very high frequency of major alleles throughout the 20 plantain accessions. These findings are consistent with previous research by GE

et al. (2005), which claimed an average Na value of 3.32, while Resmi et al. (2011) indicated an average 'Na' value of 2.70. These are values that we also observed. The SSR markers showed extremely high polymorphic information content (PIC) values. The mean PIC value obtained was higher than that reported by Resmi et al. (2011), who analyzed banana cultivars using 15 SSR markers and achieved a mean value of 0.530. In our study SSRs had PIC values greater than 0.500, indicating that they could be used to assess the genetic diversity of any plantain population. PIC values can be classified into three classes: slightly informative (PIC < 0.250), reasonably informative (0.500 > PIC > 0.250), and highly informative (PIC > 0.500) (Hayden et al., 2009). According to Ge et al. (2013), the SSR loci exhibit relatively high polymorphism and discriminative values based on this PIC scale.

Furthermore, the number of effective alleles (Ne, 1.22) was comparable to the report of Quain et al. (2018), who found a Ne value of 1.81 using SSR markers in a study of plantain accessions. In addition, Shannon's diversity index confirmed a substantial amount of genetic variation among plantain genotypes in this investigation. The results revealed that the allelic productivity of SSRs is extremely high. Shannon's information index value of close to one (1.00) indicates a high level of diversity, according to (Costa Tártara, 2014). In terms of heterozygosity traits, the present study found that the observed heterozygosity (Ho) was lower than the average expected heterozygosity (He, which reflects genetic diversity). This pattern was also apparent in unbiased expected heterozygosity (uHe), which had a higher average value than Ho. The fixation index (F) reflected this observation, with a negative value indicating an excess of heterozygote individuals within a given population and a positive value indicating an excess of homozygotes. In contrast, in research on the genetic diversity of plantain landraces from Cote d'Ivoire conducted by Cyrille et al. (2019), the average anticipated heterozygosity (He = 0.506 ± 0.008) was lower than the observed heterozygosity (Ho = 0.897 ± 0.025) across all loci and populations evaluated. Our findings were comparable to those of (Haddoudi et al., 2021).

According to our findings, the average inbreeding coefficient (FIS) value favored low observable heterozygosity. Likewise, the global genetic fixation index (FIT) found a significant positive value, indicating that homozygotes are distributed equally throughout populations. The genetic differentiation index (FST) between plantain populations based on SSR loci was very low, demonstrating unity among the three populations. In a study of B-genome-derived SSR markers in *Musa* spp., Oriero et al. (2006) found negative FIS and FIT values. Additionally, Haddoudi et al. (2021) found substantial population differentiation with an FST of 0.147 by SSR markers. Also, the value was consistent with that reported for *Musa ciliaris* (FST = 0.18) by (Badri et al., 2008). We are confident that the complete set of findings would be sufficient to demonstrate that the results were narrowed down on the predicted gene flow rate (Nm), which was accurate. The values ranged from 0.741 to 4.125, with a mean of 1.614 ± 0.511. This implies that the highest percentage of gene crossing over between populations was roughly 4.13% per generation, which is still too low to impact the genetic structure of any group. Nevertheless, Nm greater than 4.00 suggests substantial gene flow among populations, according to (Ruiz-Erazo et al., 2015).

The results of the analysis of molecular variance (AMOVA) show that there is little genetic diversity both among populations and within individuals in those groups. The primary component of genetic variation detected, on the other hand, was found among individuals (88%) across the three groups. Earlier studies on plantain diversity using SSR markers found similar minor genetic variations between populations (Changadeya et al., 2012; Kitavi, 2015; Quain et al., 2018). These findings confirm the concept that plantain morphological variety emerged predominantly through somatic mutations in a hypervariable region of the genome arising from a small number of botanically distinct clonal origins (Langhe et al., 2005). Still, the results indicate a lack of population structure, which can be attributable to the low genetic distances observed between groups.

FST values for the pairwise population matrix demonstrated low degrees of divergence among the populations. Also, the pairwise population matrix based on Nei genetic distance revealed both intimate and broad genetic links among the studied plantain accessions. As a result, the findings are corroborated

by a report on the linkages between diploid *Musa acuminata* cultivars (Changadeya et al., 2012).

Based on the Nei genetic distance matrix, the cluster analysis utilizing PCoA did not reveal a distinct cut among the three plantain populations. As a consequence, the unweighted neighbor-joining approach was used to explain the differences between plantain populations. The dissimilarity matrix was used to produce a dendrogram, which validated the linkages and dissimilarities of plantain accessions across populations. This analysis led to three cluster groups. Therefore, the report by Cyrille et al. (2019) backs up these findings.

Conclusion

The present study is one of the earliest to use SSR markers to investigate the genetic diversity of plantain genotypes in Nigeria. The use of these SSR markers revealed significant genetic variation and connections among 20 plantain accessions. The adoption of these primers was predicated on the informativeness of their high PIC values, which have been previously reported in the literature. They duplicated the same high scores on average, resulting in careful genetic variation among the 20 plantain genotypes. The main allele frequency was at its peak. The key factors seen on the genetic differentiation index scores were Shannon's information index and the inbreeding coefficient component. All of these findings correlate with the gene flow rate (Nm), which refers to the number of cross-over genes every generation. This might be thought of as the essence of the entire diversity. The results on Nm are supported by the pairwise population matrix and the AMOVA. Surprisingly, the PCoA and dendrogram generated corroborated the linkages and differences among the 20 plantain accessions. They were regrouped (rearranged inside the same initial three (3) cluster groups, but with clear mixes among the individuals throughout the three populations: Cluster 1, Cluster 2, and Cluster 3). As a result, plant breeders can choose from any of the accessions in distinct clusters for exceptional crop development schemes for sustainable agriculture. More research on the effects of co-dominant markers on genetic diversity, the assembly of desired traits, and plant improvement will be much appreciated as a precaution against any unexpected hybrid breakdown.

Supplementary Materials

The supplementary material for this article can be found online at: https://www.jpmb-gabit.ir/article_719401.html.

Supplementary Table 1. Responses of SSR markers to genetic diversity indexes across entire populations.

Supplementary Table 2. AMOVA based on SSR markers for 20 accessions of *Musa* spp.

Supplementary Table 3. Pairwise population matrix FST values (below the diagonal) and Nei genetic distance (above the diagonal).

Author Contributions

Conceptualization, D. O. and O. N.; methodology, F. O.; software, D. O. and F. O.; validation, C. O., O. U. and H. O.; formal analysis, C. O. and F. O.; investigation, D. O.; resources, D. O.; data curation, O. N. and F. O.; writing—original draft preparation, D. O. and F. O.; writing—review and editing, O. U. and H. O.; visualization, H. O. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest Statement

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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مقاله پژوهشی

بررسی تنوع ژنتیکی چنار (*Musa* spp.) در منطقه حفاظت‌شده آفریقای غربی با استفاده از نشانگرهای توالی‌های تکراری ساده

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چکیده: ارزیابی تنوع ژنتیکی ژرم‌پلاسم برای مدیریت صحیح ژرم‌پلاسم گیاهی و استفاده در برنامه‌های اصلاحی ضروری است. این مطالعه با هدف برآورد تنوع ژنتیکی در میان توده‌های چنار و برقراری ارتباط بین ژنوتیپ‌ها با استفاده از نشانگرهای توالی تکراری ساده (SSR) انجام شد. با استفاده از نشانگرهای SSR، ۲۱ آلل با فراوانی ۳/۵ آلل در هر مکان ژنی و با فراوانی آلل اصلی ۰/۸۰ ± ۰/۳۴ (SD ± میانگین) در ۲۰ توده چنار شناسایی گردید. میزان تغییرات محتوای اطلاعات چندشکل (PIC) و شاخص تنوع شانون به ترتیب ۰/۰۵۴ الی ۰/۹۱۹ و صفر الی ۱/۸۶۴ متغیر بود. تحلیل واریانس مولکولی نشان داد که ۸۸ درصد از تنوع ژنتیکی شناسایی شده، تنوع درون جمعیتی بوده در حالیکه فاصله ژنتیکی کمتری بین جمعیت‌های مورد بررسی مشاهده شد. بنظر می‌رسد این موضوع سبب اندک شدن برآورد فاصله ژنتیکی Nei و مقادیر FST در هنگام تفکیک جمعیت‌ها گردید. نرخ جریان ژنی به‌طور قاطع کارایی استفاده از نشانگرهای همبارز را نشان داد، همان‌طور که از طریق تجزیه به مولفه‌های اصلی (PCoA) و دندروگرام به اثبات رسید. این تحقیق بیانگر وجود تنوع ژنتیکی بین ژنوتیپ‌های جمعیت‌های چنار بوده و اطلاعات حاصله از گروه‌بندی ژنوتیپ‌های را می‌توان در برنامه‌های آتی اصلاحی مورد استفاده قرار داد.

کلمات کلیدی: نشانگرهای SSR، Musa spp، تنوع ژنتیکی، فاصله ژنتیکی، شدت جریان ژنی، هتروزیگوسیتی.

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Evaluation of Fructan metabolism enzymes in contrasting barley (*Hordeum vulgare*) genotypes under drought stress

Original paper

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Abstract: In terminal drought conditions, the limitations on current photosynthesis can improve the influence of cereal stem carbohydrate reserves on preserving grain yield. This study investigated how the remobilization of stem fructans affects barley yield under drought conditions. The present study performed comparative physiological and gene expression analyses using flag leaf, penultimate, and peduncle tissues from four barley genotypes (G1, G2, G3, and G4). We examined the expression levels of fructan metabolism genes in drought-stressed leaf, stem penultimate, and peduncle tissues during the grain-filling stage, comparing them to those under normal conditions. Notably, genetic variability among the cultivars influences the expression of drought-responsive genes associated with tolerance. Specifically, genotypes 1 and 3 showed an up-regulation of fructan metabolism genes in response to drought, while genotypes 2 and 4 exhibited a down-regulation. Our findings indicated that increased fructan accumulation and its subsequent remobilization significantly contribute to the yield stability of G1 and G3 under drought stress conditions. Barley genotypes that demonstrate a strong capacity for fructan production and remobilization in response to terminal drought stress could serve as valuable resources for breeding programs aimed at improving drought tolerance through the selection of these traits.

Keywords: Barley, drought tolerance, fructan metabolism, gene expression, seed filling.

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Introduction

Barley, *Hordeum vulgare*, is the fourth most significant grain crop globally, following by wheat, maize, and rice (FAO, 2020). Barley crops grown in semi-arid regions may become dehydrated during growth, especially during anthesis and seed filling, making them more dependent on water-soluble carbohydrates (WSCs). WSCs include fructan, glocuse, and sucrose, with fructans being the most important stored carbohydrates (Goggin and Setter, 2004; Hübner et al., 2015). Drought stress triggers a range of biochemical and physiological responses in plants, ultimately resulting in decreased crop production (Nosratiazar et al., 2024). Drought is a critical environmental factor that limits plant growth and productivity, particularly during the flowering and seed-filling stages of barley. It is the second leading cause of grain yield loss, following plant pathogens (Liliane and Charles, 2020). In general, seed filling depends on the supply of carbon from two sources: post-anthesis carbohydrates that are mobilized directly to the seed (current photosynthesis) and remobilization of storage material from the stem to grain (Siah et al., 2003; Esmaeilpour-Jahromi et al., 2012). In drought conditions, the role of stem reserves and their remobilization becomes increasingly vital due to restricted photosynthesis, significantly impacting the final yield (Sehgal et al., 2018). The carbohydrate reserves in barley stem tissues serve as a key carbon source during the initial grain-filling stages. Water scarcity can lead to a reduction in yield of up to 50-60%, primarily due to decreased seed weight and fewer spikes. Genotypes that are sensitive experience reductions in yield-related traits, including pollen viability, panicle number, and seed weight when subjected to stress conditions (Barnabás et al., 2008). When faced with drought, the remobilization of these stem reserves can aid in grain filling. Under terminal drought conditions, the remobilization of carbohydrates from the stem to the seeds can enhance grain yield by approximately 40% (Liu et al., 2020). To elucidate the molecular basis of genotypic variation in the concentration of WSC, a microarray analysis was conducted on the stem. Different barley genotypes exhibit varying capacities for WSC accumulation in the stem internode (Badigannavar et al., 2018). Physiological, morphological, and molecular traits can be surveyed to better understand the molecular mechanism of stem reserve remobilization in response to drought conditions. Drought stress can impede grain photosynthesis and assimilation, leading to reduced grain yield by diminishing stomatal conductance and fostering protein degradation, and causing significant declines in chlorophyll levels (Farooq et al., 2017). Chlorophyll is a pigment that plays a key role in photosynthesis by capturing light energy (Adl et al., 2023). Concurrently developing varieties with multiple stress resistance may not be possible using conventional breeding (Bita and Gerats, 2013; Saidi and Hajibarat, 2020). Fructans constitute a significant component of carbohydrate storage in cereal plants and exhibit a notable increase under stress conditions within their vegetative structures. The biosynthesis of fructans occurs through fructosyl transferases. Key enzymes that facilitate fructan biosynthesis include sucrose:sucrose 1-fructosyltransferase (*1-SST*) and sucrose:fructan 6-fructosyltransferase (*6-SFT*) (Nagaraj et al., 2004). Studies have indicated that osmotic stress leads to an upregulation of the *1-SST* and *6-SFT* gene expressions in wheat stems (Xue et al., 2008). Fructans are primarily stored in stem internodes until around the mid-grain filling stage (Schnyder, 1993). The significance of utilizing these stem reserves during drought stress is highlighted when photosynthetic efficiency declines during the grain filling phase (Blum, 1998). As the demand for grain filling intensifies and sucrose becomes scarce, fructans are broken down to release additional fructose and sucrose. The mobilization of stored carbohydrates necessitates the hydrolysis of fructans, a process facilitated by fructan exohydrolases (*FEHs*), predominantly the fructan-1-exohydrolases (*1-FEHs*). Both *FEHs* and fructosyl transferases are likely regulated at the transcriptional level (Van Laere and Van Den Ende, 2002; Zhang et al., 2020). Research by Zhang et al. (2020) revealed that the expression of the *1-FEH* w3 gene increased in wheat stems under terminal drought, approximately 20-25 days after anthesis. One investigation demonstrated that the *1-FEH* w3 gene plays a crucial role in the remobilization of stem fructans and serves as a valuable marker for selecting wheat with efficient fructan remobilization (Zhang et al., 2015). Since fructans cannot be directly

transferred from vacuoles, they must be degraded and moved as sucrose. As sucrose is transported from source to sink via the phloem, sucrose transporters (SUTs) are essential for facilitating its active transport across plasma membranes (Lalonde et al., 2003). SUT proteins are divided into two subgroups: *SUT1* and *SUT2* (Endler et al., 2006). However, few studies have explored the activity or expression patterns of these transporters in different wheat and barley internodes under terminal drought conditions. The expression of *SUT1* peaks 16-20 days after anthesis (DAA), underscoring its vital role in regulating carbon distribution during the grain filling process in cereals (Aoki et al., 2002). In this study, the accumulation and remobilization rates of fructans in the penultimate internode were analyzed in different genotypes subjected to terminal drought stress. Additionally, the research included transcriptional profiling of genes that encode vital enzymes involved in fructan metabolism, resulting in the identification of correlations between transcript levels and grain yield. This study aims to compare different barley genotypes in terms of their fructan metabolism enzymes during drought stress at the grain filling stage.

Materials and Methods

Study sites and planting materials

Four different barley genotypes were subjected to field trials at the Varamin Agricultural Research Station, Ministry of Jehad Agriculture, focusing on both normal and water-deficient conditions. Water scarcity poses a significant challenge in these regions. The plant material was supplied from seed collection of Seed and Plant Improvement Institute ([SPII), ministry of Jehad Agriculture in Karaj. The experiment included two treatments: a well-watered control and a water-stressed treatment. A randomized complete block design (RCBD) was implemented for the study, with three replications conducted across three sites. Each genotype was sown in a single-row plot measuring 5 meters in length, with a width of 25 cm and a row spacing of 75 cm. Irrigation was applied once after seeding in the fall and three times during spring for tillering, stemming, and flowering. A water shortage facilitated the achievement of 50% flowering. Irrigation was discontinued at the 50% spike stage, which is when half of the plants in the plots had begun to flower. Each plot featured six planting lines spaced 20 cm apart on two 6-meter stacks. The total area for each plot was 7.2 square meters; after excluding 0.5 meters from both ends to account for edge effects, the area designated for harvesting became 6 square meters. All plots were planted around the same time, specifically in early November. Fertilization included 80 kg of nitrogen per hectare at planting and an additional 100 kg per hectare as top dressing on April 1st. Phosphorus and potassium fertilizers were applied at 200 kg and 50 kg per hectare, respectively, at the time of planting. The seeding rate was set at 350 seeds per square meter, with the required seed weight per plot calculated based on the weight of a thousand seeds of each cultivar. To control broadleaf weeds, Granstar herbicide was employed, while Puma Extra herbicide was used to target narrow-leaved weeds. List of barley genotypes used in the study is given in Table 1.

Physiological responses to water deficit

Three types of tissues (peduncle, penultimate leaf, and flag leaf) were examined to assess the physiological responses of four barley genotypes. To study these traits, both normal and water-stressed barley tissues were collected 28 days after anthesis at the seed filling stage. Fresh samples were rinsed with distilled water in the laboratory and then left to air dry at room temperature (18 °C) for 6 hours before analyzing chlorophyll a (Chl-a), chlorophyll b (Chl-b), total chlorophyll, and carotenoid levels. A precise weight of 0.5 g of fresh plant material was homogenized in a tissue homogenizer with 10 ml of acetone as the extraction solvent. The homogenate was then centrifuged at 12,000 rpm and 4 °C for 15 minutes. One milliliter of the resulting supernatant was combined with 4 ml of acetone. The resulting solution was analyzed for Chl-a, Chl-b, total chlorophyll, and carotenoids using spectrophotometry according to the following formulas:

$Chl\text{-}a = 12.25A_{663.2} - 279A_{646.8}$

$Chl\text{-}b = 21.5A_{646.8} - 5.1A_{663.2}$

$C\ x+c = (1000A_{470} - 1.82Ca - 85.02Cb) / 198$

$Total\ chlorophyll\ (mg/g) = [20.2\ (A_{645}) + 8.02\ (A_{663})]$

Table 1. Pedigree and yield of barley genotypes used in this study.

Genotype	Pedigree	Yp	Ys
G1	Rojo/3/LB.IRAN/Una8271//Gloria"S"/Com"S"/4/Nik	4.933	5.133
G2	Triton/Yazd-5//Nik/3/Rhn03	5.383	5.181
G3	Rojo/3/LB.IRAN/Una8271//Gloria"S"/Com"S"/4/	4.947	4.719
G4	Rojo/3/LB.IRAN/Una8271//Gloria"S"… Triton/Yazd-5//Nik/3/Rhn03	4.008	3.778

Yp: Yield under normal conditions; Ys: Yield under drought stress.

Table 2. Genes ID and primer sequences of fructan metabolism-related genes in barley were used in this study.

Gene ID	Sequence of primer
1-SST	F: TATGGACTCGTCGTACAACC
1-SST	R: TGTGTCTAGGAGAAAGGGAG
SUT1	F: TATTCCTGCTGCCCAAGATC
SUT1	R: GAGAGCCACCTAGTTACGAC
1-FEH	F: TGTGGAGAAGGGTTGGAGTG
1-FEH	R: GATGGGAAGGGCTTACTAAG
6-SFT	F: ACAACCAGCTCTCCAATGAG
6-SFT	R: GTATGAATTGCCCTTCCCTG
SUT2	F: TGCAGACAAGGAAGGAAGGC
SUT2	R: GCCCTCCTACTTTTGCCC
HvActin	F: GGTCCATCCTAGCCTCACTC
HvActin	R: GATAACAGCAGTGGAGCGCT

A = Absorbance, Ch-a = Chlorophyll a, Ch-b = Chlorophyll b, C x+c = Carotenoids.

RNA extraction and expression profiles fructan metabolism genes

Samples from the penultimate, leaf, and peduncle were collected separately to isolate RNA from barley plants subjected to both normal and water deficit conditions during the grain filling stage. Total RNA was extracted from the drought-stressed and controlled penultimate and peduncle internodes utilizing the RNX-Plus kit (Sinaclone, Iran) under the manufacturer's guidelines. The RNA's purity and concentration were assessed using a Nano Drop spectrophotometer, and its integrity was verified via 1% agarose gel electrophoresis. Subsequently, cDNA synthesis was carried out following the instructions of a cDNA synthesis kit (SinaClon, Iran). Each gene was analyzed in triplicate, using the actin gene as a reference. All primers for gene expression analysis were designed using the Oligo program (Table 2). Gene expression assessments were performed with a real-time PCR instrument (ABI real-time, Agilent**)** employing SYBR green, as per the manufacturer's recommendations. After normalization, the relative expression levels of the genes were calculated using the $2^{-\Delta\Delta CT}$ method (Livak et al., 2013), with *HvActin* as the reference for determining Ct values for the fructan metabolism genes. Quantitative real-time PCR (RT-qPCR) was conducted to elucidate the expression profiles of *SUT1*, *SUT2*, *6-SFT*, *1-SST*, and *1-FEH* genes across three tissue types under both normal and water deficit conditions, with additional investigation into their expression levels during the seed filling stage.

The heatmap was created using TBtools to illustrate the differential expression of genes and the

relationship between physiological traits and gene expression. Statistical analyses were conducted with SPSS version 20.0. Significant differences between means were assessed with Duncan's test at a significance level of $P < 0.05$.

Results

Leaf fructan metabolism gene expression under drought

The expression of the *SUT1* gene in three different tissues (peduncle, penultimate and leaf) showed a significant increase in genotype 1 in the stem penultimate (Figure 1). The expression of *SUT2* gene in all tissues revealed that the penultimate had a higher expression as compared to the other tissues. The expression of the *6-SFT* gene in leaves was higher than the other tissues in response to drought stress. The expression of *1-SST* gene in penultimate and peduncle showed higher expression as compared to leaf tissue. In genotype 2, the expression of *SUT1* gene was increased in the peduncle tissue. The expression of *SUT2* gene showed increased expression in the leaf tissues. The increased expression of *the 6-SFT* gene was observed in the penultimate tissue of the stem. The expression of *1-SST* and *1-FEH* genes increased in three tissues. The expression of *SUT1* gene in three different tissues (peduncle, penultimate, and leaf) showed that this gene was increased in penultimate and leaf of genotype 3. The expression of *SUT2* gene in penultimate and peduncle tissues showed a higher expression as compared to the leaf tissue. The expression of *6-SFT* and *1-SST* genes in penultimate and leaves in response to drought stress showed a greater increase as compared to the peduncle tissue. The *1-FEH* gene showed increased expression in the leaf tissue. In genotype 4, significant differences in expression was not observed for *SUT1* and *SUT2* genes in leaf, peduncle, and penultimate tissues.

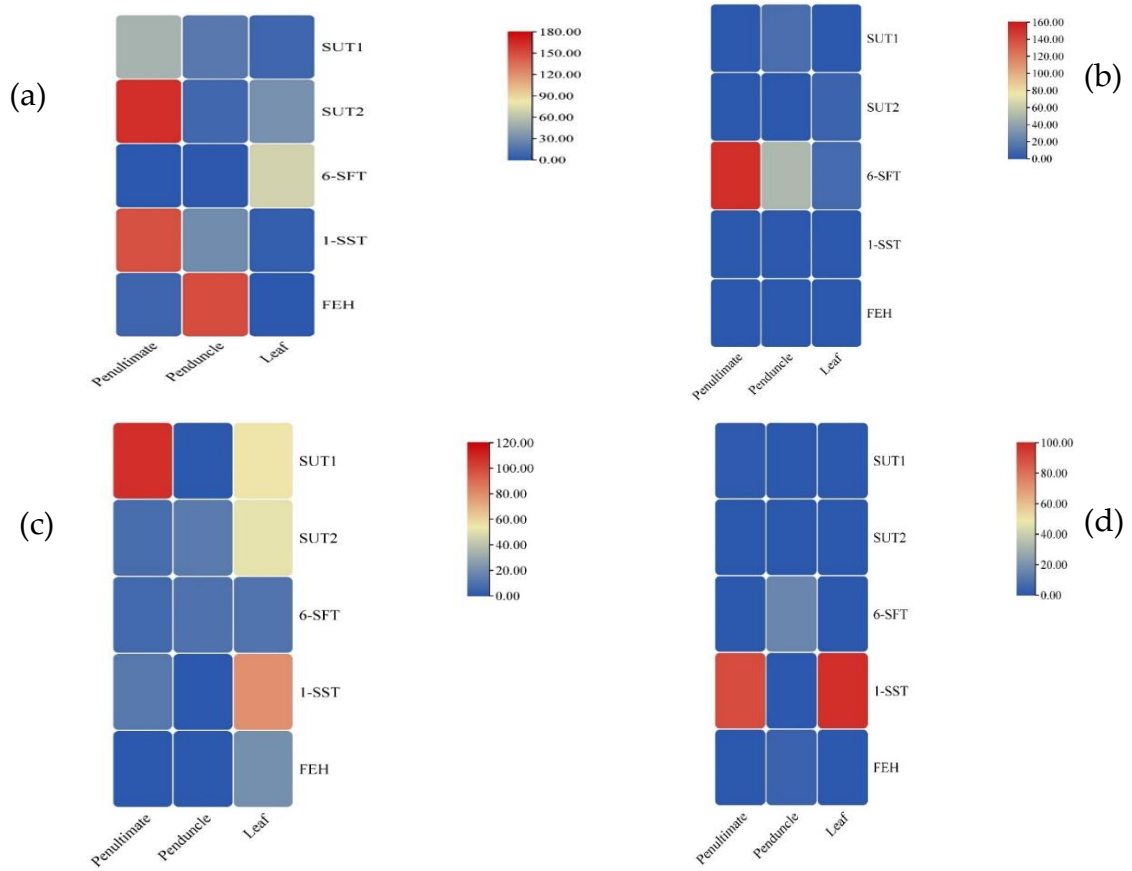


Figure 1. Fructan biosynthesis enzymes in genotype 1 (a), 2 (b), 3(c), and 4 (d) in three tissues under water deficit conditions.

The expression of *6-SFT* gene was significantly increased in the peduncle tissue. An increased expression of *1-SST* gene was observed in the penultimate tissue of stem and leaf. *1-FEH* gene expression increased in peduncle tissue.

Correlations between measured traits and gene expression levels under normal and water deficit conditions

Correlations Between Traits Under Normal Conditions

The analysis of the correlation between fructan metabolism genes and physiological traits under normal conditions revealed that chlorophyll a in the penultimate leaf had a positive and significant correlation with both chlorophyll b in the penultimate leaf and the total chlorophyll content of the penultimate leaves. Additionally, chlorophyll b exhibited a positive and significant correlation with the total chlorophyll content of the penultimate leaves, as well as with chlorophyll b present in the peduncle and leaf, and also with the *6-SFT* gene expressed in the peduncle. Conversely, chlorophyll b displayed both positive and negative correlations with the *SUT1* and the *1-SST* genes in the peduncle. Furthermore, the total chlorophyll content in the penultimate leaves showed a positive and significant correlation with both the total chlorophyll and chlorophyll a content in the leaves; however, it demonstrated a negative and significant correlation with the *SUT1* gene in the peduncle. Chlorophyll a in the peduncle exhibited a positive and significant correlation with the total chlorophyll content of the peduncle and the *6-SFT* gene in the penultimate leaf. Similarly, chlorophyll b in the peduncle showed a positive and significant correlation with the total chlorophyll content of the peduncle as well as with the *6-SFT* and *1-SST* genes in the peduncle and the leaves, specifically in relation to *1-FEH*.

Finally, the total chlorophyll content of the peduncle had a positive and significant correlation with the *6-SFT* gene in the peduncle. The chlorophyll a content in the leaves indicated a positive and significant correlation with the total chlorophyll content of the leaves and yield, while also exhibiting a negative correlation with the *SUT1* and *SUT2* genes in the peduncle, along with the *SUT1* and *SUT2* genes in the penultimate tissues (Figure 2a).

Chlorophyll b exhibited a positive and significant correlation with both leaf chlorophyll content and peduncle *6-SFT*. Conversely, chlorophyll b demonstrated a negative correlation with peduncle *SUT1* and peduncle *1-SST*. Penultimate *SUT1* revealed a positive and significant correlation with both *SUT1* and *SUT2*. Likewise, penultimate *SUT2* displayed a positive and significant correlation with peduncle *SUT2*, leaf *SUT1*, and leaf *6-SFT*. Furthermore, penultimate *1-SST* was positively and significantly correlated with both penultimate *FEH* and leaf *1-SST*. Penultimate *1-FEH* illustrated a positive and significant correlation with peduncle *1-FEH*. Peduncle *SUT1* was positively correlated with peduncle *1-SST*, while showing a negative correlation with peduncle *6-SFT*. Peduncle *SUT2* demonstrated a positive and significant correlation with leaf *SUT1*, *SUT2*, and *6-SFT*. Leaf *SUT1* showed a positive and significant correlation with leaf *SUT2* and *6-SFT*. Additionally, a positive and significant correlation was found between leaf *SUT2* and leaf *6-SFT*. The expression of the *1-SST* gene in leaves was positively and significantly correlated with leaf *1-FEH* (Figure 2a).

Correlations between traits at grain filling stage under water deficit

Correlations among various traits during the grain filling stage under water deficit conditions were analyzed. In particular, the relationship between physiological traits, yield, and the expression of fructan metabolism genes in response to stress demonstrated that penultimate chlorophyll exhibited a positive and significant correlation with total penultimate chlorophyll, peduncle chlorophyll a, chlorophyll a, total leaf chlorophyll, and yield. Conversely, chlorophyll a showed a negative and significant correlation with the *SUT2* gene in the peduncle, as well as with *SUT2* and *6-SFT* genes in the leaf. Additionally, penultimate chlorophyll b exhibited a positive and significant correlation with total penultimate chlorophyll, chlorophyll a and b, the total chlorophyll content in the peduncle, and the total chlorophyll content in the leaf. However, penultimate chlorophyll b also demonstrated a negative and significant correlation with *SUT1* in the peduncle. The total chlorophyll content in the penultimate stage exhibited a positive and significant correlation with chlorophyll a and the

total chlorophyll content in both the peduncle and leaf, including chlorophyll a and b.

Furthermore, chlorophyll a in the peduncle showed a positive and significant correlation with chlorophyll b and the total chlorophyll content in the peduncle. On the negative front, the chlorophyll of the peduncle showed a significant negative correlation with *SUT1* in the peduncle. Chlorophyll b in the peduncle was positively and significantly correlated with both *SUT1* and *1-SST* genes. Notably, the penultimate *SUT1* gene displayed a positive and significant correlation with *SUT2* in the peduncle.

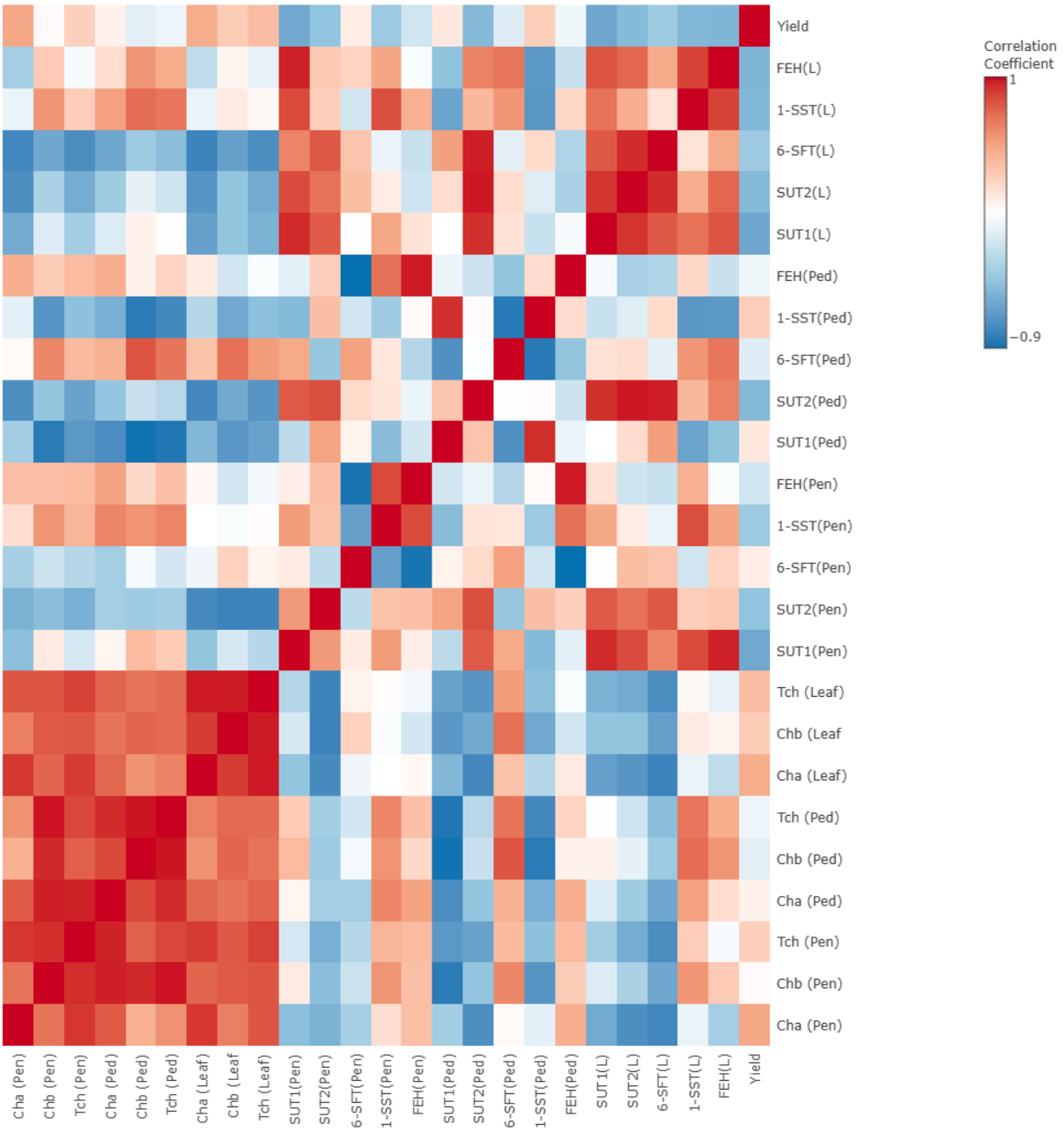


Figure 2. The correlation coefficients of physiological traits, yield, and gene expression under normal conditions.

The *SUT2* in the peduncle also revealed a positive and significant correlation with leaf *SUT1*, *SUT2* as well as leaf *1-SST* and *1-FEH*. A significant positive correlation was found between penultimate *SUT2* and peduncle *SUT2*. The penultimate *1-SST* gene offered a positive and significant correlation with both penultimate *1-FEH* and leaves *1-SST*. Furthermore, penultimate *1-FEH* exhibited a positive and significant correlation with peduncle *1-FEH*. A positive and significant correlation was observed between peduncle *1-SST* and peduncle-*1-SST*, while peduncle *SUT1* was negatively and significantly correlated with peduncle-*6-SFT*. Additionally, peduncle *SUT2* displayed a positive and significant correlation with leaf *SUT2*, *SUT1*, and *6-SFT*. In the leaves, *SUT1* was positively and significantly correlated with both leaf *SUT2* and *6-SFT*. Significant positive correlations were also noted between leaf *SUT2* and leaf *6-SFT*, as well as between leaf *1-SST* and leaf *1-FEH* (Figure 3).

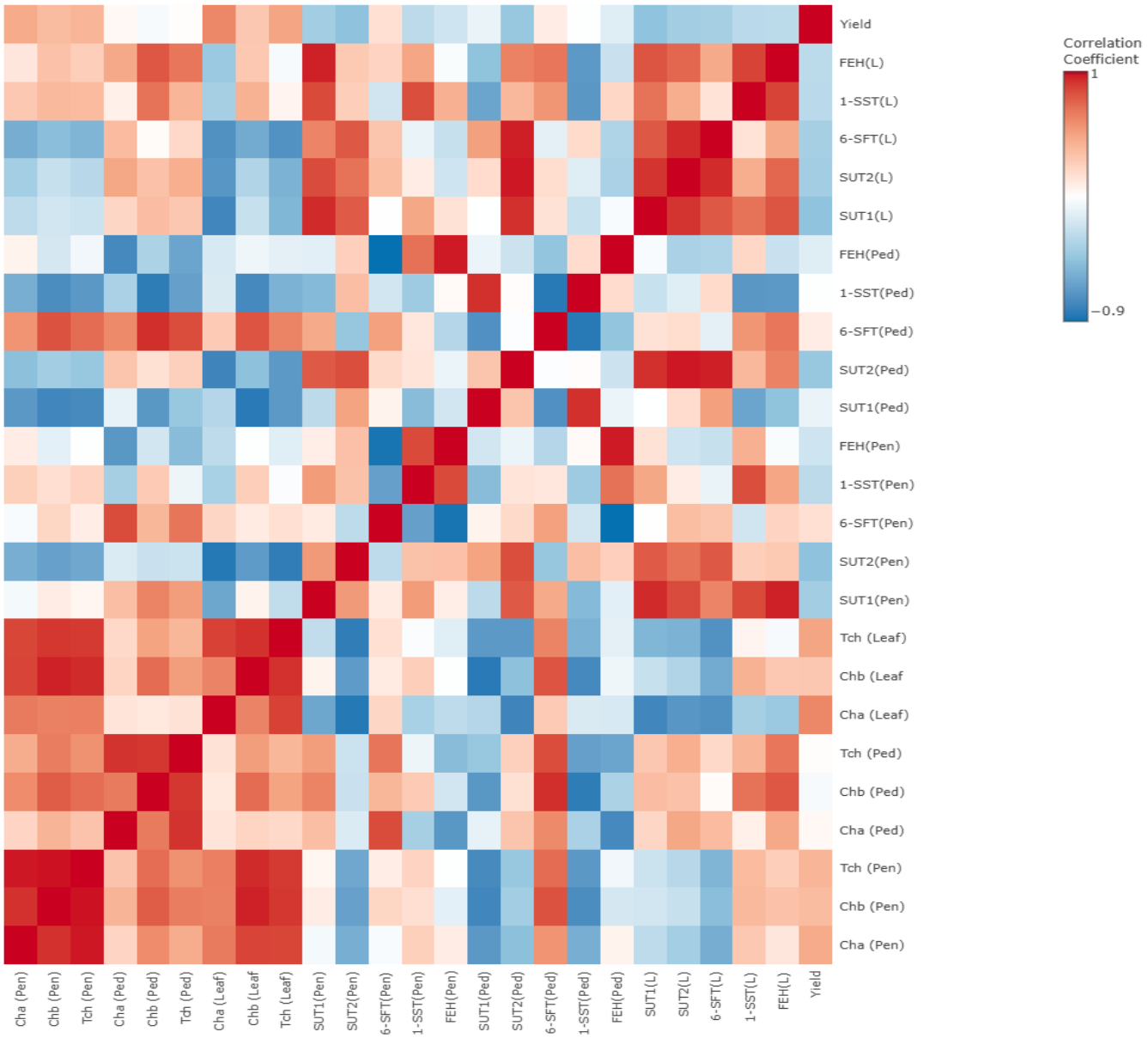


Figure 3. The correlation coefficients of physiological traits, yield, and gene expression, under drought stresses.

Discussion

Drought during the grain-filling phase significantly affects grain development and overall productivity. Breeders aim to select cultivars that possess a high capacity for nitrogen and carbon remobilization, along with genotypes that exhibit strong photosynthetic abilities (Rauf et al., 2016). Additionally, researchers must utilized the genetic foundations of yield potential (grain yield), physiological efficiency (chlorophyll content), and drought tolerance mechanisms to create cultivars capable of withstanding various stresses (Akbari et al., 2024; Hajibarat et al., 2024). Effective breeding strategies, incorporating molecular markers, should be employed to select genotypes that exhibit drought tolerance. In conditions of drought stress, stem storage serves as a crucial source for grain filling. However, the molecular processes related to the remobilization of reserves from stressed stems remain inadequately studied. Current knowledge indicates that under stress, plants draw upon water-soluble carbohydrates (WSC) from remobilized stem reserves, as well as carbon and nitrogen derived from the breakdown of leaf proteins, to support grain filling (Joudi, 2017). This process of sucrose mobilization from the stem to the wheat grain is particularly crucial for maximizing yield in conditions of terminal drought (Joudi, 2017). *1-SST* catalyzes the most important enzyme for fructan synthesis. The activity of this enzyme is related to the concentration of sucrose, which *1-SST* is affected by the activity of sucrose synthase in the stem. Fructans demonstrate a dual functionality under drought stress in barley: they contribute to osmotic adjustment, enhancing drought tolerance, and simultaneously serve as the primary polysaccharide storage in the stem, providing resources for grain filling. Previous stydy has indicated that the penultimate internode of both barley and wheat stems is the most efficient region for assimilate remobilization, with fructan and sucrose being the primary compounds involved (Zhang et al., 2015). This study further revealed that in stressed plants, genotypes 1 and 3 exhibit higher expression levels of genes crucial for fructan metabolism as compared to genotypes 2 and 4 within their internodes.

