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EDITORIAL

WELCOMING NEW SCIENTIFIC ADVISORY BOARD MEMBERS

Katarzyna Kmieć https://orcid.org/0000-0003-2272-4512

University of Life Sciences in Lublin, Poland

ABSTRACT

We welcome eighteen new Scientific Advisory Board members of Acta Scientiarum Polonorum Hortorum Cultus, who help to expand our expertise in areas of cutting-edge horticultural research and maintain our journal's high publishing standards.

In 2026, we celebrate the 25th anniversary of Acta Scientiarum Polonorum Hortorum Cultus, established in 2001. This milestone coincides with the commencement of a new term of the journal's Scientific Advisory Board (SAB), appointed for the years 2026–2030. The beginning of this new chapter offers an excellent opportunity to further strengthen the journal through the engagement of distinguished scholars whose expertise and experience represent diverse areas of horticultural sciences and related disciplines. The SAB plays an essential role in the journal's development, providing strategic guidance on scientific direction, editorial policy, and long-term vision. The active involvement of SAB members in the international scientific community help us promote best practices in research and publishing.

We warmly welcome our new SAB members and look forward to a fruitful collaboration that will further strengthen and expand journal international visibility and impact in the field of horticultural and plant sciences.

Katarzyna Bączek is a Full Professor at the Department of Vegetable and Medicinal Plants, Warsaw University of Life Sciences (SGGW), Poland. She specializes in plant science, with a focus on the genetic resources, cultivation, and phytochemical properties of medicinal and aromatic plants, contributing to both biodiversity conservation and sustainable horticultural practice. Her research focuses on extraction methods, secondary metabolite analysis, and the potential uses of essential oils and other phytochemicals. Her interests also include traditional and herbal medicine, and herbal drug development.

Sanem Bulam is an Assistant Professor at the Food Technology Program at Şebinkarahisar Vocational School, Giresun University, Turkey. Her scientific interests encompass edible and medicinal fungi, food processing, and the nutritional and bioactive properties of plant-derived foods, with a particular focus on wild mushroom species and their applications in sustainable and functional food production. She has conducted research on phenolic compounds, antioxidants and bioactive components and nutrients in foods, especially in edible wild mushrooms and their powders.

Alperen Kaan Bütüner is a Research Assistant at Bursa Uludag University, Turkey. His main research interests include entomology and pest control, with a particular emphasis on sustainable and integrated pest management strategies. He specializes in biological control approaches, especially the use of entomopathogenic nematodes, as well as precision agriculture technologies for pest monitoring and management. His scientific work focuses on host-seeking behaviour, efficacy, and environmental interactions of biological control agents, alongside the development of data-driven and technology-based solutions to improve the effectiveness and sustainability of pest management practices. He has been actively involved in both national and international research projects.

Alessandra Carruba is an Associate Professor of Agronomy and Field Crops at the Department of Agricultural, Food and Forest Sciences, University of Palermo, Italy. She received a Ph.D. in agricultural sciences. Her work focuses on sustainable cropping systems, with particular expertise in industrial field crops, and medicinal and aromatic plants in Mediterranean environments. Her research addresses cultivation under marginal and resource-limited conditions, nitrogen management, and sustainable weed control.

Barbara Frąszczak is an Associate Professor at the Department of Vegetable Crops, University of Life Sciences in Poznań, Poland. Her research focuses on cultivating horticultural crops in controlled and field environments, particularly vegetables, microgreens, and medicinal plants. She has conducted extensive studies on LED lighting, fluorescence-based stress assessment, and the quality-related traits of herbs, vegetables and microgreens. She also addresses the application of biostimulants to improve the yield and quality of plant raw materials. She is an expert in modern horticultural production systems and sustainable cultivation practices.

Gunārs Lācis is an Associate Professor, leading scientist and Head of the Unit of Genetics and Biotechnology at the Latvian Institute of Horticulture (LatHort), Latvia. He holds a Ph.D. in genetics and breeding from the Swedish University of Agricultural Sciences, and is also affiliated with the Latvia University of Life Sciences and Technologies. He has extensive experience in plant genetics and molecular biology, with research focused on the application of molecular tools in fruit crop genetics, as well as the conservation, characterization, and utilization of fruit genetic resources. His current work integrates implementation of the "One Health" concept and species interaction studies in the context of climate change, and preservation of biological diversity in agro- and forest biocenoses, ecosystem protection and services. Since 2006, he has participated in, and led 29 national and international research projects.

Mohammed Mahmood Mohammed is an Assistant Professor at the Department of Horticulture and Landscape Gardening, College of Agricultural Engineering Sciences, University of Baghdad, Iraq. His research focuses on plant physiology, plant production, and plant breeding, with particular emphasis on crop responses to abiotic stress, plant fertilization, sustainable horticulture, and vegetable production under challenging environmental conditions, including salinity. He has studied agronomic practices that enhance crop growth and yield, including research on foliar applications, nutrient management, and intercropping systems. He also participates in nationally and internationally funded research projects.

Ireneusz Ochmian is a Full Professor at the Department of Horticulture, West Pomeranian University of Technology in Szczecin, Poland. His research bridges horticultural science with practice and focuses on fruit quality and phytochemical composition, postharvest performance, and production technologies. He specializes in berry crops (notably highbush blueberry), cool-climate viticulture and enology, including strategies to improve wine quality while reducing sulphur dioxide use. He actively collaborates with industry partners, including a private winery and a large commercial blueberry plantation, translating field and storage studies into practical recommendations for growers and producers.

Mozaniel S. de Oliveira is a collaborating professor at the Postgraduate Program in Pharmaceutical Sciences, Institute of Health Sciences, Federal University of Pará (UFPA), Brazil. He holds a Ph.D. in food science and technology from UFPA, with a specialization in the supercritical fluid extraction of essential oils. He is a leading expert in natural products chemistry. He conducts research from the prospecting of volatile bioactive molecules to their *in vitro*, *in vivo*, and *in silico* evaluation for agricultural and pharmacological applications. His experience covers also food technology, natural products biotechnology and allelopathy. He is a remarkably prolific author and editor, bringing extensive editorial experience from serving on the boards of numerous international journals. His scientific impact is confirmed by his inclusion in the Stanford/Elsevier 2025 list of World's Top Scientists.

Angeliki Paraskevopoulou is an Associate Professor at the Laboratory of Floriculture and Landscape Architecture, Agricultural University of Athens (AUA), Greece. She has extensive research experience in ornamental and native plant species cultivation, green roofs, healing gardens, and Nature-based Solutions (NbS), with particular emphasis on enhancing biodiversity and local landscape character. Her work also addresses urban green and grey infrastructure, urban heat mitigation, and resilience in public spaces such as schoolyards, playgrounds, parks, as well as the protection of agricultural heritage landscapes. Prior to joining AUA, she has worked in the United Kingdom and Greece as a landscape architect and agriculturist, designing and managing public urban spaces. Her Mediterranean garden design for the Hellenic Pavilion at Horti-EXPO IGA 2003 in Rostock received a bronze award.

Bożena Pawłowska is a Full Professor at the Faculty of Biotechnology and Horticulture, University of Agriculture in Krakow, Poland. She conducts integrated research on plant biotechnology, physiology, and ornamental horticulture, with a strong emphasis on *in vitro* tissue culture and the development of efficient micropropagation systems for ornamental species and endangered plants. Her work focuses on cryopreserving germplasm to support the long-term conservation of genetic resources and protect biodiversity. She investigates plant photobiology, particularly the effects of LED light spectra on morphogenesis, physiological performance, and the synthesis of secondary metabolites under controlled light conditions. She also specializes in plant responses to abiotic stresses, such as salinity, focusing on stress physiology and biochemical adaptation mechanisms. Her research are also linked to practical applications that improve sustainability in floriculture and enable the effective use of ornamental species in landscape architecture and therapeutic horticulture.

Małgorzata Podwyszyńska is a Full Professor and researcher at the National Institute of Horticultural Research, Poland. She holds a Ph.D. in agriculture and horticulture. Her research focuses on the development of *in vitro* plant regeneration and propagation methods for the production of elite plant material and for breeding purposes. She investigates the factors affecting the efficiency of individual stages of micropropagation and the quality of plants produced through *in vitro* culture. Her work also includes the induction of polyploidy and the subsequent phenotypic and genetic evaluation of polyploid plants. In addition, she applies flow cytometry as a tool in both plant breeding and fundamental research.

Roman Rolbiecki is a Full Professor at the Department of Land Melioration, Agrometeorology and Plant Irrigation, Bydgoszcz University of Science and Technology, Poland. His extensive research address key challenges in water management, irrigation technology and sustainable agricultural systems. He has collaborated with research partners in the USA, Hungary, Turkey, and the Baltic region, and served on scientific committees including the Committee of Agronomic Sciences of the Polish Academy of Sciences and regional water partnership councils

Marta Cecilia Telesnicki is an Associate Professor at the Faculty of Agronomy, University of Buenos Aires, Argentina. She is a biologist with a PhD in agricultural sciences. Her research focuses on arthropod-plant interactions, integrating experimental evidence and statistical modelling to understand ecological processes across scales, and to enhance arthropod biodiversity and associated ecosystem services in crop and agricultural landscapes. She has contributed to national and international research projects on agroecosystems and crop management, including recent applied projects developed in collaboration with farmer-led organizations.

Kazimierz Tomala is a Full Professor at the Faculty of Horticulture, Warsaw University of Life Sciences (SGGW), Poland, and holds an honorary doctorate from the University of Life Sciences in Lublin. He is a distinguished scholar and a widely recognized authority in horticultural science. His research focuses on fruit nutrition and physiology, orchard fertilization strategies, fruit quality and shelf life, mineral composition of apples and pears, prediction of storage potential, and the application of modern storage technologies and biotechnology. The outcomes of his studies have substantial practical relevance and are widely implemented in horticultural practice, supporting growers at every stage of production, from fertilization and harvest management to advanced storage techniques.

Mehmet Tütüncü is an Associate Professor at Ondokuz Mayis University, Turkey. His research interests focus on ornamental plant breeding and biotechnology, particularly tissue culture-based propagation and *in vitro* breeding approaches. He has research experience in gynogenesis, haploid and double-haploid production, and biotechnological improvement of ornamental crops. His academic work aims to integrate classical horticultural breeding with modern biotechnological tools, supporting both scientific advancement and applied floriculture.

Sanja Fabek Uher is an Associate Professor at the Faculty of Agriculture, University of Zagreb, Croatia. Her scientific interests include the cultivation of vegetables as functional foods, integrated and hydroponic vegetable production, and the introduction of new vegetable growing technologies. Through scientific projects, she has participated in research on different factors affecting the agronomic properties and nutritional value of different vegetable and aromatic plants grown conventionally and soilless.

Evgeniya Zhekova is an Associate Professor and researcher at the Institute of Agriculture and Seed Science "Obraztsov chiflik" in Ruse, a unit of the Agricultural Academy in Sofia, Bulgaria. She is also affiliated with the "Angel Kanchev" University of Ruse, where she serves as a lecturer. Her scientific work focuses primarily on plant protection, particularly entomology, as well as plant science, seed science, general agriculture, and the development of organic and smart farming systems. Her research integrates both fundamental and applied approaches aimed at improving crop production and sustainable agricultural practices.

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REVIEW PAPER

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ADVANCING HORTICULTURAL KNOWLEDGE OF *Asparagus officinalis* L. A COMPREHENSIVE BIBLIOMETRIC AND THEMATIC ANALYSIS (1853–2025)

Siddig I. Abdelwahab[1]✉ https://orcid.org/0000-0002-6145-4466

Abdalbasit A. Mariod[2] https://orcid.org/0000-0003-3237-7948

Manal M.E. Taha[1] https://orcid.org/0000-0003-0166-8929

Khalid M. Al-Rohily[3] https://orcid.org/0009-0006-8754-7405

Adil E. Abdelnour[3]

[1] Health Research Centre, Jazan University, Jazan, Saudi Arabia
[2] College of Science, University of Jeddah, Jeddah, Saudi Arabia
[3] National Research and Development Center for Sustainable Agriculture (Estidamah), Riyadh Techno Valley, Riyadh 12373, Saudi Arabia
✉ sadiqa@jazanu.edu.sa

ABSTRACT

Background/Aim: *Asparagus officinalis* L. has attracted increasing scientific interest because of its agricultural, genetic, and pharmacological significance. This study aimed to systematically map intellectual and conceptual structures, growth patterns, and emerging trends in *A. officinalis* research from 1853 to 2025.

Methods: A bibliometric and conceptual analysis was conducted using the Scopus database following the PRISMA 2020 guidelines. A total of 1,065 original research articles were retrieved and analyzed. Bibliometric indicators, keyword mapping, co-citation clustering, and citation burst analyses were performed using Bibliometrix (RStudio), VOSviewer and CiteSpace.

Results: Scientific production exhibited a steady increase with notable surges after 2000. Japan, China, and the USA have emerged as leading contributors. Core journals were identified according to Bradford's law and key scholars were ranked by their local H-index. Thematic evolution revealed two major shifts in knowledge around 2001 and 2017, highlighting a transition toward molecular biology, genomics, and health-related studies. Eleven conceptual clusters were detected, with high Silhouette values indicating strong clustering quality. Emerging research hotspots include metabolomics, transcriptomics, and medicine.

Conclusion: This comprehensive bibliometric analysis revealed the dynamic growth and conceptual diversification of *A. officinalis* research, offering valuable insights into historical developments, current trends, and future research directions.

Keywords: *Asparagus officinalis*, bibliometric analysis, conceptual structure, thematic evolution, citation analysis

Abbreviations: ACE: angiotensin-converting enzyme; IMS: imaging mass spectrometry; PRISMA: preferred reporting items for systematic reviews and meta-analyses; RAPD: random amplified polymorphic DNA; SSR: simple sequence repeat; STS: sequence tagged site; SVG: scalable vector graphics; TLS: total link strength

INTRODUCTION

Asparagus (*Asparagus officinalis* L.) is a perennial horticultural crop of significant economic and nutritional value, cultivated globally for its edible young shoots or spears. With a rich cultivation history spanning ancient

civilizations – such as the Egyptians, Greeks, and Romans – its early use is evidenced in art and literature, though its exact applications during antiquity remain partially speculative [Moreno-Pinel et al. 2021]. The depiction of its presentation as an offering on an ancient Egyptian frieze serves as an initial indication of its utilization, although it remains ambiguous whether the plant was employed for culinary or therapeutic purposes during that period. Several literature sources have discussed its application in herbal medicine during the ancient periods of Greece and Rome [Prasad et al. 2024, Wen et al. 2024, Yadav et al. 2024]. Today, it is used by people worldwide. Furthermore, its medicinal value has been recognized in Traditional Chinese Medicine [Zhu et al. 2024].

Asparagus officinalis spears are rich in vitamins and antioxidants, and powerful in fiber, but relatively low in calories; its consumption greatly improves a healthy diet [Fang et al. 2024]. In addition, due to the presence of potassium in asparagus, some patient groups, particularly those with hypertension, can benefit from its mineral composition [Prasad et al. 2024, Wen et al. 2024]. *Asparagus* spp. has long been used in China and Korea as a source of herbal remedies. For example, vegetables are used in the management of urinary pathologies because of their diuretic properties [Kumar et al. 2010, Olas 2024]. In India, asparagus root preparations have been traditionally used to treat women's reproductive health, promote conception, and increase breast milk production [Pahuja et al. 2024]. In ancient Eastern and Greek medicine, asparagus extracts were administered as a tonic for the prevention and treatment of a myriad of illnesses, including rheumatism, liver disease, asthma, cancer, and kidney and bladder [Fang et al. 2024, Jana et al. 2025, Olas 2024, Pahuja et al. 2024, Prasad et al. 2024, Zhu et al. 2024]. Despite traditional uses, there is a lack of robust clinical data to support *A. officinalis*'s pharmacological applications, as highlighted by Olas [2024], who notes limited clinical trials for its health benefits, and Fang et al. [2024], who call for further clinical validation of its anti-proliferative effects in endometrial cancer. Traditional uses of *A. officinalis* lack robust clinical data, limiting FDA endorsement and restricting its pharmacological applications to traditional medicine [Fang et al. 2024, Zedan et al. 2025].

In the context of horticultural science, understanding the evolution of asparagus research is crucial for advancing cultivation practices, genetic improvement, and utilization strategies. Bibliometric analysis – an established method for quantitatively mapping scientific output – offers a powerful tool to evaluate global research dynamics, highlight knowledge gaps, and forecast emerging trends [Alkhammash 2023]. In the context of horticultural science, understanding the evolution of asparagus research is crucial for advancing cultivation practices, genetic improvement, and utilization strategies. Bibliometric analysis – an established method for quantitatively mapping scientific output – offers a powerful tool to evaluate global research dynamics, highlight knowledge gaps, and forecast emerging trends.

MATERIALS AND METHODS

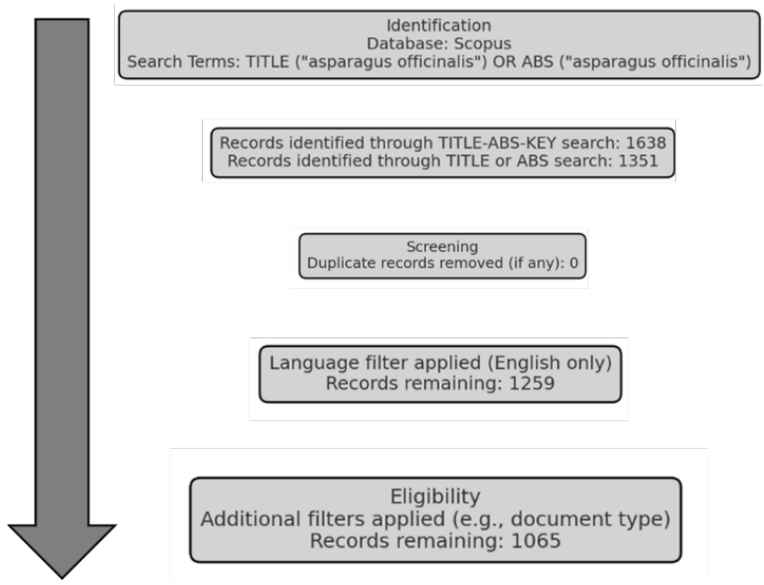
Data source and search strategy

A systematic approach using the Scopus database was employed to identify relevant studies on *Asparagus officinalis*, following PRISMA 2020 guidelines [Sarkis-Onofre et al. 2021] to ensure transparency, reproducibility, and methodological rigor (Figure 1). The search term "*Asparagus officinalis*" was used in two complementary searches to maximize retrieval of relevant articles while maintaining precision. The first search, using the TITLE-ABS-KEY field (covering title, abstract, and keywords), returned 1,638 records, capturing a broad range of studies, including those with the term in author-provided keywords. The second search, limited to TITLE or ABSTRACT fields, returned 1,351 records, focusing on articles where the term was explicitly mentioned in these fields to enhance specificity. Results from both searches were merged, and duplicate records were removed during the screening phase, yielding 1,259 records after applying a language filter to include only English-language studies. This filter was applied to ensure compatibility with bibliometric tools (e.g., Bibliometrix, VOSviewer, CiteSpace), which are optimized for English metadata, though this may introduce language bias by potentially excluding relevant non-English studies from regions like China or Japan; this limitation is acknowledged in the discussion section. During the eligibility phase, additional filters (e.g., restriction to original articles) were applied, resulting in 1,065 eligible records for bibliometric analysis. Data extracted on 31.03.2025.

Data analysis

This study employed a multi-step bibliometric methodology to analyze *Asparagus officinalis* research from 1853 to 2025. Metadata were extracted from Scopus and processed in BibTeX and CSV formats. The year 1853 marks the earliest indexed publication on this species in the Scopus database, providing a foundational point for bibliometric analysis. Including data up to 2025 ensures the incorporation of the most recent studies and preprints available at the

Figure 1. PRISMA flow diagram illustrating the search and selection process for studies *on Asparagus officinalis* identified in Scopus, including identification, screening, eligibility assessment, and final inclusion



time of data extraction (May 2024), allowing for a forward-looking perspective on emerging trends. By covering over 170 years, the analysis provides a robust and contextualized understanding of how scientific interest, methodologies, and applications of *A. officinalis* have transformed over time, highlighting both historical contributions and current frontiers in horticultural science. Descriptive statistics, including publication trends, co-authorship, international collaboration, and citation metrics, were analyzed using Bibliometrix in RStudio. Global research contributions were visualized through world maps and bar-line charts. Core journals were identified via Bradford's Law, and influential authors were ranked using the local H-index. Keyword co-occurrence networks and thematic evolutions were mapped using VOSviewer and Bibliometrix, while CiteSpace enabled co-citation clustering, burst detection, and conceptual mapping. Eleven major research clusters were identified with high silhouette values (0.831–0.997), indicating strong thematic cohesion. Together, these tools provided an integrated view of publication growth, research hotspots, collaboration patterns, and evolving themes in *A. officinalis* studies.

RESULTS

Descriptive bibliometrics

Descriptive bibliometric statistics for *A. officinalis* research were extracted from the Scopus database (1853–2025). A total of 1,065 documents authored by 2,962 researchers were analyzed across 416 sources, with an annual growth rate of 2.29%. The international co-authorship rate was 16.49% and the average number of citations per document was 21.01. Additional metrics included an average of 4.49 co-authors per document and 2,748 author keywords, reflecting a collaborative and expanding research landscape.

Growth and impact

The growth of research on *A. officinalis* has demonstrated a clear upward trajectory, particularly since the early 1990s (Figure 2). Although publication activity was sporadic and limited before 1980, a steady increase in the number of articles became evident from the late 20th century onward, with notable surges after 2000. This trend indicates rising scientific interest in the agricultural, genetic, and pharmacological properties of plants. Citation analysis revealed that earlier studies, particularly those published between the 1970s and the early 2000s, achieved higher average citations per article, suggesting their foundational influence on the field. However, in recent years (post-2017), although the volume of publications has increased, the mean total citations per article has declined, reflecting the time lag in citation accumulation, as newer publications require several years to accrue significant citations. Overall, this pattern highlights both the maturation of research on *A. officinalis* and emergence of new investigative directions (Figure 2).

Figure 2. Trends in the scientific research on *Asparagus officinalis* from 1853 to 2025. The blue line represents the mean total citations per article (MeanTCperArt), while the red line indicates the number of publications (N) per year

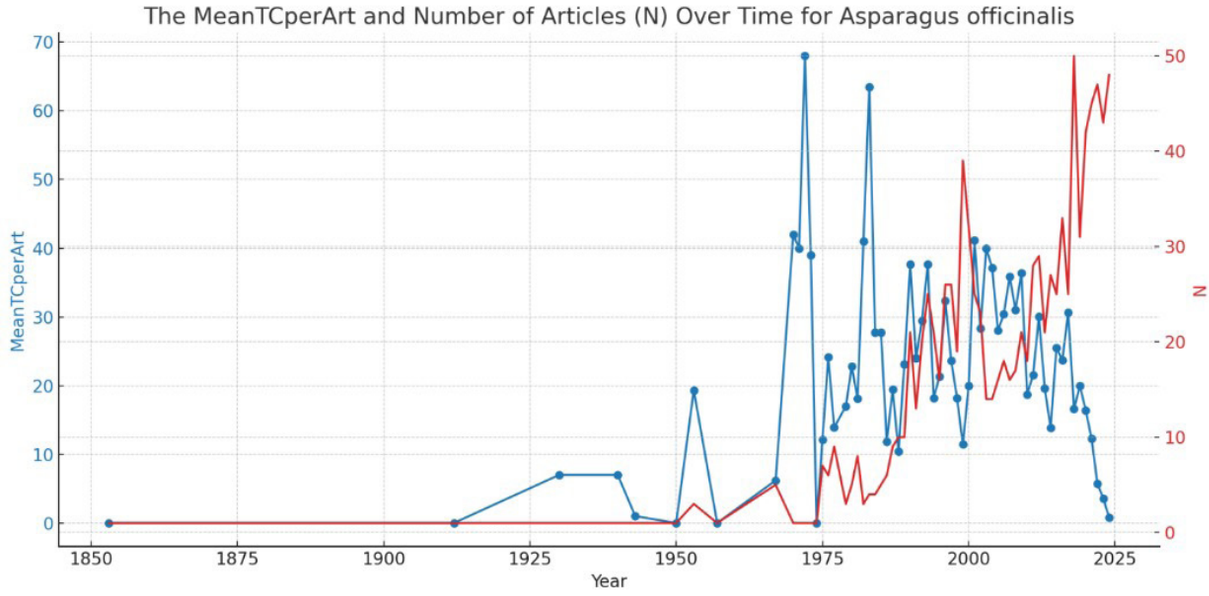
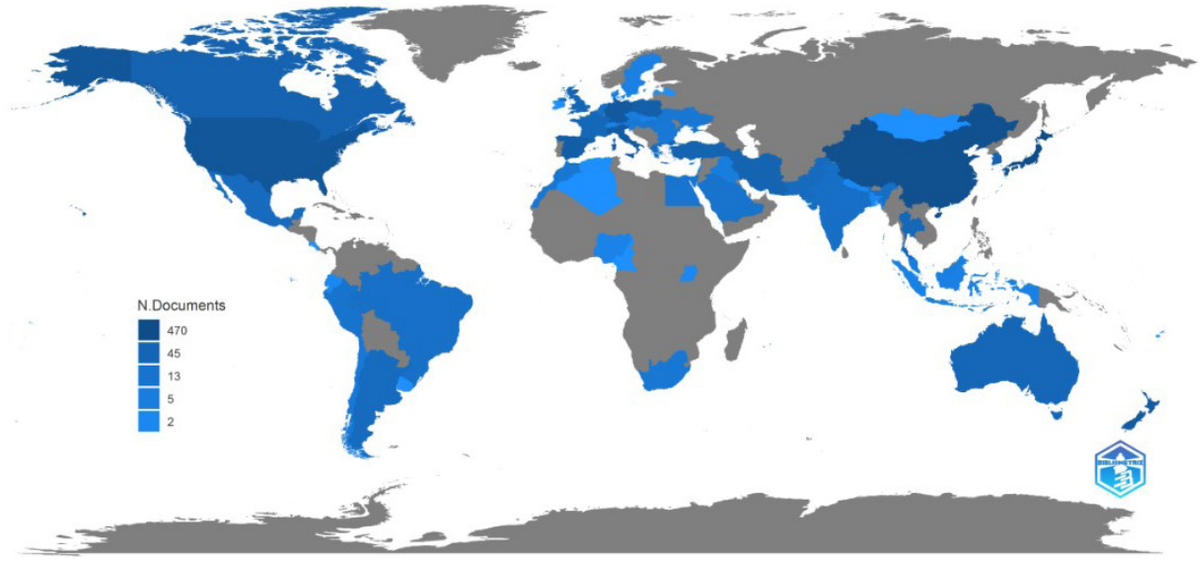


Figure 3. Global distribution of scientific publications on *Asparagus officinalis* research. Countries are shaded according to their publication output, with darker colors representing higher numbers of documents. Japan, China, and the USA are the top contributors, reflecting major centers of research activity on *A. officinalis*. Map created using Bibliometrix

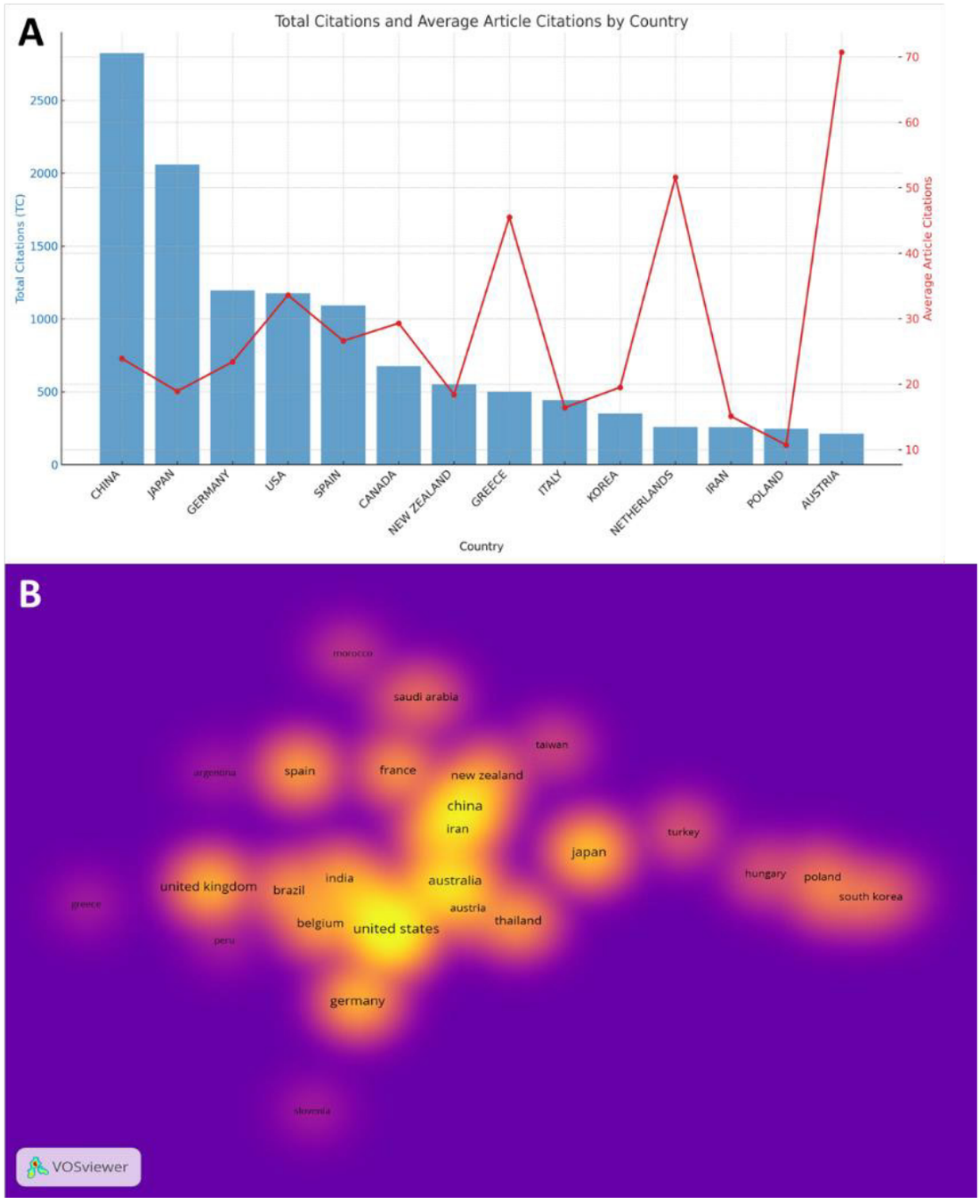


Frontier countries

The scientific production of *A. officinalis* exhibits notable geographical variation, with Japan emerging as the most prolific contributor (470 documents), followed by China (354), the USA (198), Germany (149), and Italy (127). Other countries, such as New Zealand, Spain, Poland, and South Korea, also demonstrated significant research output. Countries from Europe, North America, and Asia dominate the scientific landscape, whereas contributions from Africa, South America, and Oceania remain comparatively limited. The global distribution illustrated in Figure 3 shows dense research activity concentrated in technologically advanced and agriculturally significant nations, reflecting both the research capacity and agricultural importance of *A. officinalis* worldwide.

Figure 4A shows total citations and average article citations for *A. officinalis* research across 14 countries. China leads in total citations, followed by Japan, Germany, and the USA, while Austria has the highest citation impact per article (70.7), indicating strong academic influence despite lower output. Other high-impact countries include the Netherlands, Greece, and the USA. Figure 4B, a VOSviewer density map, highlights top collaborative nations based on total link strength (TLS), with the USA (TLS = 76), China (66), Australia (49), and Japan (46) leading. European countries like Germany, the UK, and the Netherlands also show strong research networks, along with Canada and Thailand. This underscores the central role of North America, East Asia, and Europe in global asparagus research collaboration.

