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Message from the Editor-in-Chief

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

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Aims and Scope

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

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Multiple defense layers in plant-pathogen interactions

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Review paper

Abstract: Biotic stresses always impact the yield of plants, and understanding the interaction between plants and pathogens is crucial for disease control. Plants' defense mechanisms against pathogens have various complex layers. As pathogens evolve to have more complicated and efficient effector systems during plant-pathogen coevolution, plants develop more sophisticated defense systems. The complexity of plant defense systems can be explored at different levels, including reactive oxygen species (ROS) scavenging, changes in transcription factor (TFs) expression, increased activity of PRs, and accumulation of lignin. Additionally, systemic acquired resistance (SAR) and induced systemic resistance (ISR) induction pathways play a significant role in how plants respond to biotic stresses. *ERF* and *NPR1* genes activate SAR and ISR pathways. Various protein families associated with the plant defense system, such as pathogenesis-related proteins (PRs), regulate a wide range of responses to pathogens, hindering pathogen penetration. The accumulation of certain metabolites, like lignin, helps prevent pathogen penetration and the spread of disease in plants, serving as part of the defense system. This review provides a brief overview of the diverse and essential layers of the plant's defense system against pathogens, aiding in the understanding of plant-pathogen interactions.

Keywords Biotic stress, molecular aspect, induced resistance, plant defense strategies.

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Introduction

Plants can recognize microbe-associated molecular patterns (MAMP) or damage-associated molecular patterns (DAMP) by pattern recognition receptors (PRR) and sense the presence of pathogens (Schwessinger and Ronald, 2012). Some subsequent events that cause the activation of the immune system include ion flux changes in the plasma membrane, oxidative burst, activation of the MAPK cascade, expression of defense genes, and callus deposition. This level of immunity, called (MAMPs/DAMPs)-triggered immunity (MTI) or pattern triggered immunity (PTI), is the first layer of the immune system ((Jones and Dangl, 2006); (Zipfel, 2009)). Some pathogens have developed different effectors during evolution to disrupt MTI (Dangl et al., 2013). During the co-evolution of plant and pathogen, plants developed intracellular receptors and resistance proteins (PR) to detect the presence of effectors and activate effector-induced immunity (ETI). This level of defense is considered the second layer of protection (Spoel and Dong, 2012). These two layers of immunity are usually known as the innate immunity of plants (Schwessinger and Ronald, 2012). Activating the plant's innate immunity in a specific tissue (infected tissue) leads to the transmission of defense signals to other tissues (without contamination) systemically. It promotes long-term resistance against a wide range of disease agents. This acquired immunity is known as systemic acquired resistance (SAR) (Zhou and Wang, 2018). In addition to pathogens, some chemical compounds such as salicylic acid, nitric oxide, N-hydroxy pipecolic acid, and azelaic acid can activate the SAR and, or other defense-related systems ((Chen et al., 2018); (Haghpanah et al., 2021), (Haghpanah et al., 2024); (Jung et al., 2009); (Park et al., 2007); (Wang et al., 2014)).

ROS system

The signaling network related to reactive oxygen species (ROS) is highly conserved among aerobic organisms. It controls a wide range of biological processes, such as growth, development, and response to biotic or abiotic stimuli ((Mittler et al., 2011)). In plants, the enzyme complexes generating ROS at the cell surface are still unknown, However, evidence shows that the activity of a set of enzymes, including NADPH oxidase (NOX), class III peroxidases, oxalate oxidases, amine oxidases, lipoxygenases, quinone reductase, causes the production of ROS in plant cells (Camejo et al., 2016). Additionally, cell organelles such as chloroplasts, mitochondria, and peroxisomes are important potential sources of ROS during response to biotic and abiotic stresses (Kohli et al., 2019). It is known that the production of H_2O_2 (hydrogen peroxide) and $O_2^{\bullet-}$ (superoxide) is part of the plant's defense process against pathogens. One of the first defense reactions of the plant against pathogen attack is oxidative burst, which causes the temporary production of ROS and seems to be a common feature of the plant response. ROS production during oxidative burst is associated with pathogen recognition related to the Perception of microbe/pathogen-associated molecular patterns (MAMPs/PAMPs). This process also occurs during HR (Camejo et al., 2016). Excessive production of ROS, is usually seen during the pathogen response process, causes HR or cell death, which is one of the appropriate responses to prevention of pathogen penetration. They also act as local and systemic secondary messengers to create immune responses such as the expression of defense genes or stomatal closure (Gilroy et al., 2014). The biochemical pathway of production and inhibition of oxygen free radicals illustrated that after the formation of free radicals, the activity of the superoxide dismutase (SOD) enzyme causes $O_2^{\bullet-}$ to become H_2O_2. A part of the generated H_2O_2 leaves the cell membrane and acts as a signaling molecule that can regulate cell metabolism involved in growth and response to environmental stimuli (Sagi et al., 2004); (Xia et al., 2009). However, Catalase (CAT) enzyme activity converts a significant part of the produced H_2O_2 into oxygen and water (Oliveira et al., 2016). Other critical enzymes of the ROS pathway include ascorbate peroxidase (APX) and peroxidase (POD), which are involved in the degradation of H_2O_2 and the oxidation of phenolic compounds. Treating the plant with certain chemical inducers, such as potassium phosphite and azelaic acid, stimulates the ROS pathway and triggers a defense response (Haghpanah et al., 2024); (Ramezani et al., 2018).