Based on the results, the higher expression of fructan mechanism enzyme under drought stress implied that genotypes 1 and 3 probably utilized the osmolytes like fructan for the osmotic adjustment and the stem reserve remobilization as compared to genotypes 2 and 4. These findings indicate that fructans may serve a dual function during drought stress, contributing both to osmotic adjustment and providing protective benefits, while also acting as the primary storage of polysaccharides in the barley stem for utilization during grain filling.The activity of *1-SST* enzymes related to the synthesis and remobilization of stems WSC showed that this enzyme has varing responces to the time and amount of soil moisture. *1-SST* activity in plant stems with normal irrigation decreased from 6 to 15 days after anthesis and then showed a high positive correlation with fructan concentration. Our findings align with previous studies that demonstrated a positive correlation between the expression levels of *1-SST* and *6-SFT* and stem WSC/fructan content. These earlier reports suggested a link between genetic variations in fructan accumulation and the expression levels of *1-SST* and *6-SFT* (Chen et al., 2024). Our findings showd that a significantly positive correlation was obtained between grain yield and chlorophyll content in both the leaves and the penultimate of stems under drought stress conditions. The activity of enzymes involved in the fructose mobilization pathway was investigated in several barley cultivars (Nosrat, Jonob, Nimrozo and Turkmen) under drought stress (Pureisa et al., 2019). The cultivars differed for WSC-related traits, reaching its maximum amount at 10 days after anthesis. The results revealed that sucrose activity: sucrose 1-fructosyltransferase (*1-SST*) increased WSCc in these cultivars. The change in fructan-1-exohydrolase (*1-FEH*) activity on the 15th day after anthesis was accompanied by a change in stem reserve remobilization. Cultivars with continued photosynthesis and stem reserve remobilization efficiency have higher yield stability under drought stress conditions. In this study, it was observed that the expression levels of key genes associated with fructan metabolism were higher in the internodes of tolerant genotypes than those of susceptible genotypes, regardless of whether the plants were well-watered or under stress. Drought stress notably enhanced the abundance of these transcripts in tolerant genotypes, whereas no such increase was seen in the susceptible genotypes. It

was found that wheat cultivars exhibiting drought tolerance showed a rise in fructan exohydrolase (*1-FEH*) activity, which contributed to greater resistance to drought by facilitating the remobilization of larger quantities of fructan from the stem (Yang et al., 2020). Genotypes 1 and 3 displayed increased expression levels of the *1-FEH* gene, whereas genotypes 2 and 4 showed no significant variations in *1-FEH* gene expression. Additionally, the remobilization of fructan in drought-resistant barley cultivars began earlier compared to that in susceptible varieties (Khodaeiaminjan and Bergougnoux, 2021).

Conclusion

Terminal drought stress significantly reduces grain yield, but tolerant barley genotypes exhibit resilience due to their ability to store and mobilize fructans, a type of carbohydrate. Under drought stress, tolerant genotypes increase genes related to fructan metabolism, ensuring a consistent supply of carbohydrates from stored resources, and mitigating yield loss compared to susceptible genotypes. Genes associated with the synthesis and remobilization of fructans are significantly expressed in the penultimate internode of drought-tolerant barley, and their expression levels rapidly increase when exposed to drought conditions. This reaction leads to a greater accumulation of water-soluble carbohydrates (WSC) and speeds up their remobilization, which serves as an essential energy source for the plant. There was a positive significant correlation between grain yield and chlorophyll content, suggesting relationships among yield and physiological traits in response to drought stress. A strong relationship has been observed between the expression of genes related to fructan biosynthesis (*1-SST* and *6-SFT*) and the levels of fructan content, as well as between the expression of genes related to fructan remobilization (*1-FEH* and *SUT1*) in stress-tolerant cultivars during grain filling under drought conditions. These results indicated that the increased accumulation and subsequent remobilization of fructans are crucial for sustaining yield stability in drought-tolerant barley genotypes facing limited photosynthesis. There was a positive correlation between chlorophyll content (a/b, total) of penultimate with grain yield, indicating the key role of penultimate tissues under termnial drought stress conditions. The differences in fructan accumulation and remobilization mechanisms among various barley cultivars provide opportunities for breeding programs to enhance these traits, ultimately leading to the development of drought-resistant varieties.

Supplementary Materials

No supplementary material is available for this article.

Author Contributions

Conceptualization, Z.H. and A.S.; methodology, A.S.; software, Z.H.; validation, Z.H. and A.S.; formal analysis, A.S.; investigation, Z.H.; resources, Z.H.; data curation, A.S.; writing-original draft preparation, Z.H.; writing-review and editing, A.S.; visualization, Z.H.; supervision, A.S.; project administration, Z.H.; funding acquisition, A.S. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest Statement

The authors declare no conflict of interest.

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مقاله پژوهشی

ارزیابی آنزیم‌های متابولیسم فروکتان در ژنوتیپ‌های متضاد جو (*Hordeum vulgare*) تحت تنش خشکی

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چکیده: حفظ عملکرد دانه در شرایط تنش خشکی انتهایی، با کمک انتقال مجدد ذخایر کربوهیدرات ساقه غلات ممکن می‌شود. این مطالعه به بررسی چگونگی انتقال مجدد فروکتان‌های ساقه بر عملکرد جو در شرایط خشکی پرداخته است. در مطالعه حاضر، تجزیه و تحلیل مقایسه‌ای فیزیولوژیکی و بیان ژن با استفاده از بافت برگ پرچم، پنالتیمت و پدانکل در چهار ژنوتیپ جو (G1، G2، G3، G4) انجام شد. سطوح بیان ژن‌های متابولیسم فروکتان در بافت‌های برگ، پنالتیمت و پدانکل ساقه در مرحله پر شدن دانه و در دو شرایط تنش خشکی و نرمال مقایسه شدند. شایان توجه است که تنوع ژنتیکی میان ارقام بر بیان ژن‌های مرتبط با پاسخ به تحمل خشکی، اثرگذار است.به طور خاص، ژنوتیپ‌های ۱ و ۳ در پاسخ به تنش خشکی، افزایش بیان ژن‌های متابولیسم فروکتان را نشان دادند، در حالی که ژنوتیپ‌های ۲ و ۴ کاهش بیان را بروز دادند. یافته‌های ما نشان داد که افزایش تجمع فروکتان و انتقال مجدد آن در طول دوره پرشدن دانه ممکن است به طور قابل‌توجهی به پایداری عملکرد ژنوتیپ‌های ۱ و ۳ در شرایط تنش خشکی کمک کند. ژنوتیپ‌های جو که توانایی بالایی در تولید فروکتان و بازتخصیص آن در واکنش به تنش خشکی انتهایی دارند، می‌توانند به‌عنوان منابعی ارزشمند در برنامه‌های به‌نژادی جو برای شرایط تنش خشکی مورد استفاده قرار گیرند.

کلمات کلیدی: جو، تحمل به خشکی، متابولیسم فروکتان، بیان ژن، پر کردن دانه.

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Response of durum wheat (*Triticum durum*) genotypes to cadmium stress: Growth and oxidative stress indicators at seedling stage

Original paper

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Abstract: In this study, the response of 17 durum wheat genotypes to cadmium stress was assessed using an aeroponic growth system at the seedling stage. The following parameters were evaluated: glutathione peroxidase (GPX), root dry weight (RDW), malondialdehyde (MDA), cadmium of root (CR), leaf dry weight (LDW), catalase (CAT), cadmium of leaf (CL), ascorbate peroxidase (APX), chlorophyll (Chl) based on SPAD index, seedling dry weight (SDW), electrical conductivity (EC), and glutathione S-transferase (GST). Biplot analysis revealed that the first two principal components explained 64% of the observed variabilities. The pentagon plot highlighted that genotype G15 under cadmium stress exhibited the highest values for GST, GPX, MDA, and CAT; while under non-cadmium stress, it showed high APX activity and superior RDW, LDW, Chl, SDW, and EC values. G2 under cadmium stress was notable for its high CR and CL levels. Regarding seedling dry weight performance, G2 and G15 under non-cadmium stress, followed by G14 and G16, demonstrated the highest results. APX emerged as the most discriminative trait, followed by GST, GPX, CAT, and EC, as determined by the treatment-by-trait biplot. Under cadmium-stress , genotypes G6, G8, G9, G11, and G12 were identified as superior for seedling dry weight.

Keywords: Antioxidant enzymes, biplot, genotype-trait interaction, seedling dry weight.

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Introduction

Heavy metal contamination in soil poses a critical challenge to ecosystems and living organisms, as rapid industrialization and urbanization have led to a dramatic increase in soil heavy metal concentrations (Li et al., 2019) .Also, they adversely affecting plant growth, mineral balance, metabolic functions, and agricultural productivity. Among heavy metals, cadmium is particularly concerning, ranked among the top ten most toxic heavy metals, is commonly found in the Earth's crust alongside zinc, lead, and copper (Irfan et al., 2021). Key sources of cadmium contamination include excessive use of fertilizers and pesticides, mining, metallurgy, and electroplating activities (Genchi et al., 2020). When crops grow in cadmium-contaminated soils, the metal's high mobility facilitates its absorption and accumulation in various plant organs.

Cadmium poses serious risks to both plants and animals and is considered a significant environmental pollutant.Once inside the plant, cadmium inhibits growth, disrupts mineral nutrition, and impairs photosynthesis. Seed germination, a vital phase in a plant's life cycle, is particularly vulnerable to cadmium toxicity while high soil cadmium levels decrease germination rates and impede seedling growth (Shankar, 2020). Cadmium-induced toxicity triggers a cascade of physiological, biochemical, morphological, and molecular disruptions in plants (Vijiyakumar and Prince, 2024).

Cadmium, a non-essential element without any biological effects in plants, is highly mobile in soil. This mobility allows it to be readily obtained by roots and transported to aerial parts of the plant (Cai et al., 2023). Cadmium's interference in intracellular processes leads to an increase in reactive oxygen species within plant cells (Adil et al., 2020). These molecules are vital for cellular integrity; for instance, lipids play a central role in membrane structure and metabolic regulation (Li et al., 2020). Oxidation of membrane lipids compromises membrane permeability and generates toxic byproducts (Wang et al., 2017). Even at low concentrations, triggering metabolic pathways that further damage cellular components, such as increased endonuclease activity and cytochrome c-oxidase release (Alshehri, 2024). A prominent effect of cadmium toxicity is the generation of oxidative stress resulting in the production of reactive oxygen species in various species, which prompts the induction of defense systems. This system works collectively to neutralize reactive oxygen species and mitigate damage (Dumanović et al., 2021). Non-enzymatic metabolites also contribute by regulating osmotic balance and enhancing reactive oxygen species scavenging (Zandi and Schnug, 2022). Previous researches highlight the role of antioxidant enzyme activity in plant resistance to cadmium stress (Gutiérrez-Martínez et al., 2020; Pan et al., 2021). However, most investigations on mustard plants have focused on later seedling stages, with limited research addressing germination and early seedling growth stages.

Cadmium is naturally released into the environment through volcanic eruptions, weathering, erosion, and river transport. However, human activities contribute significantly to its release. Mining, non-ferrous metal smelting and refining, fossil fuel combustion, municipal waste incineration, production of orthophosphates, and recycling process of cadmium-contained materials are major sources (Singh et al., 2024). Drainage from historic mining activities further contributes to contamination. While high soil cadmium levels are typically localized to areas near mining sites, lower to moderate magnitudes of cadmium are widespread in agricultural soils. This is largely due to the use of cadmium-contaminated fertilizers, poor-quality biowaste, and peri-urban atmospheric deposition. Although cadmium levels in these soils may not directly affect plant growth or soil fertility, the risk lies in its potential transfer to humans via food and water. Recognizing cadmium's toxicity and prevalence, some researchers identify it as the one of critical pollutant martials in farming soils, emphasizing its implications for food safety and human health (Suhani et al., 2021). Addressing cadmium pollution is essential to ensure sustainable agricultural practices and protect food security. This investigation provides comprehensive grasp into the morphological, and biochemical changes induced by cadmium toxicity during germination and early seedling development across some durum wheat genotypes.

Materials and Methods

Trial and traits

Sterile seeds from 17 durum wheat cultivars (from ICARDA, Table 1) using 0.1% SDS, stirred for 20 min, and thoroughly rinsed with deionized water were obtained and their germination was done on filter papers in darkness at 25°C for two days in the laboratory of University of Maragheh. Seedlings were then transferred to an aeroponic system and cultivated as a factorial arangment in a completely randomized design with three replicates Growth conditions in the chamber included 16:8 hours light: darkness, temperature of 25°C, 65% relative humidity, and a light intensity of 6000 lux provided by yellow and white fluorescent light sources. Seven days post-germination, plants were grown with half-strength Hoagland's protocol, which was replaced with a full-strength solution after 14 days. At the 4- to 5-leaf stage, cadmium was induced by treating the seedlings with 250 mM cadmium chloride. The seedlings were maintained under stress circumstances for 14 days. Samples were collected to evaluate several parameters including biomass measurements, leaf dry weight (LDW), root dry weight (RDW), and seedling dry weight (SDW), were taken, along with chlorophyll content (Chl) and electrical conductivity (EC). Chlorophyll was measured via Minolta SPAD-502-PLUS digital chlorophyll meter (Minolta Co., Jeapan). Cadmium concentrations in roots (CR) and leaves (CL) were determined using atomic absorption spectrometry following the procedure of (Prkić et al., 2013). Additional samples were gathered from mature, well-developed leaves, then frozen in liquid nitrogen for measuring of the next physiologic indices. These included malondialdehyde (MDA), glutathione peroxidase (GPX), catalase (CAT), glutathione S-transferase (GST) and ascorbate peroxidase (APX), which were analyzed using protocols described by (Hossain et al., 2013).

Statistical analysis

The data for all measured traits were first subjected to a normality test using the Anderson-Darling method in Minitab 22.0 (Minitab, Pennsylvania, USA). This approach evaluates the strength of alignment between the dataset and a normal distribution. Subsequently, Pearson's correlations were analyzed by calculating simple correlation coefficients with IBM-SPSS 25.0 (IBM-SPSS, Chicago, USA).

Table 1. The code and pedigree of durum wheat genotypes used.

Code	Pedigree
G1	Saji
G2	45558
G3	2Gdr
G4	Green-14
G5	1-Mna/3Marb
G6	45632
G7	75MEXICALI
G8	8081A-Mar۱۴
G9	Bisu-1//Chen
G10	Gromtel-1
G11	45667
G12	Ajaia-12/F3
G13	45704
G14	45868
G15	Mrf1/Stj2//Bcrch1
G16	Rascon-37/2*Tarro-
G17	Hydranassa30/Silver-

To further explore the relationships among traits and identification of those most significantly influencing yield performance, a genotype × trait interaction analysis was conducted. This analysis utilized a biplot model within a principal component analysis framework, based on the formula via GGEbiplot software:

$$\frac{g_{ij} - t_j}{SD_j} = \sum_{n=1}^{2} \lambda_n \alpha_{in} \beta_{jn} + \theta_{ij}$$

In the model, g_{ij} represents the observed performance of genotype i for trait j, t_j is the average performance across all g_{ij} for trait j, and SD_j is the square root of variance g_{ij} for every j. The eigenvalue corresponding to the principal component n which is denoted as lambda n, while alpha in and beta jn signify the contributions of genotype i and trait j to the principal component n, respectively. The residual variance is captured by theta ij, representing unexplained variation in the model. To ensure symmetric scaling of genotypes and traits,

the eigenvalues were normalized by adjusting vector magnitudes. This allowed for a standard presentation format and facilitated the visual interpretation of relationships between genotypes, traits, and their interactions. The dataset was transformed using a scaling factor of 1, while centering was performed with a setting of 2 to standardize the values. Singular value decomposition with a value of 2 was used to calculate singular values. The singular value partitioning parameter was set to 1, providing an optimal graphical representation of the genotype.

associations through the perfect genotype display. The abiotic tolerance index Mousavi et al. (2008) as one effective index of tolerance evaluation was calculated via formula:

$$ATI = [(Xp - Xs)/[(\bar{X}p/\bar{Y}Xs)][\sqrt{(XpXs)}]$$

where, Xp is the performance of non-stress circumstance; Xp is the performance of stress circumstance; $\bar{X}p$, is the mean of Xp; and $\bar{X}s$, is the mean of Xs. This index is identified as one of the effective stress tolerance indices for detection of the most tolerant genotypes in durum wheat based on Afrooz et al. (2021) and (Sedghiyeh et al., 2025)

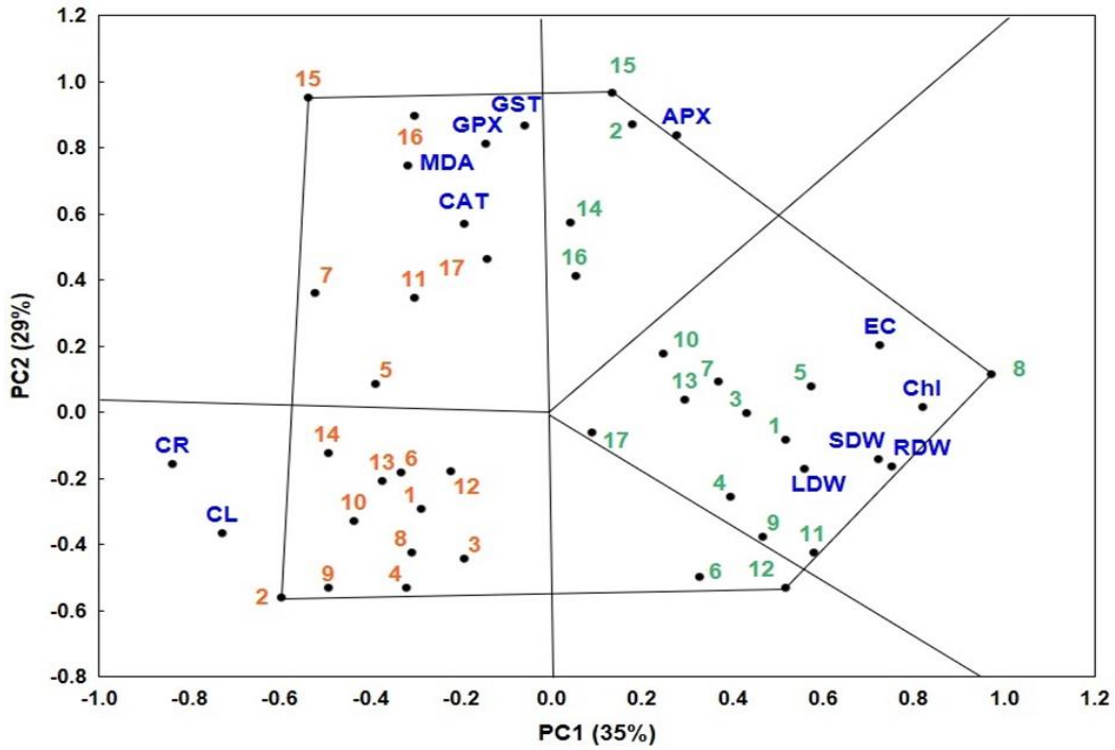


Figure 1. The pentagon of fitted model which indicated the situation of measured traits across two conditions: non-cadmium stress (green color genotypes) versus cadmium stress (orange color genotypes). Traits are: root dry weight (RDW), leaf dry weight (LDW), cadmium of root (CR), cadmium of leaf (CL), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), glutathione S-transferase (GST), malondialdehyde (MDA), chlorophyll (Chl), seedling dry weight (SDW), and electrical conductivity (EC).

otypes. Four of these genotypes exhibited superior performance in specific traits: G15 under cadmium stress showed elevated levels of glutathione peroxidase (GPX), malondialdehyde (MDA), glutathione S-transferase (GST), and catalase (CAT); G15 under non-cadmium stress excelled in ascorbate peroxidase (APX) activity; genotype 15 under non-cadmium stress also demonstrated high values for root and leaf dry weight (RDW and LDW), chlorophyll (Chl), seedling dry weight (SDW), and electrical conductivity (EC); and G2 under cadmium stress had the highest levels of cadmium accumulation in roots (CR) and leaves (CL). In contrast, G12 under non-cadmium stress did not show notable performance in any of the evaluated traits.

Results

The first two principal components of the biplot model accounted for 35% and 29% of the observed variation, respectively, capturing a combined 64% of the total variance in the wheat datasets. This proportion indicates a robust representation of the relationships among traits. Figure 1 demonstrates the utility of the biplot model in visualizing genotype performance across traits, identifying superior genotypes for specific characteristics. These insights are valuable for selecting candidates for breeding programs or commercial cultivation. A pentagon-shaped plot was observed, with perpendicular lines drawn to its edges to facilitate comparisons among the vertex genotypes. Four of these genotypes exhibited superior performance in specific traits: G15 under cadmium stress showed elevated levels of glutathione peroxidase (GPX), malondialdehyde (MDA), glutathione S-transferase (GST), and catalase (CAT); G15 under non-cadmium stress excelled in ascorbate peroxidase (APX) activity; genotype 15 under non-cadmium stress also demonstrated high values for root and leaf dry weight (RDW and LDW), chlorophyll (Chl), seedling dry weight (SDW), and electrical conductivity (EC); and G2 under cadmium stress had the highest levels of cadmium accumulation in roots (CR) and leaves (CL). In contrast, G12 under non-cadmium stress did not show notable performance in any of the evaluated traits.Also, from Fig. 1, it can be grasped that the genotypes of section G15 under non-cadmium stress (2, 14, and 16) had high amounts of APX while the genotypes of section G15 under cadmium stress (5, 7, 11, 16, and 17) had high magnitudes of some biochemical components related to antioxidants like GST, GPX, MDA, and CAT. Thus, the rection of these genotypes especially G15 and G16 was higher under stress conditions, so these genotypes are relatively tolerant genotypes to cadmium stress. For selecting of the most tolerant genotypes, the abiotic tolerance index (ATI), was used for RDW, LDW, and SDW based on Mousavi et al. (2008), and ranking of genotypes regarding this index indicated that G14 and G17 following to G4, G10, G15, and G16 were detected as the most tolerant genotypes to cadmium stress (Table 2).

Table 2. The amount and rank of the abiotic tolerance index (ATI) for some traits.

Genotypes	ATI values			Ranks			Mean
	RDW	LDW	SDW	RDW	LDW	SDW	Rank
G1	0.02189	0.00149	0.00473	8	12	2	7.3
G2	0.01112	0.00643	0.00047	13	5	9	9.0
G3	0.06477	0.00547	0.00010	2	6	16	8.0
G4	0.06190	-0.00443	0.00022	4	17	15	12.0
G5	0.06332	0.00731	0.00101	3	3	6	4.0
G6	0.02078	0.00531	0.00023	10	7	14	10.3
G7	0.01545	0.00690	0.00155	12	4	5	7.0
G8	0.07745	0.06573	0.00582	1	1	1	1.0
G9	0.04408	0.00272	0.00042	6	10	11	9.0
G10	0.01592	-0.00311	0.00061	11	16	8	11.7
G11	0.03490	0.01919	0.00267	7	2	3	4.0
G12	0.02109	0.00014	0.00201	9	13	4	8.7
G13	0.05689	-0.00242	0.00067	5	15	7	9.0
G14	-0.00025	-0.00185	0.00044	15	14	10	13.0
G15	0.00463	0.00223	0.00033	14	11	13	12.7
G16	-0.01541	0.00350	0.00037	16	9	12	12.3
G17	-0.02210	0.00516	0.00004	17	8	17	14.0

Traits are: root dry weight (RDW), leaf dry weight (LDW), and seedling dry weight (SDW).

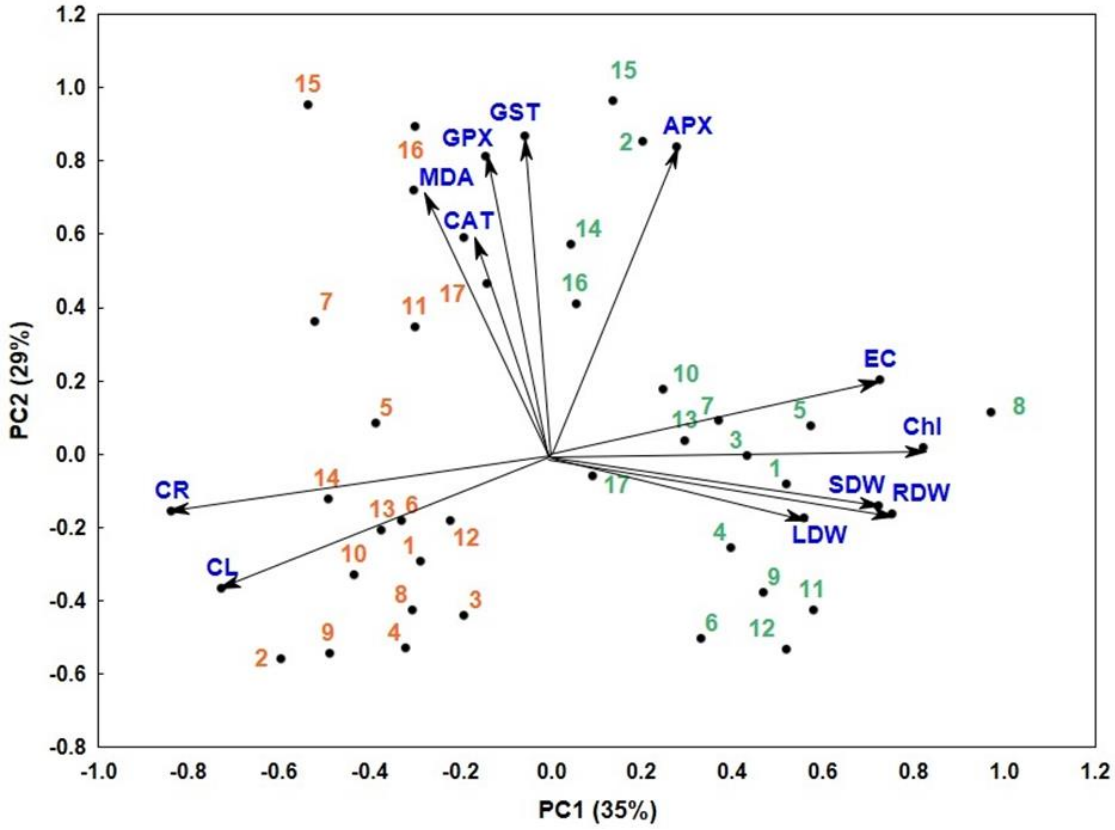


Figure 2. The vector display of fitted model which indicated the association among of measured traits across two conditions: non-cadmium stress (green color genotypes) versus cadmium stress (orange color genotypes).Traits are: root dry weight (RDW), leaf dry weight (LDW), cadmium of root (CR), cadmium of leaf (CL), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), glutathione S-transferase (GST), malondialdehyde (MDA), chlorophyll (Chl), seedling dry weight (SDW), and electrical conductivity (EC).

Comparison these results with pentagon of biplot verified the potential of G15 and G16 as the tolerant genotypes with acceptable dry yield of roots, leaves and seedlings followed by G14 and G17. Thus, these genotypes could be advised for cultivation in cadmium contaminated soils and used in future breeding projects.

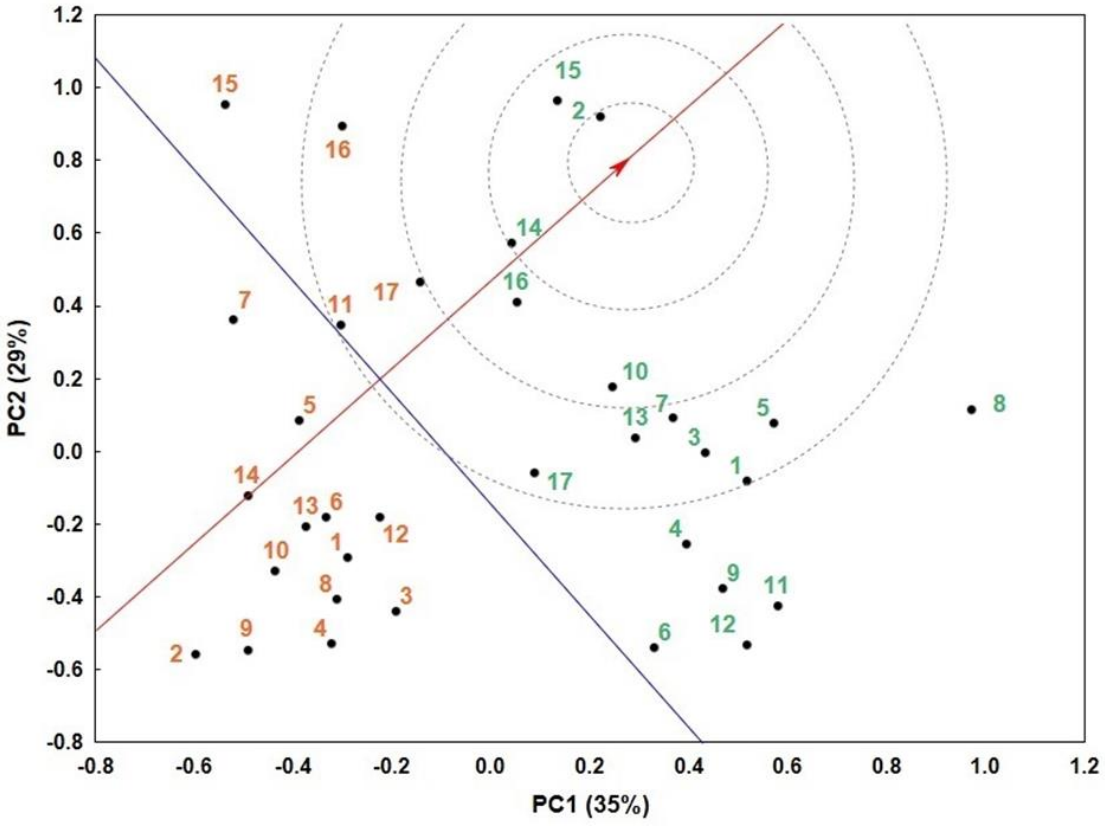
In this study, the fitted model effectively captured and predicted genotype-by-trait associations. The biplot model accounted for a substantial proportion of variation, with trait correlations revealed by the cosine of the vectors. Figure 2 illustrates significant relationships, including: (i) positive correlations between GST, GPX, MDA, and CAT, as indicated by a cosine of 0° (+1); (ii) positive correlations between RDW, LDW, Chl, SDW, and EC, suggested by close angles; (iii) a positive correlation between CR and CL, with a cosine of 0° (+1); (iv) near-zero correlations between GST, GPX, MDA, and CAT with RDW, LDW, Chl, SDW, and EC, as well as with CR and CL, indicated. by a cosine of 90° (0); and (v) Negative correlations between RDW, LDW, Chl, SDW, and EC with CR and CL, demonstrated by a cosine of 180° (-1). Finally, the predictions of the fitted model align well with the numerical associations (Table 3), while there are some inconsistencies, related to the issue that the model explains for 64% of the variation rather than total. Figure 3 shows the position of the ideal genotype, with the genotypes closest to this position considered the best performers. G2 and G15 under non-cadmium stress, followed by G14 and G16 under the same conditions, were positioned nearest to the ideal genotype, indicating their superior performance.

Table 3. Pearson's correlation coefficients among measured traits of durum wheat based on 17 data.

	RDW	LDW	CR	CL	CAT	APX	GPX	GST	MDA	Chl	SDW
LDW	0.48†										
CR	-0.48	-0.22									
CL	-0.41	-0.22	0.67								
CAT	-0.01	-0.03	-0.02	-0.10							
APX	0.11	-0.05	-0.38	-0.49	0.52						
GPX	-0.29	-0.18	-0.20	-0.27	0.44	0.66					
GST	-0.06	-0.10	-0.06	-0.35	0.48	0.77	0.75				
MDA	-0.16	0.01	0.14	-0.10	0.46	0.53	0.59	0.86			
Chl	0.57	0.22	-0.73	-0.61	0.03	0.26	-0.03	0.02	-0.20		
SDW	0.55	0.63	-0.41	-0.31	0.03	0.09	-0.24	-0.05	-0.07	0.50	
EC	0.35	0.34	-0.71	-0.59	0.02	0.25	0.19	0.14	0.10	0.47	0.37

The significant levels are 0.48 and 0.60 in 5 and 1 % level, respectively. Traits are: root dry weight (RDW), leaf dry weight (LDW), cadmium of root (CR), cadmium of leaf (CL), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), glutathione S-transferase (GST), malondialdehyde (MDA), chlorophyll (Chl), seedling dry weight (SDW), and electrical conductivity (EC).

Figure 3. The position of perfect genotype across two conditions: non-cadmium stress (green color genotypes) versus cadmium stress (orange color genotypes).



In contrast, G2 and G9 under cadmium stress were positioned farthest from the ideal genotype, making them less desirable. Most of the wheat genotypes under cadmium stress were located below the average axis, indicating less desirable performance, while a few genotypes (G11, G15, G16, and G17) were positioned above the average axis. However, it appears that the perfect genotype's ability to distinguish traits is limited under stress conditions. Therefore, it is recommended to evaluate genotypes under normal conditions to fully reveal their potential for agronomic traits. Identifying these "ideal" genotypes is essential for wheat genetic improvement programs, especially considering that leaf yield performance and yield components often show low correlations in many field crops.

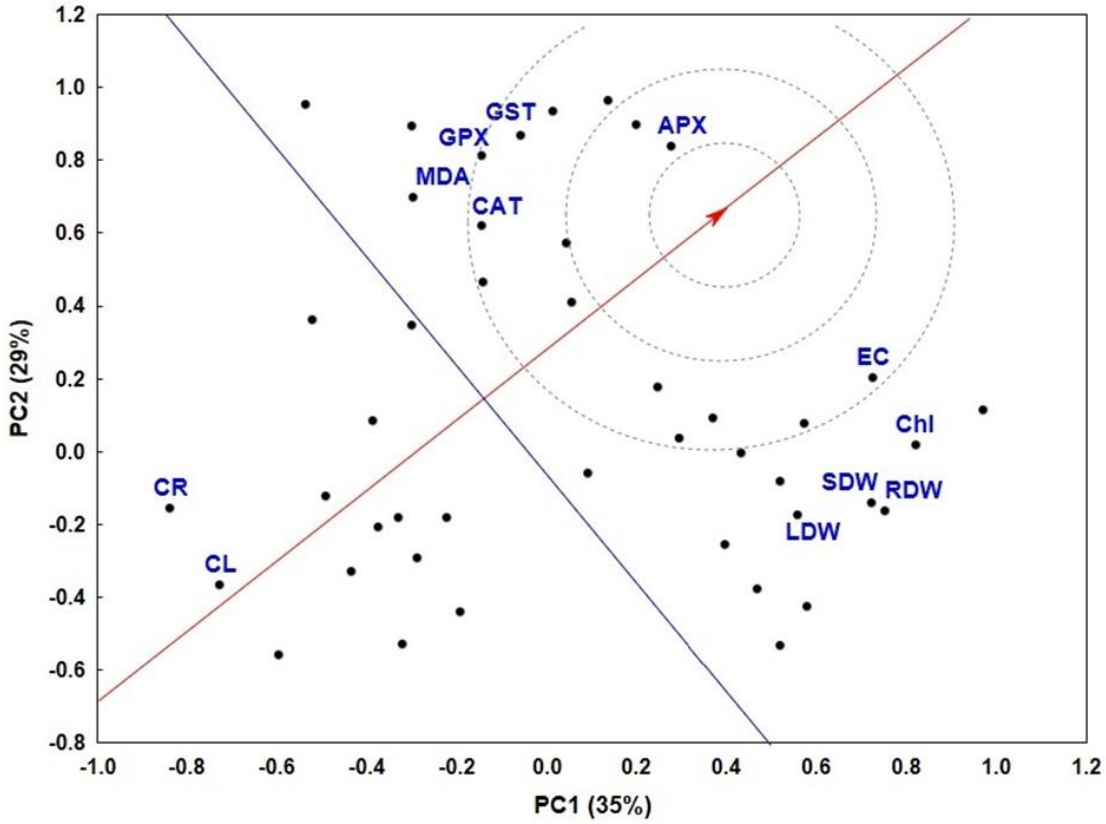


Figure 4. The position of perfect trait across two conditions: non-cadmium stress (green color genotypes) versus cadmium stress (orange color genotypes). Traits are: root dry weight (RDW), leaf dry weight (LDW), cadmium of root (CR), cadmium of leaf (CL), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), glutathione S-transferase (GST), malondialdehyde (MDA), chlorophyll (Chl), seedling dry weight (SDW), and electrical conductivity (EC).

Genotypes that performed above average for SDW are located above the average axis. The distance of each genotype from the horizontal axis represents the standard deviation, with smaller distances indicating lower variability, which makes them more desirable for selection. Consequently, G8, G9, and G11 under non-cadmium stress were recommended due to their relatively low variability compared to G6 and G12. In contrast, genotypes such as 2 and 9 under cadmium stress, which are farther from the horizontal axis, exhibit higher variability and are considered less desirable (Fig. 5). Evaluating wheat genotypes is essential for breeding under stress conditions and effectively managing genetic improvement tasks.

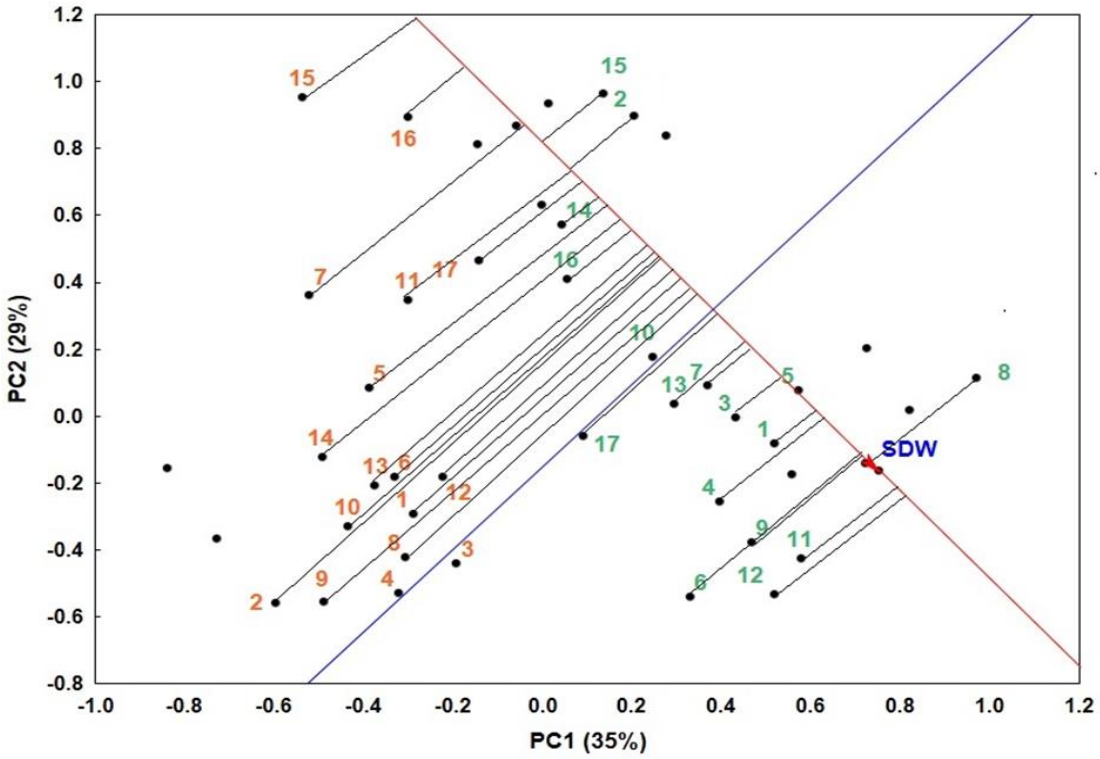


Figure 5. The examining of seeding dry weight (SDW) across two conditions: non-cadmium stress (green color genotypes) versus cadmium stress (orange color genotypes).

Discussion

The fitted biplot model accounted for 64% of the observed variation, which indicates a robust representation of the relationships among traits. The benefits of the fitted biplot model observed in this study align with findings in other crops, such as corn Santana et al. (2021) and safflower Ebrahimi et al. (2023), supporting its effectiveness in generating actionable insights for crop improvement. Cadmium stress impacts plants by accumulating in leaves, which reduces stomatal conductance and impedes carbon dioxide uptake in photosynthetic cells. This deficiency limits the production of critical metabolites required for cellular growth.

The reaction of genotypes G15 and G16 was higher under stress conditions, so these genotypes are relatively tolerant genotypes to cadmium stress. Cadmium disrupts respiration, decreasing energy production and carbon skeleton availability for essential biomolecules. It also inhibits the stabilization of nitrogen and its decrease in cells, restricting the changes of α-ketoglutarate into amino acids (Xue et al., 2023). These disruptions result in delayed and insufficient production of vital cellular components, ultimately leading to reduced the dry weight of shoots in even the more tolerant genotypes under insufficient production of vital cellular components, leading to reduced dry weight of shoots in even the more tolerant genotypes under stress. The results of the abiotic tolerance index (ATI), relatively verified the above-mentioned results whereas comparing them with the pentagon of the biplot verified the potential of genotypes G15 and G16 as the tolerant genotypes with an acceptable dry yield of roots, leaves, and seedlings. Thus, these genotypes could be advised for cultivation in cadmium-contaminated soils as well as using in future breeding projects.

To investigate the relationships among traits, vectors were drawn from the origin to each trait, which helped to clarify the interactions between them. The model revealed both additive and crossover interactions, suggesting that the ranking of genotypes based on the measured traits could change. This finding aligns with Sabaghnia et al. (2015), who highlighted the complexity of selecting genotypes without considering genotype-by-trait

interactions.. These results are consistent with Li et al. (2022), which reported a positive association between root, shoot, and leaf dry weights in wheat, and a negative association between cadmium content in roots and seedlings with yield traits. In general, the biplot model provided a clear visual representation of trait relationships in wheat, in line with findings by Chen et al. (2021), who observed positive correlations between leaf and root dry weights with seedling weight in some wheat genotypes. Although, the biplot method prepares a visual overview of trait associations based on the overall structure of the dataset, the correlation coefficients assess direct interrelationships among traits and may not always match the biplot outcomes. Cadmium disrupts the plant's water balance, leading to dry weight loss and a reduction in calcium absorption, which impairs growth and decreases biomass. The cadmium accumulation in the leaves remarkably increased under cadmium stress, which agrees with the findings of (Sabella et al., 2022). Cadmium is highly mobile in soil, making it easily absorbed by wheat roots, and its accumulation in the roots can be transferred to the leaves. This study showed that cadmium levels were significantly elevated in both the roots and shoots. Genotypes G8, G9, and G11 under non-cadmium stress were recommended due to their relatively low variability compared to genotypes G6 and G12. The effectiveness of this approach for identifying favorable genotypes for target traits has been demonstrated in studies on sweet corn Stansluos et al. (2023) and safflower (Ebrahimi et al., 2023). This study identified substantial variation among wheat genotypes, which is consistent with previous research reporting significant diversity in wheat (Qu et al., 2020; Saleh et al., 2020). The genotype × trait interaction biplot model revealed a relatively high level of variation and confirmed the presence of both simple and complex interactions among the genotypes and traits. Similar patterns of interaction have been observed in other crops, such as durum wheat Kendal (2019) and barley (Karahan and Akgün, 2020). These interactions resulted in varying rankings of genotypes across traits, making it challenging to identify the most superior genotypes. Ignoring these interaction effects complicates the selection process due to the genotype × trait interaction's influence on genetic factors. Despite reduced Calvin cycle activity and decreased dry matter production, durum wheat genotypes exhibited a unique ability to isolate and store cadmium in vacuoles. The high generation of reactive oxygen species did not necessarily cause to a corresponding increase in antioxidant enzyme activity. Bouchama et al. (2023) found that the addition of cadmium to the environment increased guaiacol peroxidase activity in the leaves of Phragmites australis. This enzyme plays a crucial role in managing oxidative stresses due to high cadmium amounts. The enhanced activity of oxidative enzymes helped mitigate the negative effects of cadmium, reducing lipid peroxidation, and allowing cells to maintain their metabolic activity.