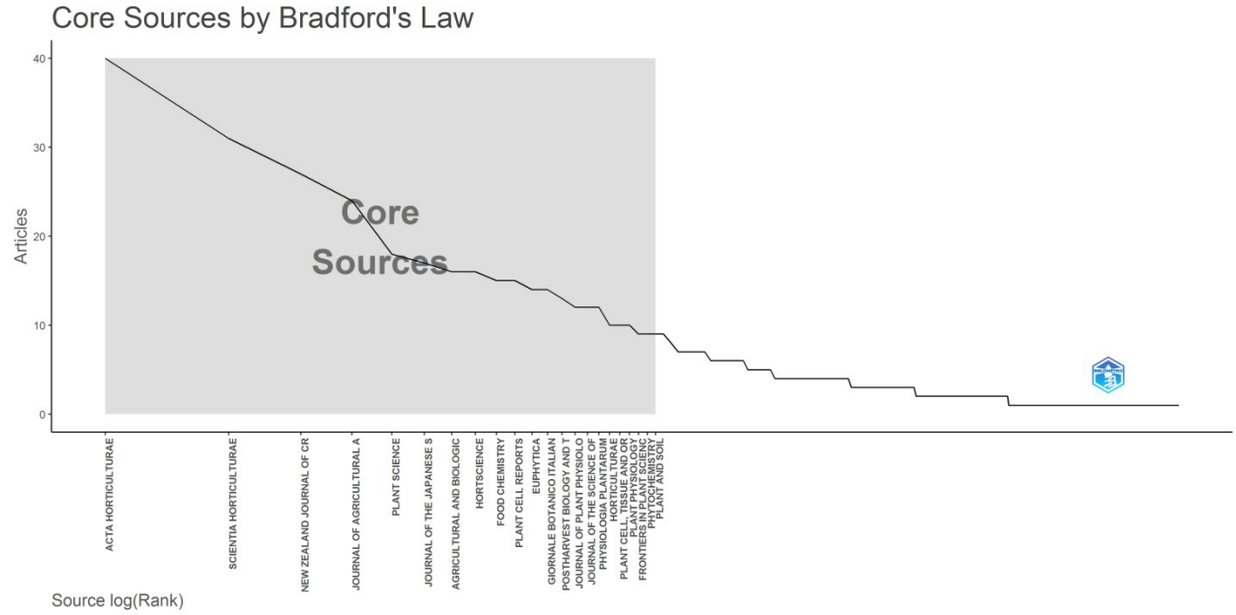
Figure 4. A: Total citations (blue bars) and average article citations (red line) for *Asparagus officinalis* research across leading countries, generated using Bibliometrix from Scopus data. B: Density visualization of international collaboration in *A. officinalis* research, generated using VOSviewer from Scopus data, with halo intensity representing total link strength (TLS**)**. Warmer colors indicate higher TLS values, reflecting greater international research connectivity



Core journals based on Bradford's law

The distribution of sources related to *A. officinalis* research follows Bradford's Law of Scattering, classifying the journals into three distinct zones (Figure 5). Zone 1 included 22 core journals that collectively accounted for a substantial portion of published articles. "Acta Horticulturae" ranked first with 40 articles, followed by "Scientia Horticulturae" (31 articles), and the "New Zealand Journal of Crop and Horticultural Science" (27 articles). Zone 2 comprised 94 journals with a moderate number of articles per source, such as "Sexual Plant Reproduction and Theoretical" and "Applied Genetics". Zone 3 contains the remaining 300 journals, each contributing a small number of documents, such as "European Food Research" and "Technology and Food Science" and "Nutrition". Overall, out of the 416 sources analyzed, a small core group was responsible for a disproportionately large share of the scientific output, highlighting the concentration of asparagus research in specialized journals.

Figure 5. Bradford's Law distribution of core journals publishing *Asparagus officinalis* research. The plot identifies three zones: a small group of highly productive core journals (Zone 1), a moderate group (Zone 2), and a large group with fewer publications (Zone 3). Analysis based on 416 sources using Bibliometrix



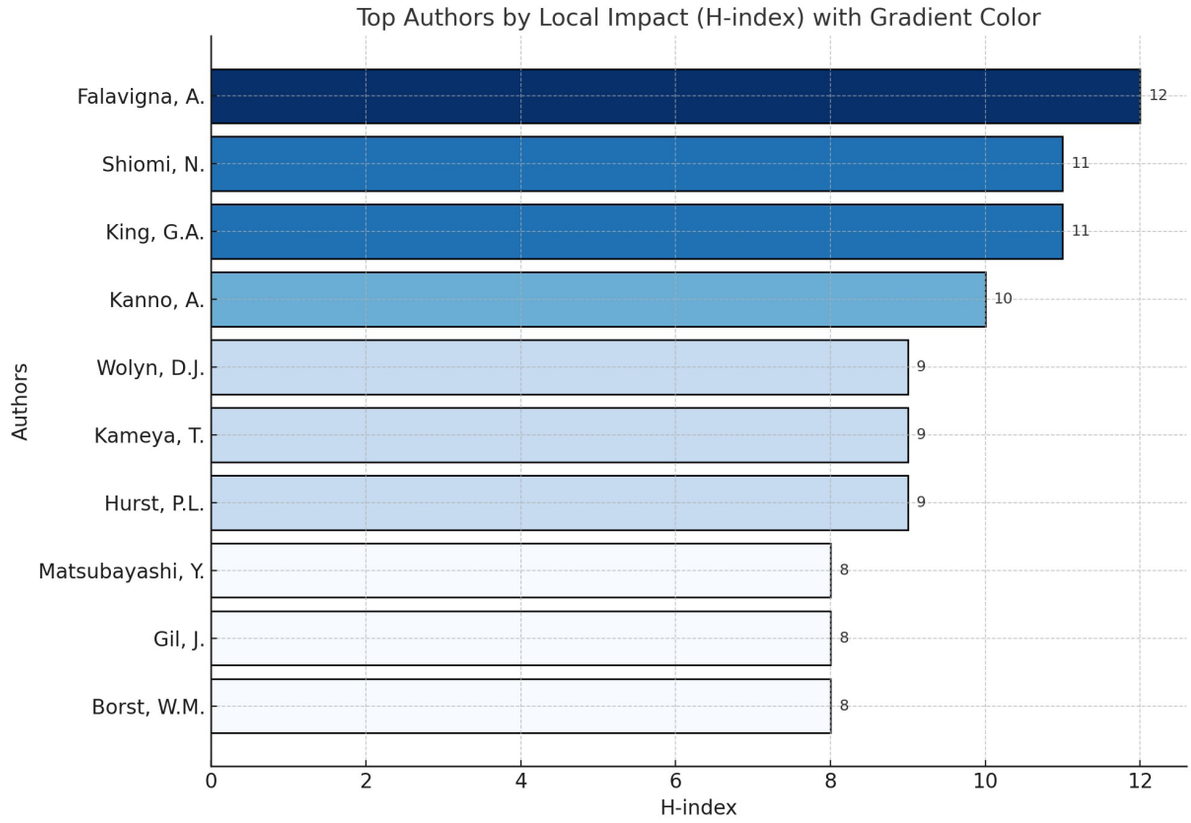
Most impactful scholars based on local H-index

Figure 6 shows the most important scholars based on their local H-index values. The H-index is a composite metric that measures both the productivity and citation impact of an author's publications; an H-index of h means that the author has h papers, each cited at least h times. In this analysis, Falavigna, A. achieved the highest local H-index (12), followed by King, G.A. and Shiomi, N. (both with 11). Kanno, A. ranked fourth with an H-index of 10, whereas several other researchers, including Hurst, P.L., Kameya, T., and Wolyn, D.J., demonstrated a strong impact with scores of 9. The gradient color scheme represents the relative strength of each author's influence, with darker shades indicating higher H-index values (Figure 6).

Keywords co-occurrence: most frequent author's keywords

The density visualization map generated using VOSviewer highlighted the most frequently occurring author's keywords in *A. officinalis* research (Figure 7). The keyword "*Asparagus officinalis*" dominates the map and is surrounded by a dense cluster of related terms. Other prominent keywords include "yield," "antioxidant activity," "storage," "flow cytometry," and "genetic diversity," indicating the major thematic areas within the field. Additionally, keywords such as "somatic embryogenesis," "metabolomics," "sucrose," "cryopreservation," and "transcriptome" emerge, reflecting research directions related to plant development, molecular biology, and biochemical profiling. The color intensity in the visualization corresponds to the frequency of occurrence, with warmer colors (yellow) indicating higher concentrations of research focus and cooler colors (blue) signifying less-frequent topics. This density map underscores the diverse yet interconnected nature of asparagus research spanning agricultural, genetic, and phytochemical domains.

Figure 6. The figure illustrates the local impact of top authors in *Asparagus officinalis* research, measured by their H-index. Authors' names are presented in the format "Surname, Initial." The length of each bar corresponds to the H-index value, while the gradient blue color intensity reflects the magnitude of scholarly impact, with darker bars indicating higher values. Falavigna, A. achieved the highest H-index (12), followed closely by King, G.A. and Shiomi, N. (both with 11). The visualization highlights variations in research productivity and influence among key contributors in the field



Thematic evolution

Chronological changes in research topics associated with *A. officinalis* demonstrate multidisciplinary diversity and its traditional branches from 1853 to 2025, as illustrated in Figure 8. This figure captures the gradual accumulation of knowledge in this area, which is tailored by two major turning points. The first turning point appeared around year 2001, where beginnings concepts like "callus," "yield," and "dioecy" transformed into expansionist monoculturalism "somatic embryogenesis," "fusarium oxysporum," "flow cytometry," and "gene expression" indicating increased focus towards plant biotechnology and pathogen relationships. The second major shift emerged in 2017, signalling an advanced phase with research expanding toward "transcriptome," "medicinal plants," "genetic diversity," and "apoptosis," indicating the integration of molecular biology, genomics, and applied health sciences into *A. officinalis* studies. This conceptual dynamics analysis underscores the continuous broadening and deepening of asparagus research, driven by technological innovations and evolving scientific priorities.

Top 25 cited authors with the strongest citation bursts

Figure 9 illustrates the top 25 most cited authors with the strongest citation bursts in *A. officinalis* research from 2014 to 2025. A citation burst indicates the period during which an author's work has received a significant increase in citations. The figure shows that authors such as Guo, Q. (7.25), Pegiou, E. (7.02), and Siomos, A.S. (6.01) exhibited the strongest citation bursts, as reflected by their high strength values. Early bursts were noted by authors such as Jamsari, A., and Nakayama, H., beginning around 2014–2015. In contrast, more recent bursts were observed for authors such as Liu, J., Mousavizadeh, S.J., and Lil, Y., suggesting emerging influential contributions in the latest research period. The red bars represent the active burst period, highlighting shifts in author influence over time.

Figure 7. Density visualization of the most frequent author's keywords related to *Asparagus officinalis* research, generated using VOSviewer. Phrases like, "*Asparagus officinalis,*" "yield," "antioxidant activity," "storage," and "flow cytometry" illustrate balancing levels of attention towards specific areas of research. The strength of shading indicates the value of the keyword's frequency

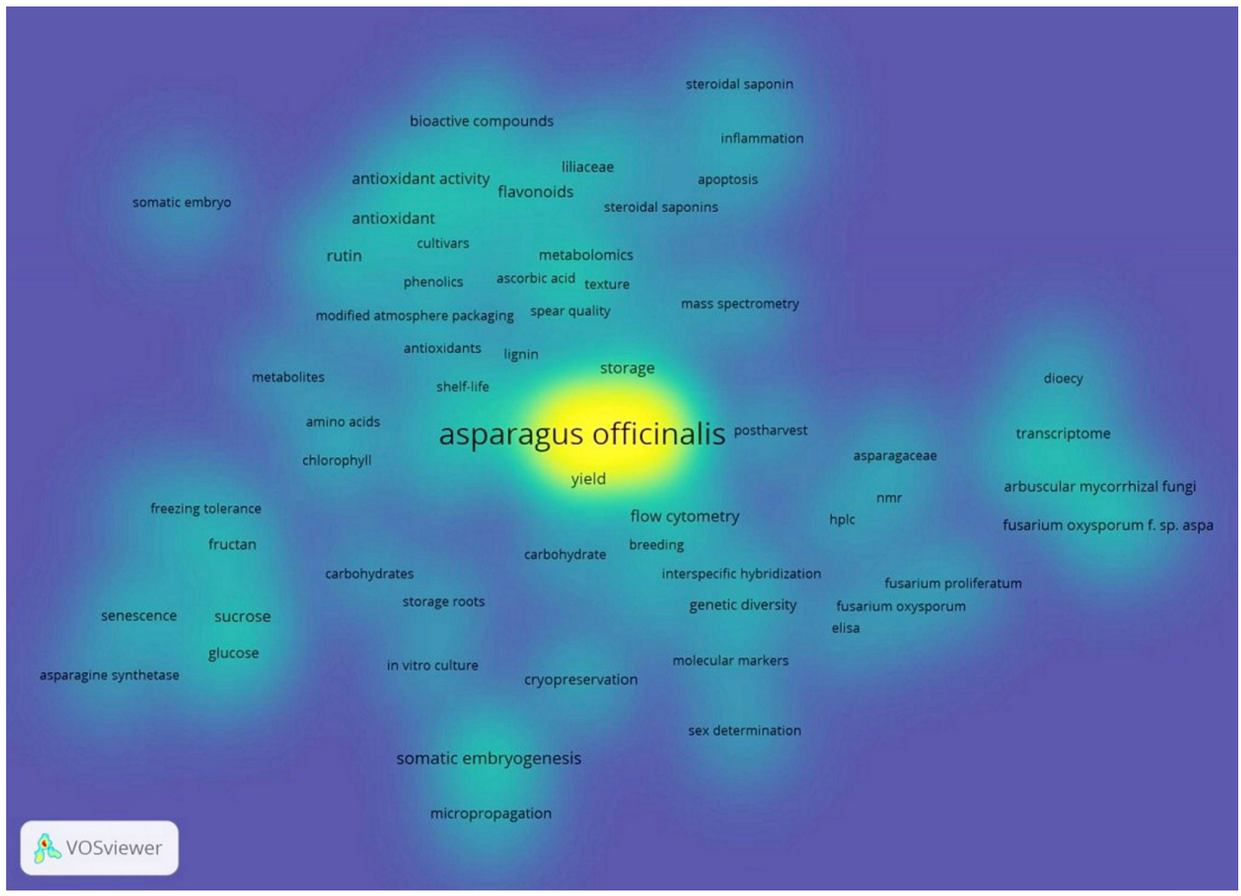


Figure 8. Thematic evolution of *Asparagus officinalis* research from 1853–2025. This figure, produced with Bibliometrix alongside a BibTeX file sourced from Scopus, showcases the most prominent changes in the focus of research over three periods, particularly emphasizing two important shifts in 2001 and 2017 that transformed the scientific frontier

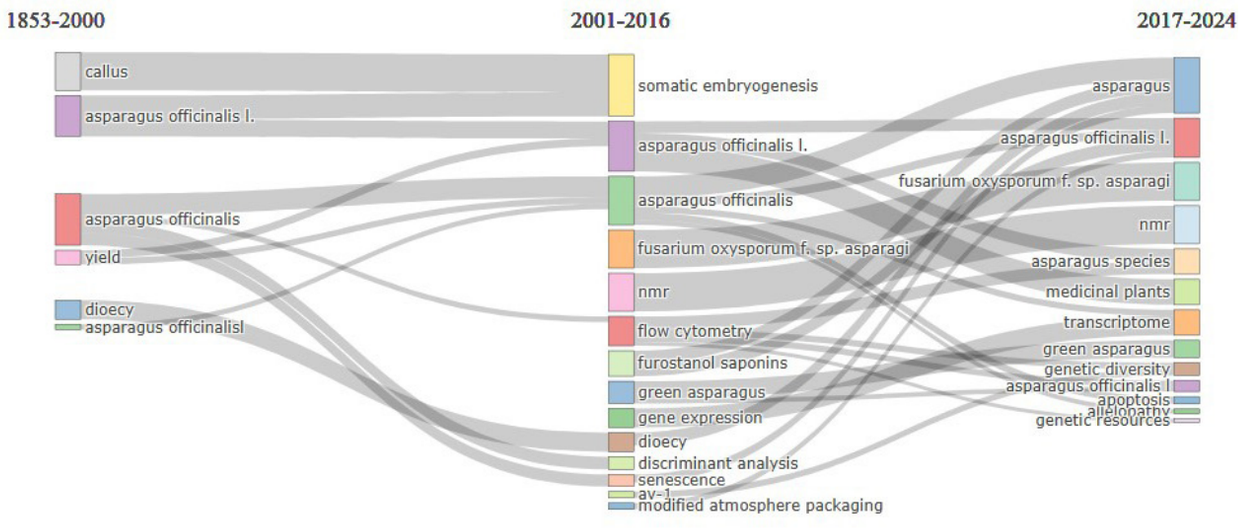


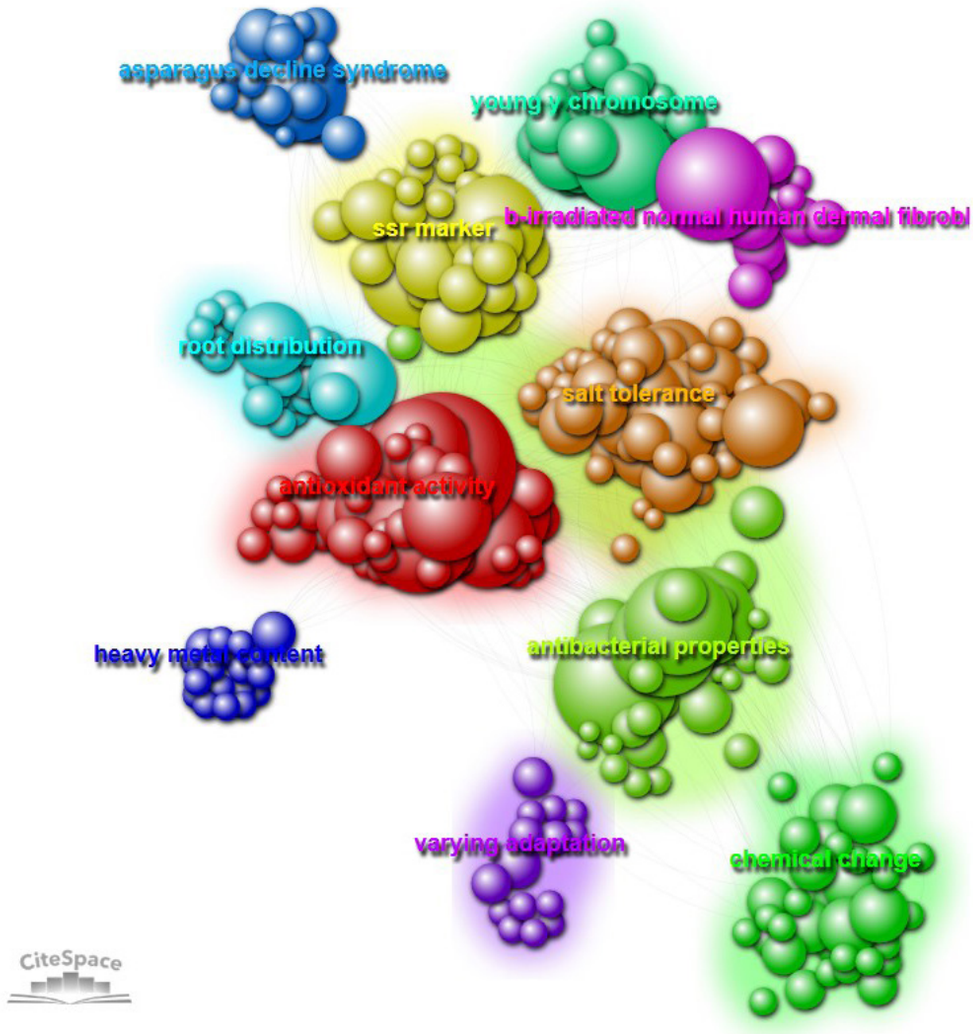
Figure 9. Top 25 cited authors with the strongest citation bursts in *Asparagus officinalis* research from 2014 to 2025. The figure, generated using CiteSpace, highlights periods of significant citation increases (red bars). Authors such as Guo, Q. (7.25), Pegiou, E. (7.02), and Siomos, A.S. (6.01) exhibited the most prominent bursts during the analyzed timeframe

Conceptual structure and clustering of *Asparagus officinalis* research

The CiteSpace-generated conceptual map (Figure 10) visualizes the knowledge structure of *Asparagus officinalis* research, identifying 11 major clusters characterized by distinct themes and research directions. The clustering quality was high, with silhouette values ranging from 0.831 to 0.997, reflecting strong homogeneity within the clusters.

Cluster #0, titled "Antioxidant Activity," was the largest with 71 members and an average of 2017. This cluster focuses on the antioxidant properties and health-promoting phytochemicals found in *A. officinalis*, with key contributions from Conversa, G. (2019) and Shahrajabian, M.H. (2022). Prominent authors in this area include Fuentes-Alventosa, J.M., Rodriguez, R., and Wang, J. Cluster #1, "Salt Tolerance," also comprises 71 members and centers on mechanisms of salt stress resistance, particularly the role of mycorrhizal associations and saponin biosynthesis, with important studies by Zhang, X. (2021) and Ying, J. (2024). The leading figures include Zhang, Y., Zhang, J., and Wang, Y. Cluster #2, "SSR Marker and Genetic Diversity," involves 58 members and highlights efforts in genetic mapping and breeding programs. Influential papers by Harkess, A. (2017) and Mercati, F. (2015) define this area, supported by key authors such as Knaflewski, M. and Kubota, S. Cluster #3, focusing on "Antibacterial Properties," encompassed 40 members, and emphasized the antimicrobial potential of asparagus extracts, including nanoparticle-mediated approaches. Noteworthy works include those by Nguyen, G.T.N. (2024) and Esfanddarani, H.M. (2025), with Guo, Q., Pegiou, E., and Zhang, H. as major contributors.

Figure 10. Conceptual Structure and Major Research Clusters in *Asparagus officinalis* Studies (2014–2025). This figure presents a cluster visualization of the conceptual structure of *Asparagus officinalis* research, based on co-citation analysis using CiteSpace (SVG format). Each color represents a distinct research cluster, with labels highlighting the dominant themes such as "Antioxidant Activity," "Salt Tolerance," "SSR Marker," and others. Node size reflects the frequency of citation, while the spatial proximity of clusters indicates thematic similarity. Data were extracted from studies published between 2014 and 2025



The "Chemical Change" cluster (#4), with 38 members, concentrates on postharvest physiology, storage, and quality preservation strategies, as seen in studies by Techavuthiporn, C. (2016) and Mastropasqua, L. (2016). Cluster #5, "Young Y Chromosome," contains 37 members and explores the evolution and genetics of sex chromosomes in asparagus, as highlighted by Harkess, A. (2017) and Murase, K. (2017).

Cluster #6, "Root Distribution," composed of 28 members, investigates the root architecture and varietal differences under diverse conditions, with Drost D (2023) as a leading figure. "Asparagus Decline Syndrome" forms cluster #7 with 19 members, concentrating on the pathology and management of viral and fungal diseases, led by studies such as those by López-Moreno FJ (2025) and Farahani-Kofoet RD (2020). Cluster #8, "Heavy Metal Content," includes 18 members and assesses the accumulation of toxic elements in asparagus grown in contaminated soils, as addressed by Conversa, G. (2019).

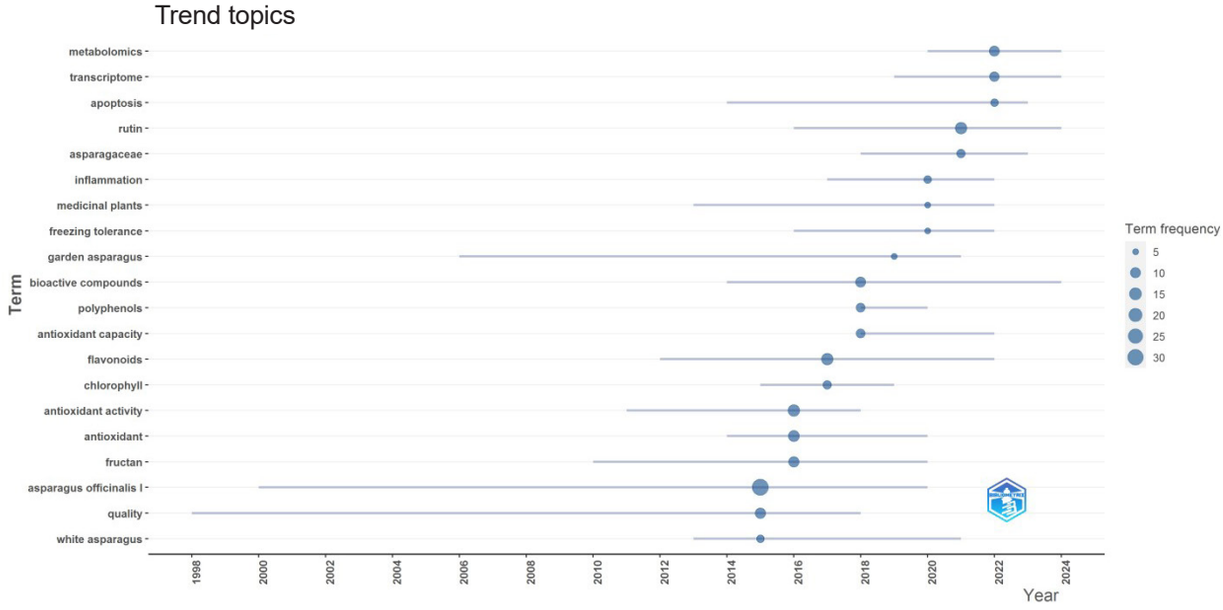
The smaller Cluster #9, "Varying Adaptation," with 15 members, explores adaptation strategies to environmental stresses, such as freezing, as represented by Panjtandoust, M. (2016). Finally, Cluster #10, "B-Irradiated Normal Human Dermal Fibroblast," consisting of 12 members, examined the biomedical effects of asparagus extracts on human cells, particularly their anti-inflammatory properties, as discussed in studies by Shirato, K. (2018, 2021).

Table 1. Conceptual structure and major research clusters in *Asparagus officinalis* studies (2014–2025)

Cluster	Size	Average year	Silhouette value	Focus
#0: Antioxidant Activity	71	2017	0.848	antioxidant properties and phytochemicals
#1: Salt Tolerance	71	2022	0.823	salt stress resistance and saponin biosynthesis
#2: SSR Marker and Genetic Diversity	58	2017	0.866	genetic mapping, diversity analysis, breeding
#3: Antibacterial Properties	40	2023	0.858	antimicrobial potential and nanoparticle applications
#4: Chemical Change	38	2016	0.831	postharvest physiology and quality retention
#5: Young Y Chromosome	37	2016	0.919	sex chromosome evolution and genetics
#6: Root Distribution	28	2020	0.972	root architecture and varietal differences
#7: Asparagus Decline Syndrome	19	2017	0.962	asparagus virus 1 and fusarium-induced decline
#8: Heavy Metal Content	18	2018	0.997	heavy metal accumulation in contaminated soils
#9: Varying Adaptation	15	2019	0.993	adaptation to environmental stresses
#10: B-Irradiated Normal Human Dermal Fibroblast	12	2019	0.962	bioactivity on human cells and anti-inflammatory effects

*The Silhouette Value is a measure used in clustering analysis to assess how well each object lies within its cluster, indicating how good or strong the clustering is. All Silhouette Values were high (approximately 0.83 to 0.99). This indicates that the clusters are well-separated, and the themes like "antioxidant activity," "salt tolerance," etc., are very cohesive and distinct.

Figure 11. Trending topics in *Asparagus officinalis* research. Using text mining in Bibliometrix, this illustration illustrates trending topics over time. Horizontal lines represent the lifespan of terms, while circle size indicates their frequency. Early research focused on "white asparagus," "antioxidant," and "quality," whereas recent attention has shifted toward "metabolomics," "transcriptome," and "medicinal plants," reflecting growing interest in molecular and health-related aspects



Trending topics in *A. officinalis* research

The trending topic analysis, generated through text mining using Bibliometrix, highlights the evolution of research interest in *A. officinalis* over time (Figure 11). Earlier topics such as "white asparagus," "antioxidant," and "quality" quality began to emerge in the early 2000s. More recent and emerging topics include "metabolomics," "transcriptome,""apoptosis," and "medicinal plants," reflecting a shift toward molecular biology, omics technologies, and health-related research. "Antioxidant capacity," "bioactive compounds," and "polyphenols" have maintained

steady attention, linking asparagus research to food quality, health benefits, and nutritional analysis. Overall, visualization emphasizes a transition from agricultural and physiological studies toward biochemical and therapeutic investigations in recent years.

From the citation analysis, Fuentes-Alventosa, J.M. emerged as the most cited scholar (Cluster #0), whereas Guo, Q. (Cluster #3) recorded the highest citation burst. The highest degree centrality was attributed to Castro, P. (Cluster #2), whereas Fuentes-Alventosa, J.M. (Cluster #0) showed the highest betweenness centrality, underscoring the pivotal role of their work within the network. Notably, Wang, J. (Cluster #0) achieved the highest Sigma score, indicating a strong combined influence of citation bursts and network centrality.

In conclusion, the research landscape of *A. officinalis* has evolved dynamically, moving from initial studies focused on antioxidant properties to advanced explorations of molecular genetics, stress adaptation, and biomedical applications. Dominant intellectual structures are anchored in clusters, such as "Antioxidant Activity," "Salt Tolerance," "SSR Marker and Genetic Diversity," while recent trends indicate a shift towards postharvest biotechnology and human health-related investigations.

DISCUSSION

This research applied bibliometric and conceptual analyses to chronologically study asparagus research from 1853 to 2025. It highlights important constituents and sheds light on new themes in the study, describing the state of the art in the field for the first time. The study, with the help of Bibliometrix, VOSviewer, and CiteSpace, discovers indicators of development, key journals, principal authors, and changes in themes. These results are important for formulating strategies for further research in plant science, biotechnology, and environmentally friendly agriculture.

The increase in research on *A. officinalis* since the early 90s can be linked to improvements in plant biotechnology, greater recognition of asparagus as a functional food, and interest in sustainable agriculture globally [Yu and Fan 2021]. Early sporadic activity prior to 1980 appears to have resulted from a combination of limited technological capacity and relatively peripheral agricultural interests. The surge after 2000 aligns with advances in genomics, molecular breeding programs, and greater appreciation for the antioxidant and phyto-pharmaceutical properties of plants [Cui et al. 2024]. Earlier studies achieved higher average citations, implying their primary character in influencing the focus of later research, whereas lower mean citations in more recent studies are a consequence of the inevitable citation time lag [Aksnes et al. 2019]. Overall, the increasing research output (Figure 2) reflects both technological advancements and broader societal emphasis on crop improvement, nutritional health, and bioactive compound discovery [Saleh et al. 2019].

Kanno and collaborators have made significant contributions to *Asparagus officinalis* research, particularly in the areas of sex determination, genetic mapping, molecular breeding, and interspecific hybridization. Early work clarified phylogenetic relationships within the genus using chloroplast DNA analysis [Lee et al. 1997] and investigated floral developmental genes, such as AODEF and GLOBOSA-like genes, refining the understanding of floral organ specification [Park et al. 2003, 2004]. Kanno et al. [2014] pioneered the development of sex-linked molecular markers, notably by converting a male-specific RAPD marker into an STS marker for early gender identification [Nakayama et al. 2006]. They also produced interspecific hybrids between *A. officinalis* and related species such as *A. schoberioides* and *A. kiusianus* to introduce disease-resistance and stress-tolerance traits [Ito et al. 2011, Kanno et al. 2014]. More recently, Kanno has contributed to transcriptomic studies revealing disease resistance mechanisms [Abdelrahman et al. 2017] and investigations into the evolution of the asparagus Y chromosome and male-specific genes [Harkess et al. 2017b, Murase et al. 2017]. Overall, Kanno's work has substantially advanced both fundamental knowledge and applied breeding strategies to asparagus research.

The conceptual structure of *A. officinalis* research revealed a well-defined and mature intellectual landscape, as evidenced by high silhouette values (0.831–0.997) across 11 distinct clusters (Figure 10, Table 1). The largest clusters, "Antioxidant Activity," "Salt Tolerance," "SSR Marker and Genetic Diversity," reflect historical and emerging research priorities in the field. Early studies have focused on the health-promoting phytochemicals of asparagus [Conversa et al. 2019, Shahrajabian and Wenli 2022], while more recent trends emphasize molecular breeding, stress adaptation, and genetic mapping [Harkess et al. 2017a, Zhang et al. 2024a]. The identification of antibacterial properties [Giang and Van Khai 2024] and investigations of postharvest physiology [Techavuthiporn and Boonyaritthongchai 2016] further demonstrate the diversification of research topics. Notably, advanced topics, such as sex chromosome evolution [Murase et al. 2017] and biomedical applications, including anti-inflammatory effects on human cells [Shirato et al. 2018], indicate the field's expansion into genomics and translational research. Citation network metrics highlight Fuentes-Alventosa, J.M. as the most influential scholar and Wang, J. as a major knowledge integrator with the highest sigma score, underscoring the central role of antioxidant studies in shaping

the discipline. Overall, conceptual mapping illustrates how *A. officinalis* research has evolved from traditional phytochemical studies to cutting-edge biotechnological and biomedical investigations.

This bibliometric and conceptual analysis of *A. officinalis* research provides critical insights that directly inform horticultural practices and strategic crop development. The identification of key research clusters – such as salt tolerance, genetic diversity, root architecture, and postharvest physiology – highlights the evolving priorities in improving stress resilience, optimizing yield, and extending shelf life. Findings related to salt stress mechanisms and mycorrhizal associations (Cluster #1) can guide breeding programs aimed at developing cultivars better suited for saline or marginal soils. The prominence of SSR markers and genomic mapping efforts (Cluster #2) suggests accelerating potential in marker-assisted selection for desirable agronomic traits. Additionally, trends in root system studies (Cluster #6) and disease management (Cluster #7) support the refinement of cultivation techniques and integrated pest management. Finally, the increasing focus on phytochemical and metabolomic profiling offers opportunities to enhance the nutritional and functional value of asparagus through targeted breeding. Together, these findings contribute a roadmap for advancing sustainable cultivation, genetic improvement, and value-added utilization of *A. officinalis* in horticultural systems.