SAR and ISR pathways

Systemic acquired resistance (SAR) can activated by most pathogens that cause tissue necrosis, either as part of the hypersensitive response (HR) or as a symptom of the disease (Conrath, 2006). When cells detect SAR signals, produce salicylic acid to activate NPR1. The NPR1 activity regulates the transcription of many genes, including pathogenesis-related (PR) genes and endoplasmic reticulum (ER) genes, which contribute to the secretion of PR proteins (Spoel and Dong, 2012); (Wang et al., 2005). As a transcription factor, nuclear NPR1 interacts with TGAs and some TFs (NIMI-interacting) to regulate the expression of downstream defense genes (Kesarwani et al., 2007). TGAs mainly activate NPR1-dependent genes, while NIMIN suppresses the expression of defense genes (Johnson et al., 2003). The SAR pathway primarily regulates the plant's defense for biotrophic pathogens (Santino et al., 2013) (Figure 1).

Induced systemic resistance (ISR) is another form of systemic immunity induced by beneficial non-pathogenic microbes (Pieterse et al., 2014). Although ISR and SAR are both systemic defense mechanisms, they differ from each other in several ways. First, the triggers for ISR and SAR are fundamentally different. SAR is triggered by compatible or incompatible pathogenic interactions, while ISR is initiated by non-pathogenic microbes (Zhou and Wang, 2018). Second, although the biochemical spectrum of both pathways is broad, there is little overlap between these two pathways, especially in exposure and impact on pathogens (Ton et al., 2002). Third, the presence and accumulation of salicylic acid are necessary for the SAR pathway. In contrast, ISR is less dependent on salicylic acid and is more regulated by jasmonic acid and ethylene (Pieterse et al., 2014) (Figure 1). Jasmonic acid and its derivatives provide immunity against necrotrophic pathogens and herbivores (Santino et al., 2013). Some inducers can activate SAR (salicylic acid, azelaic acid (Djami-Tchatchou et al., 2017); (Jung et al., 2009)) or ISR (methyl jasmonate) pathways. The type of pathogen (necrotrophic or biotrophic) can also affect the induction of the type of defense induction system. Results of a recent study showed that the use of azelaic acid can unexpectedly induce defense systems associated with jasmonic acid challenging to necrotrophic types such as *Alternaria solani* as a necrotrophic pathogen (Haghpanah et al., 2024).