Conclusion

The genotype × trait interaction biplot model explained 64% of the variation and confirmed that cadmium stress negatively impacts agronomic traits, inducing oxidative stress in wheat. G8, G9, and G11, followed by G6 and G12, exhibited the most desirable seedling dry weight performance under stress. The current investigation provided a valuable grasp for genetic improvement projects for enhancing wheat performance under cadmium stress conditions. Breeders can use these findings to prioritize effective traits in their genetic improvement programs, with the ultimate goal of increasing wheat yield.

Supplementary Materials

No supplementary material is available for this article.

Author Contributions

Conceptualization, A.P.; methodology, S.M.; software, N.SX.; validation, A.P., and N.S.; formal analysis, A.P.; investigation, S.M.; resources, A.P.; data curation, N.S.; writing—original draft preparation, N.S.; writing—review and editing, A.P.; visualization, N.S.; supervision, A.P.; project administration, A.P. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest Statement

The author declares no conflict of interest.

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اثرات تنش کادمیوم بر رشد و شاخص‌های تنش اکسیداتیو در گیاهچه‌های برخی از ژنوتیپ‌های گندم دوروم

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چکیده: در این مطالعه با استفاده از سامانه رشد هواکشت، پاسخ گیاهچه‌های ۱۷ ژنوتیپ گندم دوروم به تنش کادمیوم ارزیابی گردید. پارامترهای گلوتاتیون پراکسیداز (GPX)، وزن خشک ریشه (RDW)، مالون دی آلدئید (MDA)، کادمیوم ریشه (CR)، وزن خشک برگ (LDW)، کاتالاز (CAT)، کادمیوم برگ (CL)، آسکوربات پراکسیداز (APX)، کلروفیل (Chl) بر اساس شاخص SPAD، وزن خشک گیاهچه (SDW)، هدایت الکتریکی (EC) و گلوتاتیون S-transferase (GST) مورد ارزیابی قرار گرفتند. تجزیه و تحلیل بای پلات نشان داد که دو مؤلفه اصلی اول ۶۴ درصد از متغیرهای شناسایی شده را به خود اختصاص دادند. از نمودار پنج ضلعی می توان استنباط نمود ژنوتیپ G15 تحت تنش کادمیوم دارای بالاترین مقادیر GST، GPX، MDA و CAT بود در حالیکه این ژنوتیپ تحت تنش غیر کادمیوم، فعالیت بالای APX و مقادیر بالای RDW، LDW، Chl، SDW و EC را بخود اختصاص داد. G2 تحت تنش کادمیوم به دلیل سطوح بالای CR و CL قابل توجه بود. از نظر عملکرد وزن خشک گیاهچه، ژنوتیپ‌های G2 و G15 تحت تنش غیرکادمیمی و پس از آن ژنوتیپ‌های G14 و G16 بهترین نتایج را نشان دادند. APX به عنوان مهمترین صفت متمایزکننده شناسایی شد و پس از آن صفات GST، GPX، CAT و EC دارای این ویژگی بودند که توسط بای‌پلات ژنوتیپ در صفت تعیین شدند. در بین ژنوتیپ‌های بدون تنش کادمیوم، ژنوتیپ‌های G6، G8، G9، G11 و G12 به عنوان بهترین ژنوتیپ برای وزن خشک گیاهچه شناخته شدند.

کلمات کلیدی: آنزیم‌های آنتی‌اکسیدانی، تجزیه بای‌پلات، برهمکنش ژنوتیپ و صفت، وزن خشک گیاهچه.

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KTI and BBI protease inhibitors in seed storage protein of Iranian soybean cultivars

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Abstract: Kunitz (KTI) and Bowman-Birk (BBI) protease inhibitors are considered the most significant factors that decrease the quality of soybean proteins. In this study, the levels of these proteins were investigated using the 2D-PAGE, and their activity was evaluated through trypsin and chymotrypsin proteases in ten Iranian soybean cultivars. These results indicated that the Katol cultivar has the lowest concentration of both KTI and BBI proteins and the lowest trypsin and chymotrypsin inhibitory levels. Therefore, this cultivar is an ideal choice for soybean protein-based diets and could also serve as a valuable parent in breeding programs aimed at improving protein quality in soybean . Moreover, no significant correlation was found between KTI with BBI proteins, nor between these proteins with protease activity. Therefore, it seems that genetic control targeting either KTI or BBI proteins alone may not be an effective approach to improving the quality of soybean proteins. Additionally, no correlation was found between KTI and BBI proteins with agronomic traits. This also suggests that reducing protease inhibitors in soybean proteins does not adversely affect overall soybean performance.

Keywords: Soybean, kunitz protein, bowman-birk protein, trypsin inhibitor, chymotrypsin inhibitor, agronomic traits.

Introduction

Among oilseeds, soybean with 40% protein and 20% oil, is considered a strategic plant for oil and protein production. Soybean is the top oil producer globally and its seed protein competes with other sources of animal protein. Currently, Brazil leads in production with 169 million tons, followed by the United States with 118 million tons, and Argentina with 49 million tons. Iran ranks 34th in the world with an estimated 25,000 hectares, of soybean cultivation area and an annual production of 53,000 tons (USDA, 2024). The nutritional values of soybeans play a prominent role in human and livestock nutrition. Soy protein is increasingly consumed by humans and it also makes a relatively inexpensive protein source for livestock (Medic et al., 2014). Nevertheless, raw soybeans contain several antinutritional factors such as protease inhibitors, lipoxygenase, tannins, and phytates that could potentially impact the body's metabolism of consumers (Yasothai, 2016; Luthria et al., 2018). Among them, protease inhibitors such as trypsin and chymotrypsin inhibitors are typically found in soybean meal at very high levels (Natarajan et al., 2016; Park et al., 2023; Varga et al., 2023; Vorster et al., 2023; Kim et al., 2024). These antinutritional factors regulate the consumer's endogenous proteases and can interfere with protein digestion resulting in a negative impact on the utilization of soybean-based protein products (Varga et al., 2023). The inhibitors have been shown to induce pancreatic enzymes, hypersecretion, and a rapid stimulation of pancreas growth, which is histologically described as pancreatic hypertrophy and hyperplasia (Palacios et al., 2004). Several protease inhibitors have been identified in soybean storage proteins. Much of their activity is thought to be attributed to Kunitz (KTI) and Bowman-Birk (BBI) proteins, which represent the majority of the bioactive proteins that strongly inhibit trypsin and trypsin-chymotrypsin respectively (Gillman et al., 2015; Mittal et al., 2021; Kim et al., 2024).

The KTI is a monomeric and non-glycosylated protein encoded by a single gene in cotyledon. In contrast, the BBI is controlled by three genes and has two independent sites of inhibition, one at Lys 16-Ser 17 against trypsin and the other at Leu 43-Ser 44 against chymotrypsin (Livingstone et al., 2007; Mittal et al., 2021). It has been suggested that the levels of KTI and BBI vary among different genotypes (Pesic et al., 2007; Gu et al., 2010; Natarajan et al., 2016; Mittal et al., 2021).

A more comprehensive understanding of the variability of protease inhibitors among different soybean varieties could enhance current efforts to improve seed protein quality. However, there is limited information available regarding the relationship between genotype-specific seed levels of KTI and BBI proteins, particularly BBI, and their impact on trypsin and chymotrypsin inhibitory. Pesic et al. (2007) evaluated 12 commercial soybean cultivars and found no relationship between KTI and BBI proteins and trypsin activity. However, Zilic et al. (2011) observed a negative correlation between KTI and trypsin activity. Further evaluation of KTI and BBI protease inhibitors and activities, as well as exploring possible correlations among them could be useful for enhancing the health impacts of soybean proteins.

Unfortunately, given the importance of protease inhibitor activity in soy protein quality, complete information about the protein quality of different Iranian soybean cultivars is unavailable. Therefore, this study aimed to investigate the relationship between the level of KTI and BBI protease inhibitors in seed protein with trypsin and chymotrypsin inhibitory, as well as the most important agronomic characteristics in 10 soybean cultivars currently cultivated in Iran. Awareness of the relationship between protease inhibitors and these characteristics could enhance our knowledge of how to improve new soybean cultivars with superior quantitative and qualitative traits.

Materials and Methods

Plant material

The 10 adopted Iranian soybean cultivars: Hill, Sahar, Nekador, Caspian, Katol, Sari, Telar, Tapor, Arian, and Parto were provided by the Mazandaran Agriculture Research Center and planted in the experimental field of the Genetic and Agricultural Biotechnology Institute of Tabarestan (GABIT). The names of current Iranian soybean cultivars, breeding pedigree, year of introduction, and their grope maturity are presented in Table 1. Field experiments were conducted using a randomized

complete block design with three replications. Each plot consisted of five rows, with a distance of 50 cm between rows and 10 cm between plants. After the plants reached full maturity and any marginal effects were eliminated, ten plants were randomly chosen from each plot and assessed for key agronomic characteristics including plant height, number of branches, 100-grain weight, and seed yield.

2D-PAGE analyses

The seeds from each cultivar were peeled and finely powdered using a mortar and pestle in the presence of liquid nitrogen. 10 mg of each sample was washed with TCA/acetone 10% at -20 °C for 1 hour. The precipitated pellet was dried under vacuum and suspended in Isoelectric Focusing (IEF) buffer (7M urea, 2M Thiourea, 4% CHAPS, 1% DTT, and 0.2% ampholyte pH 3.0-10.0 as a carrier) in a ratio of 1:15 (v/w) (Natarajan, 2014; Natarajan et al., 2016). Samples were mixed in a vortex mixer for 30 minutes at room temperature. The insoluble tissue was removed by centrifugation at 14000 g for 15 minutes. Protein concentration was determined according to the Bradford method, with bovine serum albumin (BSA) as the standard (Bradford, 1976). 2D-PAGE was performed according to the instructions in the Bio-Rad IPG Ready Strips manual. A 1000 µg protein sample was loaded onto 24 cm IPG strips pH: 4.0-7.0 and rehydrated for 1 hour. The strips were then covered with mineral oil and allowed to rehydrate overnight.

The first dimension (isoelectric focusing) was performed with the following program: 50 V for 20 min, 200 V for 1 h, 500 V for 1 h, 1000 V for 1 h, 4000 V for 2 h, 8000 V for 2 h and 9500 V for 2.5 h, using the protean IEF electrophoretic chamber (Bio-Rad). For the second dimension, the IPG strips were initially incubated for 2×15 minutes with 2% DTT to reduce sulfhydryl groups, followed by alkylation with 2.5% Idoacetamide in 50 mM Tris-HCl pH: 8.8, 6M urea, 30% glycerol and 2% SDS. Equilibrated strips were then placed onto 12% polyacrylamide gels and covered with molten 0.5% Agarose in 0.375M Tris pH: 8.8, 0.192M glycine, 2% SDS and 0.01% bromophenol blue as sample buffer. They were then electrophoresed using Tris/Glycine/SDS running buffer (Bio-Rad PROTEAN Plus Dodeca). The electrophoresed gels were stained in a solution of 0.1% (w/v) Coomassie Brilliant Blue R250, 40% methanol, and 10% acetic acid for 1 hour. Subsequently, they were destined several times using a solution of 40% methanol and 10% acetic acid.

The gels were scanned separately at a resolution of 600 dpi and 16-bit TIFF grayscale pixel depth using the Bio-Rad Calibrated Densitometer GS-800. Image analysis was then conducted using SameSpot Progenesis software (TotalLab, Newcastle, England) which enables spot detection, quantification, and spot matching across multiple gels. The KTI and BBI proteins were identified using the Soybean Proteome Database, SoyProDB (Tavakolan et al., 2013; Natarajan, 2014), as well as TagIdent and ExPASy software tools (SIB Bioinformatics Resource Portal) (Table 2).

Table 1. Characteristics of the current Iranian soybean cultivars.

No.	Line	Cultivar	Breeding method	Growth type	Maturity group	Year of release
1	.....	Hill	Imported	Determinate	4	1961
2	Pershing	Sahar	Imported	Semi-determinate	5	1993
3	BP	Telar	Selection from Pershing	Determinate	5	2001
4	JK	Sari	Imported	Determinate	5	2001
5	032	Nekador	BP×Hood (Gorgan-3)	Determinate	5	2010
6	DPX	Katol	Imported	Semi-determinate	5	2010
7	033	Caspian	BP×Hill	Determinate	5	2011
8	.....	Arian	Imported	Determinate	5	2015
9	032-240-P1	Parto	Mutation from Nekador	Determinate	5	2016
10	2002	Tapoor	Sahar×JK	Determinate	5	2016

Reference: Seed and Plant Certification and Registration Institute (SPCRI)

Table 2. Characterization of protease inhibitor proteins based on soybean proteome data.

No.	Protein name	Accession number	Theoretical pI	Molecular weight (Da)
1	Kunitz trypsin inhibitor (KTI1)	P25272	4.97	22545.94
2	Kunitz trypsin inhibitor (KTI1)	NP_001237705.1	4.85	22446.81
3	Kunitz trypsin inhibitor (KTI1)	NP_001236275.2	5.39	22686.88
4	Kunitz trypsin inhibitor (KTI2)	NP_001237786.1	5.24	22676.01
5	Kunitz trypsin inhibitor (KTI3)	NP_001238611.2	4.95	24076.37
6	Kunitz trypsin inhibitor (KTI3)	NP_001237751.1	5.18	23655.24
7	Bowman-Birk protease inhibitor (BBI)	NP_001238367	5.15	12062.88
8	Bowman-Birk protease inhibitor (BBI)	KAH1233928	6.85	24020.93
9	Bowman-Birk protease inhibitor (BBI)	CAA48657	4.92	11496.28

Trypsin and chymotrypsin inhibition assay

Total protein soluble was extracted using an extraction buffer (100 mM Tris-HCl pH: 8.0 containing 20 mM $CaCl_2$) at a ratio of 1:10 (w/v) of seed powder. The suspension was shaken for 30 minutes and then centrifuged at 15000 rpm for 30 minutes at 4 °C. The clear supernatant was collected and used for the protease inhibitor assay.

The level of protease inhibition was determined by measuring the activity of trypsin (T1426) and chymotrypsin (C3142) protease using specific substrates, BAPNA (N-α-Benzoyl-DL-Arginine p-Nitroanilide) and GPNA (N-Glutaryl-L-Phenylalanine p-Nitroanilide) respectively, from Sigma Aldrich (Livingstone et al., 2007).

Preparations of trypsin and chymotrypsin were made at concentrations of 0.002% and 0.006% respectively, in 1 mM HCl at 4 °C. To prepare the substrate, dissolve 10 mg of BAPNA and 20 mg of GPNA in 200 µL of DMSO then dilute to 100 mL with 100 mM Tris-HCl pH: 8.0 containing 20 mM $CaCl_2$. To measure protease activity, 20 µl of extracted protein was incubated with 300 µl of each protease for 5 minutes. Protease activity was then measured by adding 500 µl of specific substrate and incubating for 20 minutes at 37 °C. The reaction was stopped by adding 100 µl of 30% acetic acid. For the control sample, protease activity was tested with a specific substrate in the presence of a protein extraction buffer. The absorbance of the entire reaction solution was measured at 405 nm against a reagent blank (where the enzyme and extract were replaced with the extraction buffer) using a spectrometer. The inhibition of trypsin and chymotrypsin was calculated using the following equations: Percent inhibitors = 1 – (OD_{Sample} / OD_{Enzyme}) × 100

Statistical analysis

All experimental samples were evaluated with three replications. The field experiment was designed using a randomized complete block design, while the laboratory samples were assessed using a completely randomized design. Quantitative analysis of protein subunits was conducted using the SameSpot Progenesis software program. Data analysis was carried out using the SAS statistical software and the EXCL program. After conducting an analysis of variance test (ANOVA) for independent samples, all means were compared using Duncan's multiple range test at a 5% probability level. Correlation coefficient calculations for traits were determined using Pearson's simple correlation analysis to assess the strength and significance of trait associations.

Results

Agronomic characteristics

The results of the comparison of mean agronomic traits showed that the Katol cultivar had the highest plant height, distinguishing it from the other cultivars (Table 3). Meanwhile, Parto and Sari cultivars had the lowest plant height. The Katol, Hill, and Arian cultivars had the highest number of branches. The highest number of pods was observed in Parto followed by Arian. The highest pod weight was observed in Katol followed by Telar and Arian. The highest 100-grain weight was also obtained in Arian, with the lowest in the Parto cultivar. Finally, the Katol cultivar with the highest plant height, number of branches, pod weight, and

grain weight, and the Telar cultivar with the highest number of seeds and pod weight, had the highest grain yield (Table 3).

Quantification of KTI and BBI proteins

The seed storage protein pattern and protease inhibition subunits are displayed in Figure 1. The results of the quantification of protease inhibitor proteins indicated that the studied cultivars were divided into two groups based on KTI protein content (Figure 2). Cultivars such as Hill, Sahar, Nekador, Caspian, Sari, Telar, Arian, and Parto had the highest content of KTI protein and were placed in the first group, while Katol and Tapor cultivars with the lowest content were placed in the second group. For BBI protein, the studied cultivars were divided into four groups. Sahar ranked first with the highest amount, while Caspian and Katol were ranked last with the lowest amount (Figure 2).

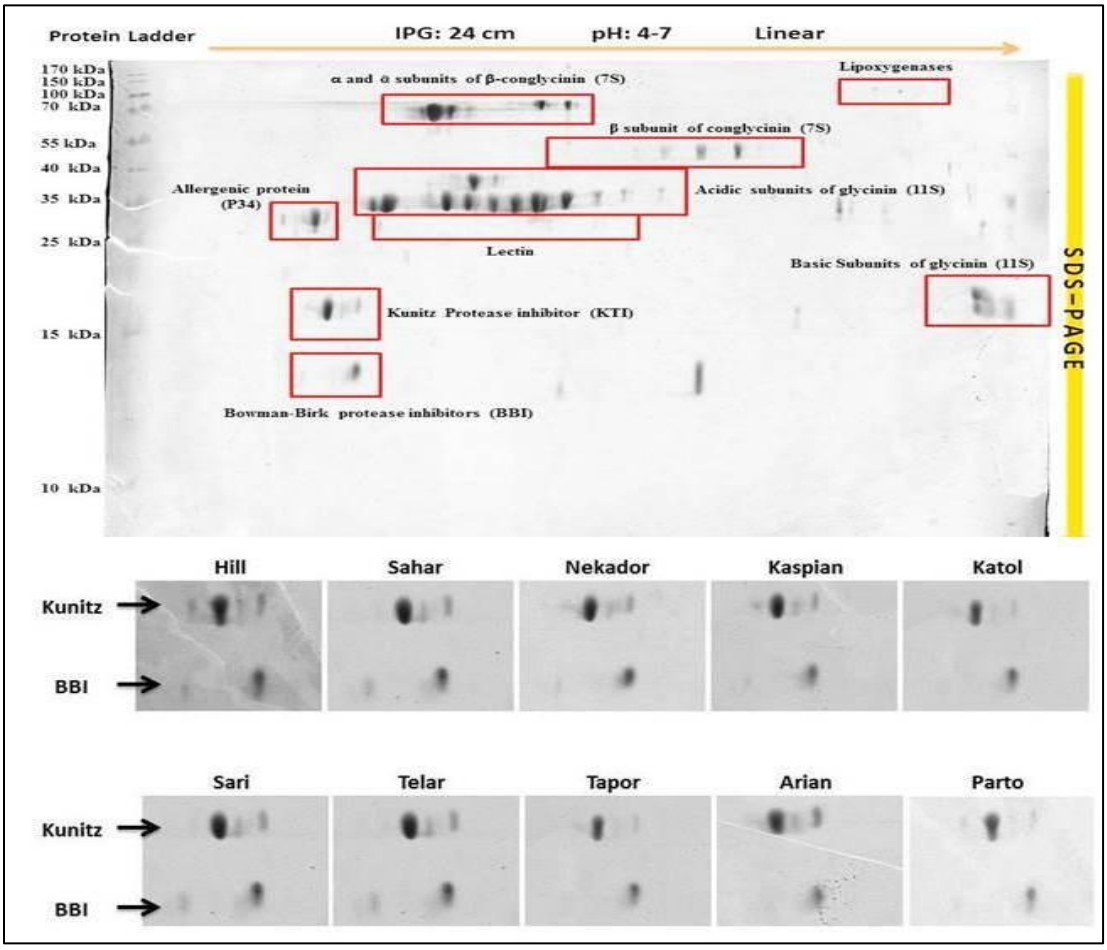


Figure 1. Seed storage proteome pattern of soybean and KTI and BBI proteins position in different Iranian soybean cultivars based on soybean proteome data.

Finally, Hill, Nekader, Sari, Tellar, and Arian cultivars which had the highest levels of KTI protein, and Sahar cultivar which had the highest levels of KTI and BBI proteins, also had the highest levels of protease inhibitors (Figure 2). Conversely, Katol with the lowest levels of KTI and BBI proteins followed by Tapor which has the lowest levels of KTI protein showed the lowest content of protease inhibitors (Figure 2).

Table 3. Mean comparison of agronomic traits of soybean cultivars.

Plant traits	Soybean cultivars									
	Hill	Sahar	Telar	Sari	Nekador	Katol	Caspian	Arian	Parto	Tapour
Plant height (cm)	121.4 cd	130.0 bc	120.6 cd	98.5 e	136.5 b	183.8 a	125.5 cd	123.3 cd	95.0 e	118.5 d
No. of branches	3.7 a	1.7 d	2.8 bc	2.8b c	1.1 d	3.7 a	1.1 d	3.5 ab	2.7 c	1.7 d
No. of pod.	67.2 cd	82.6 a-d	90.3 ab	77.2 b-d	64.3 d	80.7 b-d	62.3 d	88.3 a-c	103.3 a	77.0 b-d
Pods weight (gr)	31.0 c	35.0 bc	42.2 ab	36.3 bc	35.6 bc	46.7 a	32.2 c	38.7 a-c	32.1 c	30.8 c
100 seed weight (gr)	16.0 de	16.2 cd	15.2 de	19.0 b	17.5 c	22.0 a	16.4 cd	19.9 b	12.5 f	14.7 e
Seed yield (gr)	20 cd	22.44 b-d	27.17 ab	26 abc	22.94 b-d	32.22 a	21.22 b-d	24.83 b-d	19.28 d	21.06 b-d

Means in each row with similar letter(s) are not significantly different at 5% probability level, using Duncan's multiple range test

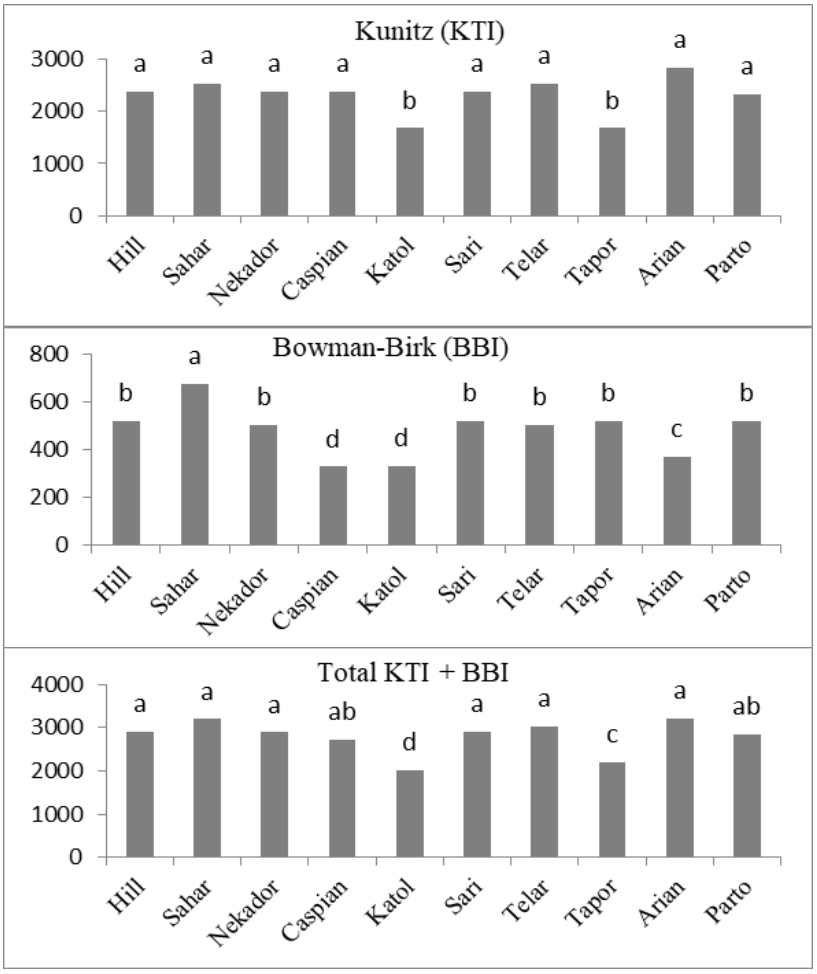


Figure 2. Quantified of Kunitz (KTI) and Bowman-Birk (BBI) protease inhibitors of 10 soybean cultivars by densitometry analyses.

Trypsin and chymotrypsin inhibition assay

The results of the protease inhibitors showed that the studied cultivars were divided into five groups based on trypsin inhibition (Figure 3). Sahar, Caspian, and Tapor cultivars had the highest levels of trypsin inhibition and were placed in the first group, while Katol, Telar, and Arian cultivars with the lowest levels were in the last group. For chymotrypsin inhibition, the Sahar, Caspian, and Tapor cultivars showed the highest inhibition rate, while the Katol was followed by Nekador, Telar, and Arian cultivars with the lowest inhibition rates. In conclusion, the Sahar, Caspian and Tapor cultivars exhibited the highest levels of inhibition for both trypsin and chymotrypsin proteases, while Katol was followed by Nekador, Telar and Arian with the lowest levels of inhibition for both trypsin and chymotrypsin proteases. Finally, Sahar, Caspian, and Tapor cultivars which have high levels of trypsin and chymotrypsin inhibition showed the highest protease inhibition. In contrast, Katol followed by Nekador, Telar, and Arian which have low levels of trypsin and chymotrypsin inhibition showed the lowest protease inhibition (Figure 3).

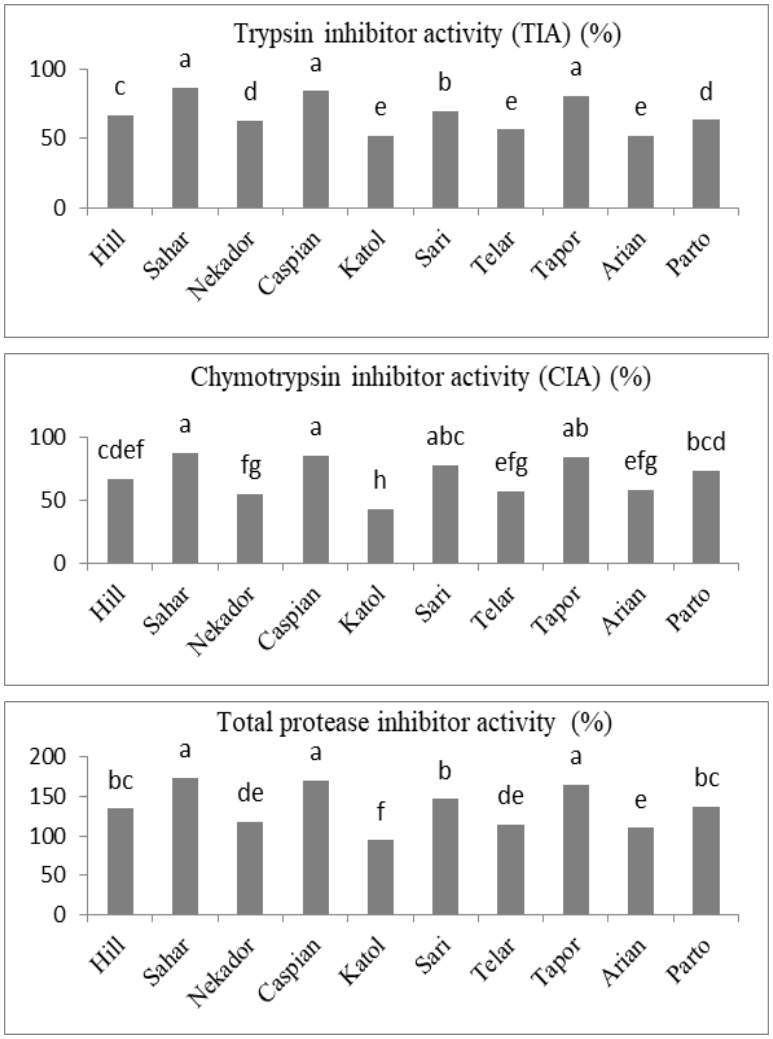


Figure 3. Protease inhibitor activity in the presence of the seed storage protein extracts of 10 soybean cultivars.

Correlation analysis

The relationships between protease inhibitory with protease activity as well as agronomic traits, were studied using correlation coefficient analyses (Table 4). These results demonstrate that the impact of the KTI protein on the level of protease inhibitor is more significant than that of the BBI protein (0.96).

Although no significant association was found between KTI with BBI protease inhibition, a very positive and significant association was observed between trypsin with chymotrypsin activity (0.92). Moreover, KTI and BBI protease inhibitory did not show a significant correlation with vegetative and reproductive traits. However, protease activity had a significant negative correlation with the number of branches, number of pods and grain yield.

Table 4. Correlation coefficients between protease inhibitors and agronomic traits.

Protease inhibitors /agronomic traits		Protease inhibitors			Protease activity		
		KTI	BBI	KTI + BBI	TIA	CIA	TIA + CIA
Protease inhibitors	KTI	1					
	BBI	0.15	1				
	KTI + BBI	0.96**	0.41	1			
Protease activity	TIA	-0.10	0.42	0.02	1		
	CIA	0.05	0.43	0.17	0.92**	1	
	TIA + CIA	0-.01	0.44	0.10	0.97**	0.98**	1
Agronomic traits	Plant height	-0.45	-0.43	-0.53	-0.29	-0.57	-0.45
	Branch	0.01	-0.24	-0.05	-0.70*	-0.57	-0.64
	Number of Pods	0.12	0.18	0.16	-0.38	-0.11	-0.24
	Pod weight	-0.09	-0.38	-0.19	-0.68*	-0.75*	-0.73*
	Seed weight	-0.08	-0.52	-0.22	-0.44	-0.54	-0.50
	Yield	-0.23	-0.40	-0.32	-0.57	-0.65*	-0.63*

* and **: Significant at 1% and 5% probability levels, respectively. KTI; Kunitz protein; BBI; Bowman-Birk protein; TIA; Trypsin inhibitor activity; CTI; Chymotrypsin inhibitor activity

Discussion

In recent years, soybean cultivars have been bred based on maximizing seed yield and oil content. Unfortunately, this strategy has led to limited genetic diversity, which may hinder advancements in enhancing seed yield, protein content, and oil content (Kisha et al., 1998). Soybean meal is increasingly being consumed by humans, and serves as a relatively inexpensive protein source for animal husbandry. Today, improving the quality of storage proteins has become one of the most important goals in soybean breeding programs (Medic et al., 2014). The quality of soybean proteins is limited by the high content of protease inhibitors, which have a negative nutritional impact in both food and feed applications (Natarajan et al., 2016; Vorster et al., 2023; Kim et al., 2024).

Several protease inhibitors have been identified in soybean storage proteins, with most of their activity attributed to Kunitz (KTI) and Bowman-Birk (BBI) proteins (Livingstone et al., 2007; Mittal et al., 2021). These proteins are known to strongly inhibit trypsin and trypsin-chymotrypsin activity respectively (Gillman et al., 2015; Mittal et al., 2021; Kim et al., 2024). These inhibitors can also cause an increase in the secretion of pancreatic enzymes and stimulate the rapid growth of the pancreas, resulting in histologically described pancreatic hypertrophy and hyperplasia (Palacios et al., 2004). Soybean KTI proteins are encoded by three genes: KTI-1, KTI-2, and KTI-3. The KTI-1 and KTI-2 genes are mainly expressed in leaves, roots, and stems, while the KTI-3 gene is specifically expressed in seeds (Krishnan, 2001). Unlike the KTI protein, which is encoded by

a single gene (21 kDa), the BBI proteins are encoded by a family of related genes and are generally expressed in seeds (8-15 kDa) (de Almeida Barros et al., 2012). Protease inhibitors play a defensive role in plants. The KTI-1 and KTI-2 proteins in vegetative tissues provide resistance to pests, while KTI-3 in seeds plays an anti-nutritional role for consumers (Gillman et al., 2015; Sultana et al., 2023; Vorster et al., 2023). The transfer of these defense genes and their expression in the leaves through genetic engineering strategies has enabled many plants to become resistant to a variety of pests (Sultana et al., 2023).

It is possible to inactivate the protease inhibitors through heat treatment or eliminate them by fractionation during food processing. Nonetheless, the BBI protein is heat stable due to the presence of seven disulfide bridges, unlike KTI which only has two disulfide bonds (Friedman and Brandon, 2001; Perez-Maldonado et al., 2003). Moreover, heat treatment reduces the solubility of proteins and availability of amino acid. Unfortunately, this approach is not only costly, but also not completely effective (Luthria et al., 2018). Many studies have reported variations in the content of KTI and BBI proteins among different soybean cultivars (Pesic et al., 2007; Mittal et al., 2020). Cultivars lacking these proteins have also been identified and introduced (Mittal et al., 2021; Dhaliwal et al., 2022). Therefore, identifying and improving new cultivars with low concentration of KTI and BBI proteins will help enhance the nutritional quality of soybean proteins, making it more suitable for consumers (Dhaliwal et al., 2022).

The evaluation of protease inhibitory proteins revealed significant differences in KTI and BBI protein concentrations among the current Iranian cultivars. The results of this study indicated that among the Iranian soybean cultivars the Sahar with the high concentration of both KTI and BBI proteins, exhibits a high level of trypsin and chymotrypsin inhibitory. Additionally, Katol with the low concentration of both KTI and BBI proteins, shows the lowest levels of trypsin and chymotrypsin inhibitory. Therefore, Katol cultivar can be an ideal cultivar for soybean protein-based diets and could also be utilized as a valuable parent in a breeding program to improve the quality of soybean proteins. It has been suggested that the KTI and BBI proteins, especially BBI, possess anticarcinogenic and cancer chemopreventive properties (Kennedy and Szuhaj, 1994; Isanga and Zhang, 2008; Krishnan et al., 2012). Therefore, the Sahar cultivar, which had the highest concentration of BBI and a high percentage of trypsin-chymotrypsin inhibition, may be a suitable source for this purpose. Although Sahar with the high level of KTI and BBI proteins and Katol with the low level of KTI and BBI proteins, characterized by the high and low activity of trypsin and chymotrypsin inhibitory respectively, nevertheless, no significant relationship was found between KTI and BBI proteins with trypsin and chymotrypsin inhibitory. For example, the Tapor cultivar, which had a low content of KTI, exhibited high trypsin inhibitory. In contrast, the Arian cultivar, which had a high content of KTI, showed low trypsin inhibitory. There have been several reports on the relationship between KTI protein with protease activity. For example, Zilic et al. (2011) observed a positive correlation between KTI and trypsin inhibitory. Moreover, Dhaliwal et al. (2022), studied soybean isolines with a null kti allele and reported that these isolines showed significant differences in protease inhibitor activity. However, Pesic et al. (2007) reported no association between KTI with trypsin inhibitory. A study also demonstrated that mutants of KTI-1, KTI-2 and KTI-3 were unable to produce adequate levels of protease activity in soybean protein (Kim et al., 2024). Therefore, there seems to be an interaction between KTI and BBI proteins for protease inhibitors in soybean storage protein. For example, the evaluation of a soybean genotype with frameshift mutations in both the KTI-1 and KTI-3 proteins showed a significant increase in BBI protein levels (Gillman et al., 2015). Whereas, an evaluation of genetic diversity in a wide range of genotypes that exhibited significant differences in trypsin inhibitors, revealed that there was no significant association between KTI and BBI proteins (Mittal et al., 2021). On the other hand, it has been reported that soybean lipoxygenase protein, which is considered a defense protein due to its ability to oxidize unsaturated fatty acids, is also capable of inhibiting the proteases trypsin and chymotrypsin (de Carvalho et al., 1999; Barros et al., 2008). In addition, unlike the Kunitz protein, the BBI protein plays an important role in inhibiting both

trypsin and chymotrypsin proteases (Livingstone et al., 2007; Mittal et al., 2021). Therefore, based on the results of this study and other reports, it appears that genetic control alone of a protease inhibitor protein cannot be an effective approach to increase the quality of soybean proteins.

Based on our results, no correlation was found between KTI and BBI proteins with agronomic traits. However, protease activity showed a negative correlation with pod weight and seed yield. Limited information is available on the relationship between protease inhibitors with agronomic characteristics in soybean. Nevertheless, Dhaliwal et al. (2022), studied soybean isolines with a null kti allele and reported that there were no significant differences in agronomic traits such as days to maturity, plant height, seed weight, protein content and oil content. Livingstone et al. (2007) also demonstrated that reducing the protease inhibitors in soybean seed protein can be achieved without making substantial modifications to seed composition or nutritional quality. Therefore, the lack of correlation between KTI and BBI proteins with agronomic traits, as well as the negative correlation between protease activity with grain yield, indicate that reducing protease inhibitors in soybean proteins has no negative impact on soybean breeding programs. On the other hand, some studies have reported that new modern cultivars of soybean have fewer protease inhibitor proteins compared to the wild and older varieties (Natarajan et al., 2007). This feature contributes to the greater resistance of wild soybean varieties to pests and diseases. For instance, studies have identified several distinct protease inhibitors in wild soybean seeds that are not as prevalent in cultivated varieties (Natarajan et al., 2007). Therefore, in order to improve new soybean cultivars, it is essential to increase the levels of KTI-1 and KTI-2 proteins in vegetative tissues while reducing the presence of KTI-3 and BBI proteins in seed storage proteins.

Conclusion

The results of this study demonstrated that, among the current Iranian soybean cultivars, Sahar with the highest concentration of both KTI and BBI proteins, exhibits a high level of trypsin and chymotrypsin inhibitory. Additionally, Katol which has the lowest concentration of both KTI and BBI proteins, exhibits the lowest levels of trypsin and chymotrypsin inhibitory. Therefore, Katol cultivar can be an ideal cultivar for soybean protein-based diets and could also be utilized as a valuable parent in a breeding program to improve the quality of soybean proteins. Based on correlation analysis there was no significant relationship between KTI and BBI proteins, as well as between these proteins with protease activity, it appears that genetic control of either KTI or BBI proteins alone may not be an effective approach to improving the quality of soybean proteins. Furthermore, the lack of correlation between KTI and BBI proteins with agronomic traits suggests that reducing protease inhibitors in soybean proteins does not have a negative impact on soybean breeding programs.

Supplementary Materials

No supplementary material is available for this article.

Author Contributions

Conceptualization, M. A., N. B. J., G. N. and A. D.; methodology, M. A. and E. G.; software, M. A. and E. G.; validation, M. A. and E. G.; formal analysis, M. A. and E. G.; investigation, M. A. and E. G.; resources, M. A., N. B. J., G. N. and A. D.; data curation, M. A.; writing—original draft preparation, M. A.; writing—review and editing, M. A.; visualization, N. B. J., G. N. and A. D.; supervision, M. A., N. B. J., G. N. and A. D. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest Statement

The authors declare no conflict of interest.

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مقاله پژوهشی

بازدارنده‌های پروتئازی KTI و BBI در پروتئین ذخیره دانه ارقام سویا ایرانی

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چکیده: بازدارنده‌های پروتئازی Kunitz (KTI) و Bowman-Birk (BBI) مهمترین فاکتورهای کاهش دهنده کیفیت پروتئین‌های دانه سویا محسوب می‌شوند. در این مطالعه، سطوح مختلف این پروتئین‌ها با استفاده از تکنیک 2D-PAGE بررسی و فعالیت آن‌ها نیز از طریق پروتئازهای تریپسین و کیموتریپسین در ۱۰ رقم سویای ایرانی ارزیابی شد. این نتایج نشان داد که رقم کتول با کمترین غلظت هر دو پروتئین KTI و BBI و کمترین سطوح بازدارندگی تریپسین و کیموتریپسین از دیگر ارقام متمایز شده بود. بنابراین این رقم می‌تواند یک منبع ایده‌آل برای جیره‌های مبتنی بر پروتئین سویا باشد و همچنین می‌تواند به عنوان یک والد ارزشمند در برنامه‌های اصلاحی برای بهبود کیفیت پروتئین‌های دانه سویا مورد استفاده قرار گیرد. علاوه بر این، هیچ ارتباط معنی‌داری بین پروتئین‌های KTI با BBI و همچنین بین این دو پروتئین با فعالیت پروتئازها یافت نشد. بنابراین، به نظر می‌رسد که کنترل ژنتیکی هر یک از پروتئین‌های KTI یا BBI به تنهایی نمی‌تواند رویکرد مؤثری برای بهبود کیفیت پروتئین‌های دانه سویا باشد. علاوه بر این، هیچ ارتباطی بین پروتئین‌های KTI و BBI با صفات زراعی یافت نشد. این همچنین نشان می‌دهد که کاهش بازدارنده‌های پروتئازی در پروتئین‌های دانه سویا تأثیر منفی در برنامه‌های اصلاحی سویا ندارد.

کلمات کلیدی: سویا، پروتئین KTI، پروتئین Bowman-Birk، بازدارنده تریپسین، بازدارنده کیموتریپسین، صفات زراعی.

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Comparative structural analysis of BRI1 receptor in monocot and dicot plants

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Abstract: Brassinosteroids (BRs) play crucial role in growth and development of plants. The BRI1 receptor is the main receptor for BR hormones, which is a member of the leucine-rich kinase-like receptors. The BRI1 receptor has been reported in multiple plant species, but structural differences between monocots and dicots remain poorly understood. In the present study, bioinformatic analyses of the *BRI* gene sequence were conducted on dicot and monocot plants with existing genomic data. The analysis of genome data from 95 plants species revealed a presence of 141 *BRI1* genes, with 92 being dicot and 49 being monocot. Different evolutionary paths have been identified for *BRI1* genes in monocots and dicots through analysis of the phylogenetic tree. Additionally, our analysis of *BRI1* orthologs based on physicochemical properties indicated a conserved structure among BRI1 proteins, with distinctions noticeable between monocots and dicots. The majority of *BRI1* genes were found to contain a single exon and the average MW and pI values for BRI1 were lower in monocots compared to dicots. However, we predicted a greater number of phosphorylation sites in BRI1 orthologs of monocots. This study offers insights for future research on BRI1 genetic modification.