Metabolomics has emerged as a major trending topic in *A. officinalis* research, reflecting a shift toward understanding the complex biochemical and physiological processes of plants. Recent studies have demonstrated the critical role of metabolomics in identifying bioactive compounds and stress responses. Nakabayashi et al. [2015] first applied targeted metabolomics to identify asparaptine, a sulfur-containing metabolite with angiotensin-converting enzyme (ACE) inhibitory activity. Further advancing the field, Nakabayashi et al. [2021] used spatial metabolomics via imaging mass spectrometry to localize asparaptine A within asparagus tissues, linking metabolite distribution to specific developmental structures [Nakabayashi et al. 2021, Nakabayashi et al. 2015]. Creydt et al. [2018] optimized extraction protocols to enhance metabolite profiling in asparagus, enabling more comprehensive untargeted metabolomic studies [Creydt et al. 2018]. Recently, integrated metabolomics and transcriptomics approaches have provided insights into the regulation of bioactive compounds, such as steroidal saponins [Cheng et al. 2023], and stress adaptation mechanisms under drought conditions [Zhang et al. 2024b]. Collectively, these studies underscore how metabolomics advances both fundamental knowledge of *A. officinalis* biology and its applications in breeding for improved nutritional and stress-resilient traits (Figure 11).

The "SSR Marker and Genetic Diversity" clusters represent a critical research focus in *A. officinalis* studies, emphasizing molecular breeding, genetic mapping, and population diversity. Simple Sequence Repeats (SSRs), also known as microsatellites, are widely used as markers because of their high polymorphism, co-dominant inheritance, and reproducibility. Studies in this area aim to assess genetic variability within and between *Asparagus* populations, facilitate the construction of linkage maps, and support marker-assisted selection (MAS) in breeding programs. Notable contributions, such as those by Harkess et al. [2017b] and Mercati et al. [2015], have advanced our understanding of genome structure and diversity, including interspecific hybrids and wild relatives. Kanno et al. have helped define the gene pools available for cultivar improvement (Kanno et al. 2014). This cluster underpins efforts to enhance disease resistance, stress tolerance, and yield traits in *A. officinalis*, making it central to the long-term sustainability and genetic improvement of this species.

Authors such as Guo, Q. (7.25) and Pegiou, E. (7.02) exhibited strong citation bursts due to their influential work in Cluster #3 (Antibacterial Properties), focusing on the antimicrobial potential of *A. officinalis* extracts, particularly through nanoparticle-mediated approaches [Francis et al. 2024]. These contributions align with emerging trends in bioactive compound research and their applications in food safety and biomedicine, driving rapid citation increases

This study had several limitations. This study focused only on the Scopus database and failed to include relevant research found in Web of Science or PubMed. They may also have suffered from bias by only accepting articles in English. Recent publications on lesser-cited works might contain perception bias. Working papers and conference proceedings were excluded, potentially omitting unpublished research. Finally, the restrictions posed by Bibliometrix, VOSviewer, and CiteSpace limit algorithmic clustering and clustering of repetitively identical keywords, leading to ambiguous theme labeling.

CONCLUSIONS

This research maps the intellectual structure of *Asparagus officinalis* research and its thematic evolution from 1853 to 2025, which has not been done before. It also contains a comprehensive bibliometric and conceptual analysis of research. It is clear that the field of asparagus research is developing with the help of biotechnology, phytochemistry, and genomic advances. Fundamental studies by Is it Kanno et al. [2014] and other scholars have advanced the foundational components of genetic research pertaining to sex determination and breeding.

Attention is shifting toward molecular and biomedical applications, particularly in metabolomics. Achievements in human health research have been made by integrating multi-omics, climate-resilient approaches, and large translational studies; however, gaps remain. Global efforts to tackle these liminal areas are essential, along with a focus on international collaboration to promote rigorous longitudinal field experiments and shift the focus to standardized multi-omics platforms. Addressing these gaps will increase the significance of research on *A. officinalis*. These actionable insights will advance the emerging importance of *A. officinalis* as a functional crop and further highlight the significance of fundamental and applied research.

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NUMERICAL AND QUALITATIVE ANALYSIS OF ASCOSPORE DISCHARGE OF *Venturia inaequalis* IN CENTRAL POLAND IN RELATION TO WEATHER CONDITIONS

Sylwester Masny[1] https://orcid.org/0000-0002-0052-7625
Paweł Jankowski[2] https://orcid.org/0000-0002-3176-8798
Arne Stensvand[3] https://orcid.org/0000-0002-8630-2434

[1] The National Institute of Horticultural Research, Konstytucji 3 Maja 1/3, 96-100 Skierniewice, Poland

[2] Department of Computer Information Systems, Institute of Information Technology, Warsaw University of Life Sciences-SGGW, Nowoursynowska 159, 02-776 Warszawa, Poland

[3] Division of Biotechnology and Plant Health, Norwegian Institute of Bioeconomy Research (NIBIO), Ås 1431, Norway

✉ sylwester.masny@inhort.pl

ABSTRACT

The study presents the results of nine years of field observations of ascospore release of *Venturia inaequalis* in the Skierniewice area in central Poland. In total, spores were trapped 221 times during 2005–2008 and 2010–2014. Of these, 142 episodes lasted for less than 8 hours, 69 lasted from 8 to 29 hours, and 10 episodes lasted from 30 to 93 hours. Spore releases started in spring from 25 March to 28 April and ended from 27 May to 17 June, and the season for ascospore release lasted from 43 to 76 days, with an average of 58 days. During 139 ascospore releases, less than 1 000 spores per cubic meter of air were collected and during 25 discharges more than 10 000 spores were trapped. Releases of ascospores were highly related to rainfall and daylight. Two-thirds (67%) of the spores were trapped during rain. Only 12% of the discharges occurred without any registered rain, accounting for 7% of all trapped spores. Nearly three fourth (73%) of all ascospore release hours occurred in daylight, and 91% of the spores were trapped in daytime. Rainy nights with constant leaf wetness were observed, during which no spore releases occurred despite the rainfall. Ascospore releases were also less prominent at the beginning and end of the season and after weak rains. Rain was most effective as the trigger of discharges at temperature between 5 and 13 °C and when global radiation coinciding with rainfall was below 700 W/m^2. In conclusion, the study confirms the dominant role of daytime rainfall in the release of ascospores by *Venturia inaequalis*.

Keywords: apple scab, computer calculations, *Malus* × *domestica*

INTRODUCTION

The most important disease in apple production in Poland, similar to that in temperate regions throughout the world, is apple scab caused by the fungal pathogen *Venturia inaequalis* (Cooke) G. Winter [MacHardy 1996]. The control of apple scab is responsible for 70% of the pesticide use in apple production [Creemers and Laer 2006].

The localization of the investigation took place close to the Grójec area, which is one of the largest regions of apple production in Poland, and was carried out in 2005–2008 and 2010–2014. In our previous studies, we focused on modelling the influence of moisture on maturation rate of ascospores of *V. inaequalis* [Jankowski and Masny 2019, 2020]. Pseudothecial development and severity of apple scab in the same area were discussed by Meszka [2015].

The predominant primary inoculum of *V. inaequalis* in spring is ascospores released from pseudothecia in leaf litter on the ground. Numerous reports have shown that rainfall is the primary reason for ascospore re-

lease in *V. inaequalis*, including Brook [1969], MacHardy and Gadoury [1986], Rossi et al. [2001], and Alt and Kollar [2010].

Another major factor affecting the quantity of ascospore discharge in *V. inaequalis* is global radiation. Again, the importance of daylight to trigger high releases of ascospores in *V. inaequalis* has been reported by many authors, such as Brook [1969], MacHardy and Gadoury [1986], Warner and Braun [1992], Gadoury et al. [1998], Rossi et al. [2001], and Villalta et al. [2002].

Another important weather parameter that influences the length of ascospore release seasons is periods of dryness. As shown, for example, by Stensvand et al. [2005], long, dry periods during the period of ascospore maturation will extend the season of ascospore release.

In this work, we aim to present a numerical and qualitative description of the influence of weather conditions on the timing of ascospore discharges of *V. inaequalis* in the Grójec area, Poland. We want to verify the observations made by other authors under climatic conditions in central Poland. Nine seasons of field observations of ascospore release by *V. inaequalis* were used to analyse the relationship between spore discharge and weather factors.

MATERIALS AND METHODS

The experimental site

Spore trapping was carried out in an experimental orchard at The National Institute of Horticultural Research (InHort) in Skierniewice (N 51°55'; E 20°6'), located in the Grójec area of central Poland. The data analysed in the study were collected during 2005–2008 and 2010–2014. Analysis of weather factors in each year suggested incorrect measurements of the leaf wetness sensor in the season 2009. Therefore, data from 2009 were excluded from the analysis.

Monitoring of weather conditions

Weather monitoring was conducted in the orchard. Air temperature (°C), precipitation (mm, with 0.2 mm as the lowest amount measured), relative humidity (RH, %), occurrence of leaf wetness (LW), and global radiation (W/m^2) were recorded continuously from January to June by an automated weather station (model Metos-Compact, Pessl Instruments, Weiz, Austria). Leaf wetness was measured at a height of 20 cm above the ground. The other weather parameters were measured at a height of 2 m. Data were collected at intervals of 12 minutes and averaged at hourly intervals. Precipitation and RH sensors were calibrated each year. All hours of the day presented in the manuscript were adjusted to Central European Time (CET). CET was used consistently during the whole year, so Central European Summer Time (CEST) was never used.

Throughout this article, hours with daylight were defined as the hours with observed global radiation and the remaining hours as night hours.

Degree-day (DD) accumulation (base = 0 °C) was computed for each year from the start to the end of the spore trapping seasons. The adjusted DD accumulation ceased after four consecutive days without measurable rain. Accumulation then resumed when a rain event terminated the "dry" interval. Following the proposal of Stensvand et al. [2005], "dry" years were defined as years in which the difference between unadjusted versus adjusted DD accumulation exceeded 200.

Ascospore trapping

A Burkard 7-day recording volumetric spore trap (Burkard Manufacturing Co Ltd., Rickmansworth, Hertfordshire, UK) was used to monitor the release of ascospores from the leaves of *Malus* × *domestica* (Suckow) Borkh. cv. McIntosh. Approximately 2 m^2 of the ground under the spore trap was covered with apple leaves heavily infected by *V. inaequali*s in the previous season. The leaves were gently arranged in autumn after most of the leaves had fallen from the trees. They were covered with plastic netting to prevent them from being moved by the wind. The orchard area surrounding the spore trap (0.2 hectares) was not treated with fungicides during the 9-year period of spore trapping. The spore trap was installed in the centre of this area and was adjusted to sample air 1 m above the ground at a rate of 0.6 m^3 h^{-1}. The tape from the Burkard spore trap (with a width of 19 mm and a length of 336 mm) was changed weekly. The weekly segments of the tape were cut into seven 48 mm long segments, representing the length covering a period of one day of sampling. The number of ascospores of *V. inaequalis* deposited on each 2 mm width of the tape corresponding to 1-hour sampling, was counted using a microscope (200× magnification). Ascospore counts were converted into counts per cubic meter volume of sampled air per hour.

The end of each discharge period was determined as the hour after which no ascospores were trapped for at least four hours. Therefore, if an interval without trapped spores between two periods with spore release lasted less than four hours, this was considered one discharge period.

Statistical analysis

Data analyses were performed using GNU Octave version 7.3.0, and R program version 4.2.2 with the R Studio version 2022.12.0.

The Spearman correlation was used to analyse the relation between diurnal cycles of precipitation, global radiation, RH, and temperature with start of spore discharges and abundance of trapped ascospores.

The exact Fisher's test was performed to analyse the relationship between observation of an ascospore discharge during a rainy hour and rainfall amount, LW occurrence, RH, temperature, and global radiation. The same test was used to examine the differences between the characteristics of ascospore releases caused by rainfall and releases not directly related to rain.

The Kruskal-Wallis analysis was applied to compare the distribution of temperature and RH in selected groups of hours of the observed seasons. The Dunn's post-hoc test was used to identify their homogenous groups. The proportions of hours with wet leaves in these sets of hours were compared using the pairwise test of proportions with the Benjamini and Hochberg adjustment method.

RESULTS

Length and abundance of the observed ascospore discharges

In 2005–2008 and 2010–2014, the seasons for ascospore release of *V. inaequalis* in Skierniewice started between 25 March and 28 April and ended between 27 May and 17 June (Table 1). The duration of ascospore trapping ranged from 43 to 76 days, with an average of 58 days. Accumulation of DDs and adjusted DDs from start to the end of each spore trapping season varied from 586 to 886 and from 433 to 612, respectively. Seasons that can be considered "dry", i.e., years with a difference between unadjusted and adjusted accumulation of 200 DDs or more, were 2005, 2006, 2008, and 2012. There were 221 periods when ascospores were trapped during the nine seasons, lasting a total of 2054 hours (16.3% of all hours), with 1797 hours (14.3%) with trapped ascospores. The majority of the discharge periods (142) lasted less than 8 hours; 55 lasted between 9 and 17 hours; and 24 over 17 hours. The 10 longest release periods lasted 30 to 93 hours. When adjusted for volume of air, the total seasonal number of ascospores trapped varied from less than 10 000 to approx. 550 000 (Table 1). During 139 release periods, less than 1 000 spores were trapped; during 57 periods between 1 000 and 10 000 were trapped; and over 10 000 spores were trapped during 25 discharge periods. The highest trapping of ascospores was approx. 213 000 over a period of 30 hours in 2013.

Table 1. Characteristics of the *Venturia inaequalis* ascospore seasons during nine years (2005–2008 and 2010–2014) in an experimental orchard in Skierniewice, Poland. The dates (month–day) of the first and last trapping of ascospores, season lengths (in days and hours), degree-days, the total number of trapped ascospores, and percentage of hours of discharge events and hours with trapped spores

Year	Date of		No. of		Degree-days		Ascospores trapped	Percentage of hours	
	start	end	days	hours	unadjusted[1]	adjusted[1]		of discharges[2]	with trapped spores[2]
2005	04–28	06–17	52	1247	667	433	48340	15.5	13.8
2006	04–16	06–17	63	1511	812	590	211901	12.2	11.3
2007	04–09	06–02	55	1327	692	543	9291	12.9	9.6
2008	03–29	06–13	77	1859	886	457	118365	13.2	11.8
2010	04–02	05–27	56	1349	586	575	328592	26.4	22.1
2011	04–04	05–28	55	1324	631	477	86455	18.5	16.0
2012	04–13	06–14	63	1521	871	593	183832	12.4	11.4
2013	04–19	06–01	44	1062	598	467	548080	25.0	23.1
2014	03–25	05–29	66	1591	730	612	40609	12.8	11.2

[1] Degree-days (base = 0 °C) accumulated from start to end of each spore trapping season. The adjusted degree-day accumulation ceased after four consecutive days without measurable rain. Accumulation then resumed when a rain event terminated the dry interval.
[2] Any interval of less than four hours without trapped spores between two hours with trapped spores was considered part of a discharge event. The end of each discharge period was determined as the hour after which no ascospores were trapped for at least four hours.

Weather during the ascospore release season

Details about the weather characteristics in each season are given in Table 2. Over the nine seasons, 972 hours (7.6% of all hours) with rainfall were observed at the location of the experiment. During 34.3% of the rainy hours, the precipitation equalled 0.2 mm. For 38.3% and 22.1% of the rainy hours, precipitation was 0.2 to ≤1 mm and >1 to 3 mm, respectively. Rainfalls higher than 3 mm were observed in only 5.3% of the events. Leaf wetness was observed during 22.5% of the analysed hours. About two-thirds (64%) of the rainy hours and 68% of the amount of precipitation were registered during daytime. The share of the hours with daylight was 66.5%.

The lowest and highest temperatures during the nine seasons were –4.7 °C and 31.8 °C, with a mean of 12.2 °C. The lowest RH during the whole period of investigation was 22.8%, with a mean of 79.1%, and the fraction of the hours with RH ≥ 99% was 23%.

Table 2. Weather characteristics for all hours of the *Venturia inaequalis* ascospore seasons, starting from the first and ending with the last trapping of ascospores, during nine years (2005–2008 and 2010–2014) in an experimental orchard in Skierniewice, Poland. Percentage of hours with observed rainfall (≥0.2 mm), recorded leaf wetness (LW), and daylight. The extremes (minimum and maximum) of temperature (°C) and minimal percentage relative humidity (RH)

Year	Percentage of hours			Temperature [°C]		RH [%]
	rainy	with LW	with daylight	minimum	maximum	minimum
2005	6.2	19.0	69.2	0.6	31.8	39.4
2006	5.8	13.2	68.2	2.8	26.8	26.2
2007	7.7	21.8	66.4	–4.1	30.6	26.0
2008	5.5	15.9	65.6	–2,2	27.7	34.2
2010	10.0	29.9	64.7	–2.2	25.2	22.8
2011	6.3	20.3	65.4	–1.4	26.4	25.4
2012	8.2	18.0	68.4	–1.4	29.4	23.4
2013	12.1	30.6	67.0	0.9	26.5	30.4
2014	8.2	21.1	64.6	–4.7	27.6	28.2
Total	7.6	22.6	66.5	–4.7	31.8	22.8

Table 3. Weather characteristics for hours when ascospores of *Venturia inaequalis* were trapped during nine years (2005–2008 and 2010–2014) in an experimental orchard in Skierniewice, Poland. Percentage of hours with observed rainfall (≥0.2 mm), recorded leaf wetness (LW), and daylight. The extremes (minimum and maximum) of temperature (°C) and minimal percentage relative humidity (RH)

Year	Percentage of hours			Temperature [°C]		RH [%]
	rainy	with LW	with daylight	minimum	maximum	minimum
2005	19.7	60.5	76.7	3.7	23.6	58.8
2006	28.1	59.8	68.6	6.6	23.2	51.8
2007	38.0	69.8	76.0	4.4	30.6	45.0
2008	31.7	75.9	69.5	0.75	19.0	69.8
2010	29.2	69.9	67.1	1.7	20.6	45.2
2011	28.2	61.9	72.2	–0.52	22.5	38.2
2012	46.3	76.4	79.8	–0.09	22.8	50.6
2013	32.7	72.9	74.1	5.0	23.1	54.0
2014	45.1	82.8	81.9	1.4	19.2	45.2
Total	32.7	74.7	73.3	–0.52	30.6	38.2

The weather characteristics during periods of ascospore release in each season are given in Table 3. One-third (33%) of the hours with trapped ascospores of *V. inaequalis* occurred during rainfall, leading to 42% of the total number of spores trapped. The rainy hours during which ascospores were trapped accounted for 61% of all rainy hours during the nine seasons. Over one-half (51%) of the discharge hours, and 67% of spores were observed from 1 hour before registered rainfall (<0.2 mm) to 1 hour after the rainfall. Moreover, nearly two in five (38%)

observed spore release periods started during a rainfall. The share of discharges that started from 1 hour before to 1 hour after rainfall was 56%, and the share of ascospores fell rapidly with the increasing distance in time from recent rainfall. Only 10.5% of the ascospores were observed 4 to 6 hours after rain and 4.5% after a dry period longer than six hours. The 49 (12% of all) discharges without any rainfall observed accounted for about 7% of all trapped spores. Of all ascospore release hours, 73.3% occurred in daylight, and 91% of the spores were trapped during those hours.

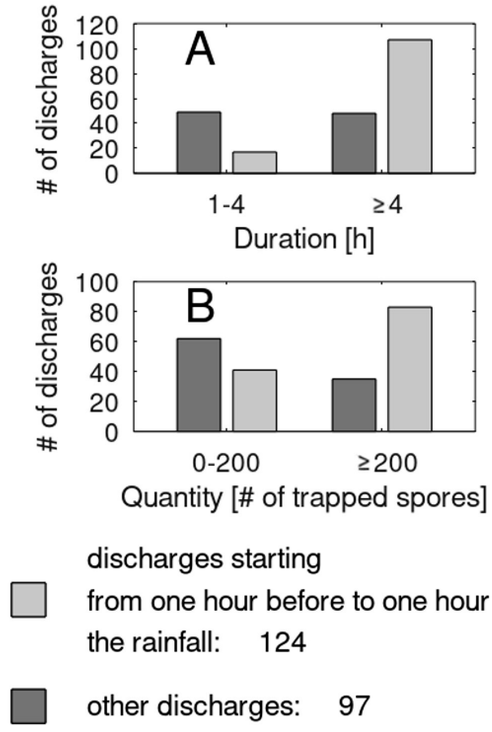
The absolute minimum and maximum temperatures of all periods during which ascospores were trapped were –0.5 and 30.6 °C, respectively, with a mean of 10.4 °C. The lowest RH during the release periods was 38.2, with a mean of 91.6%, and 44.2% of the hours had RH ≥ 99%.

Start of ascospore release either related or not related to rainfall

The start of the observed periods of spore release was divided in two groups depending on the distance in hours from rainfall. The first group consisted of 124 (56% of all) discharges in which the first ascospores were trapped from 1 hour before to 1 hour after the closest rainfall. The second group contained the remaining 97 discharges.

Trapping of ascospores from the first group lasted most of the time for 4 hours or more, and the majority of the episodes contained more than 200 spores (Figure 1). These discharges corresponded to 63% of the length of all spore release periods and to 60% of the total number of ascospores trapped. Approximately the same number of spore release periods from the second group lasted from 1 to 4 hours (49 discharges) and for more than 4 hours (48 discharges). During most of these episodes of spore release less than 200 spores were trapped. According to the Fisher's exact test, differences between the two groups of discharges were significant for both length and abundance ($p < 0.0001$).

Figure 1. Histograms comparing duration of ascospore trapping (A) and numbers of trapped ascospores (B) of *Venturia inaequalis* during discharges in which the first ascospores were trapped from 1 hour before to 1 hour after the closest recorded rainfall (light grey) and the remaining ones (dark grey)



Influence of weather on occurrence of trapped ascospores during rainfall

The comparison of weather conditions during the rainy hours with and without trapped spores is presented in Figure 2. Each observed variable was split into ranges with a higher number of rainy hours either with or without ascospores.

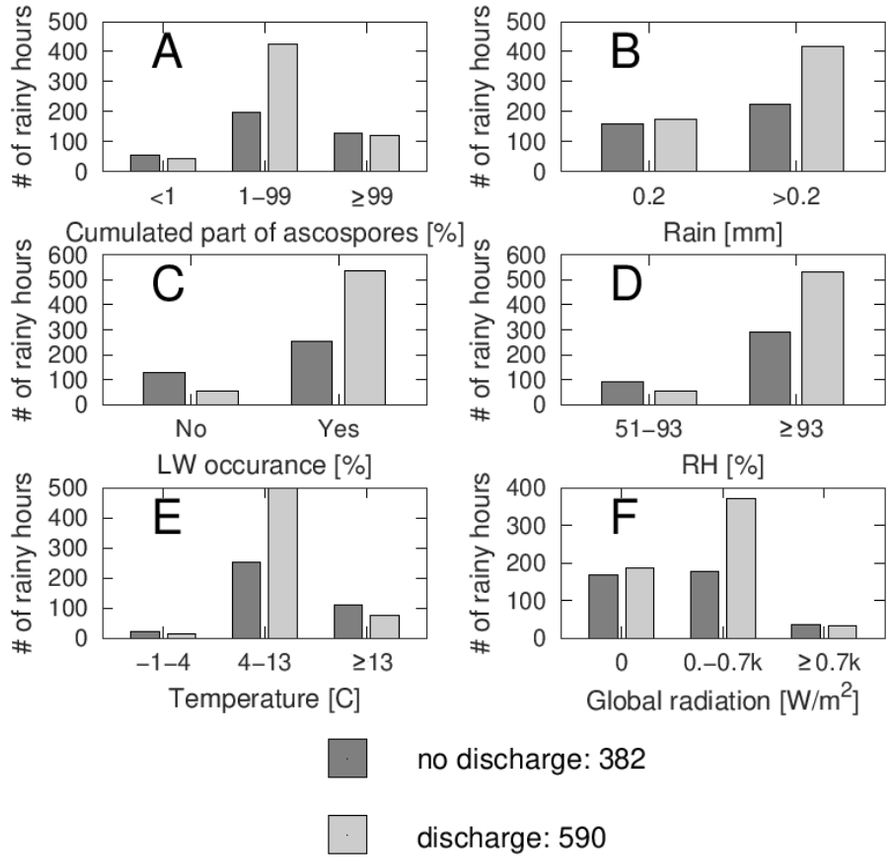
The *p*-value of Fisher's exact test for independence was computed for each variable. The immediate ascospore releases were least frequently caused by precipitation in the beginning and end of the seasons, when either less

than 1% or more than 99% of the seasonal spores had been trapped, respectively (Figure 2A). The weather conditions during a rainy hour, less favourable for immediate ascospore discharges, were as follows:

i) rain equal to 0.2 mm, with a relatively higher number of hours with vs. without trapped ascospores (Figure 2B);
ii) LW was recorded (Figure 2C);
iii) RH was below 93% (Figure 2D);
iv) temperature was below 4 °C or above 13 °C (Figure 2E);
v) during night when there was no global radiation, or
vi) when global radiation coinciding with a rainfall was above 700 W/m^2 (Figure 2F).

All the observed relations were statistically significant, at $p < 0.001$. No statistically significant impact of wind speed on the ascospore discharge during a rain was found.

Figure 2. Histograms comparing conditions during the rainy hours without (dark grey) and with (light grey) trapped ascospores of *Venturia inaequalis*. Number of hours in the two groups versus: (A) percentage of cumulated seasonal spores (%), (B) the amount of precipitation (mm), (C) occurrence of leaf wetness (LW), (D) relative humidity (% RH), (E) temperature (°C), (F) global radiation (W/m^2)



Relation between diurnal cycles of ascospore abundance and diurnal weather cycles

The share of hours with trapped ascospores increased from midnight, attaining a flat plateau between 7 and 18, when it started to decrease until 23 (Figure 3A). During the period of maximum spore trapping, there were two separate maxima around 8–12 and 16–18 (Figure 3B).

The diurnal distribution of the observed weather factors formed two groups. The first one was related to precipitation. The diurnal cycle of the number of rainy hours during the observed seasons showed two broad peaks (Figure 4A). The first peak was in the morning, with the highest values at the verge of night and day around 3–4. The second one occurred in the afternoon, with the highest number of rainy hours around 14–17. The distribution of the amount of rainfall showed the same two peaks with the maximum rate at 14, due to the largest single rainfall of 42.8 mm observed in the 9-year study (Figure 4B). The least amount of rain was observed around midnight and at 9–11.

Figure 3. Diurnal cycles averaged from nine seasons (2005–2008 and 2010–2014) of ascospore trapping of *Venturia inaequalis* at Skierniewice, Poland: (A) the percentage of hours with trapped spores, and (B) the mean number of spores. The data is divided into daytime (light grey) and nighttime (dark grey) contributions

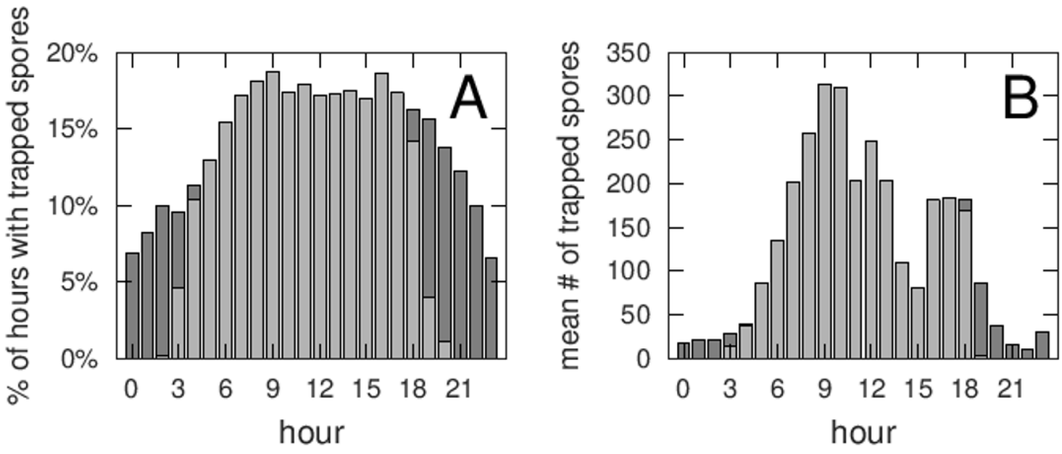
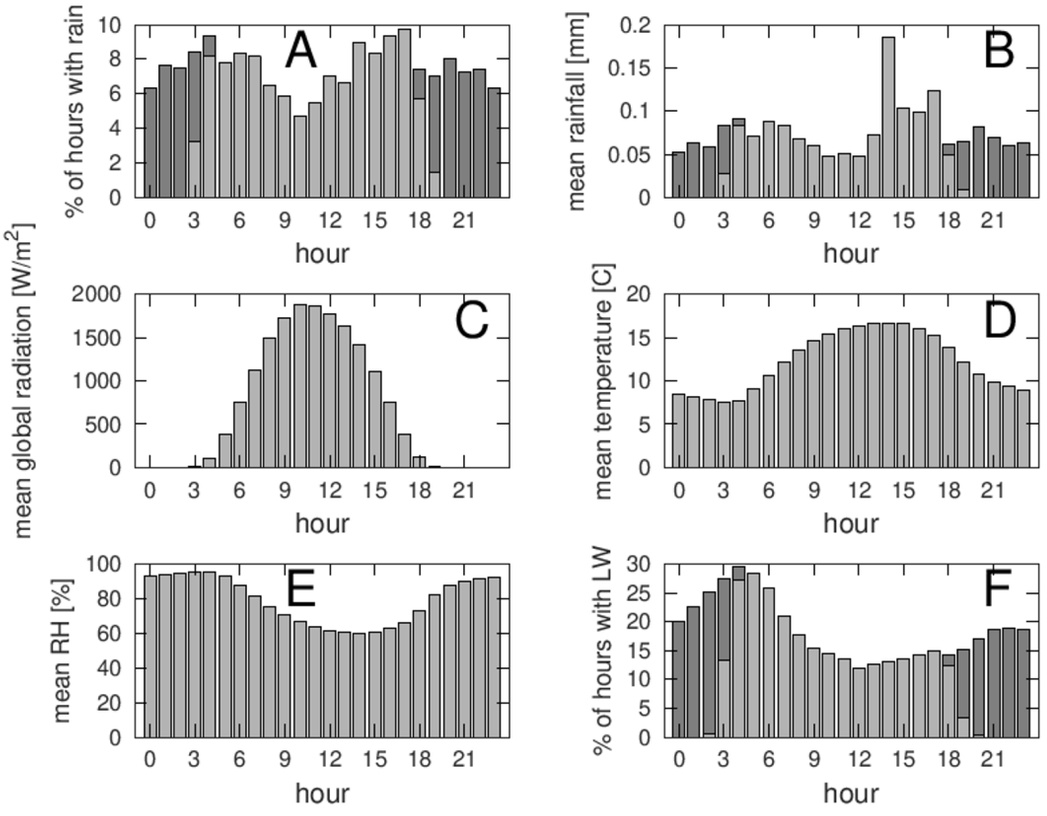


Figure 4. Diurnal cycles averaged from nine seasons (2005–2008 and 2010–2014) of ascospore trapping of *Venturia inaequalis* at Skierniewice, Poland: (A) percentage of hours with rain, (B) mean rainfall (mm), (C) mean global radiation (W/m^2), (D) mean temperature (°C), (E) mean relative humidity (RH, %), and (F) percentage of hours with observed leaf wetness (LW). The data presented in plots (A), (B), and (F) is divided into daytime (light grey) and nighttime (dark grey) contributions



The diurnal cycles of LW, RH, temperature, and global radiation formed a second group. The increase of the average global radiation started from 03 to its peak at 10–11 (Figure 4C). The mean temperatures showed a more shallow but broader peak than the average global radiation (Figure 4D), and that maximum corresponds with the lowest RH in the diurnal cycle (Figure 4E). The diurnal cycle of the number of hours with wet leaves (Figure 4F) was closely related to the cycle of RH. The exception is the strong morning peak in number of hours with wet leaves around 3–4, which corresponds to the morning peak of rain intensity.

The diurnal distribution of weather factors were highly correlated within each group (Figure 5). The diurnal cycles of the hours of ascospore release and the average number of observed ascospores were significantly correlated only with the second group of weather factors: global radiation, temperature, relative humidity, and LW occurrence (Figure 5). The observed correlations were positive for global radiation and temperature and negative for RH and LW. All correlations were statistically significant at $p < 0.001$ with two exceptions, diurnal cycle of the hours with LW occurrence was correlated at $p < 0.01$ with cycles of average global radiation and with average number of trapped ascospores.