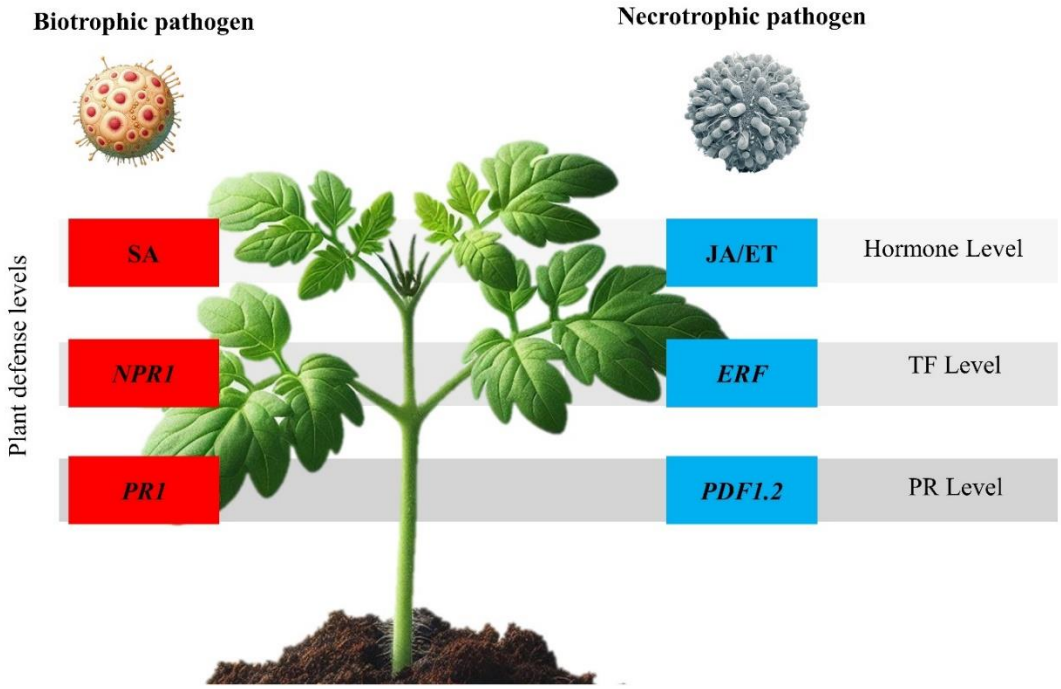

Figure 1. Plant response to biotrophic and necrotrophic pathogens.

Transcription factors

Transcription factors (*TFs*) are crucial regulators that control gene expression in all living organisms and play a significant role in plant growth, the cell cycle, signaling, and stress responses. TFs alter gene expression by binding to the cis-elements of the target gene, and in plants, TFs are encoded by approximately ten percent of genes (Inukai et al., 2017). Large *TF* families, such as *WRKY*, *NAC*, and *ERF* serve as critical regulators of various stress-related genes, aiding in the selection of genetic engineering to enhance plant resistance against various stresses (Wang et al., 2016). Studies show that inducers stimulate the defense system in plants by affecting TFs.

The WRKY family is a unique transcription factor family found in higher plants and algae, playing a crucial role in numerous biological processes, particularly in response to biotic and abiotic stresses. The structure of *WRKY* protein consists of two main parts, N-terminal DNA binding domain and C-terminal zinc-finger structure (Li et al., 2020). Based on the diversity within the structures of these parts, *WRKY* is divided into different classes (Eulgem et al., 2000). *WRKY* family members have diverse regulatory mechanisms. In summary, *WRKY* protein can efficiently combine with W-box elements and activate or inhibit the transcription of downstream genes. Additionally, it can bind to other active elements and form protein complexes, which increases the binding activity of transcription (Phukan et al., 2016). The W-box is found in the promoter regions of many defense genes, and studies show that *WRKY* causes the expression of these defense genes by binding to this region and providing resistance to pathogens (Shinde et al., 2018). The expression of the WRKY gene in tomato plants was observed to increase after inoculation with *A. solani* (Haghpanah et al., 2023).

NAC proteins constitute a large family of plant-specific transcription factors with more than 100 members in Arabidopsis and rice. *NAC* TFs are characterized by the presence of a conserved N-terminal region known as the NAC domain, which acts as a DNA-binding domain (Yuan et al., 2019). NACs play a significant role in the plant's immune system. (Rabiei et al., 2022) showed that hexanoic acid treatment increases the expression of *NAC1* and induces resistance to early blight disease in tomato plants (Rabiei et al., 2022).

ERF proteins are a subfamily of the APETALA2 (AP2)/Ethylene-responsive-element-binding protein (EREBP) family of transcription factors, specific to plants (Singh et al., 2002). According to the EST database, 112 families of AP2/ERF have been identified in tomatoes. ERF proteins have a conserved region of 58-59 amino acids (ERF domain) that can bind to two similar cis-elements, such as the GCC box (found in the promoter regions of several PR genes), and cause an ethylene response (Feng et al., 2020). Microarray expression profiling related to sensitive and resistant varieties of tomato under the stress of wave spot disease showed that ERF family transcription factors respond to pathogen attack in resistant plants. ERF transcription factors may play a role in activating PR proteins (Feng et al., 2020).