Keywords: Brassinosteroids; receptor kinase; sequence structure; evolutionary analysis.

Introduction

Brassinosteroids (BRs) are plant hormones that regulate a multitude of physiological processes, including cell elongation, vascular differentiation, reproductive development, and stress responses (Clouse and Sasse, 1998; Zhiponova et al., 2013; Zada et al., 2024). BRs have also been shown to be present in all plant parts and organs, including pollen grains, flower buds, fruits, seeds, vascular cambium, leaves, shoots and roots. BR metabolism has long been recognized as a factor that modifies plant responses to abiotic (cold, drought, salinity, etc.) and biotic (pathogens and disease-causing agents) stresses (Zhang et al., 2009; Albrecht et al., 2012; Belkhadir et al., 2012; Heidari et al., 2021). The initiation of the BR pathway is facilitated by a receptor complex consisting of BRI1, an LRR (Leucine rich repeat) RLK (receptor-like kinase) BRASSINOSTEROID INSENSITIVE 1 (Li and Chory, 1997) and has been identified in most plants. The BRI1 protein consists of a cytoplasmic portion with a juxtamembrane segment (JM), a C-terminal tail (CT), a kinase domain (KD), and a single transmembrane region, as well as an extracellular domain including an N-terminal signal peptide, 25 leucine-rich repeats, and a leucine-zipper motif (Li and Chory, 1997; Oh et al., 2000; Wang et al., 2005). Absence of BRs leads to inhibition of BRI1 kinase activity through direct physical interaction with both its C terminus and BKI1 (bri1 kinase inhibitor 1) (Wang and Chory, 2006).

Initiation of the BR signaling cascade is initiated by the interaction between BRI1 and BAK1, resulting in extensive phosphorylation of both the auto- and trans-phosphorylation sites within the intracellular kinase domains of BAK1 and BRI1. The BR signaling pathway begins with the binding of BRI to BAK, which results in extensive auto- as well as trans-phosphorylation of the intracellular BAK1 and BRI1 kinase domains (Hohmann et al., 2017). Afterward, the activated BRI1 went on to phosphorylate CDG1 (CONSTITUTIVE DIFFERENTIAL GROWTH 1) and the BSKs (BR SIGNALING KINASES), resulting in the subsequent phosphorylation of BSU1, BRI1-SUPPRESSOR 1 (Tang et al., 2008; Kim et al., 2011). Phosphorylated BSU1 deactivates Brassinosteroid insensitive 2 (BIN2), leading to the release of its inhibition on plant-specific transcription factors such as brassinazole resistant 1 (BZR1) and bri1-EMS suppressor 1 (BES1) (Li et al., 2001; He et al., 2002; Wang et al., 2002; Yin et al., 2002; Vert and Chory, 2006). In the end, this culminates in the activation of transcription factors including BES1 (Yin et al., 2002) and BZR1 (Wang et al., 2002), resulting in significant transformations in the expression of many genes downstream (Wang et al., 2002; Chen et al., 2019). Analyzing the multitude of mutant *bri1* alleles in Arabidopsis has enhanced our comprehension of how BRI1 influences plant growth and development (Vert et al., 2005). The analysis of biochemical and structural data suggests that specific *bri1* mutations can influence the interaction between BRI1 and its ligand or the co-receptor BAK1 (He et al., 2000; Wang et al., 2001; Santiago et al., 2013). BR-deficient mutants like *det2* (de-etiolated) and *cpd* (constitutive photomorphogenesis and dwarfism) share similarities in phenotype with dwarfish bri1 mutants (Chory et al., 1991; Szekeres et al., 1996). However, external application of BRs cannot reverse the mutant phenotype for the BRI receptor.

BRI1 has been identified in several plants, but there is limited information comparing the structure of this receptor in monocots and dicots. Understanding the role of BRI1 in both monocots and dicots plants has major implications for improving crop yields and stress tolerance. In this study, the *BRI1* gene sequence was extracted and analyzed from the genome of plants with available information. The results of this study are useful and applicable in future research related to engineering the BR signaling pathway. This study aims to compare the structural and functional roles of BRI1 in monocots and dicots to enhance our understanding of its impact on crop yield and stress tolerance.

Materials and Methods

Identification BRI1 in dicots and monocots

To identify the *BRI1* genes, the BRI1 protein sequences in Arabidopsis (as a model dicot plant) and rice (as a model monocot plant) were extracted from the NCBI website. In the next step, these sequences were run as a query against the genome of plants genome in the BLASTp program of

Ensembl Plants database (Bolser et al., 2017). The sequences of all orthologs that had all three domains including the leucine-rich repeat domain, protein kinase domain, brassinosteroid receptor BRI1, and island domain were collected, based on Pfam results. Besides, exon number and physiochemical properties including isoelectric point (pI), and molecular weight (MW) of identified BRI1 proteins were collected from the Ensembl Plants database.

Phylogenetic analysis

To construct a phylogenetic tree, all BRI1 sequences of monocots and dicots were aligned by the Clustal Omega (Sievers and Higgins, 2018). The aligned sequences were introduced to IQ-TREE web server (Nguyen et al., 2015) to creating phylogenetic tree based on Maximum likelihood (ML) method with 1000 replications, based on the default setting. Finally, tree was visualized using iTOL (Interactive Tree Of Life) (Letunic and Bork, 2019).

Phosphorylation region prediction

To predict the potential phosphorylation site into BRI1 proteins, amino acid sequences of all BRI1 orthologs were introduced to NetPhos 3.1 server (Blom et al., 2004) and sites with high score (potential value ≥ 0.90) were selected.

Promoter element analysis

The promoter sequences including 1500 bp of upstream region of each *BRI1* gene were collected from Ensembl Plants database. The promoter sequences were introduced to PlantCARE database (Lescot et al., 2002) to identify the *cis*-regulatory elements related to light, stress, and hormones response.

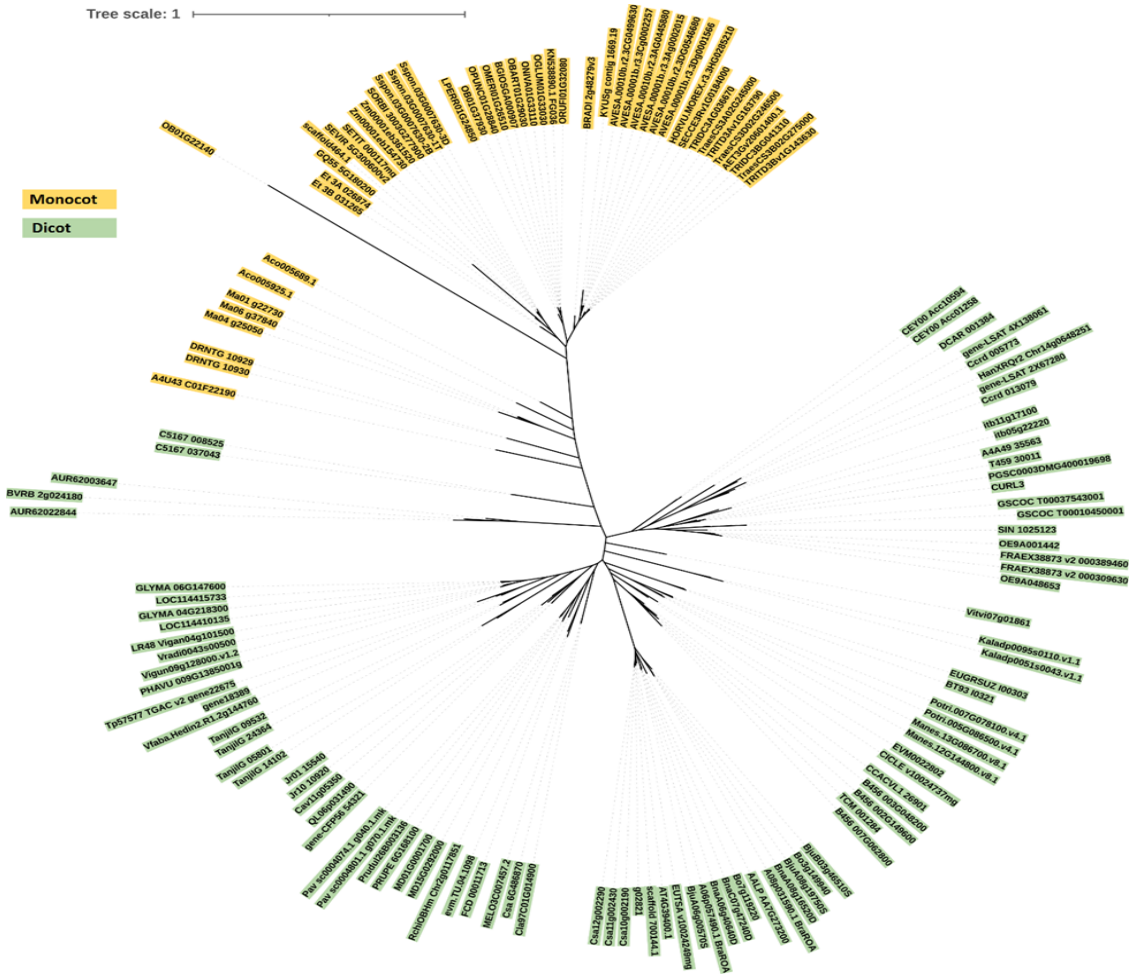


Figure 1. Phylogenetic tree of *BRI1* genes in 95 monocot and dicot plant species.

Results

Identification of BRI1 genes

In this study, the genome of 95 plants was investigated and 141 *BRI1* genes were identified in plants, 92 genes in dicots and 49 genes in monocots (Supplementary Table S1). Most plant species showed to have one *BRI1* gene however two and three copies were detected in some plants. Three copies of *BRI1* gene in *Gossypium raimondii*, *Brassica napus*, *Brassica juncea*, *Camelina sativa*, and *Lupinus angustifolius* from dicots and *Avena sativa*, *Musa acuminate*, *Saccharum spontaneum*, and *Triticum aestivum* from monocots were recognized.

Phylogenetic tree

BRI1 genes were subjected to evolutionary analysis in monocot and dicot plants (Figure 1). The phylogenetic tree showed that *BRI1* genes in monocots and dicots have undergone different evolutionary processes. According to the placement of genes in the phylogenetic tree, it can be concluded that diversity in this gene has been occurred before and after the separation of monocots and dicots. *BRI1* genes from *Oryza brachyantha*, *Ananas comosus*, *Musa acuminate*, *Dioscorea rotundata*, and *Asparagus officinalis* showed more genetic distance with other *BRI1* orthologs. Among dicots, *BRI1* genes from *Papaver somniferum*, *Chenopodium quinoa*, and *Beta vulgaris* had more genetic distance from other orthologs.

Physicochemical analysis of BRI1 in monocots and dicots

Comparison of BRI1 in monocots and dicots plants based on physicochemical properties revealed that BRI1 proteins have a nearly conserved structure, although differences were observed between monocots and dicots (Figure 2). MW of BRI1 proteins in dicots varied from 23.66 KDa (BRI1 *Coffea canephora*) to 144.56 KDa (BRI1 *Coffea canephora*) and between 101.43 KDa (BRI1 *Oryza longistaminata*) to 173.31 KDa (BRI1 *Oryza brachyantha*) in monocots (Supplementary Table S1 and Figure 2a). Besides, pI value of BRI1 in dicots ranged between 5.56 (BRI1*Gossypium raimondii*) to 8.13 (BRI1 *Lupinus angustifolius*) and from 5.33 (BRI1 *Panicum hallii*) to 7.87 (BRI1 *Oryza brachyantha*) in monocots (Supplementary Table S1 and Figure 2b). Most *BRI1* genes showed to have one exon while five exons in BRI1 *Brassica napus* (as a dicot) and 10 exons in BRI1 *Echinochloa crus-galli*, as a monocot, were observed (Supplementary Table S1 and Figure 2c). The average of MW and pI of BRI1 in monocots was less than dicots.

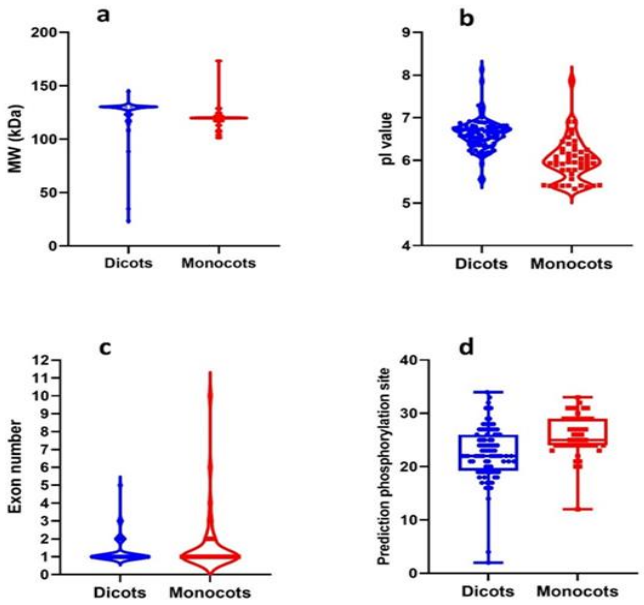


Figure 2. Comparison of BRI1 in monocots and dicots plants based on physicochemical properties including molecular weight (a), isoelectric point (b), exon number (c), and number of phosphorylation site (d).

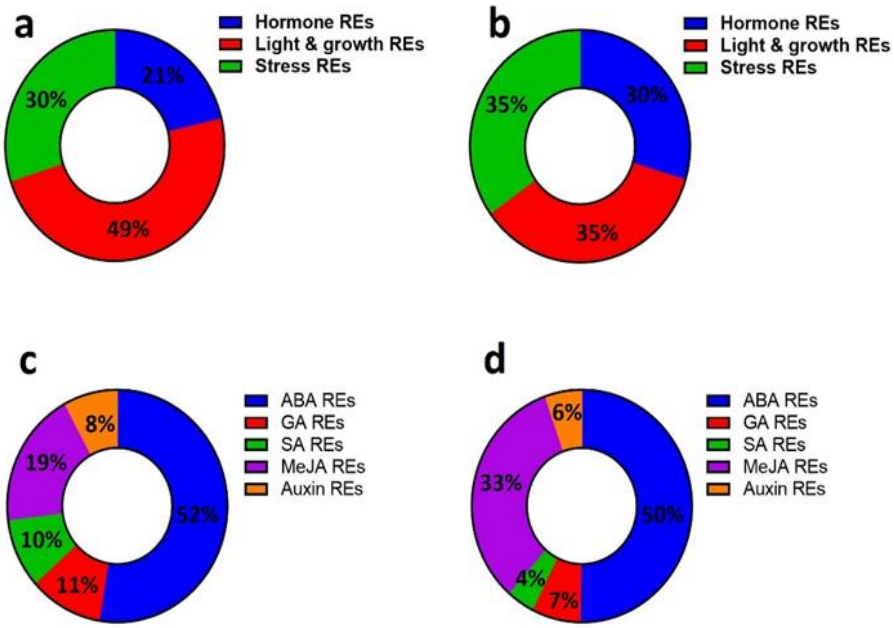


Figure 3. Comparison of BRI1 in monocots and dicots based on promoter cis-regulatory elements. a: classification of cis-regulatory elements based on their function in BRI1 dicots (a) and monocots (b). Frequency of cis-regulatory elements related to hormone responsiveness elements (REs) in dicots (c) and monocots (d).

Phosphorylation modification events

Phosphorylation modifications can affect BRI1 function. Phosphorylation modifications were predicted in BRI1 proteins (Supplementary Table S2). Phosphorylation modification sites of BRI1 in dicots ranged from 2 (BRI1 *Coffea canephora*) to 34 (BRI1 *Populus trichocarpa*) and between 12 (BRI1 *Echinochloa crus-galli*) to 33 (BRI1 *Eragrostis tef*) in monocots orthologs. Monocots showed a greater prevalence of phosphorylation modifications on BRI1 receptor compared to dicots (Figure 2d). This difference is due to changes in the protein sequence structure of BRI1 in studied monocots.

Promoter analysis

Analysis of the BRI1 gene promoter region showed that the frequency of cis-elements is different in dicots and monocots (Supplementary Table S3). Cis-regulatory elements related to abscisic acid responsiveness were more detected in up-stream region of *BRI1* gene (Supplementary Table S3). Regulatory cis-elements of the *BRI1* gene related to hormone and stress response were identified more in monocots than in dicots (Figure 3a and 3b). Separation of hormone response elements showed that their distribution varies according to plant species in the BRI1 gene (Figure 3c and 3d). MeJA response elements were significantly more abundant in the BRI1 gene promoter of monocots than in dicots. Also, salicylic acid response elements were identified more abundantly in the *BRI1* gene promoter of dicots than in monocots.

Discussion

Among plant pathways, the brassinosteroid signaling pathway stands out as one of the most well-studied for its role in BR response. BR responses are linked to the transmembrane receptor kinase known as BRI1. In all corners of the plant kingdom, BRI1 orthologs are widespread and the sensitivity to BR in these orthologs has been thoroughly examined in various plant species. However, a comprehensive study of all BRI1 orthologs has not been conducted so far, and these orthologs have not been compared in monocots and dicots based on sequence structure. In this research, the genetic data of 95 plant species (both dicots and monocots) was examined, leading to the

identification of 141 BRI1 genes within these plants. The majority of the various plant species that were analyzed demonstrated the presence of a singular *BRI1* gene; however, it was observed that in certain specific plant varieties, there were instances where two or even three copies of this gene were identified. The number of *BRI1* genes has been affected by polyploidy, and the copy number of *BRI1* gene has increased in plants undergoing polyploidy. For example, in bread wheat, three chromosome sets have been created based on polyploidy, in which three *BRI1* genes have also been identified. Evolutionary events such as polyploidy and duplication have been implicated in the development of genes in a plant species (Arab et al., 2023; Yaghobi and Heidari, 2023). Results showed that *BRI1* does not appear to have undergone duplication events in plants. The evolutionary analysis revealed that BRI1 orthologs have undergone different evolutionary processes in monocots and dicots. The arrangement of genes in the phylogenetic tree suggests that gene diversity originated at different times, both preceding and following the separation of monocots from dicots.

It was revealed that most *BRI1* genes possess only one exon. The number of exons is one of the structural characteristics of a gene that affects the speed of its induction and expression (Heidari et al., 2023a; Heidari et al., 2023b). Comparison of BRI1 orthologs in monocots and dicots revealed that the molecular size of this receptor is smaller in monocots. It is likely that deletion mutations occurred in BRI1 in monocots during evolution. These changes probably affected other physiochemical properties of BRI1 in monocots, for example, reducing its isoelectric point (pI). Post-translation modifications such as phosphorylation affect the function of target proteins. Upon receiving the ligand in the extracellular domain, the BRI1 receptor phosphorylates its intracellular domain, marking the beginning of the brassinosteroid pathways signaling cascade to downstream components (Wang et al., 2001; Santiago et al., 2013). Following the binding of BR, the intracellular region of the BRI1 receptor undergoes phosphorylation causing BKI1 to dissociate, initiating the signaling pathway with BAK1 (Vert and Chory, 2006; Santiago et al., 2013). Results disclosed that BRI1 orthologs in monocots are targeted by phosphorylation modification events. These changes can affect the brassinosteroid hormone signaling pathway and alter the rate of induction of downstream factors such as BZR1 and BES1. Overall, the brassinosteroid receptor has differences in its regulatory and structural systems in plants, and further research is needed on these differences and their role in receptor function.

Conclusion

BRI1 is a central player in BR signaling, with significant roles in regulating plant architecture and development. Understanding the structural and functional nuances of BRI1 in monocot and dicot plants provides valuable insights for crop breeding and biotechnology. BRI1 orthologs in monocots and dicots were identified and compared for the first time. The results revealed that the evolution of this receptor in monocots was different from that in dicots. It was predicted that evolutionary changes have caused this receptor to have a lower molecular weight and isoelectronic point in monocots. However, further studies such as CRISPR CAS are needed to determine the effect of these changes on receptor function.

Supplementary Materials

The supplementary material for this article can be found online at: https://www.jpmb-gabit.ir/article_720968.html

Supplementary Table S1. List of BRI1 in studied plant species.

Supplementary Table S2. Prediction of phosphorylation site in BRI1 proteins.

Supplementary Table S3. Frequency of cis-requlatory elements in promoter site of *BRI1* genes.

Author Contributions

PH and HV developed the concept, performed the database search, partially collected the data, analyzed, interpreted the results and finalized the draft. PH revised the. All authors approved the final version of the manuscript

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Conflict of Interest Statement

The author declares no conflict of interest.

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مقاله پژوهشی

گیرنده BRI1 در تک‌لپه‌ای‌ها و دو لپه‌ای‌ها: آنالیز ساختار

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چکیده: براسینواستروئیدها (BRs) نقش مؤثری در فرآیندهای رشد و نمو گیاهان دارند، زیرا در طیف وسیعی از موجودات یافت می‌شوند. گیرنده BRI1 گیرنده اصلی هورمون های BR است که عضوی از گیرنده های شبه کیناز غنی از لوسین است. گیرنده BRI1 در چندین گونه گیاهی کشف شده است، اگرچه اطلاعات کافی برای مقایسه تفاوت ساختار آن بین تک لپه‌ای‌ها و دو لپه‌ای‌ها وجود ندارد. در مطالعه حاضر، تجزیه و تحلیل توالی ژن *BRI* بر روی گیاهان با داده های ژنومی موجود انجام شد. تجزیه و تحلیل داده‌های ژنومی از ۹۵ گیاه وجود ۱۴۱ ژن *BRI1* را نشان داد که ۹۲ ژن در دو لپه ای و ۴۹ ژن تک لپه ای بودند. اکثر گونه‌های گیاهی متنوعی که مورد بررسی قرار گرفتند، دارای یک ژن *BRI1* بودند. مسیرهای تکاملی متفاوتی برای ژن‌های *BRI1* در تک لپه‌ها و دولپه‌ها از طریق تجزیه و تحلیل درخت فیلوژنتیک شناسایی شدند. تجزیه و تحلیل ارتولوگ‌های BRI1 از طریق ویژگی‌های فیزیکوشیمیایی نشان‌دهنده یک ساختار حفاظت‌شده در بین پروتئین‌های BRI1، با تمایزات قابل توجه بین تک لپه‌ها و دو لپه‌ها بود. اکثر ژن‌های *BRI1* حاوی یک اگزون هستند و میانگین MW و pI برای BRI1 در تک لپه‌ها کمتر از دو لپه‌ای بود. با این حال، سایت‌های فسفوریلاسیون بیشتری در ارتولوگ‌های BRI1 تک لپه‌ها پیش‌بینی شد.

کلمات کلیدی: براسینواستروئید، گیرنده کینازی، ساختار توالی، آنالیز تکاملی.

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The effects of salicylic acid and melatonin elicitors on the antioxidant enzymes activity and morphological characteristics of *Acorus calamus* under drought stress

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Original paper

Abstract: Medicinal plants improve human health, strengthen the immune system, reduce inflammation, and facilitate the treatment of diseases. To investigate the effect of drought stress (after 10 and 20 days of water withdrawal, representing mild and severe drought stress, respectively) and melatonin and salicylic acid on the biochemical and morphological indicators of the *Acorus calamus* medicinal plant, an experiment was conducted. Results showed that using melatonin and salicylic acid increased the activity of peroxidase enzyme under drought conditions. Also, salicylic acid increased the activity of catalase enzyme in mild drought stress. The application of melatonin and salicylic acid under severe drought stress increased the activity of the catalase enzyme. Under drought stress conditions, the use of salicylic acid and melatonin led to an increase in plant dry weight compared to normal conditions. Also using melatonin under severe drought conditions resulted in an increase in dry weight in comparison with the control treatment. In conclusion, while the plant's responses to stress and elicitors differed across various characteristics, it can generally be stated that salicylic acid and melatonin positively influence the biochemical and morphological traits of *Acorus calamus* under severe and mild drought stress conditions.

Keywords: Biochemical indicators, drought stress, foliar spraying, medicinal plant, morphological indicators.

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Introduction

Plants in natural conditions are subjected to various stresses that adversly affects their growth and production (Hosseini et al., 2019; Hosseini et al., 2021; Zulfiqar et al., 2021). Temperature, heavy metals, salinity, and water deficit are among the non-living factors that effectively affect the growth of plants. Among these factors, drought is the biggest factor limiting the production of agricultural products (Khalvandi et al., 2021). In other words, drought is one of the most important factors and the most common environmental stress that limits the growth and performance of plants worldwide (Keshavarz - Mirzamohammadi et al., 2021; Zulfiqar et al., 2021). Plants respond to drought stress at different physiological, cellular, and, molecular levels. This response depends on the species and genotype of the plant, the severity and duration of the water shortage, and the growth stage of the plant (Khalvandi et al., 2021; Zulfiqar et al., 2021; Ostadi et al., 2022).

Acorus calamus (Aghir in Turkish or small lily), is a plant that grows at riversides. This plant is valued as a "rejuvenator" for the brain and nervous system, as well as a remedy for digestive disorders. The leaves and rhizomes of this plant contain a volatile oil that has a special smell and taste (Rajput et al., 2014). The main components of the oil include beta- and alpha-asarone, saponins, lectins, sesquiterpenoids, lignans, and, steroids. Phytochemicals in plants vary based on geographic location, plant age, climate, species diversity, and extracted plant components (Rajput et al., 2014; Khalvandi et al., 2019; Hosseini et al., 2023).

In light of climate change and depletion of water resources, utilization of drought tolerant plant species or genotypes with low water demand represents an strategy for sustainable resource management and enhancing yield (Khalvandi et al., 2019; Haghpanah et al., 2024) The production of resistant plants requires a relatively long time from breeding methods, so the use of effective materials in reducing the adverse effects of stress to achieve the economic aspects of the performance of medicinal plants seems to be important (Khalvandi et al., 2019; González-Villagra et al., 2022; Khalil et al., 2022). Plants respond to different environmental conditions through changes in various biochemical and physiological processes (Adl et al., 2023; Hosseini et al., 2023; Akbari et al., 2024). Plants can produce both volatile and non-volatile compounds including plant hormones that help them adapt to environmental changes (Kaur et al., 2022; Murali-Baskaran et al., 2022). Salicylic acid is a hormone-like substance that affects plant growth and development. Salicylic acid belongs to a group of plant phenols (Zulfiqar et al., 2021). This compound plays an important role in regulating plant growth and development, interacting with other organisms, responding to environmental stresses, glycolysis, photosynthetic performance, transpiration and fruiting (González-Villagra et al., 2022). Various studies have shown that salicylic acid significantly causes ion leakage and reduces the accumulation of toxic ions in plants (Khalvandi et al., 2021; Zulfiqar et al., 2021; González-Villagra et al., 2022). According to the reports, salicylic acid has significant effects on the morphological and physiological characteristics of the plant and plays a role in stimulating the support mechanisms to increase resistance against biotic and abiotic stresses (Khalvandi et al., 2021).

Melatonin is another compound that plays an important role in regulating the physiological and biochemical activities of plants (Murch and Erland, 2021). Melatonin plays a significant role in helping plants cope with stress by regulating physiological and biochemical responses (Ali et al., 2024). It enhances antioxidant activity, protects cellular structures, and improves water retention, thus promoting plant growth under adverse conditions (Ali et al., 2024; Cano et al., 2024). Additionally, melatonin influences the production and synthesis of secondary metabolites in medicinal plants, such as flavonoids and phenolics (Ghorbani et al., 2024a). These compounds contribute to plant defense mechanisms and enhance medicinal properties, making melatonin crucial for improving both stress tolerance and therapeutic potential in medicinal herbs (Back, 2021; Murch and Erland, 2021; Ali et al., 2024; Cano et al., 2024; Ghorbani et al., 2024b).

Given the limited understanding of plant hormones, particularly melatonin, in plant responses to drought stress, it is essential to conduct extensive research on the effects of this hormone. Therefore, the present study was conducted to investigate the effects of drought stress and

exogenous application of salicylic acid and melatonin on the morphological and biochemical characteristics of the *Acorus calamus* medicinal plant.

Materials and Methods

Plant materials and treatments

The current research was conducted using two factors factorial experiment based on a completely randomized design with three replications. The first factor included two levels the drought stress, continuous irrigation (W) and, interruption of irrigation (No-W). The second factor included foliar spraying of elicitors at three levels (distilled water (control), melatonin (ML) (100 µM), and, salicylic acid (SA) (1 mM). Foliar sprays were carried out once every 5 days until harvest time. The first foliar spraying was done when stopping the irrigation.

The samples of *Acorus calamus* along with the rhizome were collected from Arzefoon wetland near Sari City, Mazandaran province, and, transferred to the greenhouse. Plants were planted on separate beds with dimensions of 40 x 200 cm, which were isolated using plastic covers. These beds were prepared with river sand plus garden soil with a ratio of 1:1 to a depth of 15 cm. The conditions for the growth of plants were provided by flooding. Each plot contained 15 plant samples. The studied plants were kept under these conditions for two months under solar light irradiance of 60000 lux, the temperature of 30-35 °C and, relative humidity of 70 % in greenhouse conditions.

Traits measurement

Sampling was done at two time points: 10 and 20 days after water withdrawal, representing mild and severe drought stress, respectively. After recording the morphological traits, the samples were transferred to -80 °C for biochemical measurements. Characters including fresh and dry weight, number of roots, length of roots, number of nodes in the rhizome and number of leaves were measured from these samples. To calculate the dry weight, the samples were kept at 76 °C for 72 hours and then weighed.

Extraction of enzyme and soluble protein

Fresh tissue was weighed and homogenized with 2 ml of potassium phosphate buffer (pH=6.8). After homogenization, the samples were transferred to two ml vials and centrifuged at 4°C at 15,000 rpm for 12 minutes. Then, the upper phase was separated and these extracts were used for enzyme assays. The Bradford method (Bradford, 1976) was used to measure soluble protein concentration.

Catalase (CAT) enzyme activity assay

The Luck method (Luck, 1971) was used to measure CAT enzyme activity. 20 µl of enzyme extract was mixed with 980 µl of phosphate buffer containing 2 mM hydrogen peroxide and their absorbance changes were read at a wavelength of 240 nm using a spectrophotometer. The enzyme activity was calculated using the Beer-Lambert law and an extinction coefficient of 40 ($\mu M^{-1}cm^{-1}$). The enzyme activity was calculated in terms of mmol per minute per mg of protein.

Superoxide dismutase enzyme activity assay

Superoxide dismutase (SOD) enzyme activity was measured using the nitroblue tetrazolium (NBT) photoreduction inhibition assay at a wavelength of 560 nm. For this purpose, 50 mM potassium phosphate buffer solution (pH=7.4) was prepared. Then, 13 mM methionine, 0.1 mM EDTA, 75 µM NBT, and 4 µM riboflavin were added to prepare the reaction mixture, respectively. Before adding these compounds to the potassium phosphate buffer, we covered the container containing the reaction mixture so that it was not exposed to light. Then, 100 µl of extract from each sample was poured into each test tube and 3 ml of the above solution was added to it and the reaction was started immediately by placing them under the light of a fluorescent lamp (W40). After 1.5 minutes, the absorbance of the samples was read at a wavelength of 560 nm.

The activity of the SOD enzyme in the samples was measured in comparison with the light control. The difference in absorbance of the samples and the light control at 560 nm indicates the inhibition of the photoreduction of NBT in the presence of the SOD enzyme present in the sample. Using this absorbance difference, the enzyme activity was calculated in terms of enzyme units per gram of fresh weight for all samples (Beauchamp and Fridovich, 1971).

Peroxidase (POD) enzyme activity assay

POD was assayed using the method of (Nakano and Asada, 1981). The reaction mixture consisted of 2.5 ml of 50 mM phosphate buffer (pH=7.0) containing 0.1 mM EDTA, 1 mM sodium ascorbate, 0.2 ml of 1% H2O2, and 0.1 ml of extracted protein extract. POD was calculated as the decrease in absorbance at 240 nm for 1 min (Extinction coefficient= 2.18 mM^{-1} cm^{-1}).

Statistical analysis

The extracted data were first normalized and then analyzed using SAS (9.1) statistical software. Comparisons of mean data were performed using the least significant difference (LSD) method at a 5 % probability level, and figures were drawn using EXCEL software. Also, XLSTAT (2016) software was used to perform principal component analysis and cluster analysis. The Varimax rotation method was also used to maximize high- and low-value factor loadings and minimize mid-value factor loadings. Euclidean distance and the UPGME method were used for cluster analysis.

Results

POD enzyme activity

Analysis of variance showed that the effects of drought stress, elicitor and their interaction were significant for the POD at mild and severe drought stress ($P \leq 0.01$) (Table S1). The results of mild drought stress demonstrated that the highest amount of POD was observed in No-W+SA (13.67 U/mg protein min) and No-W+ML treatments (15 U/mg protein min) and the lowest amount of POD was observed in W (normal) treatments (6.67 U/mg protein min), W+ML (5 U/mg protein min) and No-W (6 U/mg protein min) (Figure 1). The use of W+SA, No-W+ML, and No-W+SA treatments increased the POD by 60, 125, and 105 %, respectively, in comparison with W treatment (Figure 1). In severe drought stress, the highest amount of POD was observed in the No-W+SA treatment (47.12 U/mg protein min) (Figure 1). On the other hand, the lowest amount of POD was observed in W (7.05 U/mg protein min) and No-W (7.04 U/mg protein min) treatments, which showed no statistically significant difference with W+ML (14.33 U/mg protein min) and No-W (7.04 U/mg protein min) treatments. Using W+SA and No-W+SA treatments caused 197 and 568 % increases in POD in comparison with W treatment, respectively (Figure 1).

CAT enzyme activity

According to analysis of variance, the CAT was significantly affected by drought stress, elicitors, and their interaction at mild and severe drought stress exposure ($P \leq 0.01$) (Table S1). In mild drought stress exposure the highest amount of CAT was observed in W (2.47 U/mg protein min) and W+ML (2.5 U/mg protein min) treatments (Figure 1), however, the lowest amount of CAT was observed in W+SA treatment (1.06 U/mg protein min). Application of W+SA, No-W, No-W+ML, and No-W+SA treatments resulted in a reduction of 133, 71, 20, and 49 % for CAT in comparison with W treatment, respectively (Figure 1).

In severe drought stress, the highest amount of CAT was observed in the W+ML treatment (2.56 U/mg protein min) (Figure 1), but the lowest amount belonged to the No-W treatment (0.55 U/mg protein min). Application of W+ML treatment caused a 17 % increase in CAT in comparison with W treatment; but using W+SA, No-W, No-W+ML, and No-W+SA treatments caused decreased 121, 298, 141, and 115 % of CAT in comparison with W treatment, respectively (Figure 1).

SOD enzyme activity

Results showed that the effects of drought stress ($P \leq 0.01$), elicitor ($P \leq 0.05$), and their interaction ($P \leq 0.01$) were significant for SOD at mild and severe drought stress (Table S1).

Ten days after stress exposure illustrated that the highest amount of SOD was observed in W (0.98 U/mg protein min) and W+SA (1.02 U/mg protein min) treatments, however, the lowest amount of SOD belonged to No-W treatment (0.07 U/mg protein min) (Figure 1). In severe drought stress, the highest amount of SOD was observed in No-W+ML (1.09 U/mg protein min) and No-W+SA (1.17 U/mg protein min) treatments (Figure 1). However, the lowest activity of SOD was correlated with W (0.86 U/mg protein min), W+ML (0.77 U/mg protein min), W+SA (0.87 U/mg protein min), and No-W (0.91 U/mg protein min) treatments. Application of No-W+ML and No-W+SA treatments promoted 27 and 36 % SOD in comparison with W treatment, respectively (Figure 1).

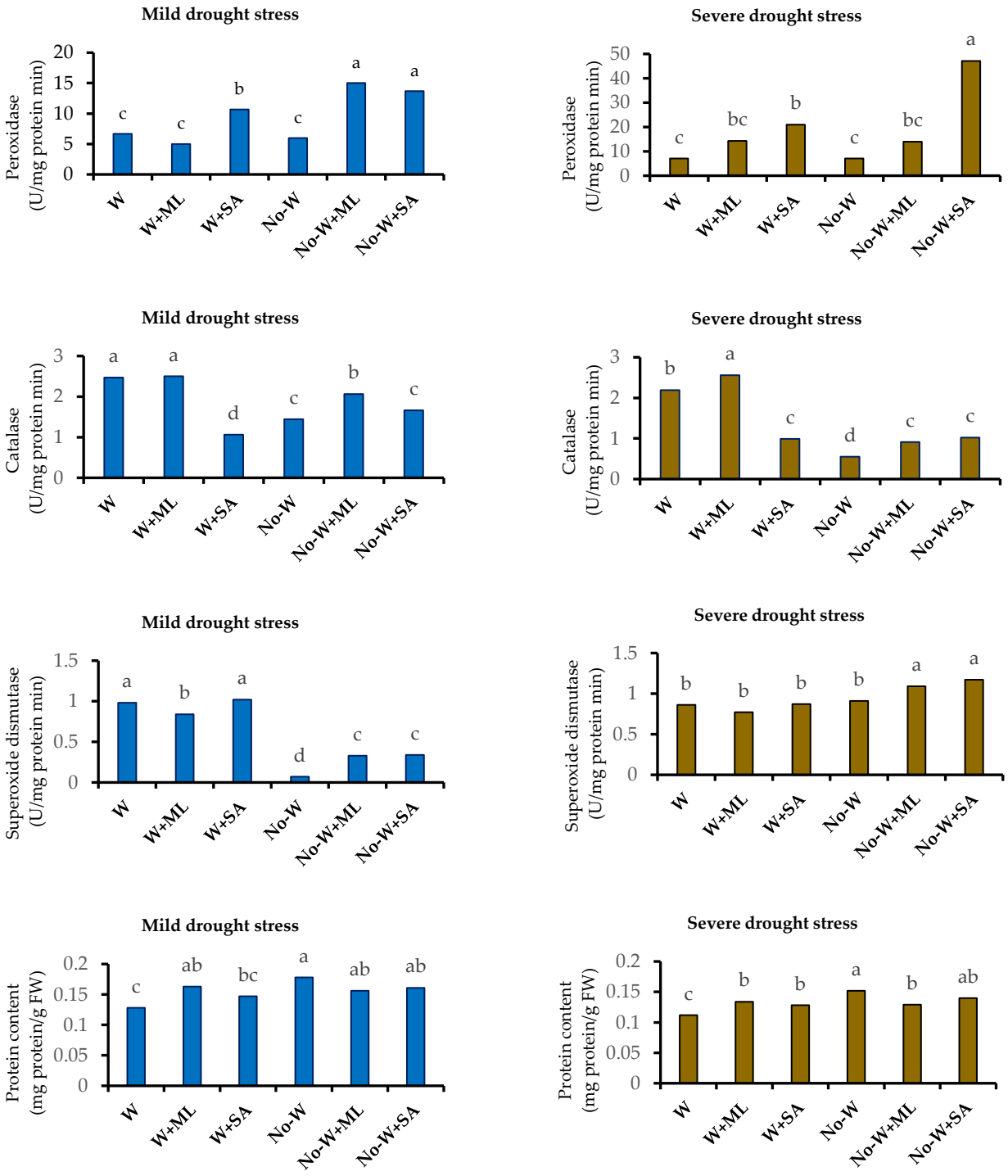


Figure 1. The effects of foliar spraying of salicylic acid (SA) and melatonin (ML) on the activity of antioxidant enzymes and protein content in *Acorus calamus* plant under continuous irrigation (W) and mild and severe drought stress (No-W). Continuous irrigation (W), continuous irrigation plus application of melatonin (W+ML), continuous irrigation plus application of salicylic acid (W+SA), mild and severe drought stress (No-W), mild and severe drought stress plus application of melatonin (No-W+ML), mild and severe drought stress plus application of salicylic acid (No-W+SA). The blue and brown charts correspond to the mild and severe drought stress, respectively.

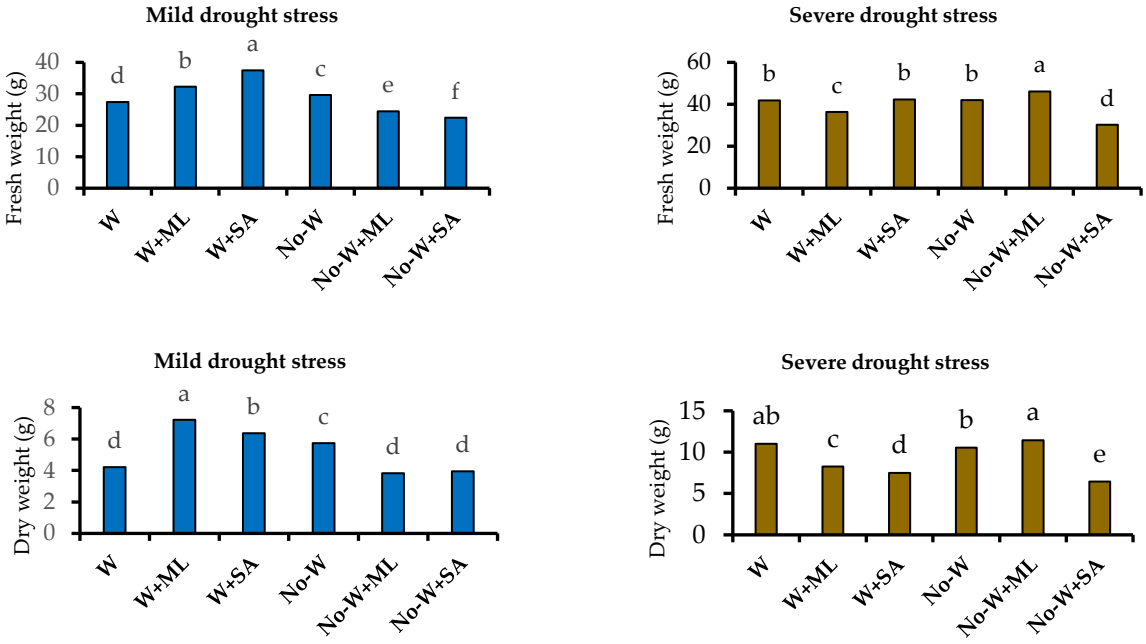


Figure 2. The effect of foliar spraying of salicylic acid (SA) and melatonin (ML) on the fresh and dry weight of the *Acorus calamus* plant under the treatment of continuous irrigation (W) and mild and severe drought stress (No-W). Continuous irrigation (W), continuous irrigation plus application of melatonin (W+ML), continuous irrigation plus application of salicylic acid (W+SA), mild and severe drought stress (No-W), mild and drought stress plus application of melatonin (No-W+ML), mild and severe drought stress plus application of salicylic acid (No-W+SA).