Periods of rainfall with no ascospores observed

Three groups of rainy hours during which no ascospores were trapped were selected after the detailed analysis.

The first group contained rainfalls which occurred directly before the start of ascospore discharges. In each case, the first rain started a period of constant leaf wetness ending up with ascospore discharge. We observed 71 such periods, varying from 1 to 10 hours, but only 17 periods were longer than 3 hours. The number of rainy hours which occurred during each period varied from 1 to 8. In the majority (41) of cases only one rainy hour was observed, and only in 9 cases their number was greater than 3 hours. In total, approximately 14% of all rainy hours occurred during these periods.

Figure 5. Spearman correlations between the diurnal cycles of intensity of spore trapping (Figure 2) and main weather factors (Fig. 3) averaged from nine seasons (2005–2008 and 2010–2014) of ascospore trapping of *Venturia inaequalis* at Skierniewice, Poland: percentage of hours with rain, mean rainfall, percentage of hours with observed leaf wetness (LW), mean relative humidity (RH), mean temperature, mean global radiation, percentage of hours with trapped spores, and mean number of trapped spores. NS stands for 'non-significant'

	% of hours with rain	mean rainfall	% of hours with LW	mean RH	mean temperature	mean global radiation	% of hours with trapped spores
mean rainfall	0.86						
% of hours with LW	NS	NS					
mean RH	NS	NS	0.92				
mean temperature	NS	NS	-0.91	-1.00			
mean global radiation	NS	NS	-0.58	-0.75	0.76		
% of hours with trapped spores	NS	NS	-0.65	-0.82	0.83	0.86	
mean # of trapped spores	NS	NS	-0.54	-0.69	0.70	0.93	0.87

The second group contained rainfalls which occurred during the ascospore-free periods separating two spore discharges by more than 3 hours but less than 16 hours. We observed 23 such intervals containing about 9% of all rainy hours. All of them were characterized by persistent leaf wetness. All but one started shortly before or during night and ended during night or in the early morning. During 14 periods the intensity of precipitation was at least 3 rainy hours. There were three very long intervals, lasting from 10 to 15 hours, with high intensity of precipitation for 10 to 12 hours. Similarly, 13 ascospore-free nightly periods between two discharges with constant leaf wetness but no precipitation were observed.

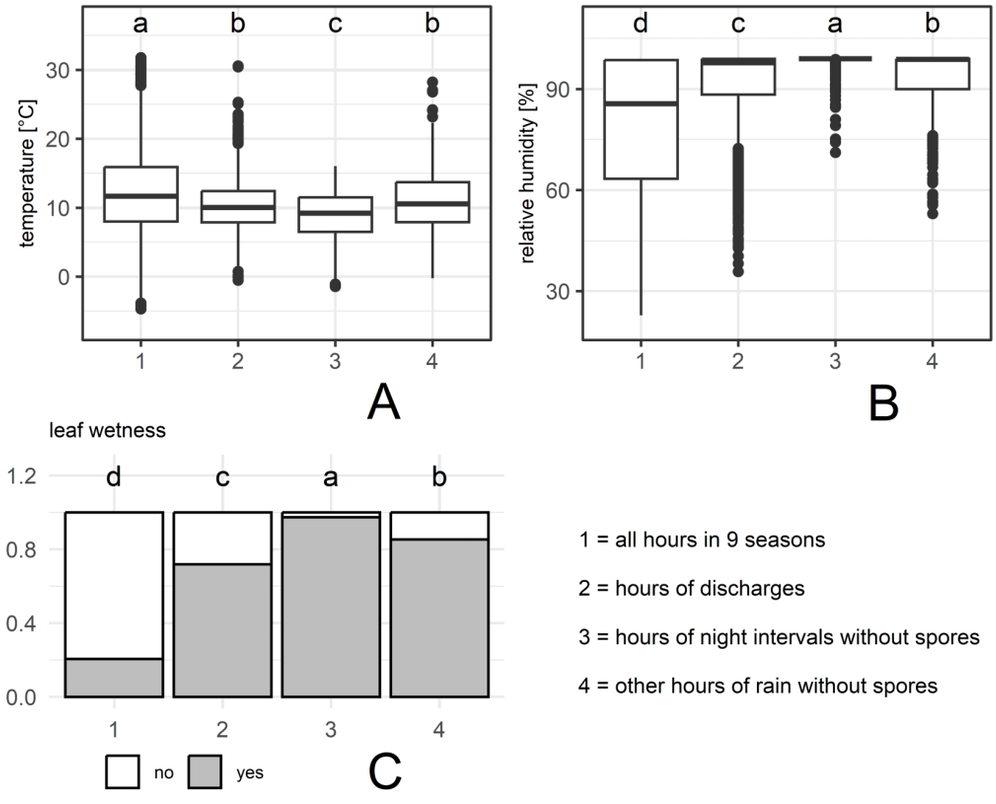
The remaining 75 hours of rainfalls (about 8% of all rainy hours) without trapped spores occurred either shortly after the ascospore discharge when leaves were still wet or when leaves had already dried out. In the latter case, on single occasions the rains were observed a few hours after an ascospore discharge but usually after ten or more hours and over ten hours since a next discharge. In 56 cases they lasted for only one hour. During 53 of the 75 hours only 0.2 mm was recorded.

The distribution of temperature (Figure 6A), RH (Figure 6B), and proportion of hours with observed LW (Figure 6C) was compared within the groups of:

i) all hours of the observed seasons,
ii) hours of discharges,
iii) hours of the 36 night intervals without trapped ascospores, and
iv) the remaining 75 hours of precipitation without trapped spores.

In case of all three weather factors, the Kruskal-Wallis analysis showed significant differences between the groups of hours ($p < 0.001$). The 36 night intervals were coldest, with highest average RH (97.9%), highest proportion of hours with wet leaves (97.4%), and the fraction of hours with RH ≥ 99% around 81%.

Figure 6. Distributions of temperature (A) and relative humidity (B), and proportions of hours with observed leaf wetness (C) within the groups of: all hours of the observed seasons, hours of discharges, hours of night intervals without trapped spores, and the remaining hours of rainfall without trapped spores. The distributions of temperature and relative humidity were compared with the Kruskal-Wallis analysis and their homogenous groups, denoted with letters, were established according to the Dunn's post-hoc test. The proportions of hours with wet leaves in these sets of hours were compared using the pairwise test of proportions with the Benjamini and Hochberg adjustment method. All differences were significant at $p < 0.001$



Supplementary material in Additional file 1

Figure S1 shows the comparison of the dates of the ascospore release seasons and average climate conditions in central Poland with similar data from three European areas where spore trapping of *V. inaequalis* had taken place. Figures S2 to S30 present selected fragments of the analysed nine seasons containing most of the significant ascospore release periods.

DISCUSSION

In the present work, we analysed nine years of ascospore release of *V. inaequalis* in central Poland. The discharge seasons started from late-March to late-April and ended from late-May to mid-June. They were significantly delayed compared to regions of Europe characterized by a climate with higher temperatures and longer periods of sunshine, such as Avignon, France or Passo Segni, Italy (Additional file 1: Figure S1). In Avignon, the spore trapping (years 1990–1995, 1997–2001 and 2003) started from 23 February to 8 April and ended from 5 May to 6 June [Roubal and Nicot 2016]. In Passo Segni, the spore releases (years 1991–1996) started from 16 March to 2 April and ended from 17 April to 12 June [Rossi et al. 1999]. On the other hand, in the colder climate of Ås, Norway (1990–1995, 1997–2001, and 2003), the first ascospores of *V. inaequalis* were on average observed later than in Skierniewice, from 2 April to 30 April, and 95% of the spore trapping took place from 29 April to 7 July [Stensvand et al. 2005]. This important difference implicates the impact of climate on beginning and end of the seasons for ascospore release of *V. inaequalis*. On the other hand, our results showed that as in the other regions mentioned above, the major factors influencing the timing and intensity of spore releases in central Poland are rainfall and daylight. The presented detailed analysis of nine spore release seasons may be useful in assessing possible changes in dates of ascospore releases with expected climate changes in central Poland. Such changes will likely lead to higher temperatures and change of rainfall pattern in spring and summer [Ghazi et al. 2023], thus influencing the two key factors in spore release, and therefore, in apple scab development.

Numerous studies have reported that rain is the most important factor affecting ascospore release in *V. inaequalis*, and a study by Rossi et al. [2001] indicated that rainfall is the sole factor leading to onset of ascospore discharge of the fungus. According to Alt and Kollar [2010], raindrops are necessary to induce ascospore discharge due to the pressure they exert on the fruiting bodies. Our study also shows that precipitation and spore discharges are highly correlated. In general, the discharges triggered by rainfall were the longest and most abundant ones. Only about one in ten of discharges were completely rainless, and these releases accounted for less than 10% of all trapped spores. Moreover, ascospores were trapped during more than half of the rainy hours during the ascospore release seasons. About half of the discharge hours occurred and two-thirds of the airborne spores were trapped in periods from one hour before to one hour after measured rainfall. In the analysis, it was assumed that the effect of a rainfall on ascospore discharges may be delayed by one hour, similar to the average 1.1 hour delay observed in northern Italy [Rossi et al. 2001]. We also assumed that the observed rain could be preceded by precipitation of an intensity below the sensitivity of the weather station. Moreover, the microscope tape can be slightly skewed in relation to time, thus leading to incorrect timing of assumed ascospore trapping. Hence, we considered the period from 1 hour before to 1 hour after a rainfall as the period of its direct impact on a spore release.

Nevertheless, not all rainfalls led to immediate onset of ascospore discharges. Firstly, spores were less often trapped during rainy hours in the early and late parts of the seasons of ascospore release. This is most likely because of lower numbers of mature ascospores ready for release at that time. The above observation is in agreement with the model of ascospore maturation by Rossi et al. [2007], where the period of low risk of discharges and the period of no risk of discharges were introduced in the model in the beginning and end of a season, respectively.

Secondly, the ability of rain to induce a discharge is affected by the intensity of the rain. In the present investigation, the probability of an immediate release decreased when rainfall intensity was at the detection limit of 0.2 mm. The relation between rain intensity and spore releases explains the relation of the latter with leaf wetness, which usually occurs following rainfall.

Thirdly, rainfalls less frequently led to ascospore release if there was no global radiation (during night) measured or when global radiation was above 700 W/m^2. Similarly, low or high air temperatures were not conducive to ascospore discharges. Although ascospores were trapped at temperatures varying from about 0 °C to more than 25 °C, more than half of the rainfalls did not result in ascospore releases if temperatures were below 4 °C or above 13 °C. As stated by Stensvand et al. [1997], temperatures of 2 °C or less nearly stop the process of ascospore dis-

charge. On the other hand, high temperatures lead to faster drying of the leaves after rain and therefore decrease the probability of ascospore discharge.

Finally, ascospore trapping seasons in 2006, 2008, and 2012, which were described as "dry" seasons (difference between unadjusted and adjusted accumulation of DDs higher than 200), were among the four longest seasons. This confirms that in central Poland the long, dry periods during ascospore maturation extend the season of ascospore release [Stensvand et al. 2005]. The season in 2005, which was also described as "dry", was much shorter due its exceptionally late start.

Daylight was the second factor with a widely recognized importance for spore discharge. Numerous authors have reported the suppression of ascospore discharges at night observed in both field [Brook 1966, 1969, MacHardy and Gadoury 1986, Warner and Braun 1992, Rossi et al. 2001, Villalta et al. 2002] and laboratory [Gadoury et al. 1998] studies. The same phenomenon was observed in the present investigation. More than 90% of the ascospores were trapped during daytime, while the share of hours with daylight was 65%. These results agree with other reports in terms of the proportion of ascospores trapped in the dark, although the numbers reported in different studies have varied. While around 9% of the spores in Skierniewice were trapped during the night, Brook [1966] in New Zealand observed only 0.51%, MacHardy and Gadoury [1986] in New Hampshire in USA below 5%, Rossi et al. [2001] in Italy around 7%, and Villalta et al. [2002] in Victoria in Australia about 18% of ascospores of *V. inaequalis* trapped during darkness.

The ascospore release in central Poland was not only suppressed by the lack of daylight but in general its intensity depended on the intensity of global radiation. When analysing the diurnal cycles of average ascospore discharge frequency and intensity and the cycles of main weather factors, the highest correlation (0.93) was found between the cycles of average global radiation and average number of trapped spores. The latter was correlated with all main weather factors: LW, RH, air temperature, and global radiation, which are strongly related to each other. Most importantly, the diurnal cycle of spore intensity was significantly unrelated to the diurnal cycle of precipitation. How is it possible if, as discussed above, rainfall is so crucial for the onset of ascospore releases?

The answer lies mainly in the role of light and its interaction with the rain [Brook 1969, Gadoury et al. 1998]. Firstly, as already discussed, high global radiation coinciding with rainfall is not conducive for ascospore release, because of the quick drying of leaves. Secondly, during our field experiments we observed night periods between discharges with rainfalls that did not trigger ascospore releases. These periods were characterized by high air humidity and constantly wet leaves that could not dry due to lack of daylight. We assume that fog deposition or dew formation were the causes of leaf wetness. Similar intervals with wet leaves but without noticeable precipitation were also observed between discharges. Although Stensvand et al. [1998] found that dew can cause significant ascospore releases, others [Brook 1969, MacHardy and Gadoury 1986] observed that only rarely small amounts of airborne ascospores were trapped during periods of dew. Rossi et al. [2001] found that nightly rainfalls followed by heavy dew deposition that persisted some hours after sunrise suppressed spore discharges. According to Brook [1969], in the case of dew, the water deposit increases too much and ascospores are discharged into the water and not into the air.

The above issue explains why two maxima in the diurnal cycle of rainy hours lead to different size maxima in the cycle of trapped spores. The smaller afternoon maximum of airborne ascospores, around 16–17 hour, follows the maximum of rainfall intensity that starts around 14 hour. As the daylight soon fades after 17 hour, the spore discharges are suppressed. The more significant morning peak of ascospore intensity, around 8–12 hour, follows the stronger maximum rainfall intensity, around 3–7 hour. This peak corresponds to the most intense radiation period, and the ascospore discharges are not limited by lack of daylight.

Study limitations and future research

A significant study limitation may be that the study only took place in a single localization and with only one apple cultivar. That, and the ongoing climate change and constant modification of the *V. inaequalis* genotype due to its high genetic variation, imply that the relationship between the *V. inaequalis* ascospore releases and weather may have to be analysed more often and, if possible, should be performed based on data originating from various localizations and apple cultivars.

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RESEARCH PAPER

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MITIGATION OF SALINITY STRESS ON PISTACHIO (*Pistacia vera* L.) SEEDLINGS THROUGH THE APPLICATION OF CARBON NITRIDE MODIFIED WITH IRON (FE/C_3N_4) NANOSTRUCTURES

Soleiman Gahramanzadeh[1] https://orcid.org/0009-0006-0602-1269
Seyed Morteza Zahedi[1] https://orcid.org/0000-0001-7766-8787
Mohammad Ali Azami[1] https://orcid.org/0000-0001-5182-6492
Farzad Rasouli[1] https://orcid.org/0000-0003-0038-5396
Fatemeh Bahrevar[1] https://orcid.org/0009-0002-6353-8038
Sezai Ercisli[2] https://orcid.org/0000-0001-5006-5687
Nesibe Ebru Kafkas[3] https://orcid.org/0000-0003-3412-5971
Salih Kafkas[3] https://orcid.org/0000-0002-9037-4764
Anna Adamkova[4] https://orcid.org/0000-0003-2692-9670
Jiri Mlcek[4]✉ https://orcid.org/0000-0002-5753-8560

[1] Department of Horticulture, Faculty of Agriculture, University of Maragheh, Maragheh 55187-79842, Iran
[2] Department of Horticulture, Faculty of Agriculture, Ataturk University, 25240 Erzurum, Türkiye
[3] Department of Horticulture, Faculty of Agriculture, Çukurova University, 01330 Adana, Türkiye
[4] Department of Food Analysis and Chemistry, Faculty of Technology, Tomas Bata University in Zlin, Vavreckova, 76001 Zlin, Czech Republic
✉ mlcek@utb.cz

ABSTRACT

Salinity has a global impact on plants by inducing biochemical and metabolic changes that lead to oxidative stress, impairing growth, yield, and productivity. The pistachio tree (*Pistacia vera* L.) is a salt-tolerant species. This study aims to investigate the effectiveness of carbon nitride nanostructures modified with iron (Fe/C_3N_4) on the Akbari pistachio variety under salinity stress levels (0, 50, 100, and 150 mM) and foliar applying (distilled water as a control, Fe_2O_3 (0.2 g L^{-1}), C_3N_4 (0.2 g L^{-1}), and carbon nitride modified with iron or Fe/C_3N_4 (0.2 g L^{-1}). The findings showed that salinity decreased relative water content (RWC), SPAD index, membrane stability index (MSI), maximum fluorescence (Fm), and variable fluorescence (Fv), and increased hydrogen peroxide (H_2O_2). However, foliar application with Fe_2O_3, or Fe/C_3N_4, improved all traits. Nevertheless, there was no significant interaction between the applied mitigating treatments and salinity levels on RWC, MSI, SPAD index, Fm, Fv, and H_2O_2. Salinity stress increased malondialdehyde (MDA), phenol, and flavonoid levels, and reduced leaf number, height, photosynthetic pigments, vitamin C, and total protein. The application of foliar treatments, especially Fe/C_3N_4, improved the influence of salinity stress. Additionally, the activity of antioxidant enzymes increased under salt stress and foliar application. Fe/C_3N_4-treated seedlings consistently exhibited higher growth and photosynthetic traits and lower oxidative damage than untreated controls across salinity levels, indicating a stable physiological benefit rather than a salinity-specific effect.

Keywords: antioxidant enzymes, carbon nanomaterials, environmental stresses, growth index

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INTRODUCTION

Pistacia vera L. is a significant crop in arid and semi-arid regions. This species is also tolerant to soil salinity, making it a suitable alternative for farming in orchards with salinity-influenced soils [Hajiboland et al. 2014]. Despite this, it is imperative to mention that the resistance of pistachio trees to salinity stress varies significantly based on the specific genotype and rootstock employed [Rahneshan et al. 2018]. The pistachio is one of the most valuable tree nuts worldwide and is deeply integrated into the economic and nutritional systems of semi-arid regions. Global pistachio production surpassed 1.2 million tons annually in the 2020s, with Iran, the United States, Turkey, and China leading, collectively accounting for over 90% of the world's exports. In Iran, pistachio orchards cover approximately 273881 ha, contributing more than 25% of total global pistachio yield and providing livelihoods to hundreds of thousands of smallholder farmers [FAO 2023]. Globally, the pistachio industry is a model for sustainable perennial crop management in salty landscapes [Akhavan and Gonçalves 2021].

Plant cultivation is subject to several stress environments, with salinity reflected as the most restricting factor. According to Kamran et al. [2019], salinity critically impacts many plant processes, especially yield. Industrial and agricultural activities, along with modern living, have increased soil and water salinity, leading to a decline in their quality worldwide [Ahmadi and Souri 2020]. Over the past decades, the rapid increase in the world's population has put significant pressure on soil fertility and agricultural productivity. High application rates of chemical fertilizers to achieve high yields have considerably promoted soil salinity [Ahmadi and Souri 2018].

Soil salinity is one of the principal abiotic stresses that reduces crop yields worldwide. It impairs crop growth, yield, and quality, particularly in arid and semi-arid areas. Recent global assessments indicate that salinity affects more than 20% of irrigated land and is spreading further due to climate change, unsustainable irrigation, industrial waste, and intensive fertilizer application [Demo et al. 2025, FAO 2023]. Increasing human activities, such as industrial effluents, over-irrigation, and excessive fertilizer use, have raised saline levels in arable soils, reducing their fertility and crop yields [Demo et al. 2025]. Munns and Tester [2008] pointed out that a plant's salinity tolerance relies on keeping low cytosolic Na^+ levels through methods such as selective ion exclusion, vacuolar sequestration, and the production of compatible osmolytes like proline and glycine betaine to maintain osmotic balance. Salinity also causes osmotic stress and ionic toxicity, mostly by causing too much Na^+ and Cl^- ions to build up, which messes up water relations, nutrient uptake, metabolic processes, and photosynthesis [Shafi et al. 2025]. Plants have developed complex physiological and molecular mechanisms to deal with salinity stress [Zhou et al. 2024]. These include selective ion exclusion, vacuolar sequestration of Na^+, accumulation of osmolytes (e.g., proline and glycine betaine), and activation of antioxidant defense systems [Mendis et al. 2025]. However, these natural processes do not always function effectively when salinity is high or persists for extended periods. This can cause oxidative damage, chlorophyll breakdown, and CO_2 assimilation to slow down, and less biomass to build up [Karpinska and Foyer 2024]. As a result, developing long-term, practical strategies to improve plant salt tolerance has become a top priority for researchers worldwide.

By enhancing nutrient balance and physiological resilience, foliar and soil nutrient application has long been acknowledged as a successful strategy to reduce damage caused by salinity [Kaya and Ashraf 2024]. When these elements are applied to plant leaves, they can enter via various pathways, including stomata, cuticular cracks, epidermal cells, and leaf hairs, as reported by Abdoli et al. [2020]. One significant problem in saline and alkaline soils is iron deficiency due to their high pH [Askary et al. 2017]. The effectiveness of Fe-containing solutions is primarily determined by evaluating the rate at which Fe is absorbed by plant tissues, the speed at which it is translocated from treated organs to other tissues, and the extent to which it enhances chlorophyll synthesis. Accurate assessment of these factors is crucial to ensure that Fe-supplemented plants are healthy and exhibit optimal growth [Fernández et al. 2008]. Nanotechnology has emerged as a promising approach in sustainable agriculture, offering innovative methods for precise nutrient delivery, enhanced bioavailability, and reduced environmental impact [An et al. 2022]. Recently, the use of nanotechnology in horticulture has been widely investigated through nanoscale manipulation of materials to improve fertilizer delivery, nutrient uptake, and crop fortification [Ma et al. 2020]. According to recent research, Fe-based nanomaterials can improve nutrient signaling and redox active interactions to control ionic homeostasis, increase chlorophyll content, and reduce oxidative stress under salinity [Ghosh et al. 2025, Singh et al. 2024]. Iron oxide nanoparticles (Fe_2O_3-NPs) are known to enhance chlorophyll content, antioxidant activity, and growth under stressful conditions, thereby supporting sustainable farming, as highlighted by Khanizadeh et al. [2024]. The use of Fe_2O_3 nanoparticles in nanofertilizers has the potential to advance the horticultural industry. By enhancing nutrient absorption and reducing soil toxicity,

these nanofertilizers can significantly promote sustainable agricultural practices and ensure food security [Verma et al. 2022]. Fe_2O_3 nanoparticles serve as both micronutrient sources and redox modulators, thereby affecting photosynthetic efficiency in saline environments, antioxidant enzyme activities, and reactive oxygen species (ROS) scavenging [Feng et al. 2022, Zhang et al. 2025]. Polymeric carbon nitride (CN), is composed of the earth-abundant elements carbon (C) and nitrogen (N) [Chen and Song 2017]. Graphitic carbon nitride nanosheets (C_3N_4) have attracted significant attention recently due to their distinctive structure and remarkable catalytic properties. C_3N_4, which consists solely of C and N, can be produced simply via low-cost N-N-supplemented composites such as melamine and urea via heat condensation [Inagaki et al. 2019]. The potential of C_3N_4-based nanostructures for biological and agro-environmental systems has been highlighted by recent international research, and these nanostructures have been extensively studied for photocatalysis, environmental remediation, and nanozyme applications [Zhuang et al. 2024].

Recent advances in agricultural nanotechnology indicate that nano-enhanced micronutrient formulations can improve nutrient-use efficiency and enhance plant stresnanos tolerance [Al-Dossary et al. 2025, Lalitha et al. 2025]. The Organization for Economic Co-operation and Development (OECD), the European Food Safety Authority (EFSA), and FAO recommend testing these nanomaterials based on observable biological indicators such as growth rate, photosynthetic traits, and oxidative stress markers to verify their safety and efficacy [EFSA et al. 2021, FAO 2023, OECD 2020]. New research shows that iron-based nanoparticles support plant growth, increase chlorophyll levels, and boost antioxidant enzyme activity under salinity stress, with reduced MDA and H_2O_2 levels and better membrane stability [Rico et al. 2015, Verma et al. 2022].

Although the positive impacts of iron-based nanoparticles like Fe_2O_3 and Fe_3O_4 on salinity tolerance, such as enhanced leaf water content, chlorophyll levels, membrane integrity, and antioxidant enzymes, have been well documented since the mid-2010s, research on iron-loaded graphitic carbon nitride (Fe/C_3N_4) nanocomposites in plants is still scarce. Specifically, there is a lack of *in vivo* physiological and biochemical data on the effects of Fe/C_3N_4 in woody perennial crops, such as pistachio, under salinity stress. This study explores the potential of combining iron-based nanoparticles and graphitic carbon nitride into a single nanocomposite (Fe/C_3N_4) to enhance protection against salinity stress in plants. Given their distinct yet potentially complementary roles in plant stress responses, we examined whether Fe/C_3N_4 confers greater benefits than Fe_2O_3 or C_3N_4 alone. We hypothesized that foliar application of Fe/C_3N_4 would more effectively reduce salinity-induced damage in pistachio seedlings by improving growth, leaf water content, membrane integrity, photosynthetic pigment levels, and efficiency. Additionally, we expected it to reduce oxidative stress by more strongly activating antioxidant enzymes (SOD, APX, and GPX) and by decreasing levels of MDA and H_2O_2. To evaluate this, pistachio seedlings were exposed to varying salinity levels and treated with Fe_2O_3, C_3N_4, or Fe/C_3N_4, and their physiological and biochemical responses were carefully compared.

MATERIALS AND METHODS

Plant material and growth conditions

The trial was conducted in the Faculty of Agriculture research greenhouse at the University of Maragheh in Iran. The study was a factorial experiment in a completely randomized design (CRD) with three replications. It included four foliar treatments (distilled water as control, Fe_2O_3, C_3N_4, and Fe/C_3N_4 at 0.2 g L^{-1}) combined with four levels of salinity (0, 50, 100, and 150 mM NaCl). One-year pistachio seedlings (Akbari cultivar) with the same height and growth were transplanted in 7-liter pots with a combination of soil and peat moss (1:1) as the culture medium. The soil was a loam-sandy clay, air-dried, sieved through a 2 mm mesh, and free of visible plant residues. According to routine laboratory analyses conducted before the experiment, the soil had a pH of 7.4, an electrical conductivity (EC) of 0.62 dS m^{-1}, potassium of 438.12 mg kg^{-1}, phosphorus of 8.67 mg kg^{-1}, nitrogen of 0.174%, and approximately 1.4% organic matter. Each pot contained one seedling. Following a 2-week establishment period under non-saline conditions, salinity treatments were applied using NaCl solutions at the designated concentrations. To avoid osmotic shock, NaCl concentration was increased gradually over three consecutive irrigations until the target salinity level was reached. Foliar treatments were applied three times to the leaves at three-day intervals, beginning two weeks after the initiation of stress. The materials (C_3N_4 and Fe/C_3N_4) used for this work were identically prepared according to the protocols mentioned by Heidarpour et al. [2020]. A concentration of 0.2 g L^{-1} for Fe_2O_3, C_3N_4, and Fe/C_3N_4 foliar applications was chosen based on prior research showing it provides physiological benefits without causing phytotoxicity in crop plants exposed to abiotic stress. Previous studies

have demonstrated that concentrations between 0.1 and 0.5 g L^{-1} can boost antioxidant activity, photosynthesis, and stress resilience, while avoiding growth suppression or oxidative damage [Kokina et al. 2020, Ma et al. 2020]. Additionally, initial observations during the study's establishment phase revealed no visible phytotoxic effects, such as leaf chlorosis or necrosis, at this concentration. Hence, 0.2 g L^{-1} was deemed both effective and safe for pistachio seedlings.

At the end of the experiment, morphological attributes, such as plant height and leaf number, were quantified. Fully developed leaves were sampled to study morphological, physiological, and biochemical traits, as well as total antioxidant enzyme activity.

Relative water content (RWC)

To determine the RWC of leaves, we followed the technique by Kumar et al. [2020]. Fresh leaf samples were collected, and their initial weight (FW) was noted immediately. The leaves were then soaked in distilled water at room temperature for 4 hours to reach turgid weight (TW). Subsequently, the samples were oven-dried at 65 °C for 24 h until their weight stabilized, and the dry weight (DW) was recorded. The relative water content was determined using the following formula:

$$RWC\% = (FW - DW) / (TW - DW) \times 100$$

Membrane stability index (MSI)

Membrane stability index (MSI) was measured according to the method described by Sairam et al. [1997]. Fresh leaf samples (0.1 g) were placed in 10 mL of double-distilled water and incubated at 40 °C for 30 minutes. The electrical conductivity (C_1) of the solution was then measured. Next, the samples were boiled at 100 °C for 10 minutes, cooled to room temperature, and the final conductivity (C_2) was recorded. MSI was calculated using the formula: 0.1 g leaf samples from each pot were used to measure electrical conductivity at 40 °C (EC_1) and 100 °C (EC_2).

$$MSI\% = [1 - (C_1 / C_2)] \times 100$$

Chlorophyll index (SPAD) determination

The chlorophyll index or greenness index was quantified in young leaves via a portable Chlorophyll Meter and expressed as SPAD (Instruments SPAD-502, Japan).

Photosynthesis pigments

Chlorophylls and carotenoids were measured using a spectrophotometer and Arnon [1949]. To extract pigments, 0.5 g of fresh leaf tissue was crushed with liquid nitrogen and mixed with 5 mL of 80% acetone. The mixture was then analyzed for absorbance at 664 nm, 647 nm, and 470 nm wavelengths through a spectrophotometer (UV-1800, Shimadzu, Japan) and expressed as mg g^{-1} FW.

Chlorophyll fluorescence parameters

The pulse amplitude modulation fluorometer (PAM-2500, Walz, Effeltrich, Germany) was exploited to record chlorophyll fluorescence parameters on fully expanded young leaves, following the technique depicted in detail by Chen et al. [2011]. The parameters measured included the minimum chlorophyll fluorescence (F0), the maximal fluorescence (Fm), the variable fluorescence (Fv), and the maximal quantum yield of PS II (Fv/Fm).

Vitamin C

To determine the amount of vitamin C, 1 g of pistachio leaves was blended with 3 mL of 1% metaphosphoric acid and centrifuged at 4 °C and 6000 rpm for 15 minutes. The resulting extract was mixed with 180 µL of 2,6-dichloroindophenol sodium hydrate, and the optical density (OD) was measured at 520 nm using a spectrophotometer. Finally, the concentration of vitamin C was measured based on a pure ascorbic acid standard and expressed as mg per 100 g^{-1} FW [Bor et al. 2006].

Total phenol content (TPC)

To measure TPC, 0.5 g of fresh leaves was extracted with acidic methanol and centrifuged at 10000 rpm for 15 min. The reaction mixture comprised water, 10% Folin-Ciocâlteu reagent, the extract, and 7.5% sodium carbonate. The OD was noted at 765 nm using a UV spectrophotometer. TPC was estimated as mg gallic acid g^{-1} FW [Singleton and Rossi 1965].

Total flavonoids content (TFC)

To determine the total flavonoid content (TFC), 1 g of the fresh pistachio sample was mixed with 80% methanol. The resultant mixture was centrifuged at 16000 rpm for 15 min to attain the supernatant. A solution was prepared by mixing 95% methanol, 10% aluminum chloride (Merck, DarmStasdt, Germany), 1 M potassium acetate, and distilled water with the supernatant. The resulting OD was quantified at 415 nm, and the TFC was calculated as mg quercetin g^{-1} FW [Chang et al. 2002].

Malondialdehyde (MDA)

To analyze MDA content in the leaf sample, 0.5 g of fresh pistachio leaves was digested in 0.1% trichloroacetic acid. After centrifugation, 0.1% thiobarbituric acid, containing 20% trichloroacetic acid, was added. The mixture was heated in a bain-marie, then placed on ice to stop the reaction. After centrifugation, the OD sample was read at 532 and 600 nm, as expressed by [Heath and Packer 1968] MDA concentration was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as nmol MDA g^{-1} FW.

Hydrogen peroxide (H_2O_2)

To perform an H_2O_2 analysis on a plant sample, a mixture was prepared by combining 10 mM KH_2PO_4 buffer (pH 6.8) and 1 M KI. The plant sample was then extracted with 0.1% w/v trichloroacetic acid. The extracted plant sample was added to the prepared combination, and the OD was measured at 390 nm using a spectrophotometer. H_2O_2 concentration was calculated using a standard calibration curve and expressed as µmol H_2O_2 g^{-1} FW [Sinha et al. 2005].