NPR1 Regulatory Protein

The *NPR1* gene is essential for the activation of the SAR pathway. It was initially discovered in Arabidopsis through the comparison of mutants of this gene, demonstrating that plants lacking *NPR1* can not activate the SAR pathway (Liu et al., 2015); (Shah et al., 1997)). NPR1 activity can be stimulated by the treatment of salicylic acid and its analogs instead of pathogen infection (Wang et al., 2006). Microarray studies have shown that increasing the concentration of salicylic acid in the cell results in the transfer of *NPR1* to the nucleus, leading to changes in the transcription of 2248 genes associated with SAR. Despite not having a second known DNA binding site, *NPR1* is believed to act as a transcriptional cofactor (Withers and Dong, 2016). In addition to its role in the SAR pathway, *NPR1* activity is also crucial for activating the ISR pathway (Pieterse et al., 1998). Nuclear NPR1 is required for the SAR pathway, while cytoplasmic *NPR1* plays a role in ISR pathway activation. Cytoplasmic *NPR1* may regulate the interaction between salicylic acid and jasmonic acid (Ramírez et al., 2010); (Stein et al., 2008).,). Research indicates that the NPR1 protein is important in determining cell death during ETI (Withers and Dong, 2016). When stimulated by salicylic acid, NPR1 regulates and activates the SAR pathway increasing the expression of genes such as

PR1, *PR2*, and *PR5* (Molinari et al., 2014). On the other hand, if jasmonic acid or ethylene stimulates *NPR1*, the ISR pathway is activated, resulting in the expression of defense factors such as *PDF1*.2, *PR3*, and *PR4* (Thomma et al., 1998).

Mitogen-activated protein kinase (MAPK)

MAPKs are signal transduction units that are highly conserved and participate in many signal transduction processes through MAPK cascades. A typical MAPK cascade consists of MAPK (MPK), MAPK kinase (MAPKK, MAP2K, MKK, or MEK), and MAPK kinase kinase (MAPKKK, MAP3K, or MEKK) (Cristina et al., 2010). In a typical MAPK signaling cascade, MAPKKKs are activated by "stimulated plasma membrane receptors" and transmit signals downstream (Cristina et al., 2010). During this molecular cascade, MAPKKK activates MAPKK by phosphorylating specific MAPKK motifs. Then MAPKK activates MAPK by phosphorylating specific areas of MAPK. Finally, MAPK activates downstream kinases, enzymes, transcription factors, and other response factors and transmits extracellular environmental signals to the cell (Cristina et al., 2010); (Zhang et al., 2018). Through step-by-step phosphorylation, the MAPK cascade can amplify signals and transmit them to downstream proteins, causing the expression of resistance genes. The MAPK cascade plays an important role in cell differentiation, cell growth, hormonal activity, and response to biotic and abiotic stresses (Komis et al., 2018). Studies on the interaction between pathogens and tomato plants show that the MAPK family, especially MAPK3, plays a critical role in the response to pathogen attacks (Kandoth et al., 2007); (Melech - Bonfil and Sessa, 2010); (Stulemeijer et al., 2007). Plant treatment with inducers can be effectively stimulate the MAPK cascade (Zheng et al., 2020).

Lignin accumulation

Lignin is one of the most important secondary metabolites produced in plant cells through the phenylalanine/tyrosine pathway. This metabolite makes up approximately 30% of the organic carbon content in the biosphere (Ralph et al., 2004). The biosynthesis of lignin is a complex process that can be divided into three main steps: 1) the biological synthesis of lignin monomers, 2) the transport of monomers, and 3) polymerization (Liu et al., 2011). Lignin, as a complex phenolic polymer, increases the stiffness of the plant cell wall, creates a hydrophobic property, and facilitates the transport of minerals through the vascular bundles in the plant (Schuetz et al., 2014). Additionally, lignin is an essential physical barrier against the penetration of pathogens (Ithal et al., 2007); (Liu et al., 2018) . The lignin wall also acts as a reservoir of antimicrobial compounds that are released when the cell wall is destroyed (Miedes et al., 2014). To overcome the plant cell wall barrier, fungi secrete various enzymes, including cellulases, pectinases, hemicelluloses, chitinases, and proteases. These enzymes break down components of the cell wall and potentially trigger plant defense responses. (Yang et al., 2018). Applying certain chemical compounds, such as silica, induces lignin formation in plant tissues. Research indicates that silicic acid (the absorbable form of silica for plants) can interact with pectins and polyphenols in cell walls (Clarkson and Hanson, 1980). Some silicones attached to cell walls likely exist as esters (such as silicic acid derivatives) that function as a link in the structural organization of polyuronides, influencing the content and metabolism of polyphenols in cell walls. Cells are impacted by these changes. Silicon may regulate lignin biosynthesis and enhance the physical barrier against pathogen attack (Song et al., 2016).