Protein content

The results showed that the protein content in mild and severe drought stress was significantly under the influence of drought stress ($P \leq 0.05$) (Table S1), however, using elicitors demonstrated cannot significantly increase the protein content. The interaction of drought stress and elicitor changed significantly ($P \leq 0.05$) the protein content (Table S1). In mild drought stress, the highest amount of protein content was observed in No-W treatment (0.178 mg protein/g FW), when compared with W+ML (0.163 mg protein/g FW), No-W+ML (0.156 mg protein/g FW) and No-W+SA treatments (0.161 mg protein/g FW), illustrating no significant difference (Figure 1). In contrast, the lowest amount of protein content was related to W treatment (0.128 mg protein/g FW), which showed no significant difference with W+SA treatment (0.147 mg protein/g FW). Results showed that using W+ML, No-W, No-W+ML and No-W+SA treatments increased the amount of protein content by 27, 39, 22 and 26 %, respectively, in comparison with the W treatment (Figure 1).

The results related to the severe drought stress showed that the highest amount of protein content was achieved by the application of No-W treatment (0.152 mg protein/g FW), which had no statistically significant difference with the No-W+SA treatment (0.14 mg protein/g FW) (Figure 1). On the other hand, the lowest amount of protein content was observed in the W treatment (0.112 mg protein/g FW). The results showed that the use of W+ML, W+SA, No-W, No-W+ML and No-W+SA treatments increased the amount of protein content by 19, 14, 35, 15 and 25 %, respectively, in comparison with the W treatment (Figure 1).

Fresh and dry weight

Analysis of variance showed that the fresh and dry weight_in the time of mild drought stress was significantly affected by drought stress ($P \leq 0.01$), elicitors ($P \leq 0.01$) and their interaction ($P \leq 0.01$) (Table S2, Figure 2). On the other hand, the drought stress had no significant effect on the fresh weight in the severe drought stress. The results of the analysis of variance showed that the fresh weight in the severe drought stress was significantly affected

by elicitors (P ≤ 0.01) and the interaction of drought stress and elicitors (P ≤ 0.01). The results of the analysis of variance showed that the dry weight in the severe drought stress was significantly changed by drought stress (P ≤ 0.01), elicitors (P ≤ 0.01) and, their interaction (P ≤ 0.01) (Table S2).

The results of mild drought stress revealed that the highest amount of fresh weight was related to the W+SA treatment (37.44 g) and the lowest amount of fresh weight was related to the No-W+SA treatment (22.41 g) (Figure 2). The application of No-W, W+ML and W+SA treatments increased the amount of fresh weight by 8, 21 and, 36 %, respectively, compared to the W treatment. In contrast, the application of No-W+ML and No-W+SA treatments decreased the fresh weight by 12 and 22 %, respectively, in comparison with the W treatment (Figure 2).

In severe drought stress the heaviest fresh weight was produced by the No-W+ML treatment (46.18 g) (Figure 2) and the lightest was obtained from the No-W+SA treatment (30.24 g). According to the results using No-W+ML treatment created 10 % more fresh weight from the W treatment; while the application of W+ML and No-W+SA treatments compared to the W treatment caused 15 and 38 % reduction in fresh weight, respectively (Figure 2).

In mild drought stress, the most dry weight was observed in the W+ML treatment (7.23 g); In contrast, the least dry weight were measured in W (4.21 g), No-W+ML (3.83 g) and No-W+SA (3.94 g) treatments. The W+ML, W+SA and No-W treatments were able to make 71, 51 and 36 % more dry weight, respectively (Figure 2). Furthermore, the severe drought stress showed the highest dry weight in the No-W+ML treatment (11.45 g), which had no significant difference with the W treatment (11 g) (Figure 2). However, the lowest dry weight was obtained from the No-W+SA treatment (6.46 g). Using W+ML, W+SA and No-W+SA treatments compared to the W treatment have yielded 33, 46 and 70 % reduction in dry weight, respectively (Figure 2).

Number of leaves

The results related to the conditions of mild drought stress showed that the highest number of leaves was achieved in the W+SA treatment (7.33) (Figure 3). On the other hand, the lowest number of leaves was achived in the No-W treatment (3.33), which was not statistically significantly different from the No-W+ML treatment (7.33). The results showed that the W+SA treatment increased the number of leaves by 29 % compared to the W treatment. Based on the obtained results, the use of No-W and No-W+ML treatments reduced the number of leaves by 70 % and 31 %, respectively compared to the W treatment (Figure 3). The results indicated that the W+SA treatment produced the highest number of leaves (9.67), while the No-W+ML treatment had the lowest (4.67), with no significant difference from the No-W treatment (5.33). Also, the application of W+SA treatment caused a 16 % increase in the number of leaves compared to the W treatment. Conversly, the application of No-W, No-W+ML and No-W+SA treatments resulted in decrease in the number of leaves by 56, 78 and 31 %, respectively, compared to the W treatment (Figure 3)

Root length

According to the analysis of variance, root length at mild drought stress was significantly influenced by drought stress (P ≤ 0.01) and the interaction of drought stress and elicitors (P ≤ 0.01) (Table S2). The highest root lengths were observed in the No-W (31.74 cm), No-W+ML (32.77 cm) and No-W+SA (33.8 cm) treatments, while the lowest were in the W (22.91 cm), W+ML (21.18 cm), and W+SA (21.24 cm) treatments (Figure 3). The No-W, No-W+ML, and No-W+SA treatments increased root length by 38, 43 and 47%, respectively, compared to the W treatment (Figure 3).

At severe drought stress, the analysis of variance indicated significant effects from drought stress (P ≤ 0.01), elicitors (P ≤ 0.01) and their interaction (P ≤ 0.01). The highest root length was achieved with the No-W+SA treatment (30.9 cm), while the lowest was in the W+SA treatment (19.1 cm). The No-W, No-W+ML, and No-W+SA treatments increased root length by 18, 17 and 34%, respectively, compared to the W treatment. Conversely, the W+ML and W+SA treatments resulted in decreases of 8 and 20% in root length, respectively, compared to the W treatment (Figure 3).

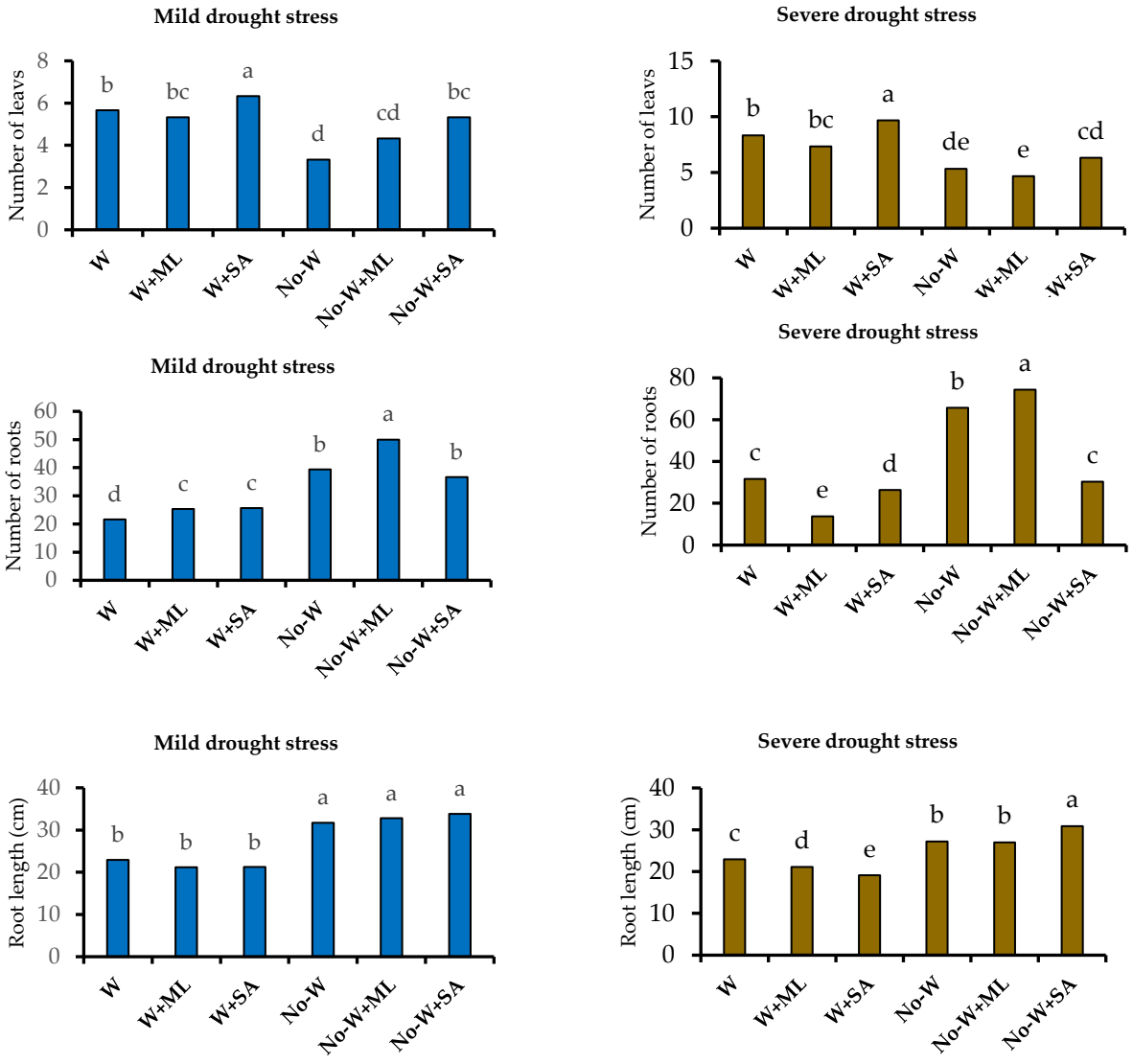


Figure 3. The effect of foliar spraying of salicylic acid (SA) and melatonin (ML) on the number of roots and leaves and root length of the *Acorus calamus* plant under the treatment of continuous irrigation (W) and mild and severe drought stress (No-W). Continuous irrigation (W), continuous irrigation plus application of melatonin (W+ML), continuous irrigation plus application of salicylic acid (W+SA), mild and severe drought stress (No-W), mild and severe drought stress plus application of melatonin (No-W+ML), mild and severe drought stress plus application of salicylic acid (No-W+SA).

Principal components analysis (PCA) and cluster analysis (CA)

Based on the results obtained from the PCA, it was observed that the four components had an eigenvalue higher than one and were known as effective components (Table 1). The first and second components had a relative variance of 48.37 and 20.65 %, respectively, and accounted for 69.02 % of the total variance (Table 1). The results showed that in the first component, SOD, fresh weight, number of roots, number of leaves and root length traits in the mild drought stress and CAT, SOD, number of roots, number of leaves and root length traits had high factor loadings at the severe drought stress (Table 1). Also, in the second component, POD, fresh weight and dry weight traits had high factor loading (Table 1).

Table 1. Factor loading related to different traits in Acorus calamus plants under salicylic acid treatments and drought stress (Mild and severe drought stress).

Characteristics	Factors				
	F1	F2	F3	F4	F5
Peroxidase (Mild drought stress)	0.561	0.443	-0.388	-0.473	0.339
Catalase (Mild drought stress)	-0.235	-0.251	-0.530	0.741	0.227
Superoxide dismutase (Mild drought stress)	-0.945	0.124	-0.252	-0.147	0.080
Protein (Mild drought stress)	0.573	-0.070	0.766	0.221	0.173
Peroxidase (Severe drought stress)	0.280	0.957	0.030	-0.004	0.064
Catalase (Severe drought stress)	-0.726	-0.028	-0.270	0.607	0.175
Superoxide dismutase (Severe drought stress)	0.812	0.471	-0.312	-0.141	0.045
Protein (Severe drought stress)	0.588	0.040	0.796	0.138	-0.029
Fresh weight (Mild drought stress)	-0.715	-0.195	0.534	-0.399	0.078
Dry weight (Mild drought stress)	-0.590	-0.159	0.753	0.069	0.232
Number of roots (Mild drought stress)	0.925	-0.164	-0.037	-0.142	0.311
Number of leaves (Mild drought stress)	-0.744	0.558	-0.265	-0.239	0.089
Length of roots (Mild drought stress)	0.989	0.108	-0.053	0.042	-0.073
Fresh weight (Severe drought stress)	-0.013	-0.813	-0.221	-0.528	0.106
Dry weight (Severe drought stress)	0.208	-0.883	-0.410	0.012	-0.089
Number of roots (Severe drought stress)	0.784	-0.549	-0.113	-0.268	-0.009
Number of leaves (Severe drought stress)	-0.889	0.276	0.006	-0.308	-0.194
Length of roots (Severe drought stress)	0.900	0.257	-0.123	0.268	-0.193
Eigenvalue	8.707	3.719	3.060	2.011	0.504
Variability (%)	48.370	20.658	17.000	11.173	2.799
Cumulative (%)	48.370	69.028	86.028	97.201	100.000

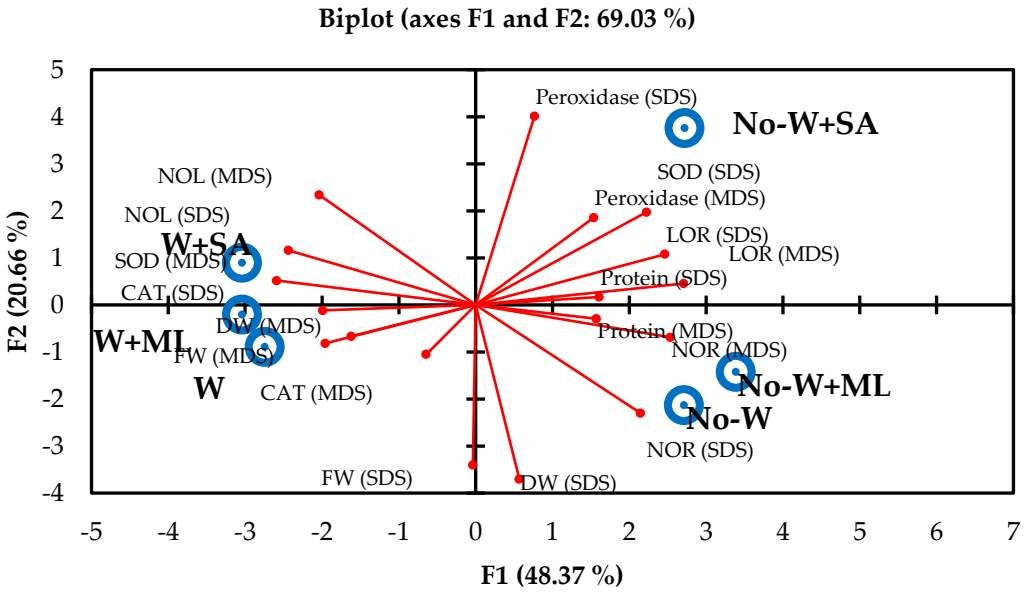


Figure 4. Bi-plot resulting from PCA based on all studied traits in *Acorus calamus* plants under the exposure of drought stress and application of elicitors treatments. (Mild drought stress (MDS); severe drought stress (SDS); catalase (CAT); superoxide dismutase (SOD); continuous irrigation (W), continuous irrigation plus application of melatonin (W+ML), continuous irrigation plus application of salicylic acid (W+SA), mild and severe drought stress (No-W), mild and severe drought stress plus application of melatonin (No-W+ML), mild and severe drought stress plus application of salicylic acid (No-W+SA).

The bi-plot diagram obtained from the first and second components showed that No-W+SA treatment had a high correlation with POD, SOD, root length and protein traits in the severe drought stress and POD and root length traits in the mild drought stress (Figure 4). The No-W+SA treatment was separated independently due to the large distance compared to other treatments. On the other hand, the W+SA, W+ML and W treatments were placed in the same group due to their high affinity and were correlated with number of leaves, SOD, dry weight, fresh weight and CAT traits in the mild drought stress and number of leaves and CAT traits in the severe drought stress. Also, No-W+ML and No-W treatments were placed in the same group and showed a strong correlation with the number of roots and protein content traits in the mild drought stress and fresh weight, dry weight and, number of roots traits in the severe drought stress (Figure 4). The results of the cluster analysis showed that all the investigated treatments were divided into three separate groups, so in the first group there were three treatments (W+SA, W+ML and, W), in the second group there were two treatments (No-W+SA and No-W+ML) and in the third group one treatment (No-W) were separated (Figure 5).

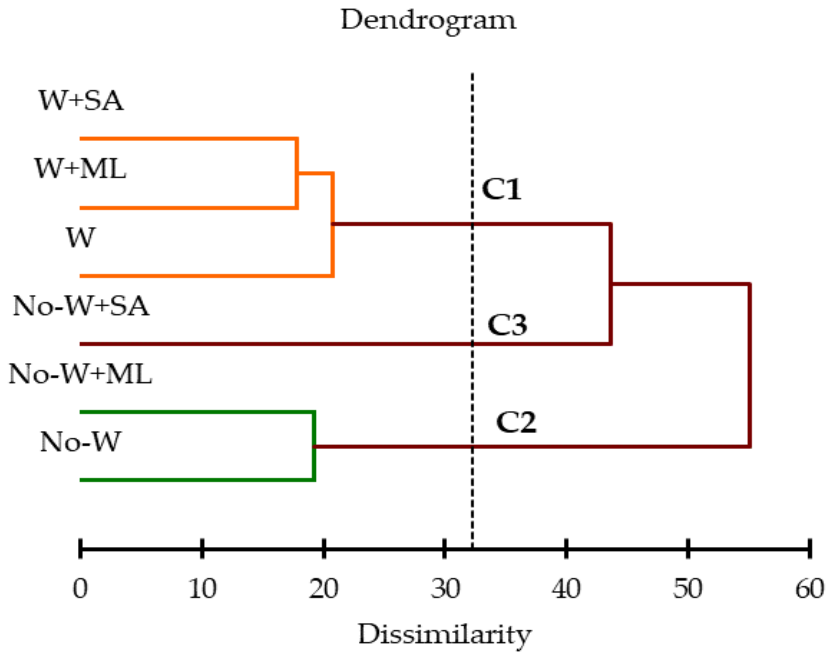


Figure 5. Dendrogram resulting from cluster analysis based on all studied traits in *Acorus calamus* plants under the exposure of drought stress and application of elicitors treatments. Continuous irrigation (W), continuous irrigation plus application of melatonin (W+ML), continuous irrigation plus application of salicylic acid (W+SA), mild and severe drought stress (No-W), mild and severe drought stress plus application of melatonin (No-W+ML), mild and severe drought stress plus application of salicylic acid (No-W+SA).

Discussion

Drought stress is one of the most important limiting factors for plant growth and changes the physiological activities of plants (Khalvandi et al., 2021). Among these, elicitors play an important role in regulating the physiological activities of plants under environmental stress conditions, especially drought stress (Moustafa-Farag et al., 2020). In the present experiment, the use of salicylic acid and melatonin led to significant changes in the morphological and physiological characteristics of the *A. calamus* L. plant.

When plants encounter drought stress, the amount of reactive oxygen species (ROS) in their cells increases. This rise in ROS leads to damage to cell membranes and the degradation of biological molecules. Therefore, the removal of ROS is one of the key strategies for plants to tolerate drought stress in (Zulfiqar et al., 2021). In our experiment, the response of antioxidant enzymes to drought stress was different; in most cases, the activity of these enzymes decreased with the application of drought stress. Additinally, the activity of antioxidant enzymes in response to environmental stress differ across various plant organs (Pan et al., 2021).

In studes conducted on mint plants (Khalvandi et al., 2019; Hosseini et al., 2023), basil (Zulfiqar et al., 2021), thyme (Rahimi et al., 2022) and rosemary (Mohammadi-Cheraghabadi et al., 2021; Ostadi et al., 2022), an increase in the activity of antioxidant enzymes in response to salinity and drought stress has been reported, which support the findings of our study. CAT, ascorbate POD and SOD are among the most effective antioxidant enzymes. the increased activity of these enzymes inhances tolerance to various stresses, including drought stress, in plants (Khalvandi et al., 2019; Hosseini et al., 2023).

On the other hand, the results of the present experiment indicated that the use of salicylic acid and melatonin increased the activity of antioxidant enzymes under both normal conditions and drought stress, however, the response to these elicitors varied somewhat different under normal conditions.

Salicylic acid not only enhances antioxidant activity but also reduces the toxicity of free radicals that have accumulated due to drought stress damage, and thus reduces the damage caused by drought stress (González-Villagra et al., 2022). Additionally, the application of salicylic acid increases the content of polyamines, which play an important role in developing tolerance to drought stress (Hasan et al., 2021). Foliar spraying of wheat plant with salicylic acid has enhanced drought stress tolerance by increasing membrane stability, reducing leakage and peroxidation of cell membrane lipids (Khalvandi et al., 2021).

Salicylic acid induces metabolic changes that increase ROS levels and enhance the antioxidant defense system, playing a role in cellular signaling under drought stress conditions (Salvi et al., 2021). By activating the enzymes involved in the biosynthesis of antioxidant enzymes, salicylic acid facilitates the removal and inactivation of ROS. Consequently, by enhancing the levels of both enzymatic and non-enzymatic antioxidants, it potentially reduces hydrogen peroxide in plant cells by ascorbate as a very important substrate for hydrogen peroxide detoxification in the photosynthetic apparatus (Hayat et al., 2021).

Increasing antioxidant activity is a crucial important mechanisms by which plants enhance their tolerance to environmental stresses, including drought. The exogenous application of salicylic acid significantly reduced malondialdehyde levels in wheat plants subjected to drought stress (Khalvandi et al., 2021). In another study, the application of salicylic acid resulted in decreased malondialdehyde production, indicating the ability of this plant growth regulator to neutralize ROS and prevent their accumulation (Damalas and Koutroubas, 2021).

Probably, salicylic acid reduced the content of superoxide and hydrogen peroxide radicals by influencing the activity of antioxidant enzymes and inhibited the activity of lipoxygenase enzyme and the breakdown of membrane fatty acids, ultimately leading to a reduction in oxidative damage to membrane lipids during drought stress (Damalas and Koutroubas, 2021; Khalvandi et al., 2021). Research has demonstrated that salicylic acid acts as a resistance mechanism by boosting the antioxidant capacity of cells. It provides greater protection to cell membranes, reduces lipid peroxidation, and prevents protein oxidation (Kaur et al., 2022). In an experiment with tomato plants, phenolic compounds accumulated after the application of salicylic acid following the increase in the activity of the phenylalanine ammonia-lyase enzyme (Fan et al., 2022). Therefore, salicylic acid plays an important role in the biosynthesis of phenolic compounds and the expression of plant defense genes (Khalil et al., 2022). The results of the present experiment demonstrated the positive effect of melatonin on some morphological and physiological characteristics. Probably, melatonin stimulates plant growth by affecting plant metabolites and increasing the biosynthesis of

phytohormones such as auxin and cytokinin, and facilitating nutrient absorption (Tiwari et al., 2021). By increasing turgor pressure and the relative water content of leaves, melatonin improves growth under drought stress conditions (Moustafa-Farag et al., 2020).

According to the results of the present experiment, drought stress reduced the number of leaves, however, the application of melatonin and salicylic acid increased the number of leaves under stress conditions. Conversly, while melatonin and salicylic acid enhanced dry weight under normal conditions, they did not have a positive effect on dry weight under drought stress. It seems that under drought stress, the composition of melatonin affects the secondary metabolites involved in the stress resistance mechanism to maintain the plant survival, but does not affect the increase in dry matter (Tiwari et al., 2021). How elicitors affect physiological processes in plants is very complex and requires further investigations in biological systems (Altaf et al., 2022). The positive effect of melatonin on photosynthetic indices is proven in various plants under drought stress such as chickpea (Ahmad et al., 2023), chrysanthemum (Luo et al., 2023) and barley (Talaat, 2023).

Drought stress causes stomatal closure and reduced internal CO2, decreasing NADPH demand in the Calvin cycle, leading to oxidation and disruption of the electron transport chain, using oxygen as an electron acceptor and generating singlet oxygen. Previous studies indicate that melatonin mitigates drought stress effects on photosynthesis by maintaining light energy balance (Khalvandi et al., 2021; Karumannil et al., 2023; Zahra et al., 2023).

Excess light energy triggers the Mehler reaction, producing superoxide and hydrogen peroxide, which oxidize membrane lipids and damage photosynthetic pigments, reducing photosynthesis. Melatonin exogenously protects chlorophyll by reducing lipid peroxidation and regulating chlorophyll-degrading genes (Zahra et al., 2023). Melatonin enhances chloroplast levels, improves the Fv/Fm ratio, and prevents chloroplast damage (Rehaman et al., 2021; Karumannil et al., 2023). Melatonin also increases compatible osmolytes and phenol, improving turgor pressure and protecting cellular structures during stress (Moustafa-Farag et al., 2020; Khalid et al., 2022). Additionally, melatonin reduces hydrogen peroxide and ion leakage, enhancing plant survival under drought stress (Tiwari et al., 2021).

When oxidative damage occurs, plants activate antioxidant systems. Melatonin enhances H+-ATPase pump activity by converting to 5-methoxytryptamine, stabilizing the plasma membrane during stress (Siddiqui et al., 2021). Additionally, phenolic and flavonoid hydroxyl groups inhibit free radicals, protecting cell membranes (Jafari and Shahsavar, 2021). On the other hand, in our experiment, root length increased under drought stress. It seems that plants increase the length of their roots to escape from drought stress conditions, so that they can overcome the stress limitation by increasing the absorption of water and nutrients (Ranjan et al., 2022) .

Conclusion

The results of the present experiment showed that salicylic acid and melatonin had a positive effect on the activity of different antioxidant enzymes such as CAT, SOD, and POD, at mild and severe drought stress. The positive effect of these elicitors on the enzyme activity was also significant in the absence of drought stress. Additionally, salicylic acid and melatonin significantly increased the dry weight of the plant in mild drought stress. The number of roots increased with the application of these elicitors at this treatment. The application of salicylic acid increased root length, while melatonin increased the number of roots. Finally, it can be concluded that although the plant's response to stress and elicitors varied in terms of different characteristics, generally, salicylic acid and melatonin have a positive effect on the biochemical and morphological characteristics of the *Acorus calamus* medicinal plants under mild and severe drought stress.

Supplementary Materials

The supplementary material for this article can be found online at: https://www.jpmb-gabit.ir/article_721544.html.

Supplementary Table S1. Supplementary Figure 1. Analysis of variance related to antioxidant enzyme activity and protein content traits in response to mild and severe drought stress and elicitors treatments.

Supplementary Table S2. Analysis of variance of fresh weight (FW), dry weight (DW), number of roots (NOR), number of leaves (NOL), length of roots (LOR) and number of internodes (NOIN) in response to mild and severe drought stress.

Author Contributions

Conceptualization, G.R. and A.A.; methodology, A.A.; software, S.J.H.; validation, G.R., V.G. and A.A.; formal analysis, S.J.H.; investigation, A.A.; resources, G.R.; data curation, G.R.; writing—original draft preparation, S.J.H.; writing—review and editing, G.R.; visualization, V.O.; supervision, G.R.; project administration, G.R.; funding acquisition, G.R. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest Statement

The authors declare no conflict of interest.

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مقاله پژوهشی

بررسی تاثیر الیسیتورهای اسید سالیسیلیک و ملاتونین بر فعالیت آنزیم‌های آنتی‌اکسیدان و خصوصیات مورفولوژیک گیاه دارویی *Acorus calamus* تحت تنش خشکی

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چکیده: گیاهان دارویی به بهبود سلامت انسان کمک می‌کنند، تقویت سیستم ایمنی، کاهش التهاب و درمان بیماری‌ها را تسهیل می‌کنند. برای بررسی تأثیر تنش خشکی و محلول‌پاشی ملاتونین و اسیدسالیسیلیک بر شاخص‌های بیوشیمیایی و مورفولوژیکی گیاه دارویی *Acorus calamus*، آزمایشی انجام شد. نتایج نشان داد که کاربرد الیسیتورهای ملاتونین و اسید سالیسیلیک موجب افزایش فعالیت آنزیم پراکسیداز در شرایط تنش خشکی ۱۰ و ۲۰ روز پس از قطع آبیاری شد. همچنین اسید سالیسیلیک در تیمار ۱۰ روز پس از تنش موجب افزایش فعالیت آنزیم کاتالاز شد. کاربرد ملاتونین و اسید سالیسیلیک در ۲۰ روز پس از تنش موجب افزایش فعالیت آنزیم کاتالاز شد. در شرایط عدم تنش، کاربرد تیمارهای اسید سالیسیلیک و ملاتونین در ۱۰ روز پس از قطع آبیاری موجب افزایش وزن خشک گیاه در مقایسه با تیمار شاهد شد. کاربرد ملاتونین در شرایط تنش خشکی (۲۰ روز بعد از تنش) موجب افزایش وزن خشک در مقایسه با تیمار شاهد شد. در نهایت، در حالی که پاسخ‌های گیاه به تنش و محرک‌ها در ویژگی‌های مختلف متفاوت بود، به‌طور کلی می‌توان گفت که اسید سالیسیلیک و ملاتونین تأثیر مثبتی بر ویژگی‌های بیوشیمیایی و مورفولوژیکی گیاه *A. calamus* تحت تنش خشکی داشتند.

کلمات کلیدی: شاخص‌های بیوشیمیایی، تنش خشکی، محلول‌پاشی، گیاه دارویی، شاخص‌های موفولوژیکی.

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Plant isoflavones as anticancer agents in nanomedicine

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Review paper

Abstract: Isoflavones are natural phytoestrogens and belong to a subgroup of flavonoids that can be found mainly in legume plants, especially soybeans (*Glycine max* L.) and the other plants of the Fabaceae family. They have a structure similar to 17β-estradiol, which allows them to bind estrogen receptors and exhibit estrogenic or antiestrogenic effects. They are found in non-fermented products mainly as glycosides. During the fermentation process, they are converted into bioactive aglycones. Isoflavones are also linked to antioxidant and therapeutic benefits, supporting bone health and cholesterol regulation. Soybean-derived isoflavones, especially genistein, show strong anticancer potential by inhibiting cell growth. Epidemiological studies indicate, that consumption of isoflavones may reduce the risk of some diseases, including breast cancer but these compounds' clinical application is limited due to poor bioavailability, solubility, and stability. Today, integrating traditional medicine and medicinal plants with modern medicine could offer new opportunities for treating various diseases. Nanocarriers such as liposomes, dendrimers, and nanoparticles have been developed to enhance the bioavailability and controlled release of soy isoflavones, enabling their targeted delivery to cancer cells while minimizing off-target effects. In this review, we examine how nanotechnology synergizes with *Glycine max*-derived isoflavones to enhance their bioavailability and biological activity, considering the challenges and limitations of this approach for therapeutic development.

Keywords: Phytoestrogens, nanotechnology, cancer, genistein.

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Introduction

Isoflavones are naturally occurring organic compounds related to flavonoids, abundantly presentin in soybeans and other legumes (Bustamante-Rangel et al., 2018; Rizzo and Baroni, 2018). They are phenolic compounds that contain hydroxyl groups attached to aromatic rings, making them a subclass of flavonoids. Isoflavones, a type of phytoestrogen, share structural similarities with 17β-estradiol, a human estrogen hormone. Because of these similarities, isoflavones can bind to estrogen receptors in the body and have either estrogenic or anti-estrogenic actions (Kurzer and Xu, 1997; Tham et al., 1998). Isoflavonoids are mostly found in leguminous plants, notably those of the Fabaceae family. Soybeans (*Glycine max*) are the richest sources, accounting for a significant portion of dietary consumption through items including soy flour, soy drinks, miso, and tempeh. Other legumes, such as red and white clover, alfalfa, and beans, contain these chemicals at various levels. In non-fermented soy products, isoflavonoids are largely found as glycosides (conjugated forms), which are transformed into the more physiologically active aglycones during fermentation procedures (Zaheer and Humayoun Akhtar, 2017; Křížová et al., 2019). Trace amounts of isoflavonoids are found in nuts, fruits, and vegetables, but their dietary contribution is minimal compared to soy-derived foods (Bustamante-Rangel et al., 2018). Isoflavonoids have attracted considerable attention for their potential health benefits, including antioxidant, estrogenic, and anti-estrogenic properties, which support their therapeutic applications (Křížová et al., 2019). They are suggested to improve bone density and prevent osteoporosis in postmenopausal women by mimicking estrogen's effects (Ye et al., 2006). Research further suggests that these substances, particularly soy isoflavones, may lower the incidence of hormone-dependent malignancies via pathways involving estrogen receptor signaling and antioxidant activity (Messina et al., 2006a; Messina and Hilakivi-Clarke, 2009). Additionally, these compounds have been associated with improved lipid profiles, including reduced LDL cholesterol and increased HDL cholesterol (Carroll, 1991; Chalvon-Demersay et al., 2017). Furthermore, these substances have been linked to better lipid profiles, including lower LDL and higher HDL cholesterol (Lethaby et al., 2007).

Nanotechnology, or the manipulation of materials at the nanoscale (1-100 nanometers), has transformed disease treatment, particularly cancer therapy, by allowing for precise, targeted, and efficient drug delivery via nanostructures such as liposomes, dendrimers, and nanoparticles (Nasrollahzadeh et al., 2019; Chehelgerdi et al., 2023). These developments enhance bioavailability, minimize toxicity, and facilitate controlled medication release, leading to significantly improved therapeutic outcomes. Nano-carriers allow for the targeted delivery of larger medication concentrations directly to sick cells, bypassing the constraints of standard therapies. This strategy reduces side effects and adverse responses, simplifies treatment processes, and improves therapeutic success rates (Emeje et al., 2012; Tran et al., 2020).

Breast cancer is the most often diagnosed disease in women and the leading cause of cancer-related death worldwide, with over 2.3 million new cases recorded each year (Sung et al., 2021; Bray et al., 2024). Significant survival inequalities persist despite improvements in targeted medicines, genetic profiling, and early detection. This is especially true in low- and middle-income countries, where timely screening and treatment are often inaccessible. Tumor heterogeneity, medication resistance, significant side effects, and the difficulty of controlling metastatic illness are among the treatment's obstacles (Lei et al., 2021). Age, genetic mutations, hormones, and lifestyle variables are among the risk factors that have been identified (Dumitrescu and Cotarla, 2005). Innovative strategies such as immunotherapy, nanotechnology, and personalized medicine are essential for overcoming these challenges, as they enhance treatment precision, reduce toxicity, and improve outcomes (Han et al., 2019; Li et al., 2021).

In this review, we examine the synergy between soy isoflavones and nanotechnology in breast cancer treatment. Although extensive research has evaluated the individual benefits of soy isoflavones and advanced nanotechnology-based drug delivery systems, there remains a notable gap in understanding their combined effects. Specifically,

it is unclear how integrating these natural compounds with nanotechnological strategies may enhance therapeutic efficacy, reduce side effects, and overcome the limitations of traditional therapies.

Application of isoflavones in medicine and human health

Anticancer properties

Isoflavones have antioxidant characteristics, suppress cancer cell growth, and are anti-inflammatory, making them promising cancer preventive medicines (Sohn et al., 2021). These chemicals have been investigated in terms of cancer prevention, including breast, uterine, and prostate cancers (Horn-Ross et al., 2003; Messina et al., 2006a; Messina et al., 2006b; Van der Eecken et al., 2023). Some research has found that in Asian communities with greater soy intake (20-30 g per day), there is an inverse link between soy isoflavone consumption and breast cancer risk. This contrasts with Western populations, where soy consumption is far lower (Xiao, 2008; Dong and Qin, 2011). As a result, soy food consumption in Western countries has increased over the past few decades, driven by the belief in their health benefits, especially their role as functional foods rich in phytochemicals (He and Chen, 2013).

Cardioprotective and anti-atherosclerotic effects

Particularly in communities who consume a lot of soy, such those in Asia, isoflavones have been strongly linked to cardiovascular health. where there are reduced cardiovascular disease rates (Gómez-Zorita et al., 2020). Some studies have shown that genistein, enhances the resistance of low-density lipoprotein cholesterol (LDL-C) to oxidation in vitro, a crucial factor since oxidized LDL-C contributes to atherosclerosis (Setchell and Cassidy, 1999). Furthermore, by raising high-density lipoprotein (HDL) cholesterol and decreasing low-density lipoprotein (LDL) cholesterol, isoflavone consumption improves the blood lipid profile, improves vascular function, and helps prevent atherosclerosis and cardiovascular diseases(Carroll, 1991; Mejia et al., 2019) Beyond lipid regulation, isoflavones promote vascular health by interacting with estrogen receptor β (ERβ), stimulating the release of nitric oxide (NO), a vasodilatory agent. This results in improved endothelial function, blood vessel relaxation, and better circulation (Silva, 2021). Furthermore, isoflavones like equol, a metabolite produced from the isoflavone daidzein by gut microbiota, may play a central role in the cardioprotective effects of soy isoflavones. According to studies, equol has vasodilatory, anti-inflammatory, and antioxidant qualities that help to maintain healthy arteries and prevent atherosclerosis. Its interaction with ERβ is the main mechanism by which these effects are mediated (Zhang et al., 2021). In general, these findings highlight the protective effects of soy isoflavones on cardiovascular health and their role in preventing atherosclerosis.

Prevention of osteoporosis

Extensive studies have been conducted on isoflavones' possible function in osteoporosis prevention, particularly among postmenopausal women. Isoflavones bind to estrogen receptor beta (ERβ) and operate as selective estrogen receptor modulators, reducing bone loss caused by low estrogen levels during menopause (Morito et al., 2001). Also, isoflavones inhibit osteoclast activity (cells responsible for bone resorption) and promote osteoblast function (cells responsible for bone formation), contributing to the maintenance or increase of bone mineral density (BMD) (Onoe et al., 1997).

Some research show that consuming more soy products, soy protein, or soy isoflavones improves bone resorption or lowers bone mass loss. For example, a meta-analysis of 18 randomized controlled trials conducted between 1995 and 2019 investigated how soy isoflavones affected bone mineral density (BMD) in menopausal women. The study discovered that a daily dose of 106 mg of isoflavones for 6-24 months significantly increased BMD in the lumbar spine, femoral neck, and total hip (Barańska et al., 2022). Also in a similar meta-analysis looked at the effects of soy isoflavone extracts (not soy protein or foods) on bone mineral density (BMD) in menopausal women. Data from 11 randomized controlled trials involving 1,240 women found that taking 82 mg of soy isoflavones daily for 6-12 months increased lumbar spine BMD by 2.38% (Taku et al., 2010).

Hormonal regulation and relief of menopausal symptoms

Phytoestrogens, particularly isoflavones, are used as an alternative to hormone replacement therapy for menopausal symptoms. Because of their structural similarity to estrogen, they can have estrogenic or anti-estrogenic effects, which can help with hot flashes and hormonal imbalances. Modulating estrogen receptors improves overall health (Howes et al., 2006; Lethaby et al., 2007). For instance, a 12-week randomized study examined the effects of a low-fat, vegan diet with daily soybeans on menopausal symptoms in 38 postmenopausal women experiencing hot flashes. The intervention group experienced a 79% reduction in total hot flashes and an 84% decrease in moderate-to-severe hot flashes, compared to 49% and 42% reductions in the control group, respectively (Barnard et al., 2021). Similarly, a meta-analysis analyzed 15 randomized controlled trials involving perimenopausal and postmenopausal women to determine the efficacy of phytoestrogens in relieving menopausal symptoms and discovered that phytoestrogens significantly reduced hot flash frequency when compared to placebo (Chen et al., 2015).

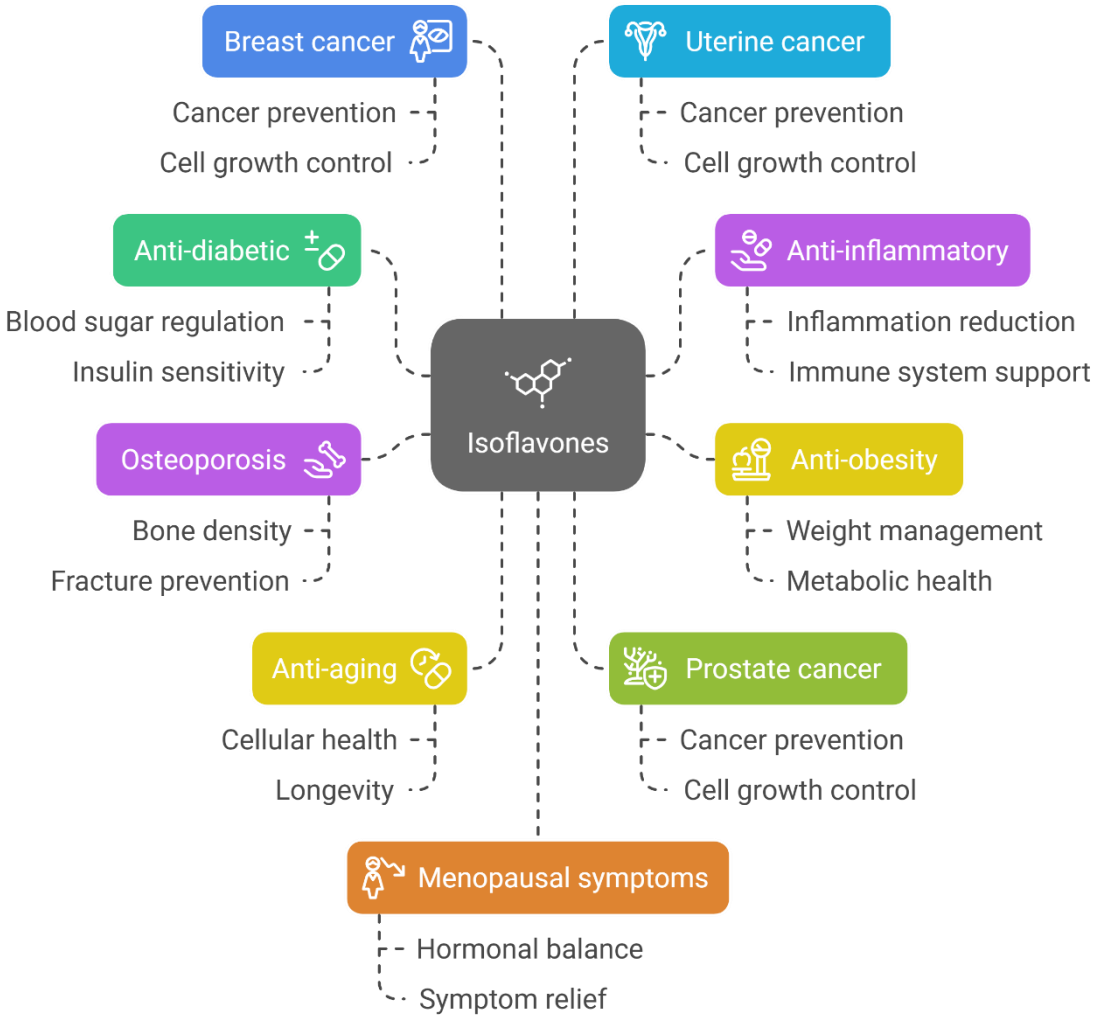


Figure 1. Health benefits and disease-preventive effects of isoflavones in humans.