Total soluble protein content

Fresh leaf tissue (0.5 g) was homogenized in 50 mM potassium phosphate buffer (pH 7.0) and centrifuged at 12,000 × g for 15 minutes at 4 °C. A 0.1 mL aliquot of the supernatant was combined with 5 mL of Bradford reagent, and absorbance was read at 595 nm with a spectrophotometer. Finally, the total soluble protein content was determined using a BSA standard curve and expressed as mg g^{-1} FW [Bradford 1976].

Enzymatic antioxidant activity

Ascorbate peroxidase activity (APX). The activity of the APX was assessed via creating a reaction combination that included 250 mM KH_2PO_4 buffer with a pH of 6.8, 1 mM H_2O_2, 0.5 mM ascorbic acid, and 0.1 mM EDTA. The reaction was initiated by adding hydrogen peroxide to the mixture. The peroxidation of ascorbic acid decreased light absorption at 290 nm, which was measured over two minutes using a spectrophotometer. At the end APX activity was calculated as µmol min^{-1} mg^{-1} FW [Yoshimura et al. 2000].

Guaiacol peroxidase activity (GPX). To determine GPX activity, 0.5 g of leaf tissue was pulverized using KH_2PO_4 buffer (100 mM, pH = 6.8), EDTA (4 mM), and 1% PVP. The mixture was subsequently centrifuged at 10,000 rpm for 20 minutes. The GPX activity was determined by measuring the amount of tetraguaiacol at 470 nm for 1 minute. Finally GPX activity was defined as µmol min^{-1} mg^{-1} FW [Yoshimura et al. 2000].

Superoxide dismutase activity (SOD). To determine SOD activity, we used the Nakano and Asada [1981] method. The reaction mixture included 1.5 mM sodium carbonate, 0.2 mM methionine, 3 mM EDTA, 0.1 M sodium phosphate buffer, and 2.25 mM NBT. Distilled water was added, and the mixture was incubated for 15 min at 24 °C under light, after which the absorbance was measured at 560 nm. SOD activity was calculated as µmol min^{-1} mg^{-1} FW.

STATISTICAL ANALYSIS

All data were analyzed using analysis of variance (ANOVA) with MSTAT-C (version 2.1; Michigan State University, East Lansing, MI, USA). Before conducting ANOVA, residuals were checked for normal distribution via the Shapiro–Wilk test and for equal variances using Levene's test. Mean comparisons were made using the least significant difference (LSD) test at $P \leq 0.05$. All results are presented as means ± standard deviation (SD) from three biological replicates.

RESULTS

Morphological parameters

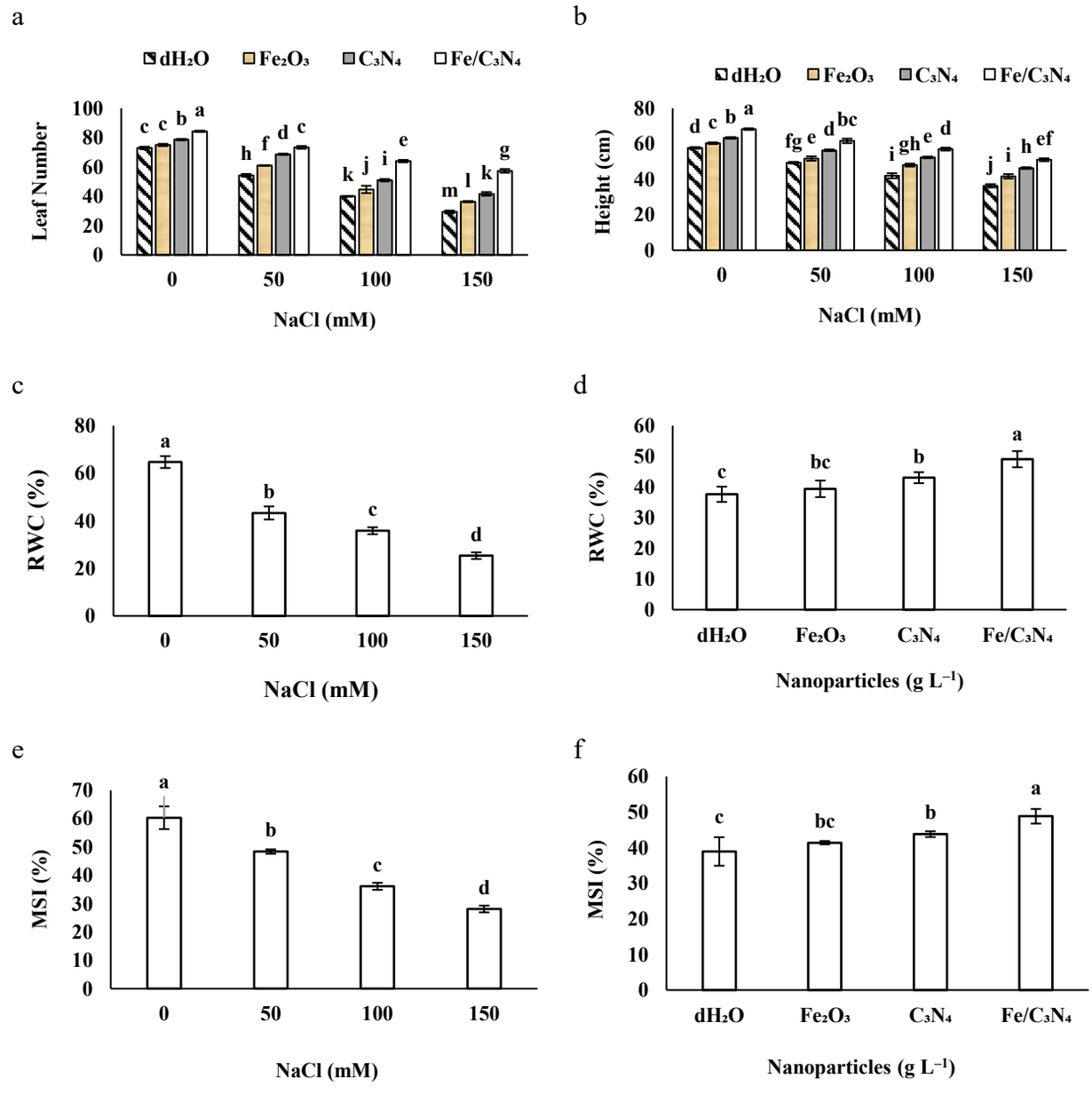
Salinity significantly ($P \leq 0.05$) reduced the number of leaves. Under both stress and non-stress conditions, all foliar treatments increased leaf number compared to the control pistachio. The uppermost leaf number was obtained under no stress and treated with Fe/C_3N_4, and the lowest was obtained in the control pistachio seedlings and under 150 mM stress. Also, the application of salt stress resulted in a 59% diminution in the number of leaves

compared to the control (Figure 1a). Salt stress reduced height, and all foliar treatments mitigated the effects of stress and increased height compared with the no-foliar-treatment condition. Based on these results, the tallest seedlings were observed in the Fe/C_3N_4 treatment and in the no-saline application condition, whereas the lowest were in control pistachio plants subjected to 150 mM salinity stress (Figure 1b).

Relative water content of leaf (RWC)

The salinity stress caused a significant lessening ($P \leq 0.05$) in RWC, and the most significant decrease was achieved with 60% at the highest salinity stress treatment compared to the control pistachio plants (Figure 1c). Foliar application of the treatments showed a 24% increase in RWC compared to the control pistachio seedlings, with no significant difference between the Fe_2O_3 and C_3N_4 treatments (Figure 1d).

Figure 1. The effects of soil salinity and iron, carbon nitride, and carbon nitride modified with Fe_2O_3 treatment (mg L^{-1}) on the number of leaves (a), height (b), RWC (c and d), and MSI (e and f) of pistachio seedlings. If the letters used to represent the data points differ, it indicates significant differences among the data points at the 5% level of significance according to the LSD test. The error bars indicate standard deviation (SD)



Membrane stability index (MSI)

Salinity stress led to a meaningful diminution in MSI, with the most significant decrease observed at 53% at 150 mM stress compared to the pistachio seedlings grown in normal situations (Figure 1e). The foliar application treatments increased MSI, with the highest increase of 20% observed in Fe/C_3N_4 compared with pistachio seedlings receiving any treatment (Figure 1f).

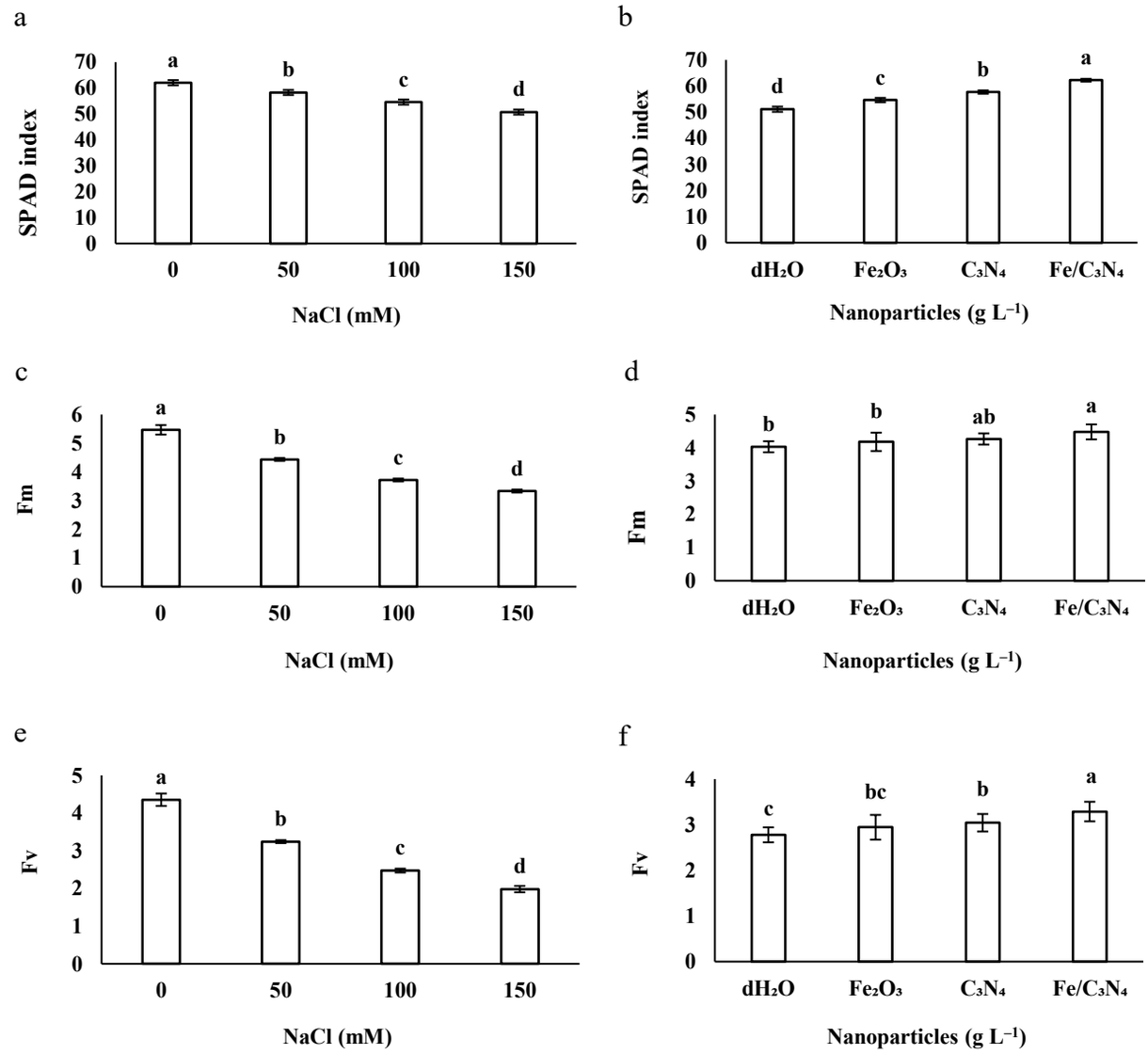
SPAD index

The chlorophyll index declined meaningfully ($P \leq 0.05$) through enhancing salinity stress. There was an 18% reduction at the highest stress as compared to the pistachio control (Figure 2a). Also, the results exhibited that foliar spray treatments significantly increased the chlorophyll index and led to a 21% increase compared to the pistachio seedlings control (Figure 2b).

Photosynthetic pigments

The concentrations of Chl a, b, total chlorophyll, and carotenoids lessened with the increase in salinity level. A significant increment ($P \leq 0.05$) in Chl a, b, total chlorophyll (Tchl), and carotenoid content was observed follow-

Figure 2. The effects of soil salinity and iron, carbon nitride, and carbon nitride modified with iron treatment on SPAD index (a and b), Fm (c and d), and Fv (e and f) of pistachio seedlings. If the letters used to represent the data points differ, it indicates significant differences among the data points at $P \leq 0.05$, as determined by the LSD test. The error bars indicate standard deviation (SD)



ing Fe_2O_3, C_3N_4, and Fe/C_3N_4 treatments in stressed and non-stressed conditions. The highest concentrations of Chl a, b, Tchl, and carotenoids were obtained in the foliar treatment of Fe/C_3N_4 without applying salinity stress. The lowest concentrations were observed at 150 mM NaCl without foliar treatments. Salt stress led to a 55% decrease in Chl a and b, a 68% decrease in Tchl, and a 50% decrease in carotenoid content (Table 1).

Fm. The salinity stress significantly decreased Fm, and the maximum decrease was achieved at 39% at the highest stress level (Figure 2c). The outcomes also exhibited that the utilization of foliar treatments increased Fm, but the foliar treatments of C_3N_4 had no significant difference with Fe_2O_3, with the control, as well as C_3N_4 and Fe/C_3N_4 with Fe_2O_3 (Figure 2d).

Table 1. Effect of Fe_2O_3, Carbon nitride, and Carbon nitride modified with iron foliar treatments on photosynthetic pigments, F0 and Fv/Fm of pistachio seedlings under salinity stress (mean ±SD)

NaCl (mM)	Treatment (g L^{-1})	Chl a (mg g^{-1} FW)	Chl b (mg g^{-1} FW)	Total Chl (mg g^{-1} FW)	Carotenoids (mg g^{-1} FW)	F0	Fv/Fm
0	ddH_2O	41.43 ±0.088 c	24.55 ±1.282 de	65.98 ±1.284 c	1.502 ±0.013 bc	1.127 ±0.001 fg	0.784 ±0.007 b
	Fe_2O_3	42.10 ±0.032 bc	25.69 ±0.463 cd	67.7 ±0.439 c	1.531 ±0.006 ab	1.127 ±0.006 fg	0.789 ±0.010 b
	C_3N_4	42.90 ±0.040 ab	27.44 ±0.446 b	70.34 ±0.453 b	1.548 ±0.006 ab	1.129 ±0.047 f	0.795 ±0.012 b
	Fe/C_3N_4	43.51 ±0.138 a	29.24 ±0.042 a	72.76 ±0.176 a	1.570 ±0.019 a	1.076 ±0.026 g	0.813 ±0.007 a
50	ddH_2O	33.98 ±0.456 f	21.65 ±0.679 f	55.63 ±0.707 e	1.362 ±0.012 e	1.217 ±0.011 cd	0.710 ±0.001 e
	Fe_2O_3	36.05 ±0.478 e	23.70 ±0.671 e	59.75 ±1.084 d	1.417 ±0.008 d	1.197 ±0.001 de	0.726 ±0.002 de
	C_3N_4	39.93 ±0.735 d	25.95 ±0.567 c	65.88 ±0.609 c	1.441 ±0.005 d	1.188 ±0.004 de	0.737 ±0.003 cd
	Fe/C_3N_4	41.75 ±0.017 bc	28.17 ±0.478 ab	69.92 ±0.487 b	1.454 ±0.003 cd	1.161 ±0.003 ef	0.750 ±0.004 c
100	ddH_2O	25.43 ±0.370 kb	16.96 ±0.247 j	42.39 ±0.616 i	1.231 ±0.002 gh	1.251 ±0.004 c	0.644 ±0.006 h
	Fe_2O_3	26.84 ±0.804 ij	18.89 ±0.437 gh	45.73 ±0.745 gh	1.269 ±0.002 fg	1.255 ±0.039 c	0.668 ±0.004 fg
	C_3N_4	27.65 ±0.949 i	18.43 ±0.633 hi	46.08 ±1.582 g	1.294 ±0.007 f	1.238 ±0.002 cd	0.663 ±0.004 g
	Fe/C_3N_4	32.49 ±0.604 g	21.66 ±0.402 f	54.14 ±1.006 e	1.421 ±0.017 d	1.235 ±0.001 cd	0.683 ±0.012 f
150	ddH_2O	18.52 ±0.865 m	11.01 ±0.454 l	29.53 ±0.656 k	0.751 ±0.044 k	1.420 ±0.041 a	0.553 ±0.008 k
	Fe_2O_3	24.34 ±1.017 l	15.56 ±1.393 k	39.91 ±2.289 j	0.997 ±0.057 j	1.375 ±0.006 ab	0.577 ±0.001 j
	C_3N_4	26.27 ±0.710 jk	17.52 ±0.473 ij	43.79 ±1.183 hi	1.095 ±0.016 i	1.338 ±0.011 b	0.602 ±0.007 i
	Fe/C_3N_4	29.72 ±0.689 h	20.15 ±0.311 g	49.87 ±0.837 f	1.186 ±0.039 h	1.269 ±0.015 c	0.640 ±0.006 h
LSD at $P \leq 0.05\%$		1.22	1.32	2.07	0.05	0.05	0.01
S.O.V.		–	–	–	–	–	–
NaCl		825.82**	298.378**	2111.262**	0.620**	0.118**	0.091**
Treatment		105.699**	246.508**	374.087**	0.081**	0.008**	0.005**
NaCl × treatment		9.561**	31.079**	22.235**	0.017**	0.003**	0.0001**
Error		0.545	20.399	1.555	0.001	0.001	0.0001
C.V. (%)		2.22	3.69	2.27	2.09	2.05	4.20

** indicates significant at $P \leq 0.01$. If the letters used to represent the data points are different from each other, it means there are significant differences between those data points at $P \leq 0.05$ of significance as per the LSD test in each column

Chlorophyll fluorescence parameters

F_0. Salinity stress significantly increased F0 and moderated its effects, whereas foliar application treatments did not. The highest F0 was observed under 150 mM saline stress without foliar application, and the lowest was observed in the control and the Fe/C_3N_4 foliar treatment (Table 1).

Fv. Salinity stress may result from a notable reduction in Fv (Table 2), with the highest decrease of 54% observed relative to the control (Figure 2e). The foliar treatments indicated an increment of Fv, and the highest value was obtained in the pistachio seedlings via the foliar treatment of Fe/C_3N_4 with 15% (Figure 2f).

Fv/Fm. Based on the results, salinity stress drastically reduced Fv/Fm ($P \leq 0.05$)/Fm, but foliar treatments partially alleviated this effect. The highest increase, up to 29%, was obtained at the highest stress level compared to the control (Table 1).

Vitamin C

Based on the results, the interaction between foliar treatments and salinity on vitamin C content in the studied variety was determined. In the absence of salinity stress, foliar application with Fe_2O_3, C_3N_4, or Fe/C_3N_4 increases vitamin C content compared to the control. Additionally, salinity stress reduced the highest level by 78% relative to the control seedlings, and foliar spray treatments mitigated its effects (Table 3).

Table 2. Result of the analysis of variance on the studied characteristics in pistachio seedlings

S.O.V.	df	Mean square											
		Number leaf	Height (cm)	SAPD	RWC (%)	MSI (%)	Fm	Fv	MDA (nmol g^{-1} FW)	H_2O_2 (µmol g^{-1} FW)	APX activity (µmol min^{-1} mg^{-1} FW)	GPX activity (µmol min^{-1} mg^{-1} FW)	SOD activity (µmol min^{-1} mg^{-1} FW)
NaCl	3	3114.139**	741.806**	283.903**	3327.383**	2390.402**	10.573**	12.822**	3.459**	4.219**	4.877**	2.575**	1677.497**
Treatment	3	935.250**	382.139**	267.157**	306.011**	213.500**	0.419**	0.546**	0.238**	0.720**	2.070**	0.522**	136.740**
NaCl × treatment	9	33.454**	3.565**	0.501ns	6.913ns	4.125ns	0.010ns	0.010ns	0.048**	0.127**	0.045**	0.053**	33.230*
Error	32	1.458	1.063	1.331	5.375	3.782	0.021	0.021	0.006	0.087	0.006	0.001	12.869
C.V (%)	–	2.07	1.96	2.04	5.48	4.50	3.39	4.76	6.51	4.37	7.84	4.24	4.36

*, ** indicate significance at $P \leq 0.05$ and $P \leq 0.01$, respectively; ns indicates non-significant differences

Total phenol and flavonoid content

Salinity significantly increased the total phenolic and flavonoid content in pistachio leaves. Under control and foliar treatment conditions, the contents of phenols and flavonoids decreased. The highest amount of phenol and flavonoid was related to the salinity stress of 150 mM without applying foliar treatments, and the lowest was related to the foliar treatment of Fe/C_3N_4 without applying salt stress (Table 3).

Malondialdehyde (MDA)

The results indicated that, under non-stress conditions, Fe_2O_3, C_3N_4, and Fe/C_3N_4 applications reduced the MDA content in pistachio seedlings. Additionally, salinity stress increased MDA accumulation by 2.9-fold in pistachio leaves compared to the pistachio control, and foliar application treatments mitigated the effects of stress. The utmost MDA content was found at 150 mM NaCl use and with no application of spraying treatments (Figure 3a).

Hydrogen peroxide (H_2O_2)

The data showed that salinity stress increased H_2O_2 accumulation, with no significant difference between the 50- and 100-mM levels (Figure 3b). The outcomes revealed that the use of Fe_2O_3, C_3N_4, and Fe/C_3N_4 foliar treat-

ments reduced the concentration of H_2O_2 compared to the pistachio seedling in normal conditions, and no significant alteration was perceived among the mentioned foliar treatments (Figure 3c).

Total Soluble protein content

Total soluble protein content decreased under NaCl stress, with the greatest reduction observed at the highest stress level (39% compared to the control seedlings). Under no-stress conditions, the application of foliar treatments significantly increased protein content and improved it across salinity treatments. The highest total soluble protein content was obtained with foliar treatment with Fe/C_3N_4 and without salinity application; the lowest was at 150 mM salinity stress without foliar treatment (Table 3).

Figure 3. The effects of soil salinity and iron, carbon nitride, and carbon nitride modified with iron treatment on MDA (a), H_2O_2 (b and c), APX (d), GPX (e), and SOD (f) of pistachio seedlings. If the letters used to represent the data points differ, it indicates significant differences among the data points at $P < 0.05$, as determined by the LSD test. The error bars indicate standard deviation (SD)

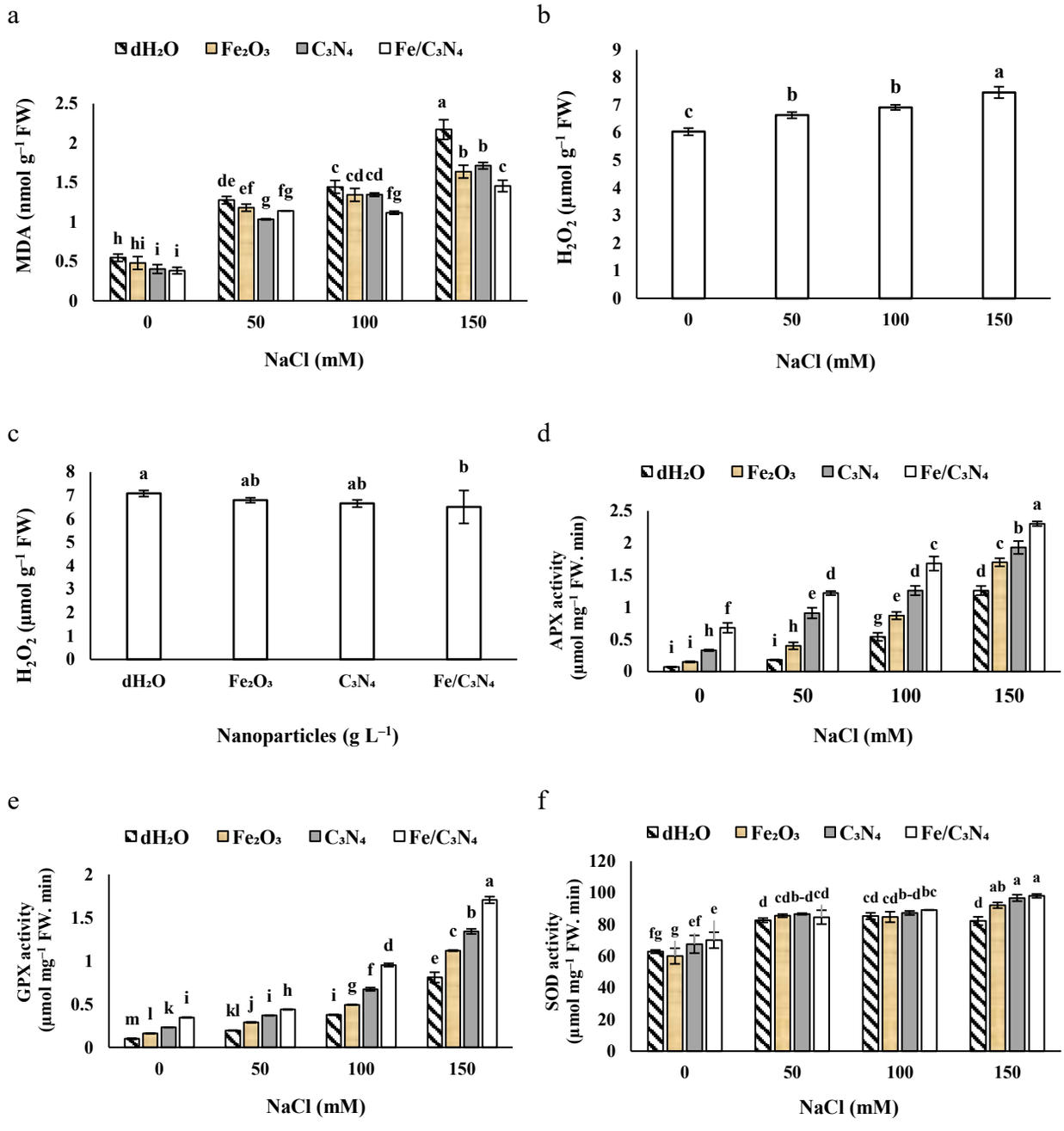


Table 3. Effect of Fe_2O_3, Carbon nitride (C_3N_4), and Carbon nitride modified (Fe/C_3N_4) with iron foliar treatments on vitamin C, Total phenol and Flavonoid content, and Total Soluble protein of pistachio seedlings under salinity stress (mean ±SE)

NaCl (mM)	Treatment (g L^{-1})	Vitamin C (mg 100 g^{-1} FW)	Total phenol content (mg g^{-1} FW)	Flavonoid content (mg g^{-1} FW)	Total Soluble protein (mg g^{-1} FW)
0	ddH_2O	14.07 ±0.585 d	681.3 ±7.716	249.9 ±8.716 gh	140.7 ±1.856 f
	Fe_2O_3	17.88 ±0.419 b	640.6 ±8.315	188.7 ±4.775 i	167.6 ±2.079 c
	C_3N_4	17.14 ±0.192 bc	617.2 ±2.400	156.2 ±5.734 j	153.9 ±0.944 de
	Fe/C_3N_4	21.15 ±1.895 a	550.3 ±10.791	125.7 ±5.835 k	194.1 ±2.283 a
50	ddH_2O	10.40 ±1.018 fg	778.3 ±11.544	347.6 ±16.462 d	132 ±1.732 g
	Fe_2O_3	12.31 ±1.166 e	734.3 ±6.687	263.2 ±6.682 g	148.8 ±0.227 e
	C_3N_4	14.07 ±0.536 d	691.2 ±15.877	251.5 ±10.113 gh	156 ±0.000 d
	Fe/C_3N_4	15.64 ±0.822 c	594.8 ±15.402	190.3 ±10.610 i	179.8 ±4.813 b
100	ddH_2O	5.292 ±0.247 ij	866.2 ±10.745	462.8 ±10.709 b	120.5 ±2.045 h
	Fe_2O_3	11.15 ±0.347 e–g	797.6 ±13.118	384.7 ±5.614 c	124.9 ±0.454 h
	C_3N_4	9.721 ±0.679 g	756.5 ±2.788	306.5 ±5.412 f	141.8 ±3.341 f
	Fe/C_3N_4	11.65 ±0.751 ef	697.8 ±8.342	236.5 ±5.027 h	170.4 ±1.487 c
150	ddH_2O	3.035 ±0.208 k	976.1 ±20.021	513.3 ±3.075 a	85.51 ±9.344 j
	Fe_2O_3	3.857 ±0.360 jk	925.5 ±6.677	448.8 ±15.386 b	123 ±0.524 h
	C_3N_4	6.113 ±0.117 hi	868.4 ±12.913	382.4 ±2.850 c	104.5 ±1.048 i
	Fe/C_3N_4	7 ±0.289 h	803.4 ±11.198	326.2 ±5.158 e	151.7 ±5.073 de
LSD at 0.05%		1.52	22.89	17.48	6.68
S.O.V.		–	–	–	–
NaCl		342.170**	161027.804**	127310.512**	5200.797**
Treatment		65.489**	57544.18**	65145.362**	6095.306**
NaCl × Treatment		3.549**	524.962*	1443.771**	190.664**
Error		0.842	189.427	110.422	16.151
C.V (%)		8.13	3.84	3.48	2.80

** indicates significance at $P \leq 0.01$. If the letters used to represent the data points are different from each other, it means there are significant differences between those data points at a 5% level of significance, as per the LSD test in each column

Enzymatic antioxidant activity

The results (Figure 3d) showed that salinity and different foliar applications of nanocomposite significantly ($P \leq 0.05$) improved the APX activity. The highest APX activity was observed with 150 mM NaCl and Fe/C_3N_4, approximately 3-fold higher than in control pistachio seedlings, which exhibited the lowest APX activity.

Also, GPX activity was considerably influenced by various levels of NaCl application and C_3N_4, Fe_2O_3, and iron-modified carbon nitride treatments ($P \leq 0.05$), see Figure 3e. The highest and lowest GPX activity was detected in Fe/C_3N_4-treated pistachio plants under 150 mM salinity stress and in control pistachio plants, respectively.

The enhancement in salinity level and the concentration of the treatments used improved the activity of SOD ($P \leq 0.05$), see Figure 3f, and the highest and lowest activity of SOD was revealed, respectively, in the salinity stress of 150 mM with Fe/C_3N_4, and the Fe_2O_3 solution treatment was obtained without applying stress.

DISCUSSION

Salinity can reduce leaf number by restricting leaf development, as salt stress hampers growth [Shahid et al. 2020]. The osmotic stress arises from the difference between the saline solution outside the cell and the internal

cellular solution within the root [Munns 2002]. Salinity-induced osmotic stress results in plasmolysis, inhibition of cell enlargement, and cell death in young leaves, stems, and roots [Arif et al. 2020]. Additionally, this stress causes stomata to close [Kiełkowska 2017]. Exposure to osmotic stress can adversely affect plants, such as inhibiting the expansion of young leaves, reducing new leaf production, and reducing stomatal conductance [Kiełkowska et al. 2019]. However, Kokina et al. [2020] found that applying nano-Fe_2O_3 increased leaf production in *Medicago falcata* L. When irrigated with saline water, plants may accumulate high concentrations of NaCl in cell walls and cytoplasm, negatively affecting photosynthesis rates, carbohydrate content, and some growth hormones [Barbieri et al. 2012]. Salinity stress has long-term effects on plant growth, reducing growth rates owing to osmotic and ionic stresses. Turgor pressure is reduced, decreasing wall extensibility and growth yield thresholds, particularly in stems and leaves. Height decreases due to reduced photosynthesis, and growth reduction is an adaptation that enhances plant endurance under stressful conditions [Bistgani et al. 2019]. Rui et al. [2016] report that specific concentrations of iron nanoparticles can increase the growth index, likely due to increased antioxidant activity and regulation of hormone levels. Ma et al. [2020] found that treating rice crops with C_3N_4 under heavy metal pollution reduced toxicity and improved plant growth. The Fe/C_3N_4 composite likely boosted antioxidant activity and maintained redox balance, reducing ROS-mediated suppression of cell division in shoot tips [Kaliyaperumal et al. 2025, Saleem et al. 2022]. Additionally, the polymeric N-rich structure of C_3N_4 offers strong coordination sites for metal ions, promoting better Fe retention and controlling its release via surface complexation and redox interactions [Chen and Song 2017, Ong et al. 2016].