Defense genes and proteins (PR)

When plants are attacked by pathogens and herbivores, biochemical and physiological changes occur in plants, such as the physical strengthening of the cell wall through the production of lignin, the production, and accumulation of phenolic compounds, phytoalexins, and an increase in related proteins to the pathogen (PR), which subsequently prevents various pathogen attacks (Bowles, 1990). Meanwhile, the production and accumulation of PR proteins in plants are vital in response to the pathogen. Studies show that some inducers cause rapid expression and significant accumulation of PRs, leading to a stronger defense response to pathogen penetration (Jung et al., 2009); (Ramezani et al., 2017) . In the past, various proteins stimulated during plant-microbe interaction were considered PR proteins, including enzymes such as PAL that exist naturally but their expression increases with plant infection. This definition led to

confusion, as a set of antifungal proteins was never considered a part of PRs (van Loon et al., 2006). Recently, 17 protein families (Table 1) have been identified as PRs (Moosa et al., 2018).

PR1 proteins

PR1 proteins are highly conserved among plants in terms of structure and are very similar in some domains, indicating that their general role is in plant response to biotic stresses (Lincoln et al., 2018). Although *PR1* is known as one of the most important members of PR, its biological function is not fully understood and only some of its properties, such as antifungal properties, have been determined (Glazebrook, 2005); (Lincoln et al., 2018). *PR1* mRNA expression has been widely used as a marker of plant disease resistance, while evidence of the protein's presence or direct effect on the disease or pathogen has been reported (Glazebrook, 2005); (Jung et al., 2009) .

Table 1. Characteristics of pathogenesis-related (PR) proteins in plants.

Family	Protein size (KDa)	Reported in plant	Properties	Reference
PR-1	15	Tobacco (PR-1a)	Antifungal	(Antoniw et al., 1980)
PR-2	30	Tobacco (PR-2)	b-1,3-glucanases	(Antoniw et al., 1980)
PR-3	25-30	Tobacco P, Q	Chitinases (I, II, IV, V, VI, VII)	(Van Loon, 1982)
PR-4	15-20	Tobacco R	Chitinases (I, II)	(Van Loon, 1982)
PR-5	25	Tobacco S	Thaumatine-like proteins (TLPs)	(Van Loon, 1982)
RP-6	8	Tomato inhibitor I	Proteinase inhibitor	(Green and Ryan, 1972)
PR-7	75	Tomato P_{69}	Endoproteinase	(Vera and Conejero, 1988)
PR-8	28	Cucumber chitinase	Chitinase (III)	(Metraux et al., 1988)
PR-9	35	Tobacco (lignin-forming peroxidase)	Peroxidase	(Lagrimini et al., 1987)
PR-10	17	Parsley (PR-1)	Ribonuclease-like proteins (RL)	(Somssich et al., 1986)
PR-11	40	Tobacco class-V chitinase	Chitinase (I)	(Melchers et al., 1994)
PR-12	5	Radish RsAFP3	Defensin	(Terras et al., 1995)
PR-13	5	Arabidopsis Thi2.1	Thionin	(Epple et al., 1995)
PR-14	9	Barley LTP4	Lipid-transfer protein (LTP)	(García-Olmedo et al., 1995)
PR-15	20	Barley OxOa (germin)	Oxalate oxidase (OXO)	(Z. Zhang et al., 1995)
PR-16	20	Barley OxOLP	Oxalate oxidase-like (OXO)	(Wei et al., 1998)
PR-17	27	Tobacco PRp27	Antiviral and antifungal	(Okushima et al., 2000)

Chitinase and glucanase are enzymes plants produce to break down molecules of chitin and glucan found in fungi cell walls. Chitin is made up of N-acetyl-D-glucosamine polymers with β-1,4 linkages that are broken down by chitinases, while glucan is composed of β-1,3-glucosidic linkages that are broken down by β-1,3-glucanase. In plants, five

classes of chitinases and three classes of glucanases have been identified.