Antidiabetic effects

Isoflavones, active compounds found in soy, help regulate glucose metabolism and improve insulin sensitivity. According to studies, consuming these compounds can help reduce insulin levels and improve blood sugar control, which is very effective in preventing the onset of type 2 diabetes. Genistein protects pancreatic β-cells from damage and improves insulin secretion. The effect is mediated by the activation of cAMP/PKA signaling pathways, which play a crucial role in maintaining β-cell function and mass. (Gilbert and Liu, 2013). Also, Soy isoflavones may improve lipid and glucose metabolism by acting as antidiabetic PPAR agonists. In obese Zucker rats, a high-isoflavone soy protein diet reduced liver triglyceride and cholesterol levels significantly more than low-isoflavone diets (Mezei et al., 2003). Also Isoflavones' antioxidative properties reduce oxidative stress, which can lead to insulin resistance and β-cell dysfunction (Umeno et al., 2016).

Antioxidant properties

In addition to the advantages listed above, isoflavones scavenge free radicals and help minimize oxidative stress in cells. This antioxidant activity promotes cell health, reduces chronic inflammation, and protects bodily tissues from oxidative damage (Figure 1) (Pietta, 2000; Sohn et al., 2021). Numerous studies have shown that eating soy and its products is particularly effective at reducing oxidative damage (Kim et al., 2021; Wójciak et al., 2024). For example, Genistein, a powerful soybean isoflavone, effectively reduces reactive oxygen species production while increasing antioxidant enzyme activities, resulting in delayed tumor development and decreased tumor multiplicity in mouse models (Wei et al., 1995).

Isoflavones as bioactive compounds with anticancer potential

Isoflavones, particularly those made from soybeans, are bioactive polyphenols of the flavonoid family that exhibit anticancer activity. Soy isoflavones, including genistein, daidzein, and glycitein, occur in both free (aglycone) and conjugated forms, such as glucosides, malonylglucosides, and acetylglucosides (Figure 2) (Wang and Murphy, 1994b; Sohn et al., 2021). High-performance liquid chromatography analyses reveal that soybeans contain 12 isoflavone isomers, with major forms being genistin, daidzin, and glycitein, along with their malonyl- and acetyl-derivatives (Wang and Murphy, 1994a; b). Isoflavones are produced through the phenylpropanoid pathway, which enables plants to synthesize various secondary metabolites, including lignin, flavone, flavonol, anthocyanin, and tannin. Isoflavones are synthesized from intermediate substrates in the phenylpropanoid pathway, specifically naringenin and liquiritigenin. Naringenin is found in most plants, and it is used to make other phenylpropanoid compounds like flavones, flavonol, and anthocyanin (Figure 3) (Sohn et al., 2021).

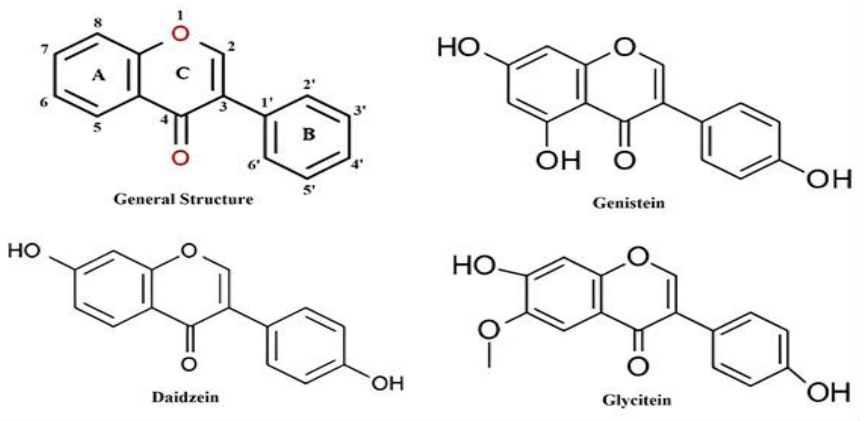


Figure 2. Chemical structure of; Genistein, Daidzein and Glycitein (Sohn et al., 2021).

Soy isoflavones, particularly genistein, have potent anti-proliferative effects in cancer cells by controlling key apoptotic and survival pathways. Pro-apoptotic mechanisms primarily involve mitochondrial-dependent pathways and the activation of caspases such as caspase-3, -7, and -9, which are critical for programmed cell death (Su et al., 2003; Yang et al., 2007; Charalambous et al., 2013). Genistein has been demonstrated to downregulate anti-apoptotic proteins such as Bcl-2 and Bcl-xL while upregulating pro-apoptotic Bax, resulting in cytochrome-c release and apoptosis (Su et al., 2003; Jin et al., 2010; Sakamoto et al., 2010). Other strategies include generating endoplasmic reticulum stress markers such as DNA damage-inducible transcript 3 (DDIT3) and activating stress response pathways (Obiorah et al., 2014). Genistein's actions are dose-dependent, with low doses regulating survival signals through pathways such as Bax/Bcl-2 and higher doses activating alternative cytotoxic pathways regardless of estrogen receptor (ER) status (Lucki and Sewer, 2011).

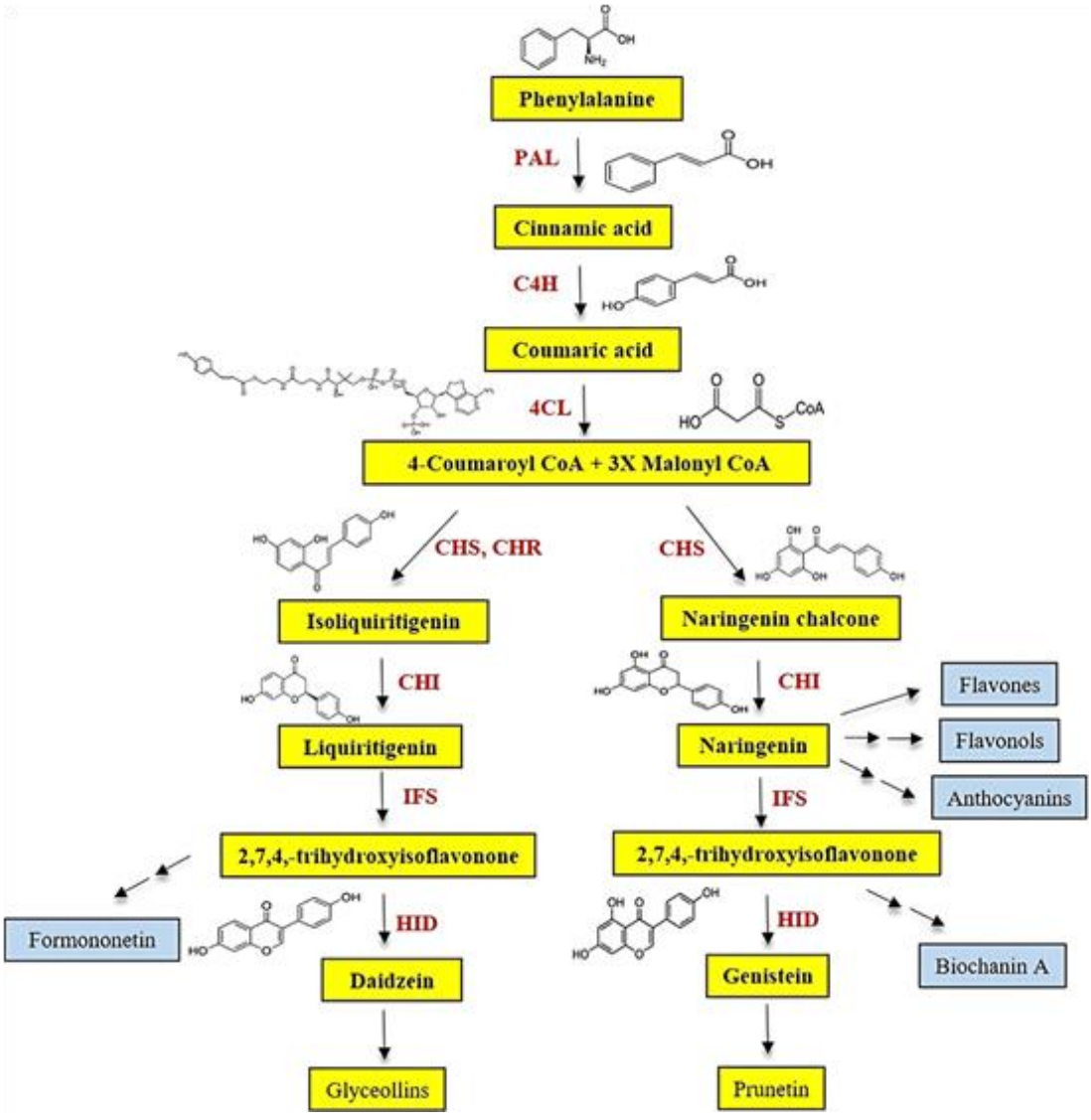


Figure 3. Partial phenylpropanoid pathway for the isoflavone biosynthesis (Sohn et al., 2021).

Genistein suppresses NF-κB pathway activation, lowering the transcription of genes linked to chemoresistance and survival, especially in aggressive breast cancer subtypes (Pan et al., 2012). Genistein's antimetastatic effects are mediated by angiogenesis inhibition and metastatic marker suppression. Genistein inhibits vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs), which are required for angiogenesis and extracellular matrix remodeling. Genistein at high concentrations (>10 μM) reduces VEGF secretion and chemoinvasion by downregulating pro-angiogenic factors and upregulating inhibitors, such as tissue factor pathway inhibitor-2 (Lee et al., 2007; Hsu et al., 2009). It has also been shown that the PI3K/Akt/mTOR and MAPK/ERK pathways, which are critical for cell survival and metastasis, are targeted by genistein (Li et al., 2008; Ahmad et al., 2013). Thus, while genistein's anti-metastatic effects are promising, further research is essential to elucidate tissue-specific mechanisms and optimize its clinical application.

Researchers have conducted significant research on soy isoflavones, plant-derived chemicals with estrogen-like action, and their function in breast cancer prevention and therapy. Several meta-analyses and observational studies have found that increased soy isoflavone intake is linked to a lower risk of breast cancer, notably in Asian communities where soy consumption is traditionally higher. A meta-analysis of 21 studies (case-control and cohort) indicated a pooled relative risk (RR) of 0.75 for breast cancer associated with soy food consumption, showing a preventive effect, with tofu and miso specifically lowering risk by up to 20% (Qin et al., 2006). A broader meta-analysis of 35 trials found that soy isoflavones were protective for pre- and postmenopausal women in Asian nations, although the benefits were less consistent in Western populations (Chen et al., 2014). studies conducted in high-soy-consuming Asian populations revealed a clear trend of decreased breast cancer risk with increasing soy isoflavone consumption, with a 29% risk reduction observed at higher intake levels (≥20 mg/day) compared to low intake levels (≤5 mg/day) (Wu et al., 2008). Similar findings were made by the 300,000-woman China Kadoorie Biobank study, which showed that moderate soy consumption did not significantly affect the risk of breast cancer, but higher intake levels showed a preventive benefit, reducing the incidence by 3% for every 10 mg/day increase in soy isoflavones (Wei et al., 2020). these findings highlight the preventive effects of soy in Asian dietary patterns, which contrast with the insignificant impact reported in Western cultures where soy intake levels are significantly lower (Brouns, 2002). Further research has looked into the impact of menopausal status and hormone receptor profiles on the soy-breast cancer link. Epidemiological research, like the Shanghai Women's Health Study, discovered that increased soy consumption during adolescence and adulthood was associated with a lower risk of breast cancer, particularly in premenopausal women (Baglia et al., 2016). Stratified analyses revealed that soy isoflavones reduced the risk of estrogen receptor-positive/progesterone receptor-positive (ER+/PR+) breast cancer in postmenopausal women and ER-negative/PR-negative (ER−/PR−) breast cancer in premenopausal women. Also these compund preventive effects were more prominent in breast cancer subtypes determined by hormone receptor status, such as ER+/PR+/HER2− tumors, highlighting the significance of receptor specificity in determining its benefits (Suzuki et al., 2008). These findings emphasize soy isoflavones as a possible dietary component for personalized breast cancer preventive methods.

Innovative nanotechnology advances for enhancing soy isoflavones stability, bioavailability, and delivery systems

Multiple studies have demonstrated innovative methods to enhance the bioavailability and stability of isoflavones through nanotechnology. The selection of these nanoformulation techniques improves biodistribution, pharmacokinetics, bioavailability, and specificity of drug transport to targeted sites (Figure 4) (Sindhu et al., 2021).

Oral bioavailability and nutraceutical enhancement

Nano spray drying has been utilized to produce stable nanoparticles of soy isoflavones with carboxymethyl cellulose, which improves water solubility, bioavailability, and penetration through

biological membranes, with up to 4.5 times the efficiency of the raw material (Del Gaudio et al., 2017). Similarly emulsification-evaporation encapsulation has created small, stable whey protein nanoparticles with high encapsulation efficiency (91.29–92.59%), significantly enhancing antioxidant activity and bioaccessibility (Liu et al., 2022). These findings indicate how nanoparticle-based systems can be used to improve the solubility and bioavailability of isoflavones, which will increase their usefulness in dietary and nutraceutical applications. Encapsulation methods for soy isoflavones using protein-based nanocarriers have demonstrated significant potential in food applications. Isoflavone encapsulation with nanoparticles made from goat milk whey protein and polymerized whey protein has shown high encapsulation efficiencies, improved stability, and better intestinal release during digestion. These systems limit isoflavone degradation under acidic and hot conditions, hence enhancing bioavailability and transport efficiency (Tian et al., 2020; Liu et al., 2022; Tian et al., 2024). Nanotubes made from α-lactalbumin can deliver soy isoflavones in functional food products, improving dispersibility and texture (Liu et al., 2023). These advancements show the potential of nanoscale systems in adding bioactive compounds like isoflavones into functional foods with increased stability and delivery capabilities.

Dermal delivery systems

Topical delivery systems have shown significant potential for enhancing the use of isoflavones in dermatological applications. Solid-lipid nanoparticles incorporated into a topical gel were found to improve skin deposition and demonstrated excellent safety, showing no irritation (Deshmukh and Amin, 2013).

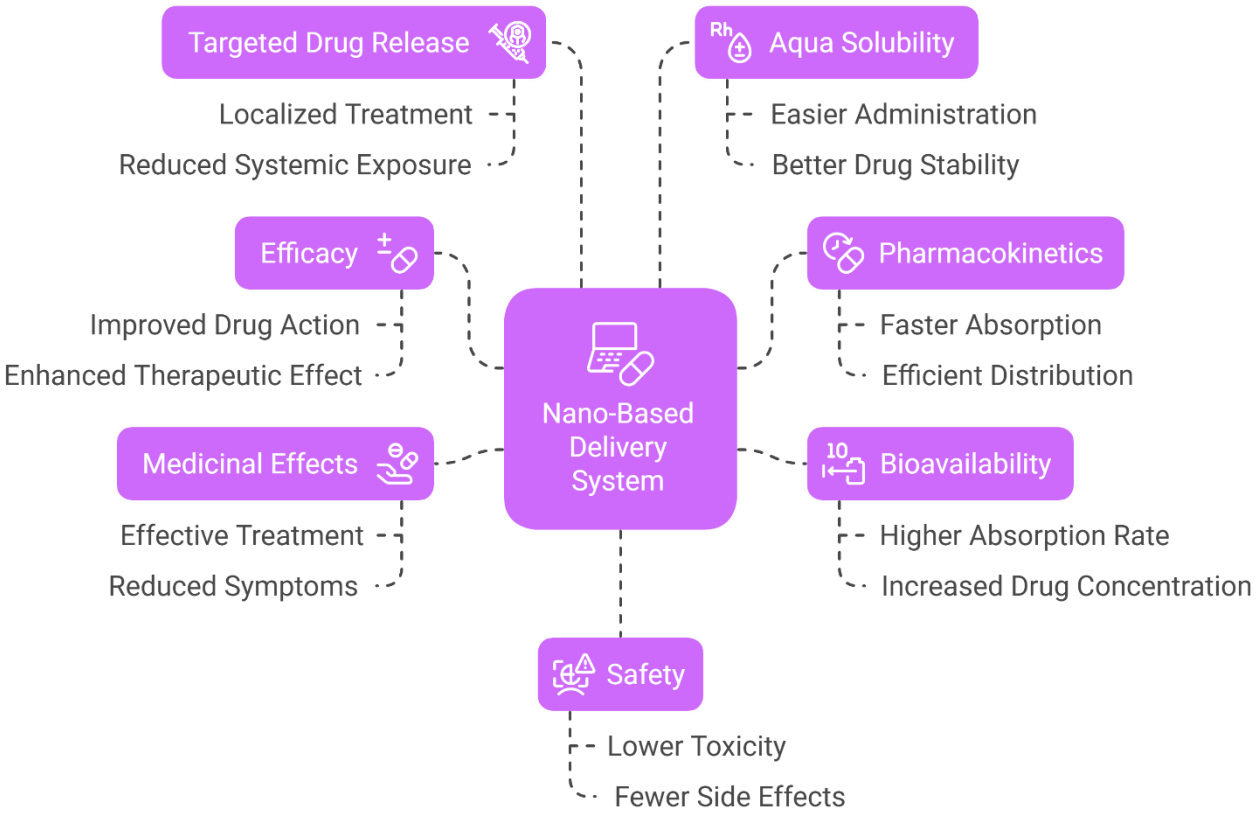


Figure 4. Benefits of nano delivery systems.

Genistein nano-emulsions created through spontaneous emulsification exhibited controlled permeation and efficient retention in the skin (Silva et al., 2009). Also, hydrogels that contain genistein-loaded nano-emulsions have been demonstrated to improve delivery through the skin while displaying desirable rheological properties (de Vargas et al., 2012). Additionally, hydrogels and nano-emulsions that incorporate isoflavones into dermal formulations promote retention within skin layers, ensuring effective delivery for topical applications (Nemitz et al., 2019). With implications in anti-aging and skin health, these results show how well nanocarriers, such as solid-lipid nanoparticles and nano-emulsions, improve cutaneous absorption and stability of isoflavones.

Systemic and multifunctional therapeutic delivery

Nanoencapsulation strategies offer significant advantages for the delivery of soy isoflavones by enhancing their pharmacokinetics and therapeutic efficacy. Slow-release formulations including polymer matrices such as hydroxypropyl-cellulose and ethyl-cellulose lengthen isoflavones residence period in plasma, resulting in a consistent therapeutic effect (Setchell et al., 2005). Similarly, spiral dextrin inclusion complexes and sustained-release microspheres provide controlled release and improved stability, making them ideal for gastrointestinal absorption and systemic effects (Setchell et al., 2005). Similarly, spiral dextrin inclusion complexes and sustained-release microspheres provide controlled release and improved stability, making them ideal for gastrointestinal absorption and systemic effects (Du et al., 2013; Wang et al., 2018).

Furthermore, hydrogel beads made from soybean husk polysaccharides (SHP) have shown effective pH-responsive release, making them appropriate for a variety of applications (Wang et al., 2020). Sustained-release microspheres made of chitosan and sodium alginate sustain steady plasma levels of isoflavones, resulting in increased biological benefits such as anti-aging qualities and antioxidant capacity (Du et al., 2013). These nanoscale systems overcome limitations such as rapid metabolism and low bioavailability, ensuring enhanced functionality. The use of soy isoflavones in multimodal delivery methods indicates their therapeutic and preventive health benefits. Soy isoflavones integrated into 3D-printed tricalcium phosphate scaffolds inhibit localized malignancies while stimulating bone regeneration, suggesting its potential in post-surgical settings (Sarkar and Bose, 2020). Pharmacokinetic studies show that isoflavones have low toxicity and rapid clearance, making dietary supplements viable for a variety of health benefits, including cancer chemoprevention (Busby et al., 2002). The encapsulation of isoflavones in rice protein nanoparticles demonstrates the compounds' versatility in achieving sustained and targeted release, which is critical for maximizing their therapeutic potential (Mo et al., 2023).

Synergistic role of nano-isoflavones in breast cancer treatment

Soy isoflavones particularly genistein, are potential anti-breast cancer therapeutic agents due to their anti-proliferative and apoptotic activities. Genistein's function in modulating molecular signaling in breast cancer cells, by downregulating estrogen receptor α (ERα) and serum response factor (SRF), has an important role in its inhibitory effects. Genistein has been shown to inhibit cell growth during the G2/M phase. also this compound reduces the proliferative S phase in MCF-7 breast cancer cells, demonstrating its efficacy in preventing cancer development. These effects are partially mediated by estrogen receptor-dependent pathways, highlighting the complexity of the process (Chen et al., 2003). Despite its therapeutic potential, the clinical application of genistein is hampered by its poor bioavailability, solubility, and stability. Nanotechnology-based solutions have addressed these challenges by developing advanced delivery systems. A study explored the formulation of PEGylated silica nanoparticles containing genistein (Gen-PEG-SiHNM), which notably enhanced

genistein's dispersion, release profiles, and cytotoxic effectiveness (Figure 5). The nanoparticles are roughly 33 nm in size and have a positive surface charge. Also enhanced genistein's water solubility and enabled pH-dependent drug release and the biological examination revealed that encapsulation increased genistein's antioxidant activity while also activating dual cell death

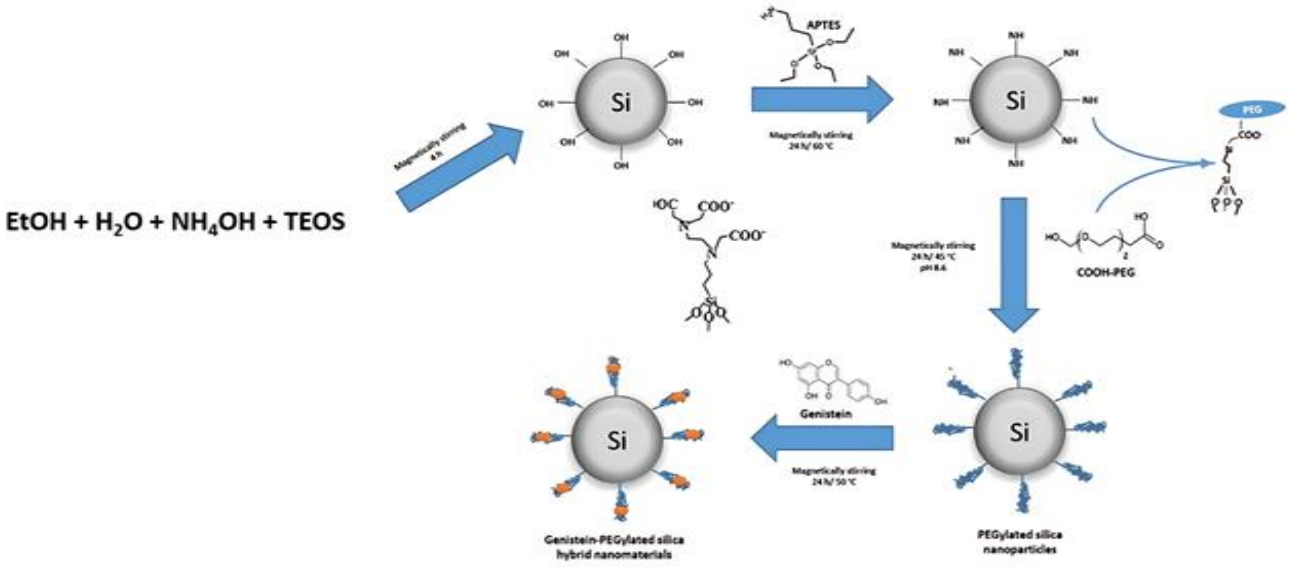


Figure 5. Schematic illustration of the development process for hybrid nanomaterials composed of PEGylated silica nanoparticles and genistein (Pool et al., 2018).

processes, apoptosis, and autophagy. But In contrast, free genistein largely triggered apoptosis, albeit with lower efficiency. This study highlights the potential of Gen-PEG-SiHNM as a more effective, multifunctional therapeutic agent with promising breakthroughs in targeted cancer therapy tactics (Pool et al., 2018; Sindhu et al., 2021). Increased genistein's solubility by more than 350 times compared to free genistein, boosting its therapeutic potential (Figure 6). This study demonstrate GenLip, provides continuous medication release while keeping genistein's antioxidant properties and in breast cancer models these liposomes had robust anticancer effects, with concentration-dependent and time-dependent cytotoxicity and much lower IC50 values than the non-encapsulated drug. Furthermore, GenLip effectively activated P53-independent apoptotic pathways through mitochondrial polarization and caspase-3/7 activation, leading in DNA fragmentation and apoptosis. The liposomal delivery technique significantly reduced off-target effects, increasing the selectivity of genistein's activity on breast cancer cells while sparing healthy cells (Phan et al., 2013b).

Additionally, metallic complexes and bioengineered derivatives of genistein show the potential to further enhance its therapeutic profile. Copper (II)-genistein complexes, for instance, showed significantly enhanced cytotoxicity and antimetastatic effects, effectively arresting the cancer cell cycle at the G2/M transition and remodeling the actin cytoskeleton to promote cell-cell adhesion and reduced cellular migration and metastasis by suppressing the expression and secretion of metastasis-associated matrix metalloproteinases, further illustrating the promise of nanotechnology-enhanced delivery systems in cancer therapy (Spoerlein et al., 2013).

Challenges and limitations

Despite isoflavones' significant therapeutic potential, poor bioavailability, quick degradation, and difficult extraction procedures prevent soy isoflavones from being used in clinical settings, despite their potential (Amawi et al., 2017). Even though nanotechnology provides ways to improve efficacy and delivery, problems including toxicity, scalability, and regulatory issues still exist and overcoming these mentioned constraints is critical for developing soy isoflavone-based cancer treatments (Gao et al., 2024).

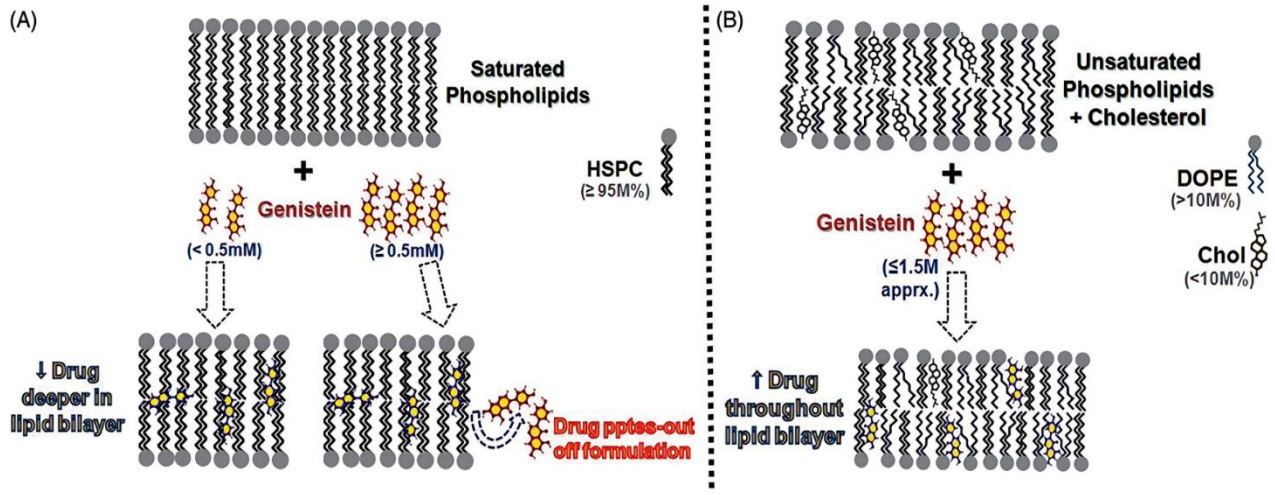


Figure 6. Schematic models illustrating genistein incorporation into liposomal membranes: (A) using rigid, highly saturated lipid components; and (B) employing flexible, unsaturated lipids combined with cholesterol (Phan et al., 2013a).

Intrinsic limitations of soy isoflavones

The use of soy isoflavones such as genistein in cancer treatment has numerous inherent problems. Poor solubility, limited bioavailability, and vulnerability to environmental conditions including temperature and pH restrict their therapeutic potential (Gao and Hu, 2010; Amawi et al., 2017; Gao et al., 2024). Furthermore, their quick breakdown by intestinal microbiota and limited oral absorption result in adverse pharmacokinetics, preventing efficient clinical application (Amawi et al., 2017). Complex techniques for isoflavone extraction, separation, and purification exacerbate the issues, with low yields and high costs limiting scalability. Traditional methods such as Soxhlet, maceration, percolation, and reflux extraction may need several kilograms of plant material to yield less than one gram of isolated chemicals (Hossain and Rahman, 2015; Blicharski and Oniszczuk, 2017). In contrast, modern techniques such as ultrasound-assisted extraction (UAE), microwave-assisted extraction, accelerated solvent extraction, supercritical fluid extraction, and negative pressure cavitation extraction offer more efficient solutions (Blicharski and Oniszczuk, 2017). For example, research found that the UAE produced considerable extraction efficiencies for target isoflavones after 45 minutes, indicating the possibility of more efficient recovery (Sun et al., 2011). Despite these breakthroughs and novel approaches, commercial-scale extraction is still economically problematic. Furthermore, the structural complexity and unpredictability of these chemicals make them susceptible to changes during manufacture, reducing their efficacy and also these issues demand novel techniques to address constraints in stability, delivery, and bioactivity (Amawi et al., 2017).

Nanotechnology-specific challenges

Nanotechnology offers promising solutions, but it introduces its own set of limitations. Formulating nanocarriers, such as liposomes and other delivery systems, to improve genistein bioavailability and therapeutic efficacy presents technological and financial obstacles. For instance, whereas platelet membrane-coated liposomes increase targeted distribution and stability, their synthesis needs precise optimization and has scaling problems (Gao et al., 2024). Furthermore, toxicological issues and the possible immunogenicity of nanocarriers remain substantial barriers (More et al., 2021; Gao et al., 2024). For instance, both the U.S. Food and Drug Administration (FDA) and European Medicines

Agency (EMA) emphasize the need for a comprehensive characterization of nanomaterials, including an assessment of their physicochemical properties, stability, and potential immunogenicity, and underscore the importance of nonclinical studies to evaluate safety and efficacy. They have created scientific recommendations to address quality, safety, and efficacy while preparing marketing authorization applications for human drugs, including nanomedicines (Hamburg, 2012; Hafner et al., 2014; Sainz et al., 2015). Moreover, cancer cell mechanisms of resistance and the necessity of advanced nanotechnology-based combination therapy hold back clinical translation (More et al., 2021). Addressing these challenge and problems is very impotante to realizing the full potential of soy isoflavones via nanotechnology.

Future directions and prospects

Future research into soy isoflavone nanotechnology should focuses on overcoming challenges such as poor solubility, limited bioavailability, and instability. Genistein, a prominent soy isoflavone, has been demonstrated to have potent anticancer properties, targeting a variety of pathways such as apoptosis, angiogenesis, and metastasis. Nanotechnology is being researched to increase the therapeutic efficacy of genistein. Nanocarriers such as liposomes, nanoparticles, and biomimetic systems show promise in terms of delivery and stability (Spagnuolo et al., 2015). However, these advancements must address concerns such as scalability, production costs, and long-term stability in therapeutic applications. Continued advancements in nanotechnology may lead to the development of formulations capable of overcoming physiological barriers, increasing drug-targeting specificity, and decreasing side effects, especially when used in combination therapy (Spagnuolo et al., 2015; Andreani et al., 2024).

Numerous clinical trials, including "Soy Isoflavones and Breast Cancer Risk Reduction" (NCT00204490, Phase II), "Effects of Soy Compounds on Breast Cancer, Prostate Cancer, and Bone Health" (NCT00200824, Phase II), and "Soy Isoflavones Supplementation in Treating Women at High Risk For or With Breast Cancer" (NCT01219075), have been conducted to examine the effects of isoflavonoids on cancer prevention and treatment. However clinical translation of soy isoflavones nanotechnology continues in its infancy, with minimal clinical studies completed to date. To close the gap between preclinical success and clinical application, future research should focus on refining nanocarrier design and investigating combination treatments that use soy isoflavones in conjunction with traditional chemotherapeutics (Andreani et al., 2024). Soy isoflavones, after nanotechnology-guided assistance and better understanding of their processes and safety profiles, have wide potential for use as low-cost, non-invasive cancer therapies, particularly concerning drug resistance and therapeutic efficacy. Development on this field requires overcoming the regulatory challenges and establishing standardized, safe preclinical and clinical research protocols (Spagnuolo et al., 2015; Andreani et al., 2024).

Conclusion

Nanotechnology-derived ERα targeting offers new hope for the future of breast cancer treatment by directly linking estrogen receptor modulation with advanced nanocarrier systems. Soy isoflavones, especially genistein, exert their anticancer effects via targeted ERα modulation, but their curative potential has been undermined by poor aqueous solubility, low bioavailability, and instability. New developments such as the synthesis of pH-responsive soybean hull hydrogel beads and liposomal drug delivery systems illustrate a new and groundbreaking approach that not only enhances site-specific targeted drug delivery but also allows for controlled release in various physiological environments. Also, these advanced nanocarriers have greatly enhanced the pharmacokinetics and clinical efficacy of soy isoflavones through delivery into the cellular environment and by initiating mechanisms such as apoptosis, inhibition of angiogenesis, and metastasis prevention. While issues of production scalability, cost, and toxicity remain, recently developed strategies such as modular nanocarrier design and more efficacious extraction processes offer promising means for overcoming these limitations. In the future, greater interdisciplinarity effort will be required to optimize these scalable delivery vehicles and incorporate them into

combination therapies. Such efforts will open new gates for nanotechnology-enhanced treatments that can transform breast cancer management and offer enduring hope for improved therapeutic outcomes.

Supplementary Materials

No supplementary material is available for this article.

Author Contributions

For this review article the Conceptualization, methodology, validation, investigation, resources, data curation and writing—original draft preparation performed by Ali Yousefian. The writing—review and editing, visualization, supervision, and project administration were performed by Fatemeh Moradian.

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Conflict of Interest Statement

The authors declare no conflict of interest.

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چکیده: ایزوفلاون‌ها فیتواستروژن‌های طبیعی هستند و به زیرگروهی از فلاونوئیدها تعلق دارند که عمدتاً در گیاهان حبوبات، به‌ویژه سویا (*Glycine max*) و سایر گیاهان خانواده *Fabaceae* یافت می‌شوند و ساختاری مشابه β-۱۷ استرادیول دارند که به آن‌ها اجازه می‌دهد تا اثرات آنتی استروژنی استروژن و گیرنده‌های استروژن را نشان دهند. آنها در محصولات تخمیر نشده عمدتاً به صورت گلیکوزید یافت می‌شوند. در طی فرآیند تخمیر، آنها به آگلیکون های زیست فعال تبدیل می شوند. ایزوفلاون ها همچنین با مزایای آنتی اکسیدانی و درمانی مرتبط هستند و از سلامت استخوان و تنظیم کلسترول حمایت می‌کنند. ایزوفلاون های مشتق شده از سویا، به ویژه جنیستئین، با مهار رشد سلولی، پتانسیل ضد سرطانی قوی نشان می‌دهند. مطالعات اپیدمیولوژیک نشان می‌دهد که مصرف ایزوفلاون ها ممکن است خطر ابتلا به برخی بیماری ها از جمله سرطان سینه را کاهش دهد، اما کاربرد بالینی این ترکیبات به دلیل دسترسی زیستی، حلالیت و پایداری ضعیف محدود است. امروزه تلفیق طب سنتی و گیاهان دارویی با طب نوین می‌تواند فرصت های جدیدی را برای درمان بیماری‌های مختلف فراهم نماید. نانوحامل‌هایی مانند لیپوزوم‌ها، دندریمرها و نانوذرات برای افزایش فراهمی زیستی و آزادسازی کنترل‌شده ایزوفلاون‌های سویا ایجاد شده‌اند، و امکان تحویل هدفمند آن‌ها به سلول‌های سرطانی را فراهم می‌کنند و در عین حال اثرات خارج از هدف را به حداقل می‌رسانند. در این مطالعه، چگونگی هم‌افزایی نانوتکنولوژی با ایزوفلاون‌های حاصل از سویا برای افزایش دسترسی زیستی و فعالیت بیولوژیکی آن‌ها بررسی و چالش‌ها و محدودیت‌های این روش در مسیر توسعه‌ی کاربردهای درمانی را نیز مورد توجه قرار گرفت.

کلمات کلیدی: فیتواستروژن ها، فناوری نانو، سرطان، جنستین.

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Graphical analysis of genetic variability in essential oil profiles of *Anethum graveolens* L.

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Original paper

Abstract: Medicinal and aromatic plants have long played a vital role in health, wellness, and culinary practices. This research aimed to assess the genetic variation in essential oil content and its components among five local landraces of dill (*Anethum graveolens* L.) from Mashhad, Ardabil, Parsabad, Bushehr, and Esfahan. Essential oil and its composition, including β-pinene, α-terpinene, β-phellandrene, carvacrol, dillapiole, neophytadiene, and hexahydrofarnesyl acetone, were analyzed. The drawn biplot explained 90% of the variation, clearly depicting genotype-trait relationships. It revealed both additive and crossover interactions, suggesting that genotype rankings were trait-dependent. Among the evaluated genotypes, Bushehr exhibited the highest essential oil yield and quality, followed by Esfahan, Mashhad, Ardabil, and Parsabad. The strongest positive correlations were observed between β-phellandrene, dillapiole, and essential oil content, while the association between α-terpinene and β-pinene was more intricate and less pronounced. The superior performance of the Bushehr genotype makes it a prime candidate for future breeding initiatives. Furthermore, carvacrol emerged as the most discriminative trait for disticnting among genotype. The study also revealed that increased in essential oil content in *A. graveolens* was associated with higher production of dillapiole and β-phellandrene, coupled with reduced levels of β-pinene, neophytadiene, and hexahydrofarnesyl acetone.

Keywords: Biplot, carvacrol, distinction ability, ideal genotype.

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Introduction

Medicinal and aromatic plants play a crucial role in health, wellness, and culinary traditions. Among them, dill (*Anethum graveolens* L.), an annual herb from the Apiaceae family, is widely cultivated for its culinary, cosmetic, and medicinal applications. Native to southwestern and central Asia(Yaldız et al., 2018) , it is grown in various regions of Iran like shourthen, northeastern, northwestern, and central areas.

Dill's leaves and seeds are used in food, cosmetics, and health products. Its seeds and flowers contain high levels of essential oils, fatty acids, phenolic acids, and flavonoids, while stems and leaves have lower concentrations (Goodarzi et al., 2016). The composition of its essential oil, particularly α-phellandrene and germacrene D, varies with drying temperature, origin, and extraction methods. These compounds contribute to dill's antioxidant, antimicrobial, and antidiabetic properties, rendering it a valuable natural additive in pharmaceutical and health-related industries.

Dill's essential oil composition and bioactivity are influenced by genetic and environmental factors, as well as post-harvest practices (Ghassemi-Golezani et al., 2022). This variation, particularly linked to genetic diversity, has attracted significant research interest, highlighting its potential for optimizing therapeutic and economic benefits.*A. graveolens*.

Genetic diversity forms the backbone of plant adaptability, resilience, and metabolic versatility. In the case of *A. graveolensA. graveolens*, the genetic variability among populations and genotypes significantly influences the synthesis and accumulation of essential oil components (Biesiada et al., 2019). This variability is critical for several reasons; because it governs the plant's ability to thrive in diverse environmental conditions, including variations in soil type, climate, and water availability. Also, genetic diversity influences the production of specific phytochemicals, which are key to the plant's medicinal efficacy and economic value. Finally, understanding the genetic factors underlying essential oil composition is essential for breeding programs aimed at enhancing specific traits, such as higher essentail oil yield, improved bioactivity, or tolerance to environmental stressors (Gurav et al., 2022). The characteristics of *A. graveolens* essential oil during drying are influenced by factors such as drying method and temperature. Understanding the effects of geographical origin on essential oil yield and composition is vital, given dill's medicinal significance. Phytochemical diversity in dill is shaped by genetic and environmental factors, including location, soil, climate, water, harvest timing, growth stage, and plant parts used.

Global studies on dill have demonstrated substantial differences in essential oil profiles among populations from various regions. For instance, research has shown that *A. graveolens* cultivated in Mediterranean climates tends to produce essential oils with higher concentrations of carvone (El-Zaeddi et al., 2016), while plants grown in arid or semi-arid regions may exhibit increased levels of limonene and α-phellandrene (Amanpour et al., 2017). These differences are often attributed to genetic factors, as well as the interaction between genotype and environment. Such findings underscore the importance of exploring the genetic diversity of *A. graveolens*, particularly in regions with diverse ecological conditions. Iran is a region of considerable ecological diversity, encompassing a wide range of climates, altitudes, and soil types. This variation creates a unique opportunity to study the genetic and environmental influences on dill essential oil composition (Biesiada et al., 2019). The northeastern regions of Iran, with their arid climate and alkaline soils, may favor the production of certain terpenoids, while the more temperate conditions of the northwestern regions could enhance the biosynthesis of phenolic compounds (Farmanpour Kalalagh et al., 2023). Such regional differences provide a valuable framework for examining how genetic and environmental factors interact to shape the essential oil profile of dill. This study examines how various landraces influence the essential oil, and its composition in some Iranian *A. graveolens* genotypes.