Plants grown in saline environments exhibit a significant reduction in RWC. This decrease can be directly attributed to salt's impact on the plasma membrane's electrical potential. This impact not only impedes the ions' absorption but also significantly hinders the absorption of water, causing a state of water stress [Babaei et al. 2017]. Plants have various osmotic mechanisms to respond to water shortages in cells. Osmotic adjustment is one of them, helping plants maintain turgor pressure by adjusting the solute concentration in their cells. As osmotic potential decreases, leaves' RWC declines. RWC is a key parameter for regulating a plant's water status. It's calculated by comparing the weight of fresh tissue to fully hydrated tissue. Higher RWC leads to better growth, improving nutrient transport and metabolic processes [Ghadakchi asl et al. 2019]. In the current study, the use of C_3N_4, Fe_2O_3, and Fe/C_3N_4 boosted the RWC. Habibi and Sarvary [2015] found that adding iron to lemon balm plants under salinity stress helps them maintain stability. Fe application is associated with thicker collenchyma cells in plant stems, which help accumulate water and, hence, yield a higher and more consistent RWC.

The first signs of salinity stress are visible in the cell membranes; therefore, MSI is a crucial parameter for plant defense against this stress [Azarmi-Atajan and Sayyari-Zohan 2020]. Measuring electrolyte leakage in leaf samples can help estimate the impact of salinity on cell membrane stability. Previous studies by Akrami and Arzani [2018] have shown that conserving the integrity of the cell membrane is crucial for salinity tolerance. Azarmi-Atajan and Sayyari-Zohan [2020] unequivocally demonstrated that MSI content in lettuce decreased significantly under salt stress. However, reported with confidence that applying nano-Fe_2O_3 effectively mitigated the adverse effects of stress. Furthermore, various treatments with C_3N_4, Fe_2O_3, and Fe/C_3N_4 significantly improved MSI in pistachio leaves under salt stress. However, foliar Fe_2O_3, C_3N_4, and Fe/C_3N_4 nanoparticles significantly restored these parameters. The improvement in RWC and MSI can be attributed to multiple mechanisms, including enhanced cuticular and stomatal uptake of Fe nanoparticles, which improve ionic regulation and osmotic balance in leaf tissues [Ghosh et al. 2025]. Additionally, Fe and C_3N_4 facilitate redox buffering at the leaf surface, reducing lipid peroxidation and preserving membrane phospholipids [Cai et al. 2021]. Improvements in RWC and MSI show that Fe/C_3N_4 strengthens cellular integrity in saline environments [Gholami et al. 2024]. While Fe_2O_3 partially restored these traits, likely through improved Fe nutrition and osmotic regulation, its protective effect decreased at higher salinity levels [Gholami et al. 2024, Zhang et al. 2025, Zhou et al. 2024]. Conversely, the present findings showed that Fe/C_3N_4 maintained significantly higher RWC and MSI across all salinity levels, suggesting a combined physiological and chemical mechanism.

The current study found that the chlorophyll index decreased in pistachio seedlings as salinity stress increased, likely due to damage caused by free radicals produced under saline conditions, which degrade chlorophyll. Salinity significantly affects photosynthetic capacity, and chlorophyll content can be measured to evaluate this impact [Sim et al. 2015]. In addition, Karimi and Sadeghi-Seresht [2018] reported that salinity reduces photosynthetic rate in pistachio seedlings by increasing stomatal resistance. Chlorophyll content is a crucial indicator of a plant's health, which is influenced by water availability and nutrient levels. Sodium toxicity often displaces essential

cations from chloroplast membranes, thereby decreasing chlorophyll stability and light-harvesting ability [Hassanpouraghdam et al. 2025]. Salinity stress adversely affected the photosynthetic pigment content of pistachio plants, and chlorophyll fluorescence features were observed in this study. The absorption of essential nutrients, for instance, Fe and Mg, was also impeded in salinity environments, thereby hindering chlorophyll biosynthesis. The decrease in chlorophyll content under salt stress primarily reduces the potential for photosystem II activity [Hassanpouraghdam et al. 2020]. Salinity reduces chlorophyll levels and impairs photosynthesis by disrupting gas transmission and stomatal conductance [Ashraf and Harris 2004, Barbieri et al. 2012]. However, the higher content of photosynthetic pigments in the present trial is consistent with previous studies on cabbage and water dropwort (*Oenanthe javanica*) [Jamil et al. 2007, Kumar et al. 2020]. Carotenoids are a class of antioxidants that help plants develop tolerance to salt stress by diminishing free oxygen radicals [Ali et al. 2017]. Salinity stress reduces carotenoid content, consistent with reports by Azarmi-Atajan and Sayyari-Zohan [2020]. The contents of Chl a, b, and Total Chl show opposing responses to salinity stress. Although salinity decreases chlorophyll concentration, the application of iron nanoparticles can mitigate the adverse effects of salt stress and increase chlorophyll concentration [Ghadakchi asl et al. 2019]. The observed restoration of chlorophyll and carotenoids after Fe/C_3N_4 spraying is due to Fe, which functions as a cofactor for protochlorophyllide reductase, a crucial enzyme in chlorophyll production [Pushnik et al. 1984]. The Fe/C_3N_4 nanocomposite gradually releases Fe in ionic form, enhancing bioavailability. The C_3N_4 polymeric matrix functions as a photoprotective shield, reducing photobleaching and providing photoelectron storage for chloroplast repair reactions. The synergy between Fe and C_3N_4 enhances the scavenging of singlet oxygen and hydroxyl radicals generated under high-salt conditions, thereby preventing pigment degradation [Ali et al. 2017].

Chlorophyll fluorescence parameters help study the impacts of various environmental stresses on photosynthesis [Kumar et al. 2020]. High salt accumulation can damage chloroplasts, affecting membrane permeability and thylakoid function. This leads to a progressive decline in photosystem activity and chlorophyll fluorescence [Tsai et al. 2019]. Salinity reduced the photosynthetic rate by reducing leaf chlorophyll content and the maximum quantum yield of PS II (Fv/Fm) [Wang et al. 2018]. Under stressful conditions, electron flux from PS II to the quinone receptor decreases significantly, reducing Fv/Fm [Rasouli et al. 2022]. According to Torabian et al. [2017], foliar application with nano-Fe_2O_3 can help sunflower plants recover from salt stress by altering chlorophyll fluorescence. Foliar Fe/C_3N_4 reversed these declines by using Fe-based nanostructures that act as extra electron carriers and help stabilize redox reactions [Kwon et al. 2017]. Maintaining thylakoid structure is associated with lower ROS production and a better chloroplast lipid composition [Foyer and Hanke 2022]. Restoring Fe–S cluster activity is essential for proper PS II photochemistry [Tiwari et al. 2016].

Vitamin C (ascorbic acid) is a key antioxidant in plants, scavenging ROS, protecting photosynthesis, and maintaining redox balance under stress. In this study, salinity stress significantly reduced vitamin C levels in pistachio leaves compared with control seedlings. This noticeable decline aligns with earlier findings that high salinity levels interfere with ascorbate biosynthesis and accelerate its oxidation via excessive ROS production, ultimately damaging the ascorbate–glutathione cycle [Hasanuzzaman et al. 2020]. Under non-stress conditions, foliar application of Fe_2O_3, C_3N_4, and especially Fe/C_3N_4 significantly increased vitamin C content compared with the untreated control. This improvement results from enhanced metabolic activity and redox balance in leaves provided with micronutrients and nanostructured materials [Elemike et al. 2019]. Enhanced Fe availability increases carbohydrate availability, which is necessary for ascorbate biosynthesis via the L-galactose pathway [Samuolienė et al. 2019]. In parallel, carbon-based nanomaterials such as C_3N_4 may contribute to redox stabilization by moderating ROS production at the chloroplast level, thereby reducing ascorbate consumption [Bi et al. 2025, González-García et al. 2019]. Reducing ROS pressure with foliar Fe_2O_3 and Fe/C_3N_4 decreases ascorbate oxidation, enabling plants to sustain elevated vitamin C levels during stress [Gholami et al. 2024]. Furthermore, Fe/C_3N_4 could offer a dual advantage by maintaining continuous Fe availability and aiding redox buffering at the leaf surface, thereby helping preserve the functionality of the ascorbate–glutathione cycle [Gholami et al. 2024, Li et al. 2024].

Plant cells are protected by phenolics, which act as water-soluble antioxidants by quenching ROS and free radicals [Ashraf et al. 2010]. Phenolics can prevent ROS production and accumulation, thereby inhibiting oxidative stress. This plays a principal role in reducing the negative effects induced by ROS [Rico et al. 2015]. Apel and Hirt [2004] found that plants increase total phenolic content under stress to activate defense mechanisms against oxidative damage caused by ions, protecting cytoplasmic structures and chloroplasts. Phenols chelate iron ions, preventing ROS and decreasing superoxide levels from the Fenton reaction [Sakihama and Yamasaki

2002]. The decline in Fe/C_3N_4-treated pistachio plants likely resulted from stress relief, reducing the need for phenolic accumulation. Similarly, flavonoid variation is associated with the normalization of oxidative status, as excess Fe can inhibit the phenylpropanoid pathway via feedback inhibition [Yin et al. 2012]. According to Moradbeygi et al. [2020], treating plants with salinity leads to the accumulation of phenolic compounds, thereby increasing antioxidant activity, findings consistent with this report. However, Hassanpouraghdam et al. [2020] showed that foliar application of iron caused a reduction in phenol content under salt stress. Determining the optimal iron level is crucial for regulating plant flavonoid levels [Chung et al. 2019]. Nourozi et al. [2019] found that increased flavonoid content can induce salt tolerance in pistachios. Fe_2O_3 nanoparticle spraying also increased flavonoid and phenolic levels in *Dracocephalum kotschyi* by upregulating genes involved in the phenylpropanoid pathway.

Salinity stress markedly increased oxidative stress in pistachio leaves, as indicated by elevated H_2O_2 and MDA levels, reflecting excessive ROS production and membrane lipid damage [Rahneshan et al. 2018]. High salinity, especially at 150 mM NaCl without foliar treatment, triggers accumulation of phenols and flavonoids, indicating activation of secondary metabolism to boost antioxidant defenses and compensate for enzymatic ROS scavenging. In contrast, foliar application of Fe_2O_3, C_3N_4, and especially Fe/C_3N_4 significantly reduced phenolic and flavonoid contents under both non-stress and salinity conditions, suggesting mitigation of oxidative stress and a reduced need for secondary antioxidant accumulation. This protective effect is closely linked to enhanced antioxidant capacity and improved redox regulation, which limit ROS-driven membrane degradation. The Fe/C_3N_4 treatment seems to encourage a stress-avoidance strategy rather than stress tolerance. Instead of building up high levels of secondary antioxidants, plants treated with Fe/C_3N_4 kept ROS levels lower, experienced less lipid peroxidation, and maintained more stable physiological states [Guo et al. 2023]. This change enables the plants to conserve carbon resources for primary metabolism and cell maintenance, thereby improving overall physiological performance in saline conditions [Arif et al. 2020].

Salinity had a significant influence on the leaf's protein content. Total soluble protein content decreased in plants exposed to salt treatments. It is widely recognized that salinity affects protein biosynthesis, the principal process in plants. Both increment and decrease in protein content are commonly observed in salt-susceptible and salt-tolerant plants, respectively [Ashraf and Harris 2004]. Protein content declined by roughly 39 % under salinity in the current research, primarily due to oxidative denaturation, protease activation, and impaired amino acid metabolism [Cao et al. 2022, Saed-Moucheshi et al. 2014]. Salinity stress can reduce protein content due to oxidative damage to protein structure. Free radicals react with proteins, altering their structures, increasing the activity of degrading enzymes, and reducing amino acid synthesis. These factors contribute to the diminution of the soluble protein content in stressful situations [Saed-Moucheshi et al. 2014]. Plants treated with nano-Fe_2O_3 and subjected to salinity exhibited better levels of soluble protein and glycine betaine, suggesting a potential role of Fe in the biosynthesis of proteins [Pain and Dancis 2016]. Foliar nanoparticles enhanced protein levels through Fe-assisted enzyme activation involved in amino acid synthesis and decreased oxidative proteolysis [Pain and Dancis 2016].

The antioxidative system maintains a balance between ROS generation and scavenging to regulate signaling levels [Ahmed et al. 2013]. Plants have evolved an antioxidant system that includes APX, SOD, and POD enzymes to combat oxidative damage triggered by harsh environmental conditions [Alam et al. 2021]. Tolerant plant varieties exhibit higher antioxidant enzyme activity than sensitive varieties under different abiotic stresses [Jamshidi Goharrizi et al. 2020]. APX and CAT are similar enzymes that use ascorbate to remove H2O2 in the glutathione-ascorbate cycle [Gharsallah et al. 2016, Rao and Shekhawat 2016]. SOD defends against ROS by converting superoxide radicals to H2O2, which is detoxified by PODs with electron donors [Gharsallah et al. 2016]. Nanoparticles may reduce Na+ uptake and oxidative stress while enhancing the antioxidant system, including enzyme activity. K+ involvement in enzyme activation may explain how NPs can increase enzyme content by lowering Na+ concentration at salinity [Khan et al. 2017]. Nanocarbons, such as graphene oxide, can affect the activities of antioxidative enzymes, including SOD, APX, and POD. Nanocarbon particles can react with specific sites on enzymes at high concentrations, thereby rendering them ineffective. Nanomaterials' small size and high permeability effectively protect plants from stressors. This is why nanomaterials are often considered beneficial [Bacakova et al. 2020]. Therefore, Fe/C_3N_4 acts as a nanobiochemical enhancer, reducing oxidative damage while enhancing nutrient metabolism [Li et al. 2025].

CONCLUSION

Salinity stress significantly affected the morphology, physiology, and biochemistry of *P. vera* seedlings, leading to reduced growth, water content, photosynthetic efficiency, and protein levels, along with increased oxidative damage and secondary metabolite accumulation. The adverse effects of rising NaCl levels confirm that, despite being relatively salt-tolerant, pistachio seedlings are strongly affected by salinity-induced osmotic and oxidative stress. Foliar application with Fe_2O_3, C_3N_4, and Fe/C_3N_4 markedly enhanced most traits measured under different salinity conditions, as shown by significant main effects in the variance analysis. These improvements included higher RWC, MSI, chlorophyll pigments, and fluorescence parameters, whereas H_2O_2, MDA, total phenols, and flavonoids decreased. The decline in oxidative markers and secondary metabolites suggests better overall physiological stability rather than stress defense. Although the interaction between salinity level and foliar treatment was not statistically significant, seedlings treated with Fe/C_3N_4 consistently exhibited higher growth and photosynthetic traits and lower oxidative stress indicators compared to untreated plants. This indicates that Fe/C_3N_4 offers a general, stable physiological benefit across both saline and non-saline conditions, rather than a specific response to salinity. The improved performance of Fe/C_3N_4-treated plants can be explained by enhanced membrane integrity, redox control, and photosynthetic function, which together reduce oxidative signaling and secondary metabolite build-up. The combination of iron and carbon nitride likely supports sustained nutrient availability and redox buffering, helping maintain cellular stability under stress. In conclusion, the study indicates that using Fe-based and C-based nanomaterials as foliar treatments can effectively mitigate the adverse effects of salinity on pistachio seedlings. The current statistical analysis shows that Fe/C_3N_4 provided the most consistent positive outcomes among the treatments. These results endorse the application of Fe/C_3N_4 as a foliar supplement to enhance the stability and growth of pistachio seedlings in saline soils, and they also establish a foundation for future field studies and long-term research in orchard environments.

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AUTHOR CONTRIBUTION STATEMENT

S.M.Z. and M.A.A.: supervised the experiment. S.G., S.M.Z., and F.R.: wrote and performed the experiments. S.G., F.B., F.R. and S.K.: analyzed data and wrote the paper. S.E., N.E.K., M.A.A., A.A. and J.M.: reviewed and checked all the details. All authors read and approved the final manuscript.

AVAILABILITY OF DATA AND MATERIALS

The data and other information will be available on request.

CONFLICT OF INTEREST

The authors have reviewed the journal's policies and have confirmed that there are no conflicts of interest to declare.

COMPETING INTERESTS

The authors declare no competing interests.

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RESEARCH PAPER

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In vitro ASSESSMENT OF DROUGHT TOLERANCE IN SUMMER SQUASH (*Cucurbita pepo* L.) CULTIVARS

Büşra Ergül[1] https://orcid.org/0000-0001-7978-0107

Ecem Kara[2] https://orcid.org/0000-0002-0118-2673

Şerife Düzen[3] https://orcid.org/0009-0006-6203-5075

Asuman Kaplan Evlice[4] https://orcid.org/0000-0002-0344-6767

Hatıra Taşkın[3] https://orcid.org/0000-0002-1784-4731

Gökhan Baktemur[2]✉ https://orcid.org/0000-0002-0362-5108

[1] Agricultural Science, Institute of Graduate Studies, Sivas University of Science and Technology, Sivas 58000, Türkiye

[2] Faculty of Agricultural Science and Technology, Sivas University of Science and Technology, Sivas 58000, Türkiye

[3] Department of Horticulture, Faculty of Agriculture, Cukurova University, Adana 01330, Türkiye

[4] Department of Field Crops, Faculty of Agricultural Science and Technology, Sivas University of Science and Technology, Sivas 58000, Türkiye

✉ gbaktemur@gmail.com

ABSTRACT

In this study, the growth performance of three different summer squash cultivars was evaluated under *in vitro* conditions using Murashige and Skoog (MS) nutrient media supplemented with varying concentrations of polyethylene glycol (PEG) at 0%, 2%, 4%, and 6%. In the study, parameters including germination rate (%), stem fresh and dry weight (g), root fresh and dry weight (g), shoot and root length (mm) were investigated. In addition, antioxidant capacity and total phenolic content in plant shoots were determined using the DPPH method. According to the findings, the highest stem fresh weight was recorded as 0.72 g in cultivar Pelin grown in control medium (without PEG). In terms of root fresh weight, the highest mean value among the media was also observed in control with 0.43 g. The longest shoot length as 48.91 mm was also determined in Pelin. Increasing concentrations of PEG were found to have a negative impact on all growth parameters.

Keywords: growth parameters, PEG, antioxidant capacity, squash, abiotic stress

INTRODUCTION

Plants are increasingly exposed to multifactorial environmental stressors as a result of global warming and climate change, which significantly affect their growth, metabolism, and productivity. Among these stress factors, drought stands out as a major abiotic constraint that disrupts intracellular water relations, impairs photosynthetic efficiency, and alters ion homeostasis. These physiological disruptions lead to the overproduction of reactive oxygen species (ROS), which induce oxidative stress and ultimately hinder plant growth and reduce yield. Recent studies indicate that climate change is intensifying both the frequency and severity of drought events across many regions of the world. This trend has emerged as a critical concern for crop productivity, plant ecophysiology,

and global food security [Ali et al. 2025]. Environmental stress factors such as drought pose a significant threat to plant growth and productivity on a global scale [Koua et al. 2021, Khan et al. 2024]. Global warming contributes to this by raising temperatures that accelerate evaporation, reduce surface water availability, and rapidly dehydrate soils. That conditions collectively intensify drought stress [Passioura and Angus 2010, Devincentis 2020]. Moreover, as a result of ongoing climate change and global warming, drought episodes are becoming more frequent and severe [Seleiman et al. 2021]. The expansion of drought-affected areas, and the subsequent loss of arable land increasingly threaten global food security. Consequently, there is an urgent need to develop drought-tolerant and high-yielding crop cultivars to ensure sustained agricultural productivity [Ali et al. 2025]. In recent years, shifts in climate models have had a substantial impact on agricultural zones, with particularly pronounced effects observed in arid, semi-arid, and coastal regions [Stringer et al. 2021, Garcia-Caparros et al. 2025]. Currently, nearly one-third of the world's cultivable land is classified as arid or semi-arid, and the intensity of drought continues to increase. Drought has emerged as one of the most critical abiotic stressors, exerting severe negative effects on crop performance and productivity worldwide [Seleiman et al. 2021, Dietz et al. 2021, Garcia-Caparros et al. 2025].

Vegetables account for approximately 12% of global agricultural production, with species from the Solanaceae and Cucurbitaceae families representing a major share of total vegetable output. However, climate change poses significant threats to both agricultural productivity and ecosystem sustainability. Drought reduces the ability of plants to absorb water, lowers photosynthetic efficiency, and disrupts key physiological processes, collectively resulting in substantial declines in crop yield [Ulas et al. 2025]. *Cucurbita pepo* L. is one of the 15 recognized species within the genus *Cucurbita*, which belongs to the Cucurbitaceae family. Its plant is characterized by large, showy, yellow to orange flowers that are insect-pollinated, as well as broad, lobed leaves typically covered with fine, bristly hairs. Although different species within the genus may vary morphologically, their fruits often exhibit similar characteristics, which has led to the interchangeable use of the terms "pumpkin" and "squash" in various contexts [Adnan et al. 2017]. *Cucurbita pepo*. is native to northern Mexico, as well as the southwestern and eastern regions of the United States. Wild forms of the species can also be found in parts of Europe and Asia. The plant is valued not only for its agricultural importance, but also for its nutritional and medicinal properties. The immature fruits are commonly consumed as vegetables, while mature fruits are used in the preparation of desserts, confections, and beverages, often roasted or cooked [Martha and Gutierrez 2016, Ratnam et al. 2017]. Furthermore, *C. pepo* holds a prominent place in traditional medicine systems due to its reported antidiabetic, antihypertensive, anticancer, immunomodulatory, antibacterial, hypocholesterolemic, antiparasitic, anti-inflammatory, and analgesic activities [Fu et al. 2006, Conti et al. 2015].

Over recent years, tissue culture–based approaches have become widely used in plant stress physiology research. Their main strength lies in enabling stress treatments to be applied under tightly controlled conditions, while also allowing rapid screening of large numbers of genotypes under uniform and reproducible exposure regimes [Vives-Peris et al. 2017, Pérez-Jiménez and Pérez-Tornero 2020]. The *in vitro* technique provides a valuable platform for studying plant development, genetic manipulation, stress responses, and mass propagation under *in vitro* conditions. Recent studies have shown that polyethylene glycol (PEG) serves as an effective osmotic agent for establishing controlled drought-like conditions *in vitro* [Sahu et al. 2023, Akram et al. 2024]. Consequently, PEG-induced osmotic stress has become a widely accepted approach for screening plant materials for drought tolerance [Zhang et al. 2018].

In this study, the drought tolerance of different squash varieties was evaluated *in vitro* under polyethylene glycol (PEG)-induced osmotic stress. Implementing such assessments within tissue culture systems provides a controlled platform that allows for the preliminary characterization and comparative evaluation of drought tolerance among the tested varieties.

MATERIALS AND METHODS

Seeds of three summer squash cultivars, namely Pelin, Nazlı, and Şebnem were used as the plant material. Masuda and Skoog [1962] nutrient medium was used supplemented with polyethylene glycol (PEG 6000; Merck/Sigma-Aldrich) at four different concentrations: 0%, 2%, 4%, and 6%, in order to simulate drought conditions. The squash seeds used in the experiment were surface sterilized by immersion in a 30% sodium hypochlorite solution for 20 minutes. Following sterilization, the seeds were rinsed 4–5 times with sterile distilled water to ensure complete removal of any residual disinfectant, and to achieve full sterility. The seeds were aseptically transferred

into the pre-prepared culture media under a laminar flow. Sowing was performed in sterile 60 mm Petri dishes, with four seeds per dish. Seeds were positioned evenly and without contact to ensure uniform exposure to the culture medium. A total of 100 seeds per treatment were used (25 Petri dishes × 4 seeds). Following sowing, the Petri dishes were incubated in a plant growth chamber at 25 ±2 °C under a 16 h light/8 h dark photoperiod, with an illumination intensity of 3,000 lux. The cultures were maintained for 21 days after sowing, and measurements were performed at the end of the culture period.

Germination was assessed by counting the number of germinated seeds, and the germination rate was expressed as a percentage (%). For biomass determination, plants were randomly selected from each group, and stems and roots were separated. Fresh stem and root weights were measured immediately using an analytical balance and recorded in grams (g). The same samples were then dried in a laboratory oven at 55 °C until constant weight and reweighed to obtain dry stem and root weights (g). In addition, stem and root lengths were measured using a digital caliper and recorded in millimetres (mm) [Adem et al. 2025].

Determination of antioxidant capacity

For antioxidant analysis, 0.25 g of powdered stem squash sample was placed into a sterile falcon tube and extracted with 20 mL of 80% methanol by shaking in a water bath at ambient temperature for 2.5 hours. The mixture was centrifuged at 7000 rpm for 15 minutes, and the resulting supernatant was filtered using blue band filter paper [Adem et al. 2025]. The clarified extracts were stored at +4 °C until further use. Antioxidant activity was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay based on the method of Masuda et al. [1999], with slight procedural adjustments. In this method, 100 µL of each extract was mixed with 1900 µL of DPPH solution and incubated in the dark at room temperature for 30 minutes. After incubation, 200 µL aliquots were transferred into microplate wells, and absorbance was recorded at 517 nm using a microplate reader (Tecan Infinite 200 Pro, Austria). The antioxidant capacity was quantified and expressed as µmol Trolox equivalents per gram of dry weight (µmol TE g^{-1} DW).

Determination of total phenolic content

For the analysis of total phenolic content, the same extracts prepared for the DPPH assay were utilized. According to the procedure, 100 µL of extract was mixed with 200 µL of 10% Folin–Ciocâlteu reagent and 800 µL of 20% sodium carbonate solution. The mixtures were incubated in the dark at room temperature for 2 hours within a microplate. Following incubation, 200 µL of each sample was transferred into new vials, and absorbance was measured at 765 nm using a microplate reader. Results were expressed as milligrams of gallic acid equivalent per 100 grams of dry weight (mg GAE 100 g^{-1} DW) in accordance with the method described by Ainsworth and Gillespie [2007].

Statistical analysis

The experiment was conducted as a two-factor (cultivar and medium) factorial experimental design in randomized plots. The data obtained from the study were subjected to two-way analysis of variance (ANOVA) using JMP statistical software, version 13.0.1 (SAS Institute Inc., Cary, NC, USA), and when statistically significant differences were detected, means were separated using the least significant difference (LSD) test.

RESULTS AND DISCUSSION

Measurements and evaluations of different squash cultivars grown under *in vitro* conditions, in nutrient media supplemented with various concentrations of PEG, were performed. The results revealed that, although germination observed in cultivars Nazlı and Şebnem on media containing 6% PEG, further plant development was not achieved under these stress conditions.

Table 1 presents the mean germination percentages of different squash cultivars grown on media containing various concentrations of PEG. Statistical analysis revealed that cultivar, medium, and the cultivar × medium interaction were significant. Regarding cultivar, the highest average germination percentage was obtained from Pelin (63.00%), followed by Nazlı (49.17%) and Şebnem (31.81%). Among the media tested, the highest average germination rate was observed in the control medium with the average value of 60.74%, while the lowest was recorded in the 6% PEG medium with 26.14%. Germination of squash seeds Pelin at the highest PEG concentration decreased by approximately 1.7-fold, Nazl 2.6-fold, and Sebne as much as 5.5-fold compared to the control.

Brdar-Jokanović and Zdravković [2015] assessed the impact of PEG-induced drought stress on 15 tomato populations and found that increasing PEG levels significantly reduced both germination percentage and energy. Akte et al. [2016] examined five rice cultivars under PEG-simulated drought stress, and reported the highest germination (100%) in Binadhan-10 at 1% PEG. Similarly, Zayova et al. [2017] studied eggplant cultivars under PEG 8000 stress, and observed a decline in plant survival rates with rising PEG concentrations. In a study by Adem et al. [2025], different tomato cultivars were cultured *in vitro* on MS media containing various concentrations of PEG (0%, 1%, 2%, 4%, and 6%) to assess their growth responses. The highest average germination rate (70.00%) was recorded in the 0% medium without PEG (overall, the results indicated that increasing PEG concentrations led to a noticeable reduction in plant growth.

These findings clearly indicate that PEG-induced osmotic stress has a strong inhibitory effect on germination, and the magnitude of this reduction differs markedly among cultivars, demonstrating the cultivar dependent nature of drought tolerance.

Table 1. Germination percentages (%) of different squash cultivars in PEG supplemented media

PEG (%)	Cultivar			Medium avarege
	Pelin	Nazlı	Şebnem	
0	75.00b	63.00c	44.23e	60.74A
2	79.00a	62.00c	38.00f	59.67A
4	53.00d	46.25e	37.00f	45.42B
6	45.00e	25.42g	8.00h	26.14C
Cultivar average	*63.00 A*	*49.17B*	*31.81C*	×
$LSD_{cultivar}$ = 1.446*** LSD_{medium} = 1.669 *** $LSD_{cultivarxmedium}$ = 2.892***				

[1] statistical differences between the averages shown in separate letters in the same column were found to be significant
[2] ***$p \leq 0.001$

Table 2. Fresh stem weight (g) of different squash cultivars in PEG supplemented media

PEG (%)	Cultivar			Medium avarege
	Pelin	Nazlı	Şebnem	
0	0.72a	0.65b	0.47d	0.61A
2	0.56c	0.46d	0.29e	0.44B
4	0.30e	0.32e	0.20f	0.27C
6	0.21f	0	0	0.07D
Cultivar average	*0.45A*	*0.36B*	*0.24C*	×
$LSD_{cultivary}$ = 0.02*** LSD_{medium} = 0.02 *** $LSD_{cultivarxmedium}$ = 0.04***				

[1] statistical differences between the averages shown in separate letters in the same column were found to be significant
[2] ***$p \leq 0.001$

The stem fresh weight values of different squash cultivars grown in media containing varying concentrations of PEG are presented in Table 2. The highest average stem fresh weight of 0.45 g was recorded in Pelin, regarding media, and cultivar × media interaction, the highest average stem fresh weight (0.61 g) was observed for Pelinin the medium without PEG. In contrast, the lowest interaction values were identified as 0.21 g for Pelin in 6% PEG medium and 0.20 g for Şebnem in 4% PEG medium. At the highest PEG concentration, the fresh shoot weight of Pelin decreased 3.5-fold compared to the control, while plants of the other cultivars died (Table 2).

Jezdinský et al. [2012] investigated the effects of drought stress on leek by evaluating various morphological parameters. Their findings revealed that drought conditions led to significant reductions in total fresh weight, leaf area, stem length, and stem diameter. Similarly, Mehmandar et al. [2023] assessed the responses of three Iranian

melon genotypes Girke, Ghobadloo, and Toghermezi to sorbitol (0.1, 0.2, and 0.4 M) and PEG (0.009, 0.012, and 0.015 M) treatments *in vitro* conditions. The genotype Girke has been determined for its drought tolerance in Iran. The study reported that both PEG and sorbitol stress increased the accumulation of proline and malondialdehyde (MDA), while reduced coleoptile length, fresh plant weight, and photosynthetic pigment contents. In another study, Kara et al. [2024a] examined the effect of drought stress on leek using PEG-induced drought *in vitro* conditions. PEG was added to the medium at concentrations of 0%, 1% , 2% , 4% , and 6% to simulate varying drought levels. The highest stem fresh weight (0.0219 g) was recorded in the control (0% PEG), whereas the lowest (0.0104 g) was observed in the 6% PEG treatment, indicating that increased PEG concentrations negatively affected stem development in leek seedlings.

Findings of the present study clearly demonstrate that increasing PEG concentrations exert a strong inhibitory effect on stem biomass accumulation, consistent with the general pattern observed in previous drought-stress studies. The marked reduction in stem fresh weight at 6% PEG, particularly in Nazlı and Şebnem where no measurable biomass was produced, suggests that severe osmotic stress limits cell expansion and water uptake to a degree that prevents normal stem development. The superior performance of Pelin across all PEG levels indicates a comparatively greater capacity for osmotic adjustment and maintenance of cellular turgor under stress. Overall, the cultivar-dependent variation observed in stem biomass under PEG-induced drought supports the conclusion that early-stage growth parameters such as stem fresh weight can serve as reliable indicators for distinguishing drought-tolerant and drought-sensitive squash cultivars *in vitro*.

Statistical analysis showed that cultivar, media, and the cultivar × media interaction had significant effect on stem dry weight (Table 3). Among the media, the highest average stem dry weight was observed in the control medium (0.08 g), while the lowest was recorded in the 6% PEG medium (0.01 g). Regarding cultivar, Pelin and Nazlı exhibited the highest average values, both at 0.06 g. The cultivar × medium interaction was statistically highly significant. The cultivars responded differently to the PEG doses. The highest individual values, belonging to the same statistical group, were obtained from Nazlı at the 2% dose (0.09 g), Nazlı at the 0% dose (0.08 g), and Pelin at the 0% dose (0.08 g).