Defensins

Defensins are a crucial family of antimicrobial peptides with a fully conserved structure. These peptides bind to the cell membrane of microbes (which have a negative charge) and interact with them (Parisi et al., 2019). The two main types of defensins are cis-defensins (found in plants and insects) and trans-defensins (found in mammals). Defensin coding genes in plants are known as *PDF1.2* (Sher Khan et al., 2019). Studies have shown that the role of this peptide in creating resistance to necrotrophic pathogens is very important (Mang et al., 2009).

Peroxidases

Peroxidases play a crucial role in multiple biochemical pathways, including scavenging reactive oxygen species (ROS), synthesizing lignin, and plant defense against pathogens (Pandey et al., 2017). Peroxidases are classified into different classes, including:

Class I, includes cytochrome c peroxidase, ascorbate peroxidase, and catalase peroxidase, which are involved in ROS scavenging.

Class II, exclusively contains fungal peroxidases and plays a major role in the biological degradation of lignin.

Class III peroxidase, perhaps the most important class of plant peroxidase, is involved in processes such as cell wall metabolism, ligninization, suberin processes, auxin-related metabolism, wound healing, ROS scavenging, and defense against pathogens (Pandey et al., 2017). In plant-pathogen confrontations, Class III peroxidases strengthen the cell wall to hinder pathogen penetration. These enzymes also participate in the production of phytoalexins and antimicrobial compounds. The effect of inducers on increasing the expression of genes and enzymatic activity of PRs has been discussed in previous studies (Jung et al., 2009); (Ramezani et al., 2017; Ramezani et al., 2018).

Conclusion

In general, the response of plants to pathogen penetration can be investigated in different layers. In the first stage, the plant senses the presence of the pathogen. Plants recognize MAMPs and DAMPs through pattern recognition receptors (PRRs), which activate rapid defense responses such as ion flux changes and oxidative bursts, activating MAPK cascades and defense gene expression. In response to pathogens that weaken MTI, plants produce resistance (R) proteins as intracellular receptors. This secondary defense mechanism enhances local and systemic immunity. SAR involves salicylic acid signaling through the NPR1 gene to orchestrate defenses primarily against biotrophic pathogens. On the other hand, ISR is activated by beneficial microbes and relies on the jasmonic acid and ethylene pathways, protecting against necrotrophic pathogens and herbivores. The role of ROS-related pathways is also important in plant-pathogen interactions. ROS act as signaling molecules and antimicrobial agents. Their production induces protective reactions, including the hypersensitivity response (HR), which increases the overall resilience of the plant. Transcription factors (TFs) such as WRKY, NAC, and ERF families regulate defense gene expression. WRKY proteins respond to various stressors, while NAC and ERF proteins are mainly involved in responding to pathogen attacks.

The final layers in the plant's response to the pathogen are PR proteins and physical barriers such as lignin accumulation. PR proteins induced during pathogen encounters include different classes with roles in antifungal activity and enzymatic functions. Their structural and functional diversity is vital to strengthen the plant's defense system. Lignin is a physical barrier that strengthens plant tissues and prevents pathogen invasion. Promoting lignin biosynthesis can significantly increase pathogen resistance.

The interconnectedness of these components reveals how plants use a multi-layered defense strategy to combat biotic stresses. A deeper understanding of MTI, ETI, ROS signaling, and the function of TF and PR proteins can inform innovative agricultural practices, including genetic engineering and breeding approaches aimed at increasing plant resilience. Such advances are critical to ensuring sustainable agriculture, allowing crops to grow amid challenges posed by climate change and emerging pathogens. Overall, this multifaceted view of plant immunity enriches our knowledge of plant biology and provides critical insights for future agricultural strategies.

Supplementary Materials

No supplementary material is available for this article.

Author Contributions

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

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Conflict of Interest Statement

The authors declare no conflict of interest.