Materials and Methods

Trial and traits

In the present study, five local landraces of dill (*A. graveolens* L.), originating from diverse regions of Iran, were evaluated. These regions included Mashhad (36° 19′ N, 59° 32′ E; altitude 995 m) in

the northeast, Ardabil (38° 15′ N, 48° 18′ E; altitude 1500 m) and Parsabad (39° 38′ N, 47° 54′ E; altitude 47 m) in the northwest, Bushehr (28° 55′ N, 50° 51′ E; altitude 8 m) in the southern part of the country, and Esfahan (32° 39′ N, 51° 40′ E; altitude 1590 m) in the central region. The research was conducted at a designated experimental field station in Ardabil, Iran, which is characterized by loamy soil. The research aimed to evaluate the genetic variation and phytochemical diversity of essential oils extracted from these landraces to assess their potential for medicinal and agricultural applications. Seeds of the five dill genotypes were collected and subjected to rigorous seed vigor testing to ensure uniformity in germination and initial growth potential. The seeds were sown in a research field following a completely randomized block design using three replications to ensure reliability. The field experiment was carefully managed, with plants irrigated twice a week to maintain optimal soil moisture levels conducive to dill growth. The plants were allowed to grow until they reached the full flowering stage, as this stage is known to yield the highest concentration of essential oils in dill. At this point, the leaves and flowers were harvested for subsequent analysis. The dried samples were ontined in room tempruture (25°C) for thrre days. The extracted essential oil was injected into a GC-MS system, which separates and identifies chemical constituents. A capillary column was used to achieve high-resolution separation. The sample was heated in a controlled manner, and components were detected based on their retention times and mass spectra. The primary compounds, including β-pinene (B-Pinene), α-terpinene (A-Terpinene), β-phellandrene (B-Phell), carvacrol, dillapiole, neophytadiene (Neoph), and hexahydrofarnesyl acetone (Hex-Ace), were identified using reference standards and spectral libraries. The relative percentage of each compound was calculated using peak area normalization (Farmanpour Kalalagh et al., 2023). Essential oil yield (EO) was determined as the percentage of extracted oil relative to the dry weight of the plant material.

Statistical analysis

The obtained data were subjected to normality testing using the Anderson-Darling test. Once data normality was confirmed, further statistical evaluations were conducted to examine the genetic and phytochemical variability among the dill genotypes. The principal component analysis (PCA) results were visualized using a genotype × trait interaction biplot model, which offers a graphical representation of the relationships between genotypes and traits. This analysis was performed using the equation:

$$\frac{x_{ij} - y_j}{SD_j} = \sum_{n=1}^{2} \lambda_n \xi_{in} \eta_{jn} + R_{ij}$$

where x_{ij} is showing the performance of genotype i in trait j, y_j is the average of all x_{ij} for trait j, SD_j is the standard deviation for trait j, λ_n is the singular value for PCAn, ξ_{in} and η_{in} are scores for genotypes i and trait j on PCAn, respectively, and R_{ij} is the residual of model. To compute symmetrical scales of traits and genotypes, the eigenvalue is adjusted through vectors' absorption. This adjustment ensures that the testers and entries are displayed in a normal and standardized manner. This approach also allows for a clear graphical representation of the relationships among genotypes and traits, along with their interactions, providing insightful visual interpretations. For the data scaling and centering process, specific parameters were applied to ensure proper analysis. Scaling was set to 1, which ensures that the data is appropriately adjusted for comparative analysis. Centering was applied using a factor of 2, allowing the data to be centered around the mean of each variable. The singular value decomposition (SVD) method was employed with an SVP value of 2 to calculate the singular scores. This method enables a deeper understanding of the underlying structure of the data. Additionally, an SVP value of 1 was used to generate singular scores that are ideal for visually interpreting the correlations among genotypes.

Results

The genotype × trait interaction biplot model explained 61% and 29% of the variation in the standardized two-way dataset (Figure 1), respectively, highlighting a substantial portion of the variation that underscores the direct associations among traits. To facilitate the interpretation of these relationships, vectors were plotted from the origin of the graph to each trait.

This approach allowed for the visualization of trait interactions, regarding additive and crossover interactions, indicating that the ranking of genotypes across traits may shift.

The biplot model demonstrated both additive and crossover interactions, indicating that the ranking of genotypes across traits may shift. This observation aligns with the findings of Sabaghnia and Jamalluddin et al. (2021) in *Triticum aestivum* L. and Yari et al. (2018) in *Secale cereale* L., who noted the complexity of genotype selection when entry-by-tester interactions are not considered. In the current study, the biplot model, as recommended by Sedghiyeh et al. (2025), proved to be an effective tool for exploring and visualizing genotype-trait relationships. The model successfully captured a remarkable 90% of the total variability, with trait associations determined by the cosine of the vectors. Specifically, a cosine of 0° corresponds to +1, 90° to 0, and 180° to -1. The biplot presented considerable variability, as indicated by the long vectors (Figure 1). Notably, β-phellandrene (B-Phell), dillapiole, and essential oil (EO) showed a positive association, as evidenced by the relatively close angles of their vectors. Similarly, neophytadiene (Neoph) and β-pinene (B-Pinene) were positively correlated due to their acute angles. In contrast, α-terpinene (A-Terpinene) was positioned at relatively right angles to B-Phell and EO, as well as to Neoph and B-Pinene, indicating no strong positive or negative relationships among them (Figure 1). This pattern was similarly observed in the interactions involving carvacrol, which exhibited negative associations with the aforementioned groups of traits. A notable negative correlation was observed between carvacrol and A-Terpinene, as their vectors formed an obtuse angle.

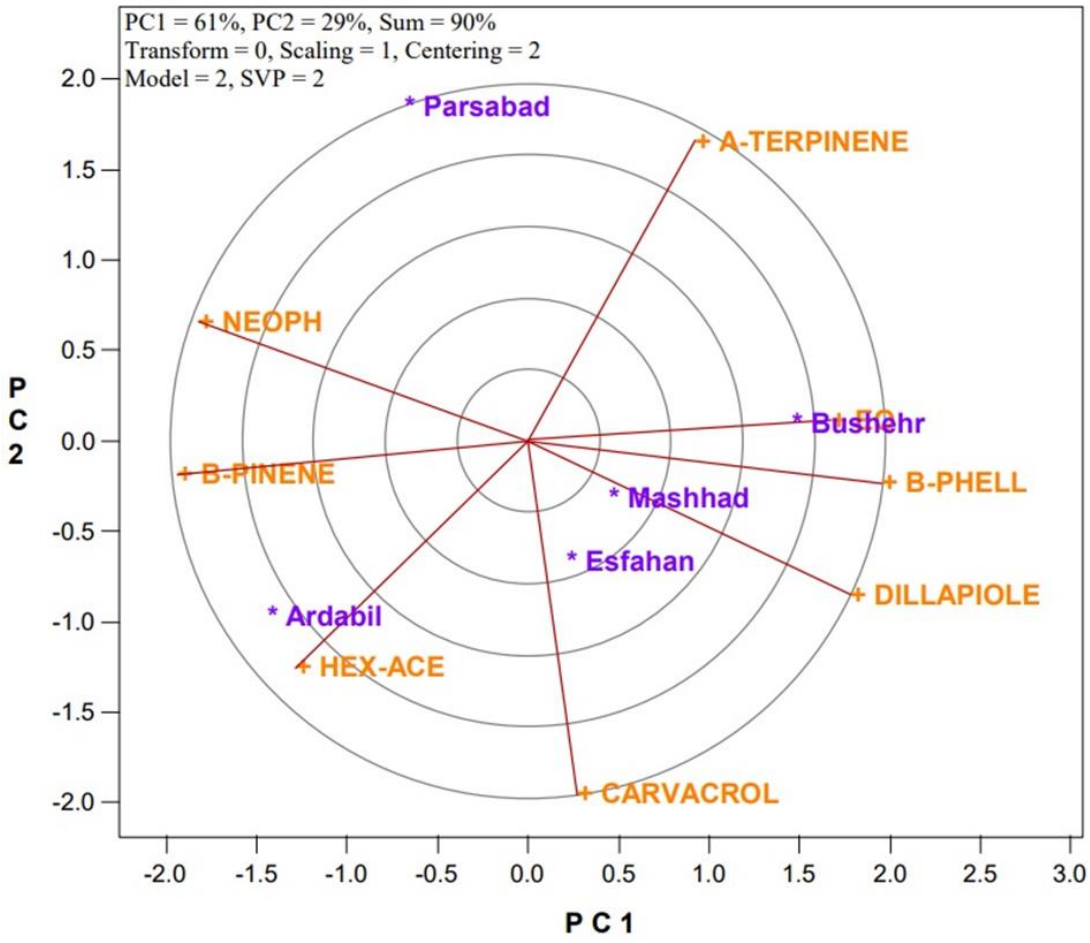


Figure 1. The relationships among traits of dill (*Anethum graveolens* L.) genotypes.

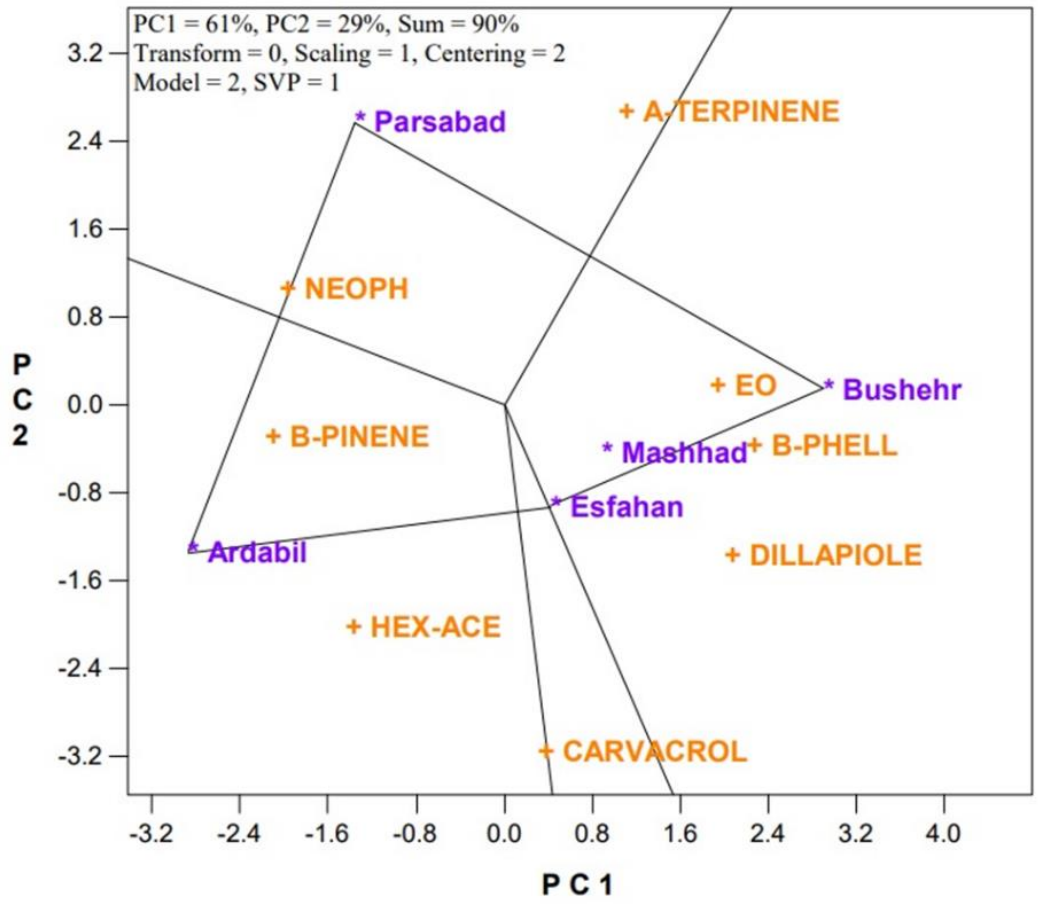


Figure 2. Superior phytochemical traits of elite Anethum graveolens genotypes

Additionally, a significant negative association was noted between B-Phell and EO with Neoph and B-Pinene, and between hexahydrofarnesyl acetone (Hex-Ace) and A-Terpinene (Figure 1). The results suggest that increasing the levels of essential oil in *A. graveolens* enhances the production of dillapiole and β-phellandrene, while concurrently reducing the generation of β-pinene, neophytadiene, and hexahydrofarnesyl acetone. However, these changes did not significantly alter the levels of carvacrol or α-terpinene. The fitted biplot model thus provided a clear visual representation of trait associations in *A. graveolens*, supporting the findings of Farmanpour Kalalagh et al. (2023), who reported positive correlations between β-phellandrene, dillapiole, and essential oil, as well as between neophytadiene and β-pinene. It is noteworthy that while the biplot method visually illustrates trait associations based on the overall structure of the dataset, correlation coefficients specifically measure the direct interrelationships of traits. Therefore, the results from the biplot may not always align precisely with the direct pairwise correlations between traits.

Figure 2 illustrates how the biplot model aids in the evaluation of genotypes based on their trait performance, identifying those that excel in specific traits and thereby serve as suitable candidates for breeding or cultivation. A tetragon was constructed with perpendicular lines to its sides, facilitating easier comparisons among the genotypes. Four key genotypes; Ardabil, Bushehr, Esfahan, and Parsabad; were identified as the vertex genotypes. Genotype Ardabil exhibited the highest values for hexahydrofarnesyl acetone (Hex-Ace) and β-pinene (B-Pinene), whereas genotype Parsabad showed superior performance in neophytadiene (Neoph) and α-terpinene. Genotype Bushehr, on the other

hand, recorded the highest values for β-phellandrene (B-Phell), dillapiole, and essential oil (EO). Carvacrol was positioned at the boundary between the vertex genotypes of Ardabil and Esfahan (Figure 2). The performance of genotype Mashhad was found to be similar to that of genotype Bushehr, albeit with relatively lower levels of the aforementioned traits. The classification of these genotypes into four distinct groups highlights their genetic diversity, which can be attributed to their adaptation to different geographical regions. This variation among local landraces is consistent with findings by Mohebodini et al. (2024), who utilized the genotype × trait interaction biplot model to assess genetic variation in ten native *Portulaca oleracea* L. genotypes, reporting similar distinct variations among local landraces.

Figure 3 illustrates the position of the ideal genotype, with the genotypes closest to its location being considered the most favorable. Based on this assessment, the *A. graveolens* genotypes were ranked as follows: Esfahan > Mashhad > Bushehr > Ardabil > Parsabad, with Esfahan identified as the most desirable and Parsabad as the least desirable. The identification of these ideal genotypes is crucial for future genetic improvement programs in dill. This issue is particularly given that essential oil content and its quality characteristics often exhibit low or even negative correlations in many vegetable crops. Thus, the application of multivariate statistical tools, combined with graphical representations, is essential for identifying ideal genotypes and refining breeding strategies for *A. graveolens*. In other words, using Esfahan as the ideal genotype provides a reliable benchmark for evaluating the yield of essential oil and its biochemical components. The results obtained from Esfahan are considered more reliable compared to those of the other genotypes due to its desirable characteristics.

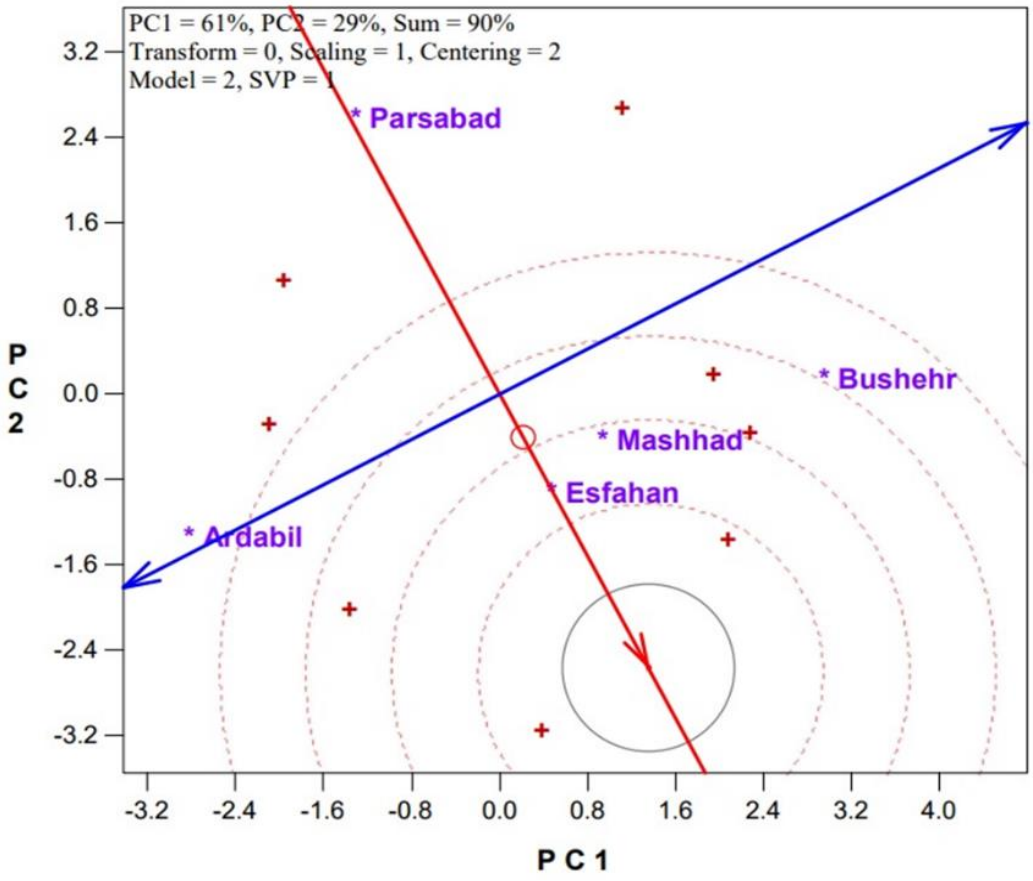


Figure 3. The ideal genotype of dill (*Anethum graveolens* L.).

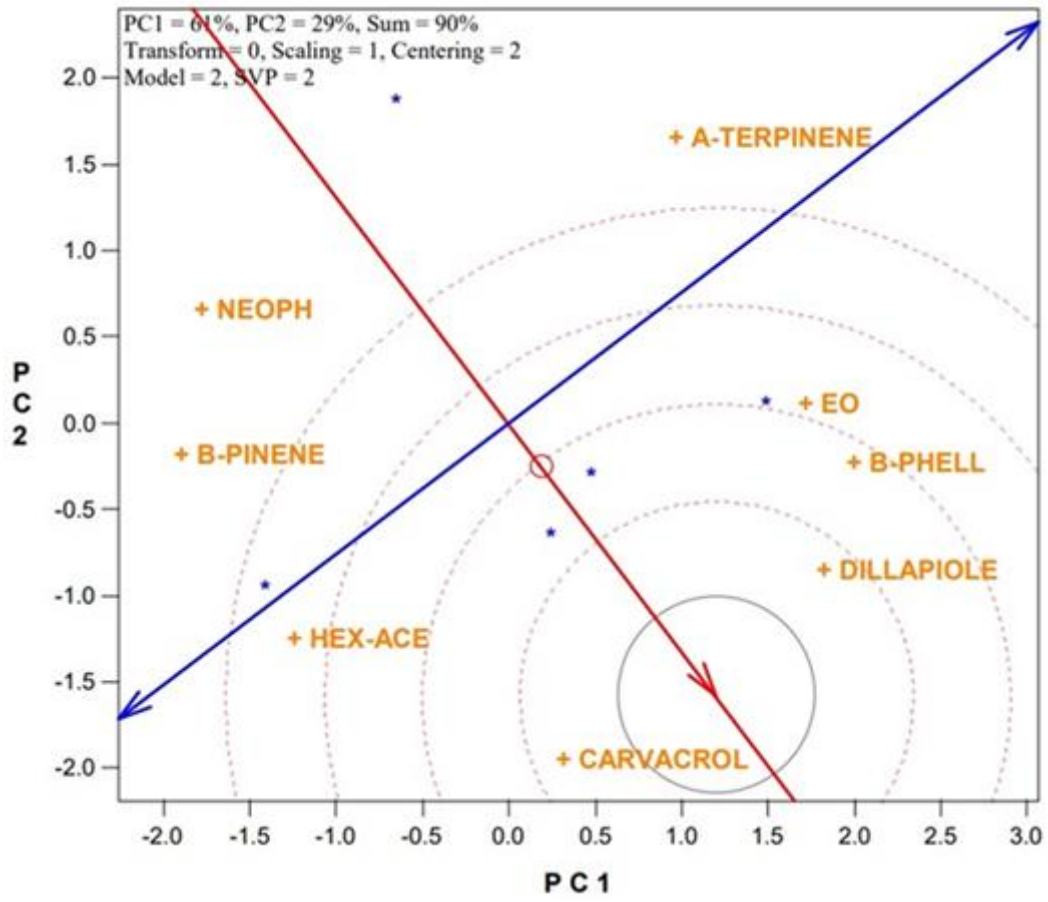


Figure 4. Target traits for ideal dill (*Anethum graveolens* L.) genotype selection.

Sabaghnia et al. (2024) suggested that ideal genotypes, such as Esfahan, should be used as reference genotypes in future studies to better understand genetic variability across traits, as they demonstrate a high potential based on the traits evaluated. The discriminative property of a trait is determined by the value of its standard deviation, with higher values indicating a greater ability to distinguish between genotypes. This property is illustrated by the hypothetical ideal trait shown in Figure 4, where traits closer to the ideal location exhibit high discriminative power, while those further away are less effective. Based on the current analysis, carvacrol is identified as the most discriminative trait. In contrast, traits such as β-phellandrene, dillapiole, hexahydrofarnesyl acetone, and essential oil demonstrate above-average discriminative potential, indicating their effectiveness in differentiating *A. graveolens* genotypes (Figure 4). Conversely, traits like α-terpinene, β-pinene, and neophytadiene show low discriminative potential and are therefore not recommended for future investigations. The typical ability of a trait, reflecting its symbolic role, is evaluated by the angle between the trait's vector and the axis of the average trait. Smaller angles indicate greater typical ability. In this context, carvacrol, followed by dillapiole, exhibits the highest typical ability, while hexahydrofarnesyl acetone shows a larger angle with the axis, reflecting its lower typical ability. When evaluating traits, ideal genotypes should be able to differentiate from other genotypes based on the representativeness of the ideal trait. Biplot analysis provides valuable insights by considering the traits with strong representativeness, offering useful information for future selection and breeding efforts.

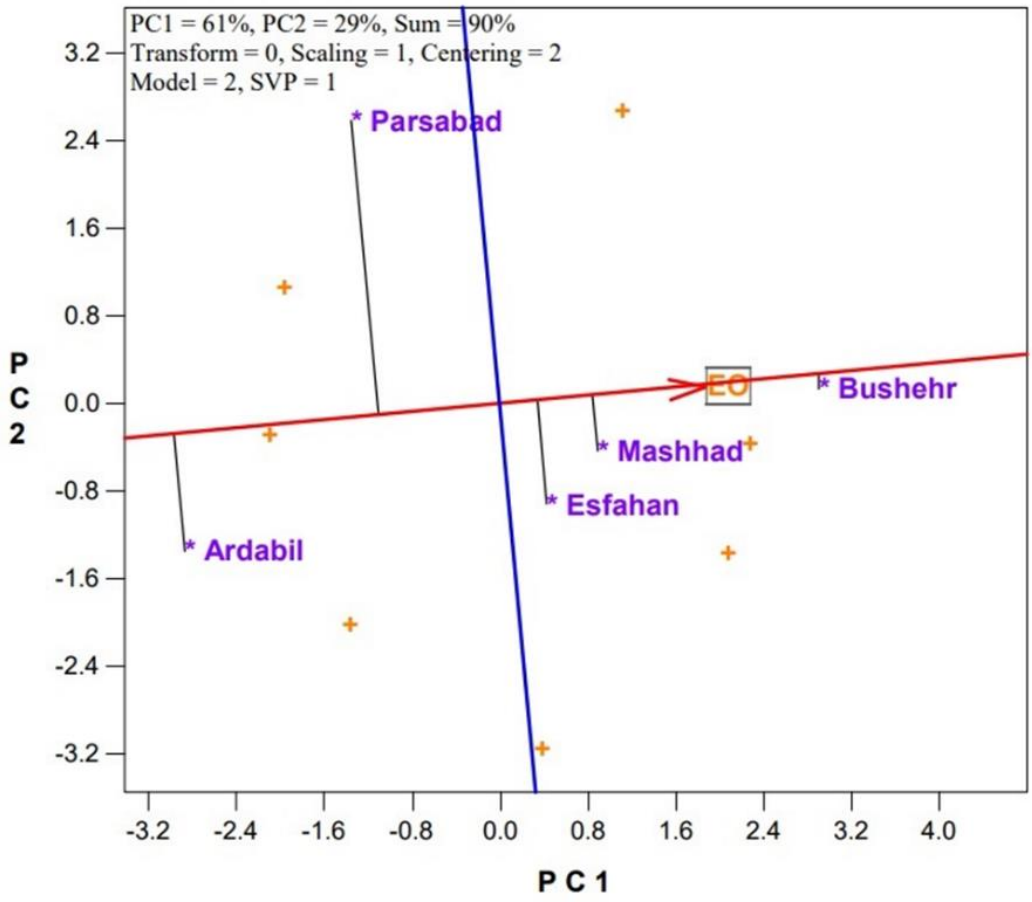


Figure 5. The performance of dill (Anethum graveolens L.) genotypes for essential oil (EO) production.

Figure 5 assesses the performance of *Anethum graveolens* genotypes in terms of EO yield, with the horizontal axis representing EO content and the direction indicated by an arrow. Genotype Bushehr demonstrated the highest performance for essential oil yield, while genotype Ardabil exhibited the lowest. Genotypes Esfahan and Mashhad showed above-average performance for EO, as they lie above the average axis, while genotype Parsabad was identified as below-average in terms of essential oil production. The distance of each genotype from the horizontal axis serves as an indicator of variability; smaller distances suggest minimal variance, making those genotypes preferable for selection. Fortunately, high-yielding genotypes such as Bushehr, Esfahan, and Mashhad are recommended for their low variance. In contrast, genotypes Parsabad and Ardabil, which exhibit lower essential oil yields and are positioned further from the horizontal axis, demonstrate greater variability and are considered less desirable. Evaluating *A. graveolens* genotypes is essential for preventing genetic erosion and optimizing genetic improvement efforts. Our study revealed considerable variation among the examined genotypes, a finding that aligns with previous studies highlighting significant diversity within dill populations (Weisany et al., 2015; Farmanpour Kalalagh et al., 2023). The genotype × trait interaction biplot model indicated a high level of variability and confirmed the presence of both simple and complex interactions between genotypes and traits. Similar patterns of genotype-trait interactions have been observed in various vegetable crops, such as *Spinacia oleracea* (Sabaghnia et al., 2015) and *Amaranthus tricolor* (Jamalluddin et

al., 2021). These interactions led to differing genotype rankings across traits, complicating the selection of the most desirable genotypes. Ignoring genotype × trait interactions can complicate the selection process due to their influence on the performance of genetic components. However, the obtained results were according to a single trial, and outcomes may vary with other datasets. To address this, an alternative model suggests replacing the first principal component with predictions derived from a linear regression model applied to tester-centered data on genotype effects, which improves the interpretability of the model.

Discussion

The present study explored the genetic variability of essential oil components across five different genotypes of *A. graveolens*, originating from various regions of Iran. These findings align with previous reports indicating significant differences in the profile of essential oil expected from geographically distinct populations of medicinal plants (Karimi et al., 2020). The analysis of essential oil components, using genotype × trait interaction biplot models, revealed notable interactions between genotype and trait, emphasizing the complex nature of selecting the most favorable genotypes with high essential oil in *A. graveolens*. One of the key outcomes of this study was the identification of the Bushehr genotype as the most favorable for high essential oil production. Bushehr exhibited elevated levels of β-phellandrene and dillapiole, both of which are considered valuable bioactive compounds. This finding is consistent with those of (Ozliman et al., 2021), who highlighted the variation in essential oil yields among different genotypes of *A. graveolens*. Additionally, this result aligns with similar studies conducted on other medicinal plants, such as fennel (*Foeniculum vulgare*) and basil (*Ocimum gratissimum*), where genotype variability significantly influenced essential oil content (Shojaiefar et al., 2015; Sharma et al., 2024). Furthermore, current analysis revealed that increasing essential oil production in *A. graveolens* was related by increasing in the levels of dillapiole and β-phellandrene, while the concentrations of β-pinene, neophytadiene, and hexahydrofarnesyl acetone were reduced. These findings are significant as they suggest that optimizing essential oil production in dill can lead to an improved balance of bioactive compounds that enhance its therapeutic properties. Similar correlations between essential oil content and specific bioactive compounds have been reported in *A. graveolens* and other medicinal plants (Gholizadeh et al., 2021; Sabaghnia et al., 2024), where increases in specific oil components correspond to better pharmacological activity. The use of the genotype × trait interaction biplot model in this study proved invaluable for exploring and visualizing the relationships between genotypes and their associated traits. The biplot clearly demonstrated how genotype rankings across traits could change, indicating the complex nature of genotype × trait interactions. This aligns with (Sabaghnia et al., 2015), who highlighted the difficulties in selecting genotypes for breeding without considering these interactions. The use of the genotype × trait interaction biplot allows for the visualization of both additive and crossover interactions, providing breeders with a tool to evaluate multiple traits simultaneously and make more informed decisions.

In terms of discriminative power, carvacrol emerged as the most effective trait for differentiating between genotypes, with its position on the biplot indicating a strong association with high essential oil content and favorable bioactive profiles. Current results are in good consistent with past reports, which has confirmed carvacrol as a critical compound in various medicinal plants, including *Origanum vulgare* and *Thymus vulgaris*, where it is known for its antioxidant and antimicrobial properties (Naghdi Badi et al., 2017). By focusing on carvacrol, breeders can identify and select genotypes that are more likely to produce high-quality essential oils with therapeutic potential. The results from the discriminative and representative property analysis also highlighted the role of carvacrol in guiding future breeding efforts. As a highly discriminative trait, carvacrol can serve as a key marker in genetic improvement programs aimed at enhancing essential oil yield and quality. This approach is supported by similar findings in other crops, where the identification of key discriminative traits has facilitated more targeted breeding strategies.

Another important observation in our study was the identification of Esfahan as the most ideal genotype

for essential oil production. The proximity of Esfahan to the ideal genotype position on the biplot indicated its superior performance in producing high-quality essential oil. This aligns with previous research that has used ideal genotype modeling to assess the genetic potential of medicinal plants for essential oil production (Sabaghnia et al., 2024). The potential to identify ideal genotypes is critical for the development of new cultivars with enhanced essential oil performance, particularly in crops like dill, where essential oil yield and quality often have low or even negative correlations with other traits. The variability observed in the essential oil components across the five genotypes supports the hypothesis that environmental factors, such as geographic origin and soil conditions, had a meaningful effect in generation of the phytochemical profile of *A. graveolens*. Thus, the importance of benefiting genetic as well as environmental issues in selecting genotypes for genetic improvement projects targeted at increasing essential oil production. Finally, relatively valuable insights were provided into the genetic variability of *A. graveolens* essential oil components and offers practical recommendations for breeding programs. The identification of Bushehr as the most favorable genotype, combined with the discriminative power of carvacrol and the ideal genotype modeling, offers a promising strategy for enhancing essential oil yield and quality in dill. Future research should focus on further exploring genotype × environment interactions and their effects on essential oil production, as well as investigating the stability of these traits across multiple growing seasons and locations. Moreover, integrating molecular markers associated with desirable traits, such as carvacrol, could accelerate the breeding of new cultivars with improved essential oil profiles. The usefulness of the ideal item for assessing discriminative ability has been demonstrated in studies by Baljani et al. (2015) in *Carthamus tinctorius* and Pourmohammad et al. (2025) in *Triticum durum* L.

To further highlight the superiority of essential oil compounds, it is essential to emphasize their enhancing properties, including their aroma, taste, and medicinal benefits. The bioactive components identified in this study, particularly β-phellandrene, dillapiole, and carvacrol, contribute significantly to the sensory and therapeutic qualities of dill essential oil. β-Phellandrene is known for its fresh, citrus-like scent, enhancing the aromatic complexity of dill, making it a valuable ingredient in culinary applications, flavoring, and perfumery. Dillapiole contributes a mildly spicy and warm note, further refining the oil's flavor profile. Carvacrol, widely recognized for its antioxidant, antimicrobial, and anti-inflammatory properties, enhances the oil's pharmaceutical value, making it beneficial for natural remedies and functional foods. The reduction in β-pinene, neophytadiene, and hexahydrofarnesyl acetone may contribute to a smoother, more refined essential oil profile, potentially improving consumer preference and therapeutic efficiency. By selecting superior genotypes that optimize these key compounds, breeding strategies can enhance both the sensory appeal and medicinal efficacy of dill essential oil, increasing its demand in pharmaceutical, cosmetic, and food industries.

Conclusion

Increasing essential oil content in *A. graveolens* enhances the production of dillapiole and β-phellandrene, coupled with β-pinene, neophytadiene, and hexahydrofarnesyl acetone reduction. The Bushehr genotype was identified as the most favorable, making it a strong candidate for cultivar development. Regarding discriminative and representative properties, carvacrol emerged as the most influential trait, effectively differentiating the most desirable genotypes. The results establish a foundation for targeted breeding strategies to increase both essential oil productivity and beneficial phytochemical compounds in new dill cultivars.

Supplementary Materials

No supplementary material is available for this article.

Author Contributions

Conceptualization, M.M.; methodology, M.M.; software, N.S.; validation, N.S., and N.S.; formal analysis, K.F.; investigation, K.F.; resources, K.F.; data curation, N.S.; writing—original draft preparation, N.S.; writing—review and editing, M.M.; visualization, K.F.; supervision, M.M.; project

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Conflict of Interest Statement

The authors declare no conflict of interest.

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مقاله پژوهشی

تجزیه و تحلیل گرافیکی تنوع ژنتیکی اجزای اسانس برخی از ژنوتیپ‌های شوید (*Anethum graveolens*)

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چکیده: گیاهان دارویی و معطر از دیرباز به دلیل سهم قابل توجهی که در سلامت، تندرستی و شیوه‌های آشپزی دارند، مورد توجه بوده‌اند. این تحقیق با هدف بررسی تنوع ژنتیکی محتوای اسانس و اجزای آن در پنج توده بومی شوید (*Anethum graveolens*) از شهرهای مشهد، اردبیل، پارس آباد، بوشهر و اصفهان انجام شد. اسانس و ترکیب آن، از جمله β-pinene، α-terpinen، β-phellandren، carvacrol، dillapiole، neophytadiene و hexahydrofarnesyl acetone، مورد تجزیه و تحلیل قرار گرفت. بای پلات ۹۰ درصد از تغییرات را توضیح داد و نمایش واضحی از روابط ژنوتیپ × صفت ارائه داد. مدل biplot هر دو برهمکنش افزایشی و ضرب‌پذیر را نشان داد، که نشان می‌دهد رتبه‌بندی ژنوتیپ می‌تواند در بین صفات تغییر کند. ارزیابی‌های اختصاصی ژنوتیپ نشان داد که بوشهر از نظر عملکرد و کیفیت اسانس بهترین عملکرد را داشته و پس از آن اصفهان، مشهد، اردبیل و پارس‌آباد قرار دارند. قوی‌ترین همبستگی مثبت بین β-phellandren، dillapiole و محتوای اسانس مشاهده شد، در حالی که ارتباط بین α-terpinen و β-phellandren پیچیده تر و کمتر مشخص بود. همچنین ژنوتیپ بوشهر به دلیل عملکرد برتر، بیشترین پتانسیل را برای برنامه‌های اصلاحی آتی نشان داد. علاوه بر این، کارواکرول به‌عنوان متمایزترین صفت برای تمایز بین ژنوتیپ ظاهر شد. این مطالعه همچنین نشان داد که افزایش محتوای اسانس در A. graveolens با افزایش تولید dillapiole و β-phellandren مرتبط است، در حالی که به‌طور همزمان سطوح β-pinene، neophytadiene و hexahydrofarnesyl acetone را کاهش می‌دهد.

کلمات کلیدی: بای پلات، توانایی تمایز صفات، کارواکرول، ژنوتیپ ایده‌آل.

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Methanol foliar spraying improves biochemical and physiological attributes of coriander (*Coriandrum sativum*) plants under salt stress

Original paper

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Abstract: Salinity stress is one of the most significant factors limiting plant growth; therefore, using compounds such as methanol to mitigate the harmful effects of stress is of great importance. This study aimed to investigate the effect of methanol spraying on the physiological and biochemical characteristics of coriander (*Coriandrum sativum*) plants under salinity stress conditions in a greenhouse experiment. Salinity treatment was applied at two levels (control and 80 mM NaCl) and methanol was exogenously applied at four concentrations (control, 10%, 20%, and 30%). The results indicated that under salinity stress conditions, the application of methanol led to a significant increase in various traits. Superoxide dismutase enzyme activity increased by 23% with 30% methanol spraying under salinity stress. Additionally, polyphenol oxidase enzyme activity rose by 51% with 30% methanol at 80 mM salinity, while catalase activity increased by 29%. Proline content also increased by 124% under salinity stress, while malondialdehyde content decreased by 63% in these conditions. Overall, 30% methanol enhances stress tolerance in plants under salinity conditions by improving antioxidant enzyme activities, protein content, and osmoprotective mechanisms. These findings suggest the role of methanol's in mitigating the effects of salinity stress on plant physiology.

Keywords: Electrolyte leakage, enzyme, malondialdehyde, pigment, sodium chloride.

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Introduction

Coriander (*Coriandrum sativum*) is a valuable herb and aromatic plant known for its medicinal and nutritional properties. This plant is rich in vitamins, minerals, and antioxidants, particularly serving as a good source of vitamins C and K, potassium, and fiber (Gantait et al., 2022). The medicinal properties of coriander include improving digestion, reducing inflammation, and helping in blood sugar control. Additionally, coriander exhibits antibacterial and antifungal effects and may assist in detoxifying the body. Consumption of coriander may also help reduce anxiety and enhance mental health (Diederichsen et al., 2020; Gantait et al., 2022).

Salinity stress is one of the most significant environmental factors affecting plant growth and development, particularly in arid and semi-arid regions (Hosseini et al., 2023). Soil salinity can arise from groundwater extraction or irregular rainfall, leading to decreased soil fertility and disrupted plant growth (Singh, 2022). Plants under salinity stress experience reduced water uptake capacity, diminished greenness, and, in severe cases, death (Khalvandi et al., 2019; Rabiei et al., 2020; Hosseini et al., 2023). Salinity causes the accumulation of toxic ions such as sodium and chloride in plant tissue, which can disrupt metabolic activities (Hosseini et al., 2023).

On the other hand, methanol, as a simple organic compound, can stimulate and improve physiological processes in plants. Some studies have shown that foliar spraying of methanol can act as a stimulant in correcting the negative effects of salt stress (Wei et al., 2015). We hypothesize that methanol enhances reactive oxygen species (ROS) scavenging under salinity stress, helping to maintain the internal balance of the plant by affecting the activity of enzymes and antioxidant systems and preventing the harmful effects of stress factors (Tavassoli and Galavi, 2011; Dorokhov et al., 2018). One of the most important effects of methanol is to improve the nitrogen and carbon status in plants, which can lead to an increase in chlorophyll levels and an improvement in the photosynthetic capacity of the plant (Wei et al., 2015; Dorokhov et al., 2018).

Antioxidant enzymes, including peroxidase, catalase, and superoxide dismutase, play a crucial role in mitigating the damaging effects of free radicals in plant cells (Singh et al., 2022). Salinity stress induces oxidative stress, increasing the production of free radicals, which necessitates a more robust antioxidant defense system (Yilmaz et al., 2023). Investigating changes in antioxidant enzyme activity in response to salinity stress, along with the positive effect of methanol, can enhance our understanding of plant responses to these conditions.

Another important parameter in assessing the effects of salinity stress is the relative water content (RWC) of leaves (Patanè et al., 2022). Reduced water content in stressed plants often leads to an imbalance in water and nutrient uptake. This is one of the primary reasons for the reduction of photosynthetic energy and, consequently, reduced growth and yield in plants (Browne et al., 2020). Studying changes in RWC can provide a valuable tool for investigating the effects of salinity stress on plant water status (Patanè et al., 2022).

Proline, considered a non-essential amino acid, plays a signifinant role in plant resistance to environmental stresses such as salinity and drought (Yan et al., 2024). In fact, the accumulation of proline in plant tissues is recognized as a marker of stress tolerance, as this compound can help stabilize proteins and cell membranes (Shahid et al., 2022). Electrolyte leakage (EL) is another measure for assessing damage caused by salt stress in plants (Abdelaal et al., 2021). Increased EL indicates damage to the cell membrane and the irreversibility of stress-induced damage (Kovaleski and Grossman, 2021). Studying EL can provide further insights into the level of cllular damage caused by salt stress (Abdelaal et al., 2021).

Malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) are other important indicators for assessing oxidative stress and its resulting damage (Goncharuk et al., 2022). Increased levels of these compounds usually occur due to increased free radical activity and environmental stresses such as salt stress (Hosseini et al., 2023). Examining the levels of these compounds can provide a more accurate assessment of the effects of methanol as a protective factor against salt stress. Total soluble sugar (TSS) also plays an important role as an energy and metabolic source in plants and can help regulate plant metabolism under stress conditions

(Hosseini et al., 2023). Changes in soluble sugar levels under stress can indicate the metabolic status of the plant and serve as a marker for assessing the effects of salinity stress (Hosseini et al., 2023).

In summary, this research uniquely investigates the relationship between salinity stress and methanol foliar application, focusing on its effects on antioxidant enzyme activity, chlorophyll content, relative water content (RWC), proline levels, electrolyte leakage (EL), malondialdehyde (MDA), hydrogen peroxide (H_2O_2), and total soluble sugars (TSS). Unlike previous studies, which often explored individual aspects of plant responses to either salinity or methanol, this study provides a comprehensive analysis of multiple physiological and biochemical traits in coriander plants under combined stress conditions. This research not only enhances our understanding of coriander plant responses to salinity stress but also offers innovative strategies to improve plant performance under saline conditions and amid climate change challenges.

Materials and Methods

Plant materials and treatments

To investigate the effect of methanol foliar spraying on the coriander medicinal plant under salinity stress, the present experiment was conducted in greenhouse conditions at the National Skills Training Center for Girls in Amol-(Tohid). A pot experiment was designed as a factorial experiment in a completely randomized design with three replications. The first factor was salinity stress, which was applied at two levels (control and 80 mM salinity) from a sodium chloride source. The second factor was methanol spraying, which included four levels (control, methanol at concentrations of 10%, 20%, and 30%).

After physical purification, the seeds were disinfected and sterilized in sodium hypochlorite solution for 1 minute, followed by several washedwith distilled water. The seeds were then soaked in distilled water for 2 hours to facilitate the dehydration process.