Table 3. Dry stem weight (g) of different squash cultivars in PEG supplemented media

PEG (%)	Cultivar			Medium avarege
	Pelin	Nazlı	Şebnem	
0	0.08a	0.08a	0.07b	0.08A
2	0.07cd	0.09a	0.07bc	0.07B
4	0.06d	0.07bc	0.06e	0.06C
6	0.04f	0	0	0.01D
Cultivar average	*0.06A*	*0.06A*	*0.05B*	×
$LSD_{cultivar}$ = 0.003*** LSD_{medium} = 0.003*** $LSD_{cultivarxmedium}$ = 0.007***				

[1] statistical differences between the averages shown in separate letters in the same column were found to be significant
[2] ***$p \leq 0.001$

Kara et al. [2024b] tested the development of white cabbage seeds in the MS media supplemented with varying concentrations of PEG (0%, 1%, 2%, 4%, 6%) to determine drought tolerance levels. The highest stem dry weight was observed in the control as 0.0110 g, indicating a negative effect of drought stress on biomass accumulation. In a related study, Rehman et al. [2024] reported that drought conditions significantly suppressed plant growth, with cabbage showing notable reductions in both fresh and dry weights of shoots and roots. Similarly, Torun and Sarı [2025] subjected 192 melon genotypes from the Cucurbitaceae genetic resources collection at Çukurova University to drought stress induced by 5% PEG 6000 for two months. They assessed parameters such as stem length, stem and root dry weights, leaf number, and a drought tolerance score (0–5 scale). Most parameters showed a decrease under stress, except root dry weight with 3% increase. Kaya [2025] investigated the effects of increasing concentrations of PEG-6000 on the morpho-physiological parameters of melon seedlings (*Cucumis*

melo Kırkağaç 589). Seedlings with two true leaves were grown for 30 days in a peat:perlite:vermiculite (6:1:1) substrate containing 5%, 10%, 15%, and 20% PEG-6000. Measurements included plant height, stem diameter, fresh/dry weights, dry matter content, leaf area, leaf temperature, chlorophyll content (SPAD), relative water content, turgor loss, and electrolyte leakage. The findings indicated that increasing drought severity led to significant reductions in plant height, biomass, and relative water content. Our results clearly demonstrate that increasing PEG concentrations markedly restricted dry matter accumulation, indicating that stem dry weight is highly sensitive to osmotic stress. In our experiment, the superior performance of the cultivars Pelin and Nazlı, particularly at lower PEG levels, suggests a greater capacity to maintain tissue hydration and carbon allocation under drought-like conditions. Overall, the cultivar-dependent variation observed in this study reinforces the value of stem dry weight as a reliable indicator of drought tolerance in squash seedlings.

Table 4 summarizes the root fresh weight responses of squash cultivars exposed to increasing PEG concentrations in vitro. Root fresh weight differed significantly among treatments and cultivars. The PEG-free control produced the highest mean root fresh weight (0.43 g). When averaged across media, Pelin had the greatest root fresh weight (0.35 g), whereas Nazlı and Şebnem showed markedly lower values (0.12 g and 0.08 g, respectively). In a related study, Yıldırım et al. [2020] examined the effect of drought stress on growth, nutrient content, and various physiological and biochemical traits in bean under irrigation levels at the 100%, 80%, and 60% of field capacity. Results indicated that drought stress adversely affected leaf area as well as fresh and dry weights of leaves, stems, and roots. Similarly, Tajaragh et al. [2022] assessed the physiological and biochemical responses of three Iranian local *Cucurbita* cultivars (Tanbal Ajili – *C. maxima*, Ajili Razan – *C. pepo*, Balghabakhi – *C. moschata*) under osmotic stress induced by PEG 6000 and mannitol *in vitro*. Seedlings were transferred to MS media supplemented with varying concentrations of PEG (0.009–0.015 M) and mannitol (0.1–0.4 M). The treatments significantly increased shoot and root dry weights, malondialdehyde (MDA), proline, and various antioxidant components, while reducing coleoptile length, shoot and root fresh weights, and photosynthetic pigment contents especially under higher stress levels. Kara et al. [2024c] evaluated onion growth performance under *in vitro* drought conditions induced by PEG concentrations (0%, 1%, 2%, 4%, 6%) added to MS medium. The highest root fresh and dry weights were recorded in the control (0.383 g and 0.031 g, respectively), while the lowest were observed in 6% PEG (0.02 g and 0.001 g, respectively). These findings clearly demonstrate that increasing PEG concentrations negatively affect root biomass and indicate increasing sensitivity to drought stress. Our results clearly indicate that root fresh weight is highly sensitive to PEG-induced osmotic stress, with marked reductions occurring even at moderate stress levels. The superior performance of Pelin suggests a stronger root system capable of maintaining water uptake under declining water availability, highlighting its potential drought-tolerance advantage.

Table 4. Fresh root weight (g) of different squash cultivars in PEG supplemented media

PEG (%)	Cultivar			Medium avarege
	Pelin	Nazlı	Şebnem	
0	0.78a	0.26bc	0.24c	0.43A
2	0.32b	0.12d	0.06def	0.17B
4	0.20c	0.09de	0.004ef	0.11C
6	0.11d	–	–	0.03D
Cultivar average	*0.35A*	*0.12B*	*0.08B*	×
$LSD_{cultivar}$ = 0.036*** LSD_{medium} = 0.042*** $LSD_{cultivarxmedium}$ = 0.072***				

[1] statistical differences between the averages shown in separate letters in the same column were found to be significant
[2] ***$p \leq 0.001$

The highest root dry weight in term of media was recorded in the medium without PEG (Table 5). In the control medium, the cultivar Pelin exhibited the highest root dry weight (0.043 g), followed by the cultivars Şebnem (0.032 g) and Nazlı (0.029 g).

Table 5. Dry root weight (g) of different squash cultivars in PEG supplemented media

PEG (%)	Cultivar			Medium avarege
	Pelin	Nazlı	Şebnem	
0	0.043a	0.029b	0.032b	0.030A
2	0.023c	0.014d	0.006e	0.010B
4	0.020c	0.009de	0.006e	0.010B
6	0.011de	–	–	0.004C
Cultivar average	*0.02A*	*0.01B*	*0.01B*	×
$LSD_{cultivar}$ = 0.002*** LSD_{medium} = 0.002*** $LSD_{cultivarxmedium}$ = 0.005***				

[1] statistical differences between the averages shown in separate letters in the same column were found to be significant
[2] ***$p \leq 0.001$

Table 6. Stem length (mm) of different squash cultivars in PEG supplemented media

PEG (%)	Cultivar			Medium avarege
	Pelin	Nazlı	Şebnem	
0	70.39a	51.05c	48.50c	56.65A
2	51.15b	42.95d	31.76e	43.30B
4	43.45d	32.86e	18.17g	31.49C
6	26.64f	–	–	8.90D
Cultivar average	*48.91A*	*31.71B*	*24.61C*	×
$LSD_{cultivar}$ = 1.958*** LSD_{medium} = 2.261*** $LSD_{cultivarxmedium}$ = 3.915***				

[1] statistical differences between the averages shown in separate letters in the same column were found to be significant
[2] ***$p \leq 0.001$

Turan and Samur [2024] examined the effects of pre-treatments with gibberellic acid (GA_3) and boric acid (BA) on germination and seedling development of rapeseed (*Brassica napus* L.) under PEG-induced drought stress. Their findings indicated that the highest seedling dry weight (0.046 g) was obtained in treatments combining the control with GA_3 and BA at concentrations of 1.00, 1.5, and 2.00 mg L^{-1}. Our results indicate that root dry weight is highly sensitive to increasing PEG concentrations, as reductions were evident even at moderate stress levels. The superior performance of Pelin suggests a more robust root system capable of maintaining structural integrity under osmotic stress, underscoring its potential drought tolerance.

Among the media, the highest stem length was observed in the 0% medium as 56.65 mm, while the lowest value was recorded in the 6% PEG medium as 8.90 mm, indicating a clear reduction in stem length with increasing PEG concentration (Table 6). Pelin showed the greatest average stem length (48.91 mm), whereas Şebnem had the shortest (24.61 mm). These findings highlight the significant influence of both cultivar and PEG concentration on stem development.

In support of these findings, Ayaz et al. [2015] induced artificial drought stress using five PEG-6000 concentrations in two tomato cultivars (Nagina and 17905) and reported that, especially under 8% PEG treatment, seed germination in Nagina dropped to 60%. Additionally, phenotypic traits such as shoot length, leaf length, leaf number, and leaf area decreased significantly along increasing PEG levels. Similarly, Dolgun and Çifci [2018] investigated the effects of drought stress on germination and early seedling development in wheat varieties using four levels of PEG-induced stress (2.5, 5.0, 7.5, and 10.0 bars) and distilled water as the control. Morphological parameters including germination rate, vigor index, root and seedling length, and biomass were significantly suppressed by increasing drought severity. Tran et al. [2020] studied the effects of drought stress induced by mannitol (35 g L^{-1} in

1/2 MS medium) and heat pre-treatment (45 °C for 120 minutes) on *in vitro* shoot development in *Solanum lycopersicum* L. Their results revealed that shoot length, leaf number, and total leaf area were reduced by nearly 50% compared to the control, emphasizing the substantial limiting effects of osmotic stress on plant development. In our study, stem elongation was highly sensitive to PEG-induced osmotic stress, with substantial reductions observed even at moderate stress levels. Among the cultivars evaluated, Pelin maintained the greatest stem length, suggesting a higher capacity to sustain cell expansion under limited water availability and, consequently, comparatively greater drought tolerance.

The highest average root length was recorded in the control (62.37 mm), while the shortest was observed in the 6% PEG medium (10.75 mm); see Table 7. In terms of cultivar, Pelinexhibited the longest average root length (51.92 mm), whereas Şebnem showed the shortest (27.99 mm). These results indicate that root development was significantly influenced by both variety and PEG concentration.

Kumar et al. [2017] reported that increasing PEG concentration proportionally reduced root growth, highlighting the critical role of the root system in plant survival under drought stress. They suggested that changes in root development may reflect the level of drought tolerance, as drought-tolerant genotypes typically possess more extensive root systems. Similarly, Wickramasinghe and Seran [2019] assessed the effects of PEG at 30, 60, and 90 g L^{-1} on germination and seedling development of the tomato cultivar KC-1 in *in vitro* conditions. Their results showed a significant negative correlation ($p < 0.0001$) between PEG concentration and all morpho-physiological traits, including shoot and root length, fresh and dry weight, and chlorophyll a, b, and total chlorophyll content. In the control group, after 4 weeks of culture, shoot and root lengths were 2.88 and 1.67 cm, respectively, while fresh and dry weights were 60.37 and 8.93 mg, respectively, and total chlorophyll content was 1.86 mg g^{-1}. Clearly, the 60 and 90 g L^{-1} PEG treatments restricted germination and seedling development. Bouchyoua et al. [2024] aimed to identify drought-tolerant *Brassica napus* L. genotypes under *in vitro* conditions using PEG-6000 to simulate osmotic stress at three levels (–0.7, –0.9, –1.1 MPa). Parameters such as germination percentage (GP), germination rate (GR), mean germination time (MGT), root length (RL), shoot length (SL), root-shoot ratio (RSR), seedling vigor index (SVI), and elongation rates (SER, RER) were evaluated. Results indicated significant effects of genotype, drought level, and their interaction on all parameters. Under severe drought, only mean germination time and root-shoot ratio increased by 56% and 76%, respectively, while.reductions of 53–96% in other measured parameters were recorded. These findings confirm that root elongation is highly sensitive to PEG-induced osmotic stress, with severe reductions occurring as water availability declines. The superior root length of cultivar 1 suggests a greater capacity for root system maintenance under drought-like conditions, indicating a potential advantage for soil moisture foraging and overall drought resilience.

Table 7. Root length (mm) of different squash cultivars in PEG supplemented media

PEG (%)	Cultivar			Medium avarege
	Pelin	Nazlı	Şebnem	
0	68.92a	64.22b	53.98c	62.37A
2	57.18c	40.98e	32.52f	43.55B
4	49.34d	33.93f	25.48g	36.25C
6	32.26f	–	–	10.75D
Cultivar average	*51.92A*	*37.78B*	*27.99C*	×
$LSD_{cultivar}$ = 2.115*** LSD_{medium} = 2.442*** $LSD_{cultivarxmedium}$ = 4.229***				

[1] statistical differences between the averages shown in separate letters in the same column were found to be significant
[2] ***$p \leq 0.001$

DPPH based antioxidant capacity measured in stem tissues of in vitro grown squash cultivars exposed to PEG is summarized in Table 8. Both the main effects (cultivar and PEG level) and the cultivar × medium interaction were statistically significant, indicating that antioxidant capacity varied according to genotype and its response to osmotic stress. When averaged across treatments, Pelin showed the highest antioxidant capacity (2.23 µmol TE g^{-1} DW), whereas Şebnem had the lowest (1.46 µmol TE g^{-1} DW). Across media, the PEG-free control yielded the greatest mean antioxidant capacity (2.93 µmol TE g^{-1} DW).

For example, Popović et al. [2016], reported that PEG 6000-induced water stress elicited marked genotype-specific variation in antioxidant capacity and polyphenol metabolism, with certain genotypes maintaining or enhancing DPPH radical-scavenging activity through increased activation of the phenylpropanoid pathway. Similarly, our significant cultivar × medium interaction suggests that the ability to maintain stem antioxidant capacity under PEG differs among squash cultivars, supporting the use of DPPH-based antioxidant capacity as a complementary indicator of drought-related stress tolerance in this study.

Table 8. Determination of antioxidant capacity in shoots of different squash varieties grown in PEG containing nutrient media by the DPPH method (µmol TE g^{-1} DW)

PEG (%)	Cultivar			Medium avarege
	Pelin	Nazlı	Şebnem	
0	2.85b	2.72c	3.21a	2.93A
2	3.10b	2.76bc	1.38f	2.41B
4	1.64d	1.50e	1.23g	1.46C
6	1.33fg	–	–	0.44D
Cultivar average	*2.23A*	*1.74B*	*1.46C*	×
$LSD_{cultivar}$ = 0.056*** LSD_{medium} = 0.065*** $LSD_{cultivarxmedium}$ = 0.112***				

[1] statistical differences between the averages shown in separate letters in the same column were found to be significant
[2] ***$p \leq 0.001$

Table 9 presents the total phenolic content measured in the stems of different squash cultivars grown in nutrient media containing various concentrations of PEG. Statistical evaluations showed that cultivar, media, and the cultivar × media interaction were all significant factors. Among media, the highest total phenolic content was recorded in the control medium (0%) as 1.27 mg GAE 100 g^{-1} DW. Ahmad et al. [2020] reported that PEG-induced drought stress substantially increased phenolic compound accumulation in *Stevia rebaudiana*, with the highest total phenolic content occurring under the 4% PEG treatment. This observation suggests that *Stevia* enhances phenolic biosynthesis as a protective strategy against ROS generated under water-deficit conditions. In contrast, our findings revealed an opposite pattern in squash cultivars, where increasing PEG concentrations resulted in a gradual and marked reduction in total phenolic content. The highest TPC value was recorded in the control treatment (1.82 mg GAE 100 g^{-1} DW in Pelin), while phenolic levels steadily declined with rising PEG concentrations, reaching their minimum under the 6% PEG treatment. Collectively, these contrasting outcomes indicate that phenolic compound production is strongly dependent on species-specific stress response mechanisms. Whereas PEG functions as an elicitor that stimulates phenolic accumulation in *Stevia*, the same osmotic stress appears to suppress phenolic biosynthesis in squash, underscoring fundamental physiological and metabolic differences in how plant species cope with drought-induced oxidative stress.

Table 9. Determination of total phenolic content in the shoots of different squash varieties grown in PEG containing nutrient media (mg GAE 100 g^{-1} DW)

PEG (%)	Cultivar			Medium avarege
	Pelin	Nazlı	Şebnem	
0	1.82a	1.17b	0.81d	1.27A
2	1.13c	0.49g	0.42h	0.68B
4	0.55e	0.42h	0.28ı	0.41C
6	0.51f	–	–	0.17D
Cultivar average	*1.00A*	*0.52B*	*0.38C*	×
$LSD_{cultivar}$ = 0.007*** LSD_{medium} = 0.008*** $LSD_{cultivarxmedium}$ = 0.014***				

[1] statistical differences between the averages shown in separate letters in the same column were found to be significant
[2] ***$p \leq 0.001$

CONCLUSION

It is an expected result that the highest values in all tested parameters were obtained in the PEG-free, (control) environment. This result confirms that the osmotic stress induced by PEG exerted a suppressive effect on both morphological and physiological traits. Especially, the reductions observed in shoot and root length, as well as in fresh and dry biomass with increasing PEG concentrations were directly proportional to the severity of the imposed stress. Among the cultivars evaluated, Pelin showed the best growth performance. These findings indicate that this cultivar can have a relatively higher level of adaptation and tolerance to drought stress. Its superior performance for both morphological parameters (shoot/root length, fresh and dry weight) and physiological parameters (antioxidant capacity, total phenolic content) highlights its potential as a promising candidate for future breeding studies aimed at enhancing drought resilience. The PEG treatments employed successfully simulated drought under *in vitro* conditions, thus enabling the effective assessment of drought tolerance among the tested genotypes. This supports the use of PEG-induced osmotic stress as a practical and reproducible method for evaluating plant responses to drought stress, particularly during early developmental stages. Transferring drought tolerance studies to tissue culture conditions to get preliminary idea will be advantageous in terms of time and cost.

AUTHORSHIP CONTRIBUTION STATEMENT

B.E.: setting up experiments and performing all analyses, statistical analyses and writing; E.K.: data analysis-review, editing and writing; Ş.D.: all analyses, A.K.E.: antioxidant analyses, H.T.: data analysis-review, editing and writing; G.B.: designing of the study, controling of all analysis and measurements, supervising and writing.

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DATA AVAILABILITY

All data of this work are available in this paper.

CONFLICT OF INTEREST

All authors have nothing to disclose.

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RESEARCH PAPER

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QUANTITATIVE METABOLOMICS-DRIVEN ELUCIDATION OF FLAVONOID DIVERSITY AND NOVEL QUALITY ASSESSMENT STRATEGY IN *Artemisia argyi* Levl. ET VAN GERMPLASMS

Lanjie Xu[1,2] https://orcid.org/0000-0001-8355-9640

Sufang An[1,2] https://orcid.org/0009-0003-1599-1759

Yongliang Yu[1,2] ✉ https://orcid.org/0009-0006-2922-2813

Wei Dong[1,2] https://orcid.org/0009-0001-4162-0316

Huizhen Liang[1,2] https://orcid.org/0009-0008-4146-9303

Qing Yang[1,2] https://orcid.org/0009-0008-1068-7332

Xiaohui Wu[1,2] https://orcid.org/0009-0009-0554-9493

[1] Institute of Chinese Herbal Medicines, Henan Academy of Agricultural Sciences, Zhengzhou 450002, China

[2] Provincial Key Laboratory of Conservation and Utilization of Traditional Chinese Medicine Resources in Henan, Zhengzhou 450002, China

✉ yyl790721@126.com

ABSTRACT

This study employed quantitative metabolomics to conduct a comprehensive and systematic analysis of the diversity and accumulation patterns of flavonoid compounds in the leaves of six different genotypes of *Artemisia argyi* Levl. et Van (*A. argyi*) germplasms. The aim was to establish a metabolite-marker-based quality evaluation system and provide theoretical underpinnings for germplasm conservation and targeted development. Flavonoids were quantitatively analyzed using ultra- performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS). A combination of principal component analysis (PCA) and hierarchical cluster analysis (HCA) of heatmaps was applied to reveal the disparities in metabolic profiles among different germplasms. Orthogonal partial least squares discriminant analysis (OPLS-DA) was utilized to identify differential metabolites, followed by Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis to explore the underpinnings of metabolic pathways. The findings demonstrated that a total of 76 flavonoids belonging to 11 categories were identified. Flavones (24 compounds) and flavonols (20 compounds) were the predominant classes, accounting for 57.9% of the total. Aa36 and Aa60 all displayed the highest diversity with 64 compounds. The total flavonoid content ranged from 8.70 to 14.01 μg/g, and Aa41 had the highest content. Seven flavonoids of jaceosidin, eriodictyol, eupatorin, hispidulin, chrysosplenetin, scutellarin, quercimeritrin consistently ranked among the top 10 components in six germplasms, thereby constituting the common pharmacodynamic foundation. PCA and HCA classified six germplasms into two metabolic types. Group I, composed of Aa9, Aa13, Aa36, Aa38, and Aa41, was abundant in methoxylated flavonoids. Group II, only Aa60, had a distinctive profile dominated by scutellarin, which accounted for 52.4% of the total content (34.7 μg/g). The differential metabolites were significantly enriched in the secondary metabolite biosynthesis pathway (ko01110), flavonoid biosynthesis pathway (ko00941), and flavone/flavonol biosynthesis pathway (ko00944), which uncovered the regulatory mechanisms. The seven identified core flavonoids can function as stable metabolic markers for the quality assessment of germplasms. Meanwhile, the scutellarin dominant profile of Aa60 offers a distinct resource orientation for the development of *A. argyi* cultivars with cardio-cerebrovascular protective functions.

Keywords: *Artemisia argyi* Levl. et Van, flavonoid diversity, differential metabolites, quality assessment

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INTRODUCTION

Artemisia argyi Levl. et Van (*A. argyi*), a perennial herbaceous plant within the Asteraceae family, has a medicinal application history extending over 3,000 years. The dried leaves of *A. argyi* are rich in flavonoids, amino acids and volatile oils, and possess antioxidant, anti-cancer and antibacterial activities [Moacă et al. 2019, Wei et al. 2024, Orege et al. 2023, Al-Hajj et al. 2025, Bsharat et al. 2025a, 2025b]. Modern pharmacological studies suggest that the medicinal value of *A. argyi* is closely correlated with its rich flavonoid content.

Flavonoids, including quercetin, rutin, kaempferitrin and apigenin have been isolated and identified from *A. argyi* [Wang et al. 2024]. These flavonoids exhibit multiple biological activities, such as antioxidant, anti-inflammatory, antibacterial and antitumor effects, through the regulation of signaling pathways like NF-κB and MAPK [Xiang et al. 2018, Lee et al. 2018, Zhang et al. 2023, Wang et al. 2024]. For instance, quercetin significantly inhibits the lipopolysaccharide-induced secretion of inflammatory factors TNF-α and IL-6 in RAW264.7 macrophages, with a half maximal inhibitory concentration (IC50) of 12.3 μM [Liu et al. 2022]. Meanwhile, rutin enhances antioxidant capacity by activating the Nrf2/ARE pathway, and its 2,2-diphenyl-1-picrylhydrazyl radical scavenging efficiency (half-maximal effective concentration, EC50 = 0.18 mg/mL) is significantly superior to that of vitamin C [Li et al. 2024]. Flavonoids are considered the core pharmacodynamic substances of *A. argyi*, and their content and composition directly determine the quality grade of *A. argyi* medicinal materials.

With the further implementation of the "Healthy China" strategy, the *A. argyi* industry has witnessed explosive growth. Statistical data indicate that the market scale of *A. argyi* products in China reached 18.2 billion yuan in 2023, with an annual demand exceeding 500,000 tons [Yu et al. 2023]. Therefore, conducting a systematic analysis of the flavonoid composition in *A. argyi* and comprehensively exploring the differential accumulation patterns of natural products among different provenances hold significant importance for the quality assessment, conservation of genetic resources, efficient development and utilization, and breeding of high quality varieties of *A. argyi* resources. In light of this, the present study selected six representative *A. argyi* germplasms from different genotypes. Ultra performance liquid chromatography electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS) technology was employed to conduct untargeted metabolomics analysis and systematically establish a quantitative fingerprint of flavonoid components in its leaves. In combination with OPLS-DA analysis and cluster analysis, characteristic differential metabolites capable of distinguishing different germplasms were screened. Through KEGG pathway enrichment analysis, the synthetic biological basis of its metabolic differences was disclosed.

Markedly distinct from conventional methodologies in experimental design, research framing and analytical rigor, this study advances beyond the technical constraints of traditional flavonoid profiling strategies. Whereas prior approaches have typically depended on spectrophotometry to measure total flavonoid content or high-performance liquid chromatography to quantify a restricted set of known analytes-techniques hampered by limited detection throughput and narrow metabolite coverage-the present work overcomes these drawbacks to achieve a holistic characterization of flavonoid metabolic diversity [Kabré et al. 2023, Gong et al. 2019]. For the first time, this study integrates UPLC-ESI-MS/MS with an untargeted metabolomics strategy, enabling high-throughput and systematic analysis of both known and unknown flavonoids in *A. argyi* and breaking through the technical limitations of conventional targeted analysis. In terms of research perspective, previous studies have mostly focused on the effects of exogenous factors such as producing area and harvesting period on chemical components [Gong et al. 2019, Nie et al. 2019, Zhang et al. 2023], while insufficient attention has been paid to the genetic differences among germplasms. Taking six *A. argyi* germplasms with clear genetic backgrounds and distinct geographical origins as research objects, this study explores the natural variation patterns of flavonoid accumulation from the perspective of genetic essence, providing metabolome-based chemical classification criteria for variety identification and breeding. In terms of analytical depth, previous studies have been largely confined to descriptive comparisons or quantification of individual components, lacking a holistic interpretation of metabolic differences [Jiang et al. 2009, Dong et al. 2016, Gong et al. 2019]. By employing OPLS-DA analysis and hierarchical clustering analysis, this study screens out differential metabolic markers with discriminative significance. Combined with KEGG pathway enrichment analysis, it systematically reveals the underlying biosynthetic and regulatory networks. This approach elevates the evaluation of *A. argyi* germplasms from superficial chemical description to a mechanistically linked, index-based discrimination level, providing a quantifiable and interpretable new strategy for breeding high-value varieties and standardizing quality.

MATERIAL AND METHODS

Plant materials. The experiment was carried out on April 20, 2025. Six *A. argyi* germplasms (codes: Aa09, Aa13, Aa36, Aa38, Aa41, Aa60) were cultivated in the modern agricultural research and development base of Henan Province. On May 20, 2025, the upper middle leaves of robust and disease of free plants were harvested, with three biological replicates for each germplasm (a total of 18 samples). Subsequent to sampling, these leaves were rapidly frozen in liquid nitrogen and stored at –80 °C for subsequent utilization. The leaf morphology of each material is presented in Figure 1, and the germplasm origins are Bainiu Town, Dengzhou City, Henan Province (Aa9); Tongbai County, Nanyang City, Henan Province (Aa13); Potou Town, Jiyuan City, Henan Province (Aa36); Caohe Town, Qichun County, Hubei Province (Aa38); Guanyao Town, Qichun County, Hubei Province (Aa41); Tangyin County, Anyang City, Henan Province (Aa60).

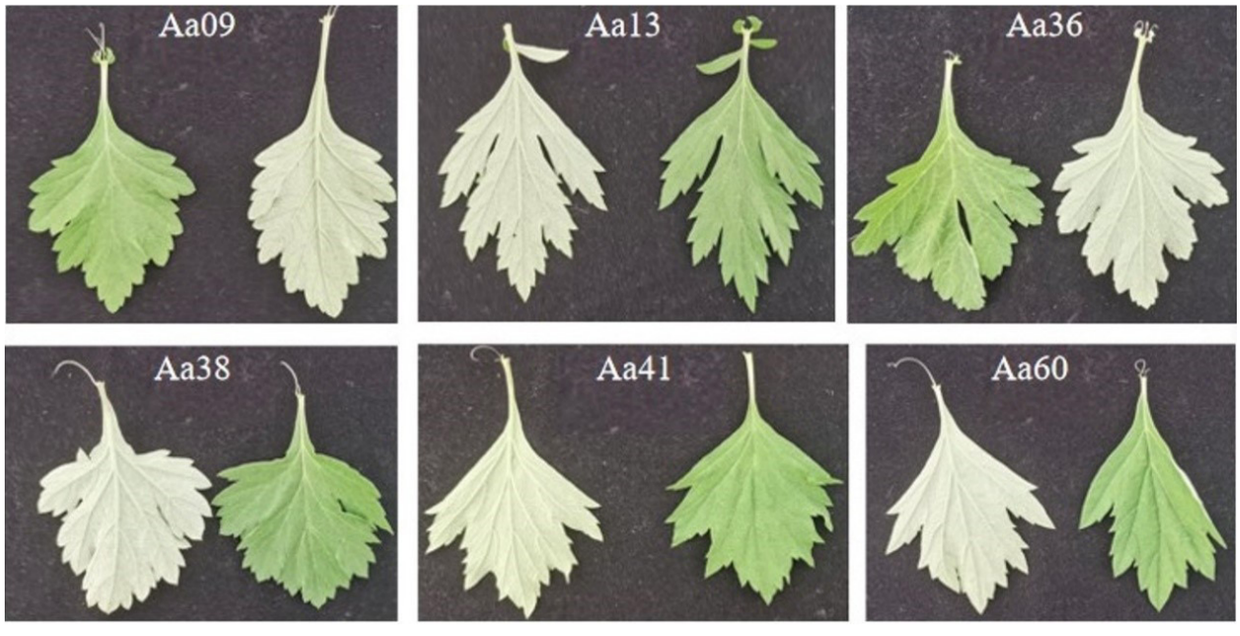
Main instruments. Liquid chromatography tandem mass spectrometry (LC-MS/MS) (model: QTRAP 6500+, SCIEX); high speed refrigerated centrifuge (model: 5424R, Eppendorf); precision electronic balance (model: AS60/220.R2, RADWAG); ball mill (model: MM400, Retsch); multitube vortex oscillator (model: MIX-200, Shanghai Jingxin); ultrasonic cleaner (model: KQ5200E, Kunshan Shumei).

Main reagents. Methanol (Chromatographic grade, Merck); acetonitrile (Chromatographic grade, Shanghai Xingke); formic acid (Chromatographic grade, Sigma-Aldrich); flavonoid standard substances (Purity > 98%, Med Chem Express); 70% methanol (Containing internal standard working solution with an internal standard concentration of 4000 nmol/L).

Extraction of flavonoid compounds. Leaves (1 × 2 cm) samples preserved at –80 °C was retrieved and transferred to a ball mill pre-cooled with liquid nitrogen (30 Hz). Subsequently, the samples were comprehensively ground for 1.5 minutes until a powdered form was achieved. Subsequently, 20.0 mg of the powder was accurately weighed into an EP tube, and 10 µL of 4000 nmol/L internal standard mixed working solution and 500 µL of 70% methanol aqueous solution were added. Ultrasonic extraction was carried out for 30 min under ice-bath conditions. The extract was then centrifuged at 4 °C and 12,000 r/min for 5 min. The supernatant was aspirated, filtered through a 0.22 µm microfiltration membrane, and transferred to a sample vial for subsequent LC-MS/MS analysis.

Chromatographic conditions. Column waters ACQUITY UPLC HSS T3 C18 column (1.8 µm, 100 × 2.1 mm); column temperature of 40 °C; mobile phase-phase A is a 0.05% formic acid aqueous solution, and phase B is an acetonitrile solution containing 0.05% formic acid; flow rate of 0.4 mL/min; injection volume of 4 µL. Gradient elution program: at 0 min (90%A:10%B), at 1.0 min (80%A:20%B), at 9.0 min (30%A:70%B), at 12.5 min (5%A:95%B), at 13.5 min (5%A:95%B), at 13.6 min (90%A:10%B), at 15.0 min (90%A:10%). Mass spectrometry conditions were as

Figure 1. Leaf morphology of 6 *Artemisia argyi* germplasm



follows: the ion source was electrospray ionization, with an ion source temperature of 550 °C. In the positive ion mode, the ion spray voltage was 5500 V, while in the negative ion mode, it was 4500 V. The curtain gas pressure was set at 35 psi, the nebulizer gas pressure at 55 psi, and the auxiliary gas pressure at 55 psi. The scanning mode employed was multiple reaction monitoring (MRM). The declustering voltage (DP) and collision energy (CE) corresponding to specific analytes were determined in accordance with the optimized parameters for each compound.

Data processing. The raw mass spectrometry data were gathered and processed by means of the analyst 1.6.3 software. Qualitative analysis was carried out based on the MWDB (Metware database) of Wuhan Metware Biotechnology Co., Ltd. Quantitative analysis employed the MRM mode, and standard curves were plotted by analyzing the MRM chromatographic peak intensities of standard products at each concentration gradient. The peak areas of each chromatographic peak in the samples were inserted into the corresponding standard curves to compute their actual contents (unit: μg/g).

Principal component analysis (PCA). This method was employed to assess the overall disparities and within group variations of flavonoids among distinct germplasm samples. OPLS-DA analysis was utilized to screen differential metabolites between pairwise groups of different germplasms. The screening criteria were as follows: the variable importance projection value > exceeded 1.0, and the fold change (FC) was either greater than or equal to 2.0 or less than or equal to 0.5. Heat map and hierarchical clustering analysis (HCA): based on the euclidean distance of flavonoid contents and ward's clustering strategy, this approach was used to visualize the similarity and differential accumulation patterns among germplasms. KEGG enrichment analysis: the KEGG database was applied to conduct metabolic pathway enrichment analysis on significantly differential metabolites, with a significant enrichment threshold of $p < 0.05$. Bar charts were generated using Excel 2007.