Soil preparation

To prepare the cultivation medium, a layer of sand was poured into each pot (20 cm deep and 10 cm wide) for drainage, followed by the addition of 5 kg of soil. The results related to the physical and chemical properties of the soil are presented in Table 1.

Nutrient supplementation

To meet the nutritional needs of the plants, specific amounts of different fertilizers were calculated for each pot, which included 0.6 g of phosphorus (P_2O_5), 0.6 g of potassium sulfate (K_2SO_4), and 0.3 g of nitrogen (from urea), based on the plants' fertilizer requirements.

Greenhouse conditions

The greenhouse conditions during the experiment were carefully maintained, with a minimum temperature of 17°C and a maximum temperature of 23°C. The light intensity was approximately 300 µmol m^{-2} s^{-1}, and the photoperiod was set to 16 hours of light and 8 hours of darkness, with humidity levels kept around 60-70% to ensure optimal growth conditions.

Experimental design

For the experimental design, a total of 30 coriander plants were used, with 10 plants assigned to each treatment group and three replicates for each treatment to ensure statistical reliability. During seed selection for the experiment, weak seeds were discarded, as they have lower germination rates and viability, exacerbated by salt stress.

Treatment application

Two weeks after planting, salinity stress was applied to the plants, and methanol foliar spraying was conducted every 7 days until the end of the growth stage.

Sampling

At the end of the growing season, samples were collected from the leaves of the coriander plants to measure various biochemical and physiological parameters. These samples were immediately placed in liquid nitrogen and transferred to a -80°C refrigerator for further analysis.

Characteristic measurements

The amount of hydrogen peroxide (H_2O_2) was determined using the method of Alexieva et al. (2001), based on the reaction of H_2O_2 with potassium iodide. Protein content was measured using the Bradford method (Bradford, 1976). The activity of catalase (CAT) and superoxide dismutase

(SOD) activity were assessed according to Keshavarz and Khodabin (2019), ascorbate peroxidase (APX) using the method of Nakano and Asada (1981), and polyphenol oxidase (PPO) enzymes using the method of Lee et al. (2021). Electrolyte leakage (EL) was measured following the protocol of Shi et al. (2006). The proline amino acid content was determined using the method of Bates et al. (1973) and malondialdehyde (MDA) levels were measured according to the method of (Zhang et al., 2008).

Statistical analysis

At the end of the experiment, data were tested for normality using the Kolmogorov-Smirnov method and subsequently analyzed using SAS 9.1 statistical software. Comparisons of means were conducted using the least significant difference (LSD) method at a statistical significance level of 5%, and then graphs were drawn using EXCEL software. Also, XLSTAT (2016) software was utilized for principal component analysis and cluster analysis, employing the varimax method for component rotation. the UPGMA (unweighted pair group method with arithmetic mean) and Euclidean distance were used to construct dendrograms, and boxplots were created to compare groups resulting from the cluster analysis.

Results

SOD activity

The analysis of variance revealed that SOD enzyme activity was significantly influenced by salinity stress, methanol application, and their interaction ($P \leq 0.01$) (Table 2). The interaction results indicated that the application of 30 % methanol under salinity stress condition resulted in the highest SOD enzyme activity (38.65 U mg^{-1} protein min^{-1}); In contrast, the lowest SOD activity was observed in the treatments of no methanol application under no salinity condition (8.12 U mg^{-1} protein min^{-1}) and the application of 10% methanol under no salinity condition (8.94 U mg^{-1} protein min^{-1}) (Figure 1).

Table 1. Physiochemical properties of soil.

Soil texture	K (mg/kg)	P (mg/kg)	Total N (%)	OM (%)	pH	EC	Clay (%)	Silt (%)	Sand (%)
Silt loam	153	7.4	0.08	0.56	7.83	1.12	17	54	29

Table 2. Mean squares of SOD, PPO, CAT, APX, protein and proline affected by salinity stress treatment and methanol foliar spraying in coriander plant.

Source of variation	DF	SOD	PPO	CAT	APX	Protein	Proline
Salt stress (A)	1	2272.05**	0.8978**	0.952**	11250**	14.85**	14248.4**
Methanol (B)	3	415.33**	0.207**	0.2033**	2444.94**	6.08**	938.58**
A × B	3	51.17**	0.0774**	0.0459**	671.39**	2.14**	186.06**
Residual	16	0.84	0.0007	0.0004	2.35	0.01	2.57
CV (%)		4.39	5.16	4.65	3.88	5.07	4.12

** indicates significance at the 1% probability level. superoxide dismutase (SOD), polyphenol oxidase (PPO), catalase (CAT), and ascorbate peroxidase (APX).

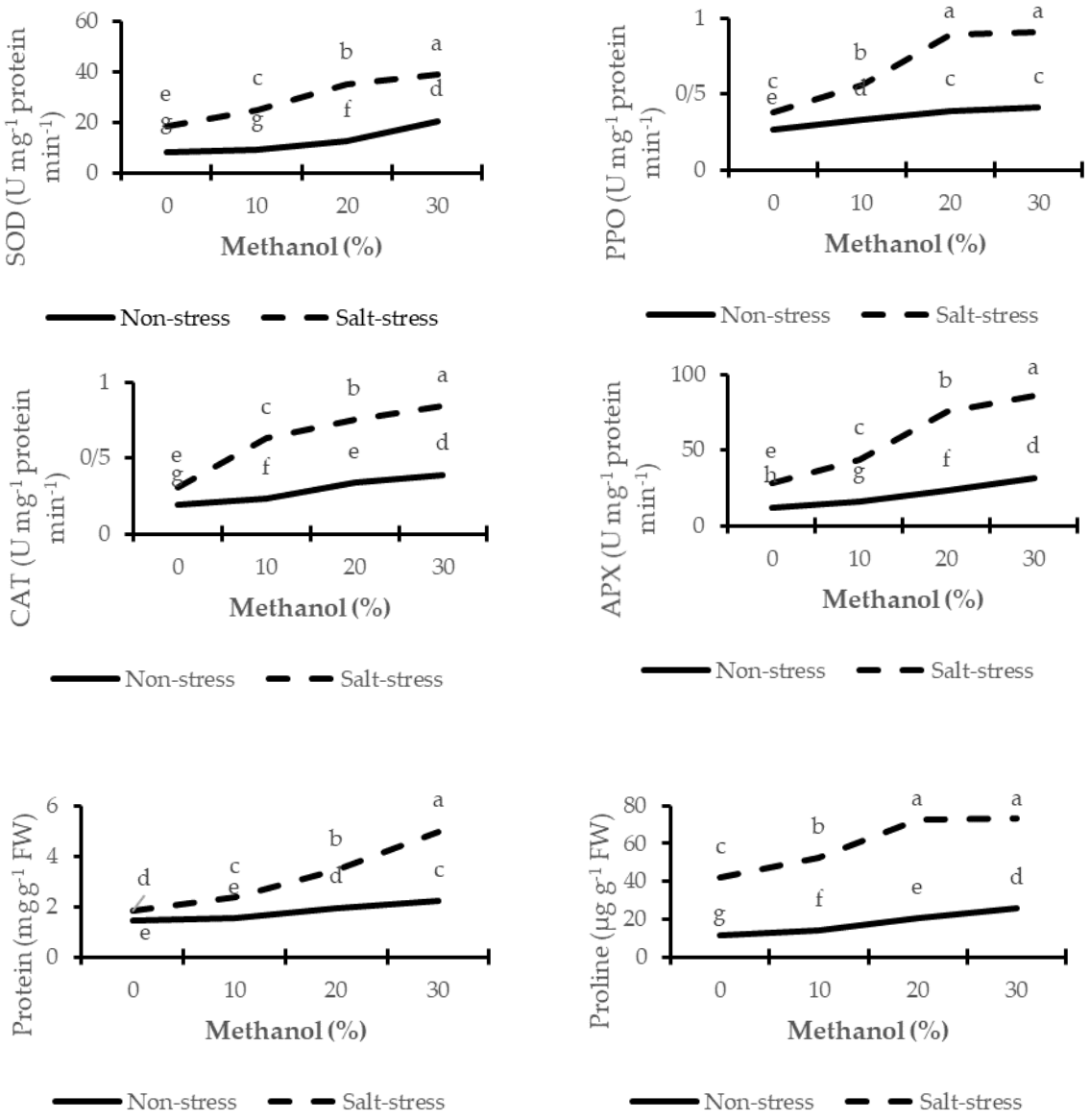


Figure 1. Results of mean comparison of superoxide dismutase (SOD), polyphenol oxidase (PPO), catalase (CAT), ascorbate peroxidase (APX), protein and proline characteristics under salinity stress (normal and 80 mM salinity stress) and methanol foliar spraying (non-spraying of methanol, 10% methanol, 20% methanol and 30% methanol) in coriander plants. The columns in the chart represent the mean ± SE. Columns with the same letters do not statistically differ from each other.

PPO activity

Data from the experiment indicated that the effects of salinity stress, methanol, and their interaction were significant for the PPO enzyme activity ($P \leq 0.01$) (Table 2). In the absence of salinity stress, the application of 20 and 30% methanol treatments improved the PPO enzyme activity by 44% and 51%, respectively compared to the control treatment without methanol (Figure 1). The highest PPO activity was recorded with 20% and 30% methanol

under 80 mM salinity conditions, measuring 0.89 and 0.91 U mg^{-1} protein min^{-1}, respectively. In contrast, the lowest PPO enzyme activity was observed in the treatment without methanol application and salinity stress (0.27 U mg^{-1} protein min^{-1}) (Figure 1).

CAT activity

Changes in CAT enzyme activity was significantly influenced by salinity stress, methanol, and their interaction ($P \leq 0.01$) (Table 2). The results indicated that the treatment using 30% methanol under 80 mM salinity conditions had the highest CAT activity (0.84 U mg^{-1} protein min^{-1}); while the lowest CAT activity was found in the control treatment without salinity stress and methanol application (0.19 U mg^{-1} protein min^{-1}) (Figure 1).

APX activity

Analysis of variance showed that APX enzyme activity was significantly affected by salinity stress, methanol, and their interaction ($P \leq 0.01$) (Table 2). Mean comparison results showed that the treatment of 30% methanol under 80 mM salinity conditions had the highest APX enzyme activity (85.69 U mg^{-1} protein min^{-1}), while the treatment without methanol application under no salinity conditionshad the lowest APX enzyme activity (12.32 U mg^{-1} protein min^{-1}) (Figure 1).

Protein content

The analysis of variance revealed that protein content was significantly affected by salinity stress, methanol application, and their interaction ($P \leq 0.01$) (Table 2). The results showed that applying methanol at concentrations of 10%, 20%, and 30% under salinity stress conditions increased protein content by 28%, 87%, and 171%, respectively, compared to the treatment without methanol application under salinity stress conditions (Figure 1). Also, based on the results obtained under non-salinity stress conditions, it was determined that there was no significant difference in protein content between the treatments without methanol application and the application of 10% methanol (Figure 1). Under non-salinity stress conditions, the application of 20 and 30% methanol improved the protein content by 32 and 51%, respectively, compared to the treatment without methanol application. The highest protein content was observed in the treatment with 30% methanol application under salinity stress conditions (4.98 mg g^{-1} FW); However, the lowest protein content was observed in the treatment without methanol application and salt stress (1.47 mg g^{-1} FW) (Figure 1).

Proline content

Analysis of variance showed that the proline content was significantly affected by salinity stress, methanol application, and their interaction ($P \leq 0.01$) (Table 2). In non-salinity stress conditions, the application of 10%, 20%, and 30% methanol increased proline levels by 25%, 73%, and 75%, respectively, compared to the treatment without methanol application (Figure 1). Also, in salinity stress conditions, the application of 10, 20, and 30% methanol treatments increased proline by 21, 78, and 124%, respectively, compared to the treatment without methanol application (Figure 1). The results showed that the treatments of 20% methanol application in salinity stress conditions (72.54 μg g^{-1} FW) and 30% methanol application in salinity stress conditions (73.25 μg g^{-1} FW) had the highest proline content; while the treatment of no methanol application in non-salinity stress conditions (11.41 μg g^{-1} FW) had the lowest proline content (Figure 1).

MDA content

Variance analysis results indicated that the MDA content was significantly affected by salinity stress, methanol, and their interaction ($P \leq 0.01$) (Table 3). Based on the results, it was observed that the MDA content decreased with the application of different methanol treatments in salt stress and non-salt stress conditions. A comparison of the mean data in non-salt stress conditions showed that the application of 30% methanol had the lowest MDA content (13.52 nmol g^{-1} FW); while the highest MDA content was related to the treatment of no methanol application (31.25 nmol g^{-1} FW) (Figure 2). Based on the results obtained in salt stress conditions, it was determined that the treatment of no methanol application had the highest MDA content (67.54 nmol g^{-1} FW); in contrast, the treatment of 30% methanol application had the lowest MDA content (24.82 nmol g^{-1} FW).

Table 3. Mean squares of MDA, EL, H_2O_2, TSS, RWC, and TChl affected by salinity stress treatment and methanol foliar spraying in coriander plant.

Source of variation	DF	MDA	EL	H_2O_2	TSS	RWC	TChl
Salt stress (A)	1	7598.97**	8516.43**	8.1**	3111.81**	1722.25**	36.63**
Methanol (B)	3	1346.25**	643.22**	2.11**	1996.2**	257.16**	23.15**
A × B	3	353.04**	1433.39**	0.58**	681.45**	57.63**	1.62**
Residual	16	1.34	3.11	0.06	6.79	6.92	0.24
CV (%)		3.23	4.56	10.7	4.87	3.64	7.25

** indicates significance at the 1% probability level. Malondialdehyde (MDA), electrolyte leakage (EL), total soluble sugar (TSS), relative water content of leaf (RWC), and total chlorophyll content (TChl).

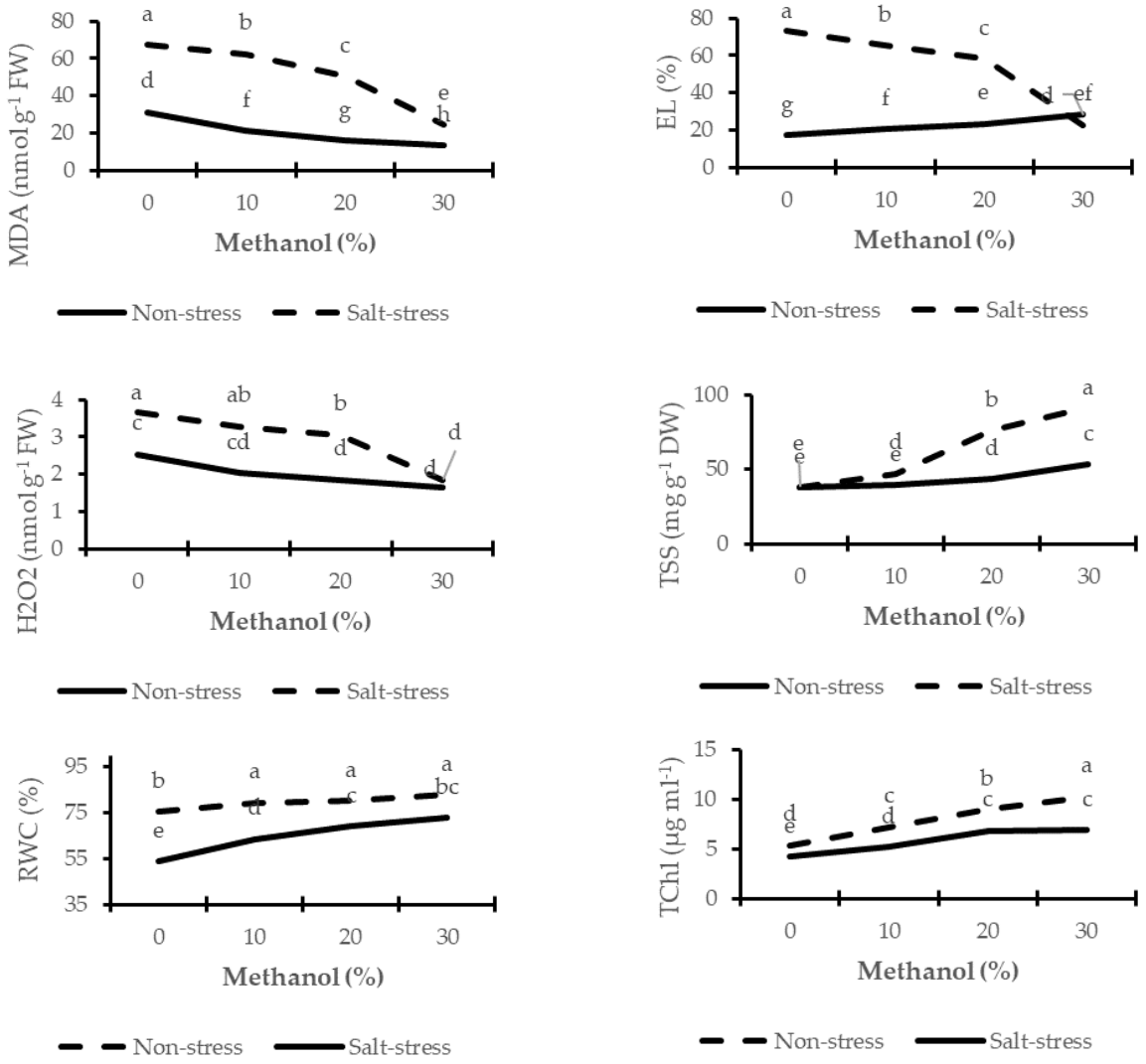


Figure 2. Results of comparison of mean related to malondialdehyde (MDA), electrolyte leakage (EL), H_2O_2, total soluble sugar (TSS), relative water content of leaf (RWC) and total chlorophyll content (TChl) characteristics under salinity stress (normal and 80 mM salinity stress) and methanol foliar spraying (non-spraying of methanol, 10% methanol, 20% methanol and 30% methanol) in coriander plants. The columns in the chart represent the mean±SE. Columns with the same letters are not statistically significantly different from each other.

Electrolyte leakage (EL)

The changes in the content of EL were affected by the treatments used; so the effect of salinity stress, methanol, and their interaction was significant for the EL content ($P \leq 0.01$) (Table 3). Based on the results obtained from comparing the mean data, it was observed that the highest content of EL was related to the treatment of not using methanol under salinity stress conditions (73.48%); while the lowest amount of EL was observed in the treatment of not using methanol and no salinity stress (17.21%) (Figure 2).

H_2O_2 content

Based on the results obtained from the analysis of variance of the data, it was determined that the effect of salinity stress, methanol, and their interaction was significant for the H_2O_2 content ($P \leq 0.01$) (Table 3). The highest content of H_2O_2 was observed in the treatment of no methanol application under salinity stress conditions (3.65 nmol g^{-1} FW), which was not statistically significantly different from the treatment of 10% methanol application under salinity stress conditions (3.28 nmol g^{-1} FW) (Figure 2). On the other hand, the lowest amount of H_2O_2 was observed in the treatments of 20% methanol application under non-salinity stress conditions (1.85 nmol g^{-1} FW), 30% methanol application under non-salinity stress conditions (1.65 nmol g^{-1} FW) and 30% methanol application under salinity stress conditions (1.85 nmol g^{-1} FW), which were not statistically significantly different from the treatment of 10% methanol application under non-salinity stress conditions (2.03 nmol g^{-1} FW) (Figure 2).

Total soluble sugars (TSS)

Analysis of variance showed that TSS was significantly affected by salinity stress, methanol and their interaction ($P \leq 0.01$) (Table 3). Based on the results of mean comparison, it was determined that the treatment of 30% methanol application under salinity stress conditions (91.84 mg g^{-1} DW) had the highest TSS content; while the treatments of no methanol application under non-salinity stress conditions (37.74 mg g^{-1} DW), 10% methanol application under non-salinity stress conditions (39.45 mg g^{-1} DW) and no methanol application under salinity stress conditions (38.25 mg g^{-1} DW) had the lowest TSS content (Figure 2).

Relative water content of leaf (RWC)

Based on the results obtained from the present experiment, it was observed that RWC was significantly affected by salinity stress, methanol, and their interaction ($P \leq 0.01$) (Table 3). Under non-salinity conditions, methanol application at 10%, 20%, and 30% increased RWC to 79.35%, 80.21%, and 82.84%, respectively, while the lowest RWC (53.71%) was observed in the no-methanol treatment under salinity stress (Figure 2).

Total chlorophyll content

Based on the results of variance analysis, it was determined that the effects of salinity stress, methanol, and their interaction were significant for the total chlorophyll content ($P \leq 0.01$) (Table 2). The use of methanol under stress and non-salinity stress conditions increased the total chlorophyll content. Based on the results, it was determined that the treatments of applying 30% methanol under non-salinity stress conditions (10.23 µg ml^{-1}) and not applying methanol under salinity stress conditions (4.23 µg ml^{-1}) had the highest and lowest total chlorophyll content, respectively (Figure 2).

Principal component analysis (PCA)

The PCA results indicated that the first and second components had eigenvalue higher than one and were identified as influential components (Figure 3). The relative variance in the first and second components was 57.90 and 36.61%, respectively, and accounted for 94.52% of the total variance (Figure 3). The biplot from the first and second components showed that the treatments of 80 mM salinity stress with no methanol and 80 mM salinity stress with 10% methanol treatments were grouped together, showing strong correlation with the H_2O_2, MDA and EL traits (Figure 3). On the other hand, the 80 mM salinity stress with 20% methanol and 80 mM salinity stress with 30% methanol treatments were separated into an independent group and showed a strong correlation with the proline, SOD, TSS, PPO, APX, CAT and protein traits (Figure 3). The biplot evaluation obtained from the first and second components showed that the no stress with non-spraying of methanol, no stress with 10% methanol, no stress with 20% methanol and no

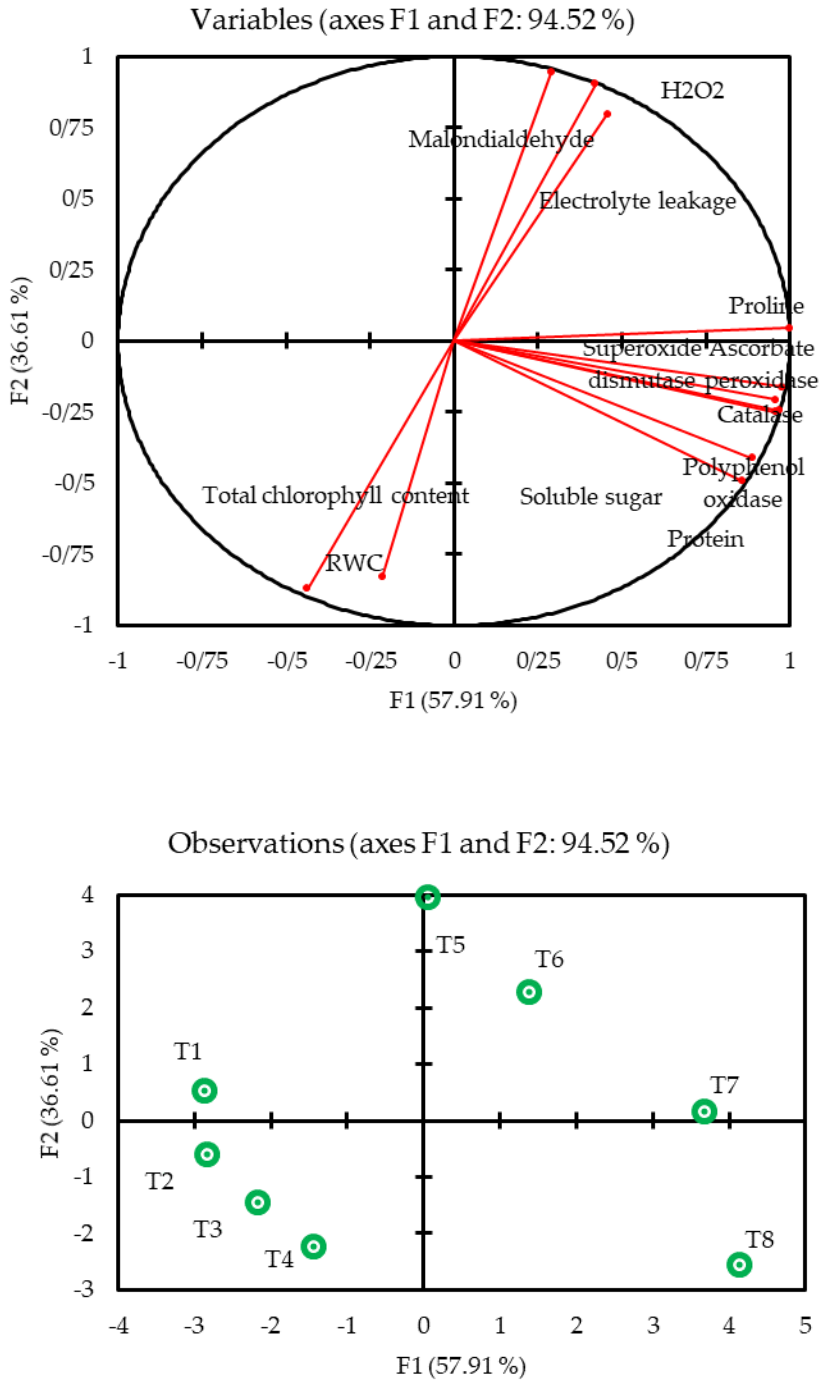


Figure 3. Biplot of the first and second components based on superoxide dismutase (SOD), polyphenol oxidase (PPO), catalase (CAT), ascorbate peroxidase (APX), protein, proline, malondialdehyde (MDA), electrolyte leakage (EL), H_2O_2, total soluble sugar (TSS), relative water content of leaf (RWC) and total chlorophyll content (TChl) traits in coriander plants under salt stress and methanol foliar spray treatments. T1: no stress with non-spraying of methanol, T2: no stress with 10% methanol, T3: no stress with 20% methanol, T4: no stress with 30% methanol, T5: 80 mM salinity stress with non-spraying of methanol, T6: 80 mM salinity stress with 10% methanol, T7: 80 mM salinity stress with 20% methanol and T8: 80 mM salinity stress with 30% methanol.

stress with 30% methanol treatments were placed in the same group and showed a strong correlation with the total chlorophyll content and RWC traits (Figure 3).

Cluster analysis

The dendrogram resulting from cluster analysis showed that the eight treatments were divided into three distinct groups; The first group included treatments without stress and non-spraying of methanol, treatments with 10%, 20%, and 30% methanol (Figure 4). The second group comprised the treatments of 80 mM salinity stress with no methanol and 80 mM salinity stress with 10% methanol. The third group included the treatments of 80 mM salinity stress with 20% and 30% methanol (Figure 4). Comparison of the groups derived from cluster analysis showed that the RWC and total chlorophyll content were significantly higher in the first group compared to the other groups (Figure 5). On the other hand, the second group was superior in terms of MDA, EL and H_2O_2 compared to the first and third groups. Box plots showed that in the third group, the SOD, PPO, CAT, APX, protein, proline and TSS traits were superior compared to the first and second groups (Figure 5)

Discussion

Salt stress significantly influences the physiological and biochemical activities of plants, and effective management strategies are crucial to mitigate its detrimental effects. In the present experiment, the exogenous application of methanol demonstrated multiple positive effects on the studied characteristics of coriander plants.

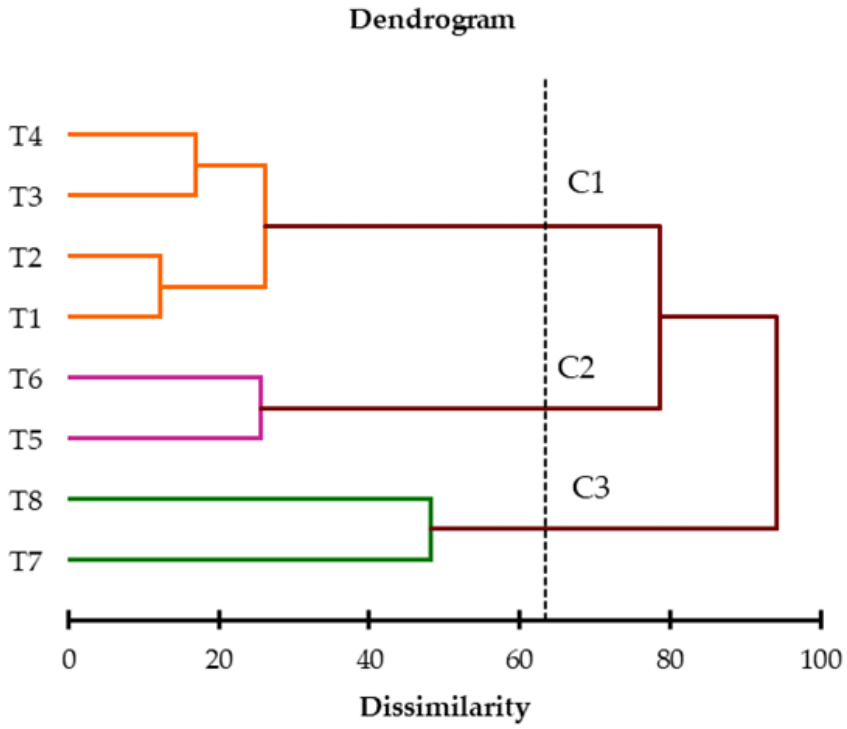


Figure 4. Dendrogram resulting from cluster analysis based on superoxide dismutase (SOD), polyphenol oxidase (PPO), catalase (CAT), ascorbate peroxidase (APX), protein, proline, malondialdehyde (MDA), electrolyte leakage (EL), H_2O_2, total soluble sugar (TSS), relative water content of leaf (RWC) and total chlorophyll content (TChl) traits in coriander plants under salt stress and methanol foliar spray treatments. T1: no stress with non-spraying of methanol, T2: no stress with 10% methanol, T3: no stress with 20% methanol, T4: no stress with 30% methanol, T5: 80 mM salinity stress with non-spraying of methanol, T6: 80 mM salinity stress with 10% methanol, T7: 80 mM salinity stress with 20% methanol and T8: 80 mM salinity stress with 30% methanol.

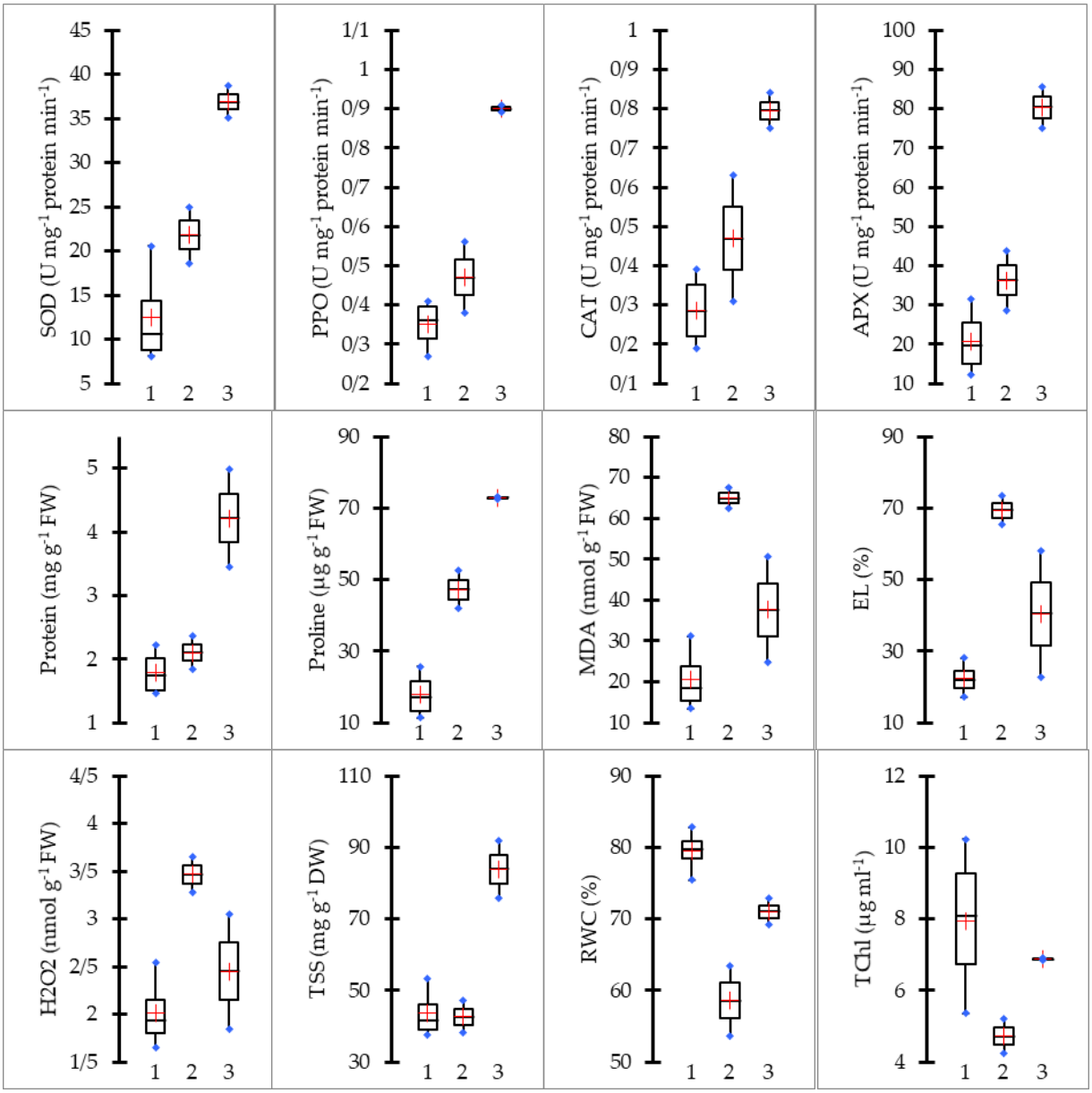


Figure 5. Box plot of comparison between groups resulting from cluster analysis in superoxide dismutase (SOD), polyphenol oxidase (PPO), catalase (CAT), ascorbate peroxidase (APX), protein, proline, malondialdehyde (MDA), electrolyte leakage (EL), H_2O_2, total soluble sugar (TSS), relative water content of leaf (RWC) and total chlorophyll content (TChl) traits in coriander plants under salt stress and methanol foliar spray treatments.

Our findings indicated that salt stress increased the activity of antioxidant enzymes, including SOD, PPO, CAT, and APX. These results align with those of (Hosseini et al., 2023), who reported elevated antioxidant enzyme activity in various mint species subjected to salt stress. Similar increases in antioxidant enzyme activity have also been documented in coriander (Rabiei et al., 2020), peppermint (Khalvandi et al., 2019), basil (Yilmaz et al., 2023), and sage (Bayat et al., 2022) under saline conditions, further corroborating our findings.

Salinity stress induces the production of ROS, which can damage cellular components and lead to oxidative stress (Hosseini et al., 2023). In response, plants enhance the production of antioxidant enzymes to mitigate this damage (Yilmaz et al., 2023). These enzymes are crucial for protecting cell membranes and organelles by neutralizing ROS (Rabiei et al., 2020). Additionally, salinity stress

activates specific signaling pathways, stimulating the production of antioxidant enzymes (Azeem et al., 2023). Under saline conditions, plants require more energy, and antioxidant enzymes play a vital role in maintaining healthy metabolism (Yilmaz et al., 2023). An increase in antioxidant enzyme activity indicates an active plant response to environmental stresses (Hosseini et al., 2023), allowing for better tolerance to saline conditions and improved resistance to various stressors. Moreover, antioxidant enzymes help regulate the levels of essential nutrients and metabolites in response to salinity. By reducing oxidative stress, these enzymes can prevent DNA damage and genetic destruction (Hosseini et al., 2021; Kesawat et al., 2023; Yilmaz et al., 2023). Enhanced antioxidant enzyme activity can also lead to improved crop quality under salt stress conditions (Khalvandi et al., 2019).

Furthermore, our experiment demonstrated that methanol application positively influenced the activity of antioxidant enzymes. Methanol can serve as a valuable carbon source for the synthesis of secondary metabolites, which support the production of antioxidant enzymes (Dorokhov et al., 2015; Wei et al., 2015; El Moukhtari et al., 2020). By activating signaling pathways and increasing oxidative stress levels, methanol stimulates the production of antioxidant enzymes (Dorokhov et al., 2015; Wei et al., 2015; Dorokhov et al., 2018). Moreover, methanol can help regulate plant osmotic pressure, creating better conditions for antioxidant enzyme activity (Tavassoli and Galavi, 2011; Dorokhov et al., 2018). This compound also acts as a stimulator of plant responses to environmental stresses, including salinity and drought (Dorokhov et al., 2018). In our results, methanol application also increased chlorophyll content under salt stress conditions, likely providing the necessary carbon for photosynthesis and improving carbon fixation (Wei et al., 2015) .

Additionally, proline content increased in response to both salt stress and methanol application. Proline, a non-protein amino acid that accumulates in plants under environmental stresses, particularly salt stress, helps mitigate salinity's negative effects (Yan et al., 2024). By enhancing water balance in cells, proline increases the relative water content of leaves and reduces transpiration caused by salt (El Moukhtari et al., 2020). Furthermore, proline contributes to improved photosynthesis by supplying energy for metabolic processes under stress conditions (Yan et al., 2024). Under salt stress, proline accumulation can reduce oxidative damage and protect cell membranes (Shahid et al., 2022). This amino acid also boosts the activity of antioxidant enzymes, which are crucial for reducing oxidative stress, aligning with our experimental results (Singh et al., 2022). Additionally, proline is linked to the accumulation of carbohydrates in cells, serving as an energy source and providing protection against stress (Mohammadi Alagoz et al., 2023). In summary, proline not only mitigates the adverse effects of salinity but also promotes growth and development under salt stress by improving photosynthetic conditions and regulating metabolism (Hnilickova et al., 2021).

Lastly, in our experiment, the application of methanol under salt stress conditions resulted in a reduction of MDA content. It appears that methanol improved antioxidant enzyme activity and proline synthesis, reduced ROS levels, and helped prevent membrane damage, which in turn led to a decrease in MDA content (Wei et al., 2015; Hnilickova et al., 2021; Hosseini et al., 2023).

Conclusion

The results of our experiment investigating the effects of methanol foliar spraying on the physiological and biochemical traits of coriander plants under salt stress showed that methanol positively influences various characteristics of this plant. Firstly, we observed a significant increase in the chlorophyll content of the leaves in methanol-treated plants. This enhancement in chlorophyll levels likely improves the photosynthesis process, indicating greater efficiency in light absorption by the plant. On the other hand, the measured amounts of proline in methanol-treated plants also increased. This is related to the enhancement of the plant's ability to cope with oxidative stress and maintain water balance within the cells. In addition, the levels of antioxidant enzymes in plants that received methanol were significantly higher than those in control plants. This increase in the levels of antioxidant enzymes indicates a better ability of the plant to cope with damage caused by salt and oxidative stress. Furthermore, the relative water

content of leaves in methanol treatment was improved, indicating the positive effect of methanol in maintaining plant water balance under salt stress. Finally, the results showed that methanol had a positive effect on reducing the level of MDA, an indicator of oxidative damage. This improvement in the level of MDA can help reduce the damage caused by salt stress and maintain the plant's cellular structure. In summary, our findings indicate that methanol foliar spraying can serve as an effective strategy to improve salt stress resistance in coriander plants, leading to increased chlorophyll content, enhanced antioxidant enzyme function, improved proline and sugar accumulation, and better water balance, while also reducing the level of MDA. To maximize these benefits and ensure a cost-effective approach, we recommend that a concentration of 20% methanol be employed for foliar spraying under saline soil conditions. This specific concentration may provide the best balance between cost and the enhancement of physiological traits, thereby optimizing the agronomic management of coriander plants facing environmental stress. By implementing this recommendation, growers can effectively improve the resilience of their crops to salt stress, leading to better yield and quality

Supplementary Materials

No supplementary material is available for this article.

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Author Contributions

Conceptualization, M.M. and Y.N.; methodology, M.M.; software, E.H.; validation, M.M., Y.N. and M.G.; formal analysis, M.M.; investigation, M.G.; resources, E.H.; data curation, M.M.; writing—original draft preparation, M.M.; writing—review and editing, Y.N.; visualization, M.G.; supervision, M.M.; project administration, E.H.; funding acquisition, Y.N. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest Statement

The authors declare no conflict of interest.

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مقاله پژوهشی

بررسی تاثیر محلول پاشی متانول بر بهبود خصوصیات بیوشیمیایی و فیزیولوژیکی گیاه گشنیز (*Coriandrum sativum*) تحت تنش شوری

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چکیده: تنش شوری یکی از عوامل مهم محدودکننده رشد گیاهان است بنابراین، استفاده از ترکیباتی مانند متانول برای کاهش اثرات مضر تنش اهمیت زیادی دارد. این تحقیق با هدف بررسی تأثیر اسپری متانول بر ویژگی‌های فیزیولوژیکی و بیوشیمیایی گیاه گشنیز در شرایط تنش شوری در گلخانه انجام شد.. تنش شوری در دو سطح (شاهد و ۸۰ میلی‌مولار از منبع کلرید سدیم) و اسپری متانول در چهار سطح (شاهد، محلول‌پاشی متانول در غلظت‌های ۱۰٪، ۲۰٪ و ۳۰٪) اعمال شد. نتایج نشان داد که در شرایط تنش شوری، استفاده از متانول منجر به افزایش معنی‌داری در ویژگی‌های مختلف شد. فعالیت آنزیم سوپراکسید دیسموتاز با ۳۰٪ متانول در شرایط تنش شوری ۲۳٪ افزایش داشت. همچنین، فعالیت آنزیم پلی‌فنول اکسیداز با ۳۰٪ متانول در شرایط شوری ۸۰ میلی‌مولار ۵۱٪ افزایش و فعالیت کاتالاز ۲۹٪ افزایش یافت. محتوای پرولین نیز در شرایط تنش شوری ۱۲۴٪ افزایش و محتوای مالوندی‌آلدهید ۶۳٪ کاهش یافت. به طور کلی، اسپری ۳۰٪ متانول تحمل به تنش را در گیاه تحت شرایط شوری افزایش می‌دهد و فعالیت آنزیم‌های آنتی‌اکسیدانی، محتوای پروتئین و محافظت اوسموتیکی را بهبود می‌بخشد. این یافته‌ها نقش متانول را در کاهش اثرات تنش شوری بر فیزیولوژی گیاه نشان می‌دهند.

کلمات کلیدی: آنزیم، رنگدانه، کلرید سدیم، مالون‌دی‌آلدهید، نشت الکترولیت.

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