RESULTS

Overall composition of flavonoid compounds in *Artemisia argyi*. A total of 76 flavonoid compounds, categorized into 11 groups, were identified from the leaves of six *A. argyi* germplasms through UPLC-MS/MS (Figure 2). An examination of the compound category distribution indicated that flavones (24 compounds) and flavonols (20 compounds) were the most prevalent groups, accounting for 31.58% and 26.32% of the total identified compounds respectively, and jointly constituted the main framework of flavonoid metabolism. The sub-prevalent groups encompassed dihydroflavones (8 compounds, 10.53%), isoflavones and chalcones (6 compounds each, 7.89%), and dihydroflavonols (4 compounds, 5.26%). Flavanols, xanthones, flavonoid C-glycosides, and other categories displayed lower abundances, with 1–2 compounds detected in each category (accounting for ≤ 2.63%).

Flavonoid compound types of different *Artemisia argyi* germplasms. As presented in Table 1, eleven categories of flavonoid compounds were detected in the leaves of all six *A. argyi* germplasms, yet notable disparities were observed in the composition of each category. Regarding the total number of compounds, the leaves of Aa36 and Aa60 exhibited the highest number of flavonoid categories, both reaching 64. The Aa13 samples contained 63 types of flavonoids, while the leaves of Aa13, Aa41 and Aa38 harbored 62 flavonoid compounds.

An analysis of the distribution characteristics of key categories indicated that flavones and flavonols were the predominant groups among all six germplasms. Specifically, the quantities of flavones detected in Aa36, Aa9, Aa13, Aa41, Aa38 and Aa60 were 20, 21, 22, 22, 21 and 21 respectively, accounting for 31.25%, 33.87%, 34.92%, 35.48%, 33.87% and 32.81% of their total identified compounds. The quantities of flavonols detected in Aa36, Aa9, Aa13, Aa41, Aa38 and Aa60 were 17, 17, 18, 16, 15, 2 and 18 respectively, accounting for 26.56%, 27.42%, 28.57%, 25.81%, 25.81%, 24.19% and 28.31% of their total identified compounds. Dihydroflavones (7–8 species), chalcones (4–5 species) and dihydroflavonols (3–4 species) were sub-predominant groups, accounting for 4.76–12.90%. Flavonoid C-glycosides, xanthones, etc., were rare groups, with only 1–2 species detected (accounting for ≤ 3.23%).

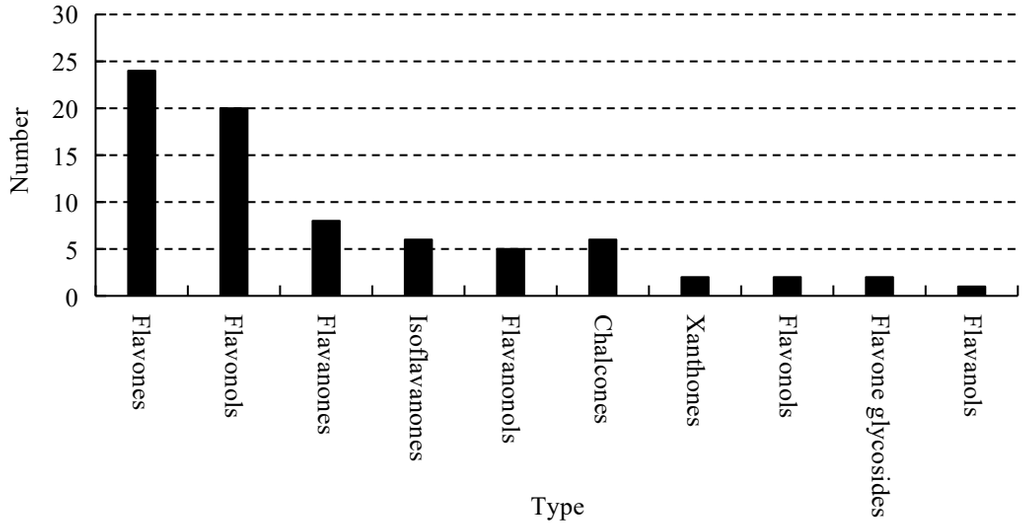
The specific category differences among the six germplasms were as follows: flavonol glycosides were not detected in sample Aa13, whereas one species was detected in the other five germplasms. Regarding chalcones, five species were detected in each of Aa36, Aa38 and Aa41, while four species were detected in each of Aa9, Aa13 and Aa60. For dihydroflavones, eight species were detected in each of Aa9, Aa36 and Aa38, and seven species were detected in each of Aa13, Aa41 and Aa60. In the case of dihydroflavone glycosides, only one species was detected in germplasm Aa36. Concerning flavones, the largest number of detected species (22) was observed in Aa13 and Aa41, followed by Aa9, Aa38 and Aa60 (21), and the fewest (20) was in Aa36. With respect to flavonols, the most de-

tected species (18) were found in Aa13 and Aa60, followed by Aa9 (17) and fewer (16 or 15) were in Aa41, Aa36 and Aa38. Other flavonoid compounds also demonstrated certain variations among the germplasms (Table 1). These differences suggest that *A. argyi* germplasms with distinct genotypes display specific synthesis and accumulation patterns of flavonoid compounds.

Table 1. Categories and quantities of flavonoid compounds in different *Artemisia argyi* germplasm

Type	Aa36	Aa9	Aa13	Aa41	Aa38	Aa60
Flavonol glycoside	1	1	0	1	1	1
Chalcone	5	4	4	5	5	4
Flavanon	8	8	7	7	8	7
Dihydroflavonol	4	4	3	3	4	4
Flavanon glycoside	1	0	0	0	0	0
Flavone	20	21	22	22	21	21
Flavonol	17	17	18	16	15	18
C-Glycosyl flavone	1	2	2	1	2	2
Flavanols	1	1	1	1	1	1
Xanthone	2	1	1	1	1	1
Isoflavone	4	3	5	5	4	5
Total	64	62	63	62	62	64

Figure 2. Categories of flavonoid compounds in *Artemisia argyi* germplasm



Flavonoid compound contents in *Artemisia argyi*. The mean content of flavonoid compounds in different *A. argyi* germplasms spanned from 8.70 to 14.01 μg/g, specifically presenting the following order: Aa41 (14.01 μg/g) > Aa13 (12.80 μg/g) > Aa38 (10.25 μg/g) > Aa60 (9.84 μg/g) > Aa36 (9.23 μg/g) > Aa9 (8.70 μg/g). The average flavonoid content in Aa41 was notably higher than that in other germplasms ($p < 0.05$), whereas there was no significant disparity between Aa60 and Aa36 (Figure 3).

Analysis of the top ten flavonoid compounds in each sample indicated that both the contents and specific compositions of the top ten compounds differed among germplasms (Figure 4). Seven compounds consistently emerged in the top ten of all six germplasms: jaceosidin, eriodictyol, eupatorin, hispidulin, chrysosplenetin, scutellarin, and quercimeritrin, which reflects the relative conservatism of the dominant flavonoid components in *A. argyi*.

Certain flavonoids were only present within the top 10 rankings of specific germplasms. Specifically, luteolin was not among the top 10 in Aa60, being substituted by kaempferol 3-neohesperidoside. Cynaroside failed to rank among the top 10 in Aa41 and Aa9, replaced by hyperoside and diosmin respectively. Rutin did not make it into the top 10 in Aa36 and Aa38.

PCA analysis. PCA demonstrated that the flavonoid metabolic profiles of the six *A. argyi* germplasms were genotype-dependent (Figure 5). The cumulative contribution rate of the first principal component (PC1) and the second principal component (PC2) attained 56.88%, where PC1 accounted for 36.52% and PC2 for 20.36%.

Distinct separation trends were discerned among diverse germplasm groups. Along the PC1 of Aa9, Aa36 and Aa38 group was characterized by an enrichment of methoxylated flavonoids, including jaceosidin, hispidulin and eupatilin in the upper – right quadrant; the Aa13 and Aa41 group was predominantly composed of jaceosidin,

Figure 3. The content differences of total flavonoids in different *Artemisia argyi* germplasm (different *A. argyi* germplasm indicate significant differences in treatments ($p < 0.05$))

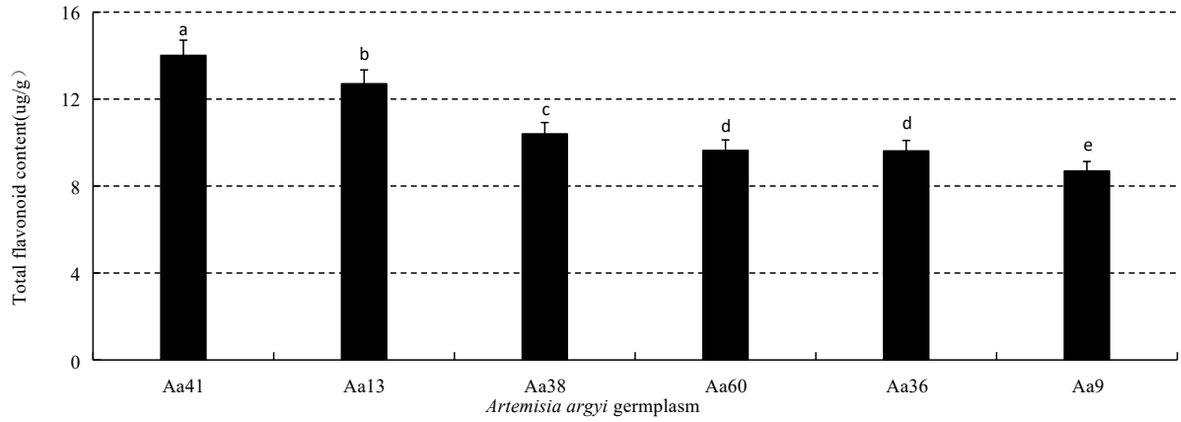


Figure 4. The tip 10 types and their contents of flavonoid compounds in leaves of *Artemisia argyi*

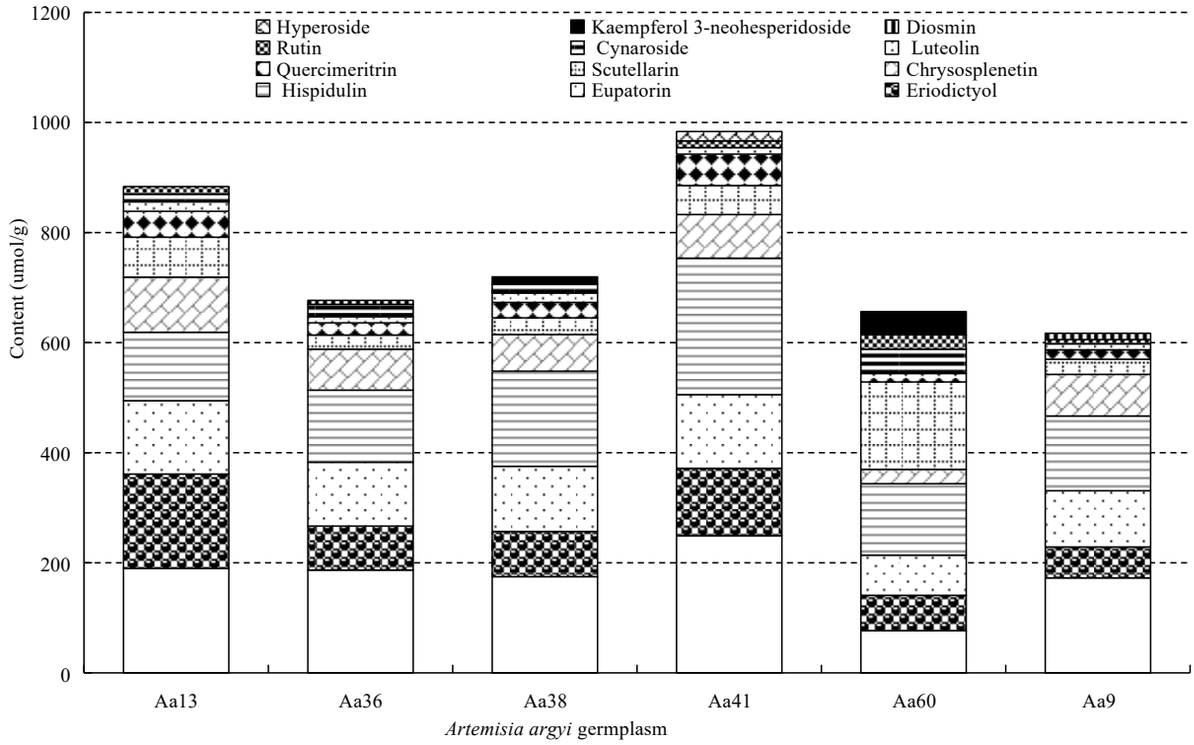
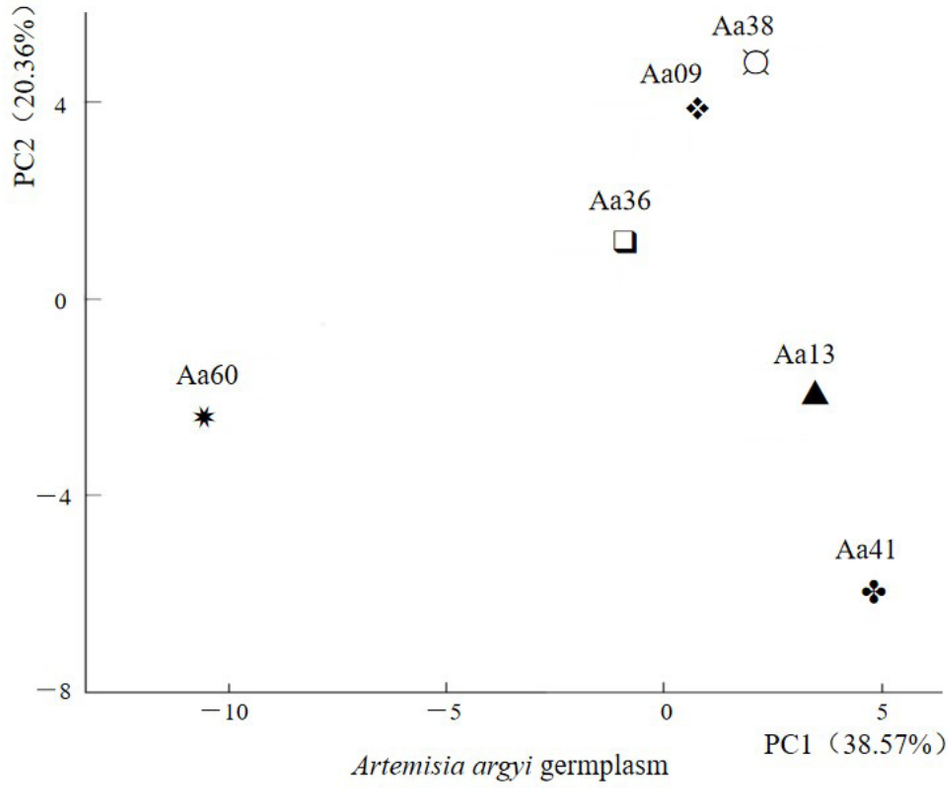


Figure 5. PCA analysis of flavonoid accumulation patterns in leaves of different *Artemisia argyi* germplasm



eriodictyol and eupatilin in the lower-right quadrant. Along the PC2 of Aa60 was separately distributed on the far-left side, which exhibited a marked distinction from the other five germplasms, primarily driven by scutellarin with a loading value of 0.92. The scutellarin content in Aa60 reached 158.73 μg/g, accounting for 52.4% of the total flavonoids, which was 2.19–6.17 times higher than that in other germplasms, thereby contributing to its unique metabolic profile.

Cluster analysis. Cluster analysis based on the contents of 76 flavonoid compounds, indicated that the six *A. argyi* germplasms were classified into two major distinct groups (Figure 6). Main group I encompassed five germplasms of Aa9, Aa13, Aa36, Aa38 and Aa41, which could be further partitioned into two subgroups. Subgroup 1 consisted of Aa9 and Aa36 originated from Henan, sharing 9 characteristic flavonoids, including methoxylated derivatives such as jaceosidin and eupatilin (average content >7.77 μg/g). Subgroup 2 included Aa13 (from Henan), Aa38 (from Hubei) and Aa41 (from Hubei), characterized by the co-accumulation of eriodictyol and acacetin (correlation coefficient $r = 0.53$). Despite the influences of regional environments, metabolic convergence was propelled by the conservatism of key flavonoid synthase genes.

Main group II contained only germplasm Aa60, with its core distinguishing attribute being that scutellarin alone accounted for over 52% of the total flavonoids (8.3-fold higher than group I).

Screening of differentially accumulated flavonoid metabolites in *Artemisia argyi* germplasm. Differentially accumulated metabolites (DAMs) were screened among paired germplasm through an OPLS-DA model (VIP > 1 & $|\log_2 FC| \geq 1$). Noticeable disparities were observed in the quantity of DAMs across diverse germplasm comparisons. The Aa38 vs Aa60 group exhibited the highest number of DAMs (42), succeeded by the Aa60 vs Aa41 group (40) and the Aa13 vs Aa36 group (37). In each comparison group, the numbers of up-regulated and down-regulated metabolites were approximately balanced (e.g., Aa38 vs Aa60: 26/26, Aa60 vs Aa41: 21/19, Aa13 vs Aa36: 24/13); see Figure 7.

Twenty DAMs exhibiting the most substantial fold-change disparities between groups were further compared. Key DAMs encompassed apigenin-7-O-glucuronide, apigenin-7-glucoside, hispidulin glycoside, apigenin dimethyl ether, astilbin, sakuranetin, isorhamnetin-3-O-glucoside, *etc*., demonstrating highly significant accumulation differences within specific groups:

Figure 6. Heat map cluster analysis of different *Artemisia argyi* germplasm based on the content of flavonoids

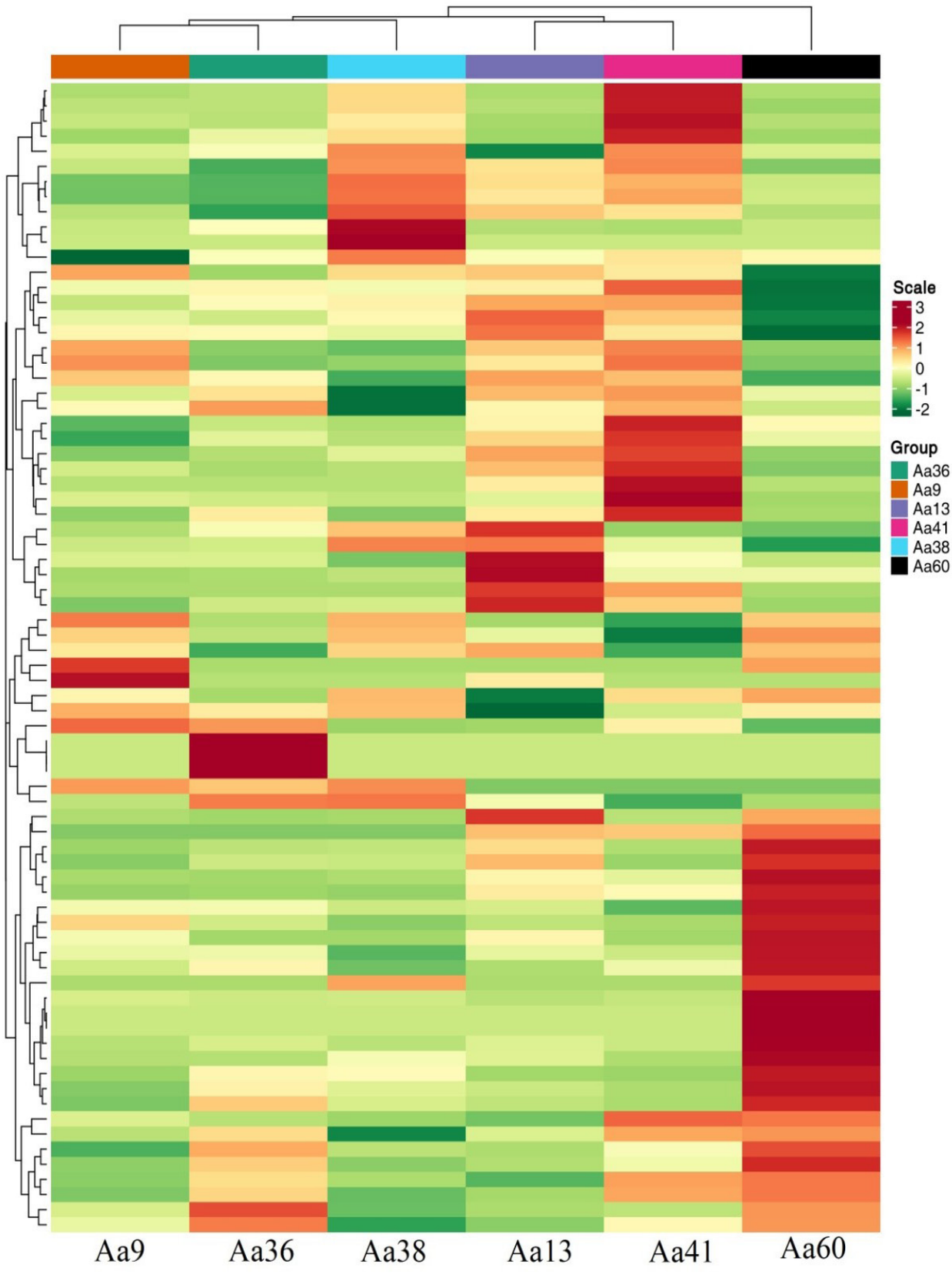
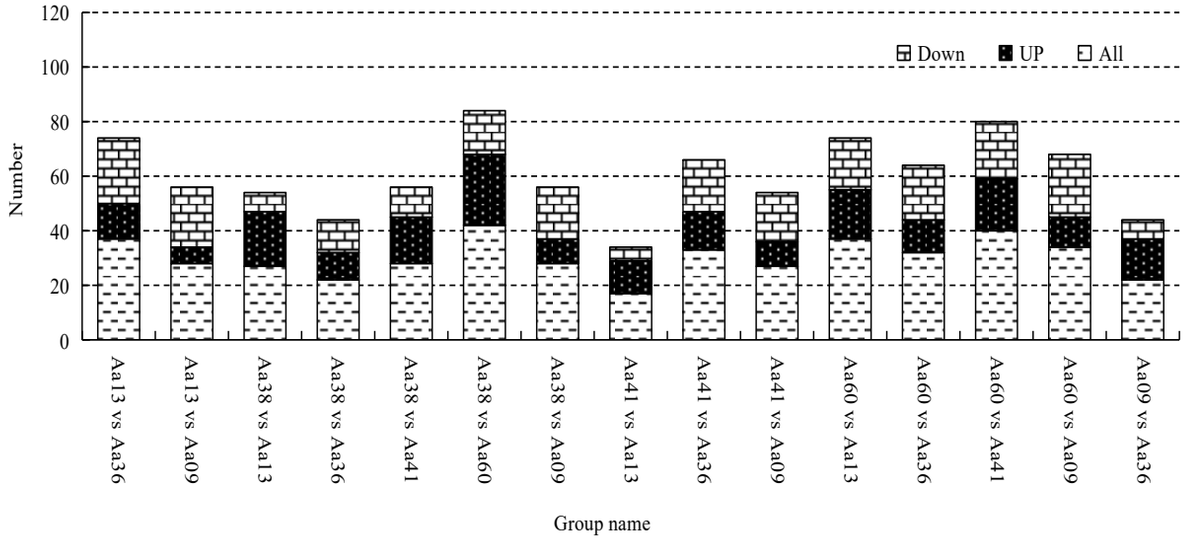


Figure 7. Distribution pattern of flavonoids among *Artemisia argyi* germplasm



Aa13 vs Aa36: in Aa36 leaves, only apigenin dimethyl ether and naringenin chalcone were up-regulated, whereas the others were down-regulated.

Aa13 vs Aa9: in Aa9 leaves, only epimedin B and (–)-catechin gallate were down-regulated, while the others were up-regulated. Among them, apigenin-7-glucoside and apigenin-7-O-glucuronide exhibited the most pronounced up-regulation.

Aa13 vs Aa36: in Aa36 leaves, astilbin, sakuranetin, hispidulin glycoside, isorhamnetin-3-O-glucoside and catechin gallate were down-regulated, and the others were up-regulated. Apigenin-7-O-glucuronide showed the highest degree of up-regulation.

Aa38 vs Aa9: in Aa9 leaves, catechin gallate, linarin and apigenin dimethyl ether were down-regulated, and the others were up-regulated. Hispidulin glycoside presented the most significant up-regulation.

Aa38 vs Aa13: in Aa13 leaves, only hispidulin glycoside was up-regulated, and the others were down-regulated. Apigenin dimethyl ether and apigenin-7-O-glucuronide had the most notable down-regulation.

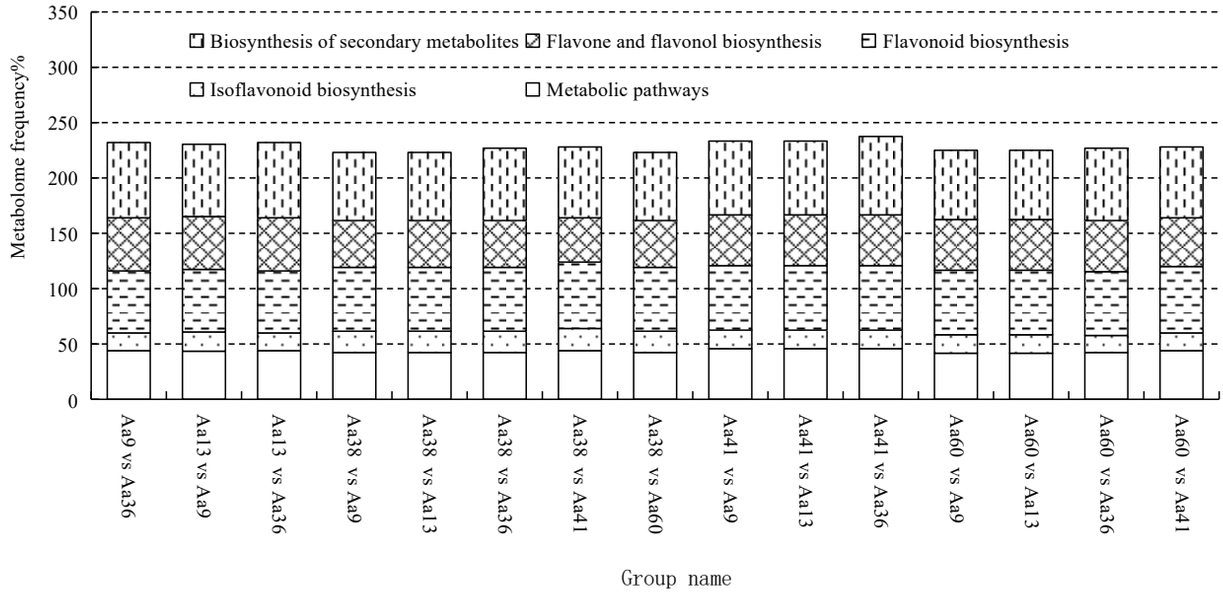
KEGG enrichment analysis of flavonoid differential metabolites in *Artemisia argyi* germplasm. KEGG metabolic pathway enrichment analysis was conducted on all the previously screened significant DAMs1 (Figure 8). The findings indicated that the differential metabolites were significantly enriched ($p < 0.05$) in five core metabolic pathways. The enrichment intensity, ranked in descending order of the rich factor, was as follows: secondary metabolite biosynthesis (ko01110, Rich Factor (RF) = 0.38, $p = 4.2 \times 10^{-5}$), flavonoid biosynthesis (ko00941, RF = 0.32, $p = 8.7 \times 10^{-4}$), flavone and flavonol biosynthesis (ko00944, RF = 0.28, p = 0.006), phenylpropanoid biosynthesis (ko00940, RF = 0.25, $p = 0.013$), and isoflavonoid biosynthesis (ko00943, RF = 0.21, $p = 0.039$).

DISCUSSION

Flavonoids, as one of the principal active constituents of *A. argyi*, make substantial contributions to its traditional therapeutic effects, such as warming the meridians to arrest bleeding and dispelling cold to alleviate pain [Chen et al. 2017, Zhang et al. 2023, Yang et al. 2024]. In this study, the diversity of flavonoids in six *A. argyi* germplasms was comprehensively analyzed via quantitative metabolomics. A total of 11 categories of flavonoids, encompassing the major branches of the flavonoid metabolic pathway, were identified, including flavones, flavonols, dihydroflavones, isoflavones and chalcones. Specifically, 24 flavones and 20 flavonols were detected, which is consistent with the previous research findings that "flavones and flavonols are the most predominant classes of flavonoid compounds in plants" [Shen et al. 2022].

Further analysis revealed seven core flavonoids that dominated the content in all six germplasm: eupatilin, eriodictyol, eupalin, hispidulin, chrysosplenetin, scutellarin and quercetin-7-O-β-D-glucoside. These core components exhibit clear biological activities: quercetin derivatives show significant anti-inflammatory effects [Li et al. 2016];

Figure 8. Results of KEGG enrichment analysis for differentially accumulated flavonoids



hispidulin has attracted attention for its neuroprotective [Wang et al. 2015, An et al. 2018], scutellarin is widely studied for its cardiovascular and neuroprotective effects [Chen et al. 2020]; eupatilin is renowned for its potent anti-inflammatory, gastric mucosa-protective and potential antitumor activities [Park et al. 2018, Hong et al. 2023]. Thus, these seven compounds likely constitute the key material basis of *A. argyi* efficacy, and their high abundance and stability may serve as core markers for quality evaluation of *A. argyi*. Notably, scutellarin had the highest content in germplasm Aa60, suggesting its potential for developing cardiovascular protective varieties [Chen et al. 2020].

Notable disparities in the quantities of specific flavonoid categories and sub-classes were discerned among distinct genotypes. For instance, dihydroflavone glycosides were solely detected in Aa36, and flavonoid C-glycosides were concentrated in Aa60, which suggests subtle genetic differentiation within the species. These disparities are directly associated with the regulation of key enzyme gene expression, and Aa36 might possess a dominant allelic variation of this gene. The number of flavonols also differed among three germplasm species (18 in Aa41 compared to 21 in Aa60), indicating that the regulation of flavonoid biosynthesis pathways is genotype-dependent, mirroring the genetic diversity of *A. argyi* germplasm [Zhou et al. 2022].

Although germplasm Aa60 (originating from Tangyin, Henan) did not exhibit the highest average flavonoid content, PCA and HCA revealed its distinctive flavonoid accumulation profile, which was clearly distinguishable from those of the other five germplasms. This distinctiveness may be attributed to its unique genetic background or adaptive disparities to the micro ecological environment of its provenance.

According to relevant research, the disparities in flavonoids predominantly stem from the biosynthetic pathway of secondary metabolites, particularly the flavonoid and its downstream flavone/flavonol branch pathways. There exist allelic variations and spatio-temporal expression differences in the key synthase genes (such as the chalcone synthase gene, chalcone isomerase gene, flavone synthase gene, flavanone 3-hydroxylase gene and flavonol synthase gene) or modifier enzyme genes (such as glycosyltransferase genes) involved in flavonoid synthesis [Tohge et al. 2013, Doyle et al. 2024a, 2024b].

While this study systematically revealed the accumulation patterns and potential biosynthetic pathway differences of flavonoids among *A. argyi* germplasm resources through UPLC-ESI-MS/MS untargeted metabolomics, two main limitations remain. First, the identification of metabolites is highly dependent on the coverage of existing mass spectrometry databases, and the structural confirmation of rare or novel flavonoids lacking reference standards still requires orthogonal techniques such as nuclear magnetic resonance. Second, although the study uncovered germplasm-specific metabolic phenotypic differences, it has not yet established a direct causal link at the molecular level between genetic variations in key enzyme genes and flavonoid biosynthesis phenotypes using techniques such as transcriptomics, genome-wide association analysis, or gene editing. As genomic resources

for *Artemisia* species continue to expand [Wang et al. 2023], future research could integrate multi-omics and functional genomics approaches (e.g., GWAS, CRISPR) to precisely identify the genetic loci regulating the synthesis of key flavonoids [Doyle et al. 2024a, 2024b, Chen et al. 2014], thereby advancing the in-depth elucidation of *A. argyi* quality formation mechanisms and promoting the practice of molecular design breeding.

CONCLUSIONS

Based on quantitative metabolomics analysis, this study systematically revealed the accumulation patterns and regulatory mechanisms of flavonoids in leaves of six *A. argyi* germplasm: 11 categories and 76 species of flavonoids were identified, and seven core flavonoid clusters stably and highly expressed in all germplasm were defined for the first time (eupatilin, eriodictyol, eupalin, hispidulin, chrysosplenetin, scutellarin, quercetin-7-O-β-D-glucoside). This cluster covers key active components (anti-inflammatory, antioxidant, neuroprotective) of *A. argyi* efficacy in warming meridians and dispelling cold, and can serve as universal metabolic markers for cross-germplasm quality evaluation. Additionally, Aa60 exhibited a unique scutellarin-dominant metabolic profile, providing characteristic resources for developing neuroprotective *A. argyi* varieties.

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