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مقاله مروری

لایه‌های مختلف دفاعی در تعامل گیاه-پاتوژن

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چکیده: تنش‌های بیولوژیکی همیشه بر عملکرد گیاهان تأثیر می‌گذارد و درک تعامل بین گیاهان و پاتوژن‌ها برای کنترل بیماری بسیار مهم است. مکانیسم‌های دفاعی گیاهان در برابر عوامل بیماری‌زا دارای لایه‌های پیچیده مختلفی است. همانطور که پاتوژن‌ها برای داشتن سیستم‌های موثر پیچیده‌تر و کارآمدتر در طول تکامل همزمان گیاه و بیماری زا تکامل می‌یابند، گیاهان نیز سیستم‌های دفاعی پیچیده‌تری را توسعه می‌دهند. پیچیدگی سیستم‌های دفاع گیاهی را می‌توان در سطوح مختلف، از جمله مهارROS ، تغییرات در بیان فاکتورهای رونویسی (TFs)، افزایش فعالیت PRs و تجمع لیگنین بررسی کرد. علاوه بر این، مسیرهای القای SAR و ISR نقش مهمی در نحوه واکنش گیاهان به تنش‌های بیولوژیکی دارند. همچنین *ERF* و *NPR1* مسیرهای SAR و ISR را فعال می‌کنند. خانواده‌های پروتئینی مختلف مرتبط با سیستم دفاعی گیاه مانند PRs، طیف وسیعی از پاسخ‌ها را به پاتوژن‌ها تنظیم می‌کنند و مانع از نفوذ پاتوژن می‌شوند. تجمع برخی متابولیت‌ها مانند لیگنین به جلوگیری از نفوذ بیماری و گسترش آن در گیاهان کمک می‌کند و به عنوان بخشی از سیستم دفاعی عمل می‌کند. این بررسی مروری کوتاه بر لایه‌های متنوع و ضروری سیستم دفاعی گیاه در برابر پاتوژن‌ها ارائه می‌کند و به درک تعاملات گیاه و بیماری‌زا کمک می‌کند.

کلمات کلیدی: القای مقاومت، تنش زنده، جنبه‌های مولکولی، ساز و کارهای دفاعی گیاه.

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Callus induction and growth, as well as metabolite variations, of two *Taxus* spp. under in vitro conditions

Original paper

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Abstract: The present study investigated the effect of growth regulators on the quantity and quality of calli of two yew tree species (*Taxus* spp). A factorial experiment based on a completely randomized design was performed using 2,4-D and Kinetin on leaf and stem explants of *T. baccata* and *T. brevifolia*. Calli traits, including fresh weight, total phenol, total alkaloids, and paclitaxel content, were investigated. The simple effect of hormonal treatments on total phenolic content, total alkaloids, and fresh weight of calli was significant. Total phenol content and fresh weight were not affected by the interaction of hormonal treatments, whereas total alkaloid content was. Paclitaxel content did not significantly differ between explants. The highest paclitaxel content was found in the leaf explant of *T. baccata* at 30 µg/g, compared to 5 µg/g and 10 µg/g in *T. baccata* stem, *T. brevifolia* stem, and *T. brevifolia* leaf, respectively. The fresh weight and total alkaloid content of stem calli of both species were higher than the leaves. Yew is a valuable endangered medicinal plant that responds well to in vitro treatments. Therefore, it is possible to manage the production of its valuable metabolites under in vitro conditions, and more research is recommended on the production of paclitaxel using *T. baccata*.

Keywords: Alkaloid, metabolite, paclitaxel, taxol, tissue culture.

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Introduction

Plants have been an important source of medicine for humans since ancient times, and their importance in modern medicine has only increased. The World Health Organization estimates that more than 80 percent of people use plants in traditional and/or modern medicine, and many synthetic drugs are formulated based on plant chemicals (Tripathi and Tripathi, 2003).

The yew tree (*Taxus* spp.) is a valuable and rare endangered conifer species found in the ancient Hircanian forests of northern Iran (Alavi et al., 2020). Paclitaxel, which has been approved for the treatment of uterine and breast cancer by the USFDA since 1997, is considered the most important anticancer drug (Abbasi Kajani et al., 2012). However, ten tons of skin and wood from yew trees are required to produce one kilogram of paclitaxel. This means that a large number of trees are needed to provide this amount, and periodic consumption of a patient requires the cutting of around 8 60-year-old trees. This puts the endangered species at risk of complete destruction (Malik et al., 2011).

In vitro cell and tissue culture of yew organs is one alternative method for paclitaxel supplementation. Callus, a parenchymal cell that grows in situ and in vitro conditions, can be used for this purpose. Previous studies have shown that the B5 media containing 4 mg/l of 2.4-D and 0.5 mg/l of Kin provides the most suitable conditions for callus formation in *T. baccata* explants (Zhiri et al., 1995; Ashrafi et al., 2010; Toulabi et al., 2015).

The aim of this study is to determine the yield and yield components of paclitaxel in *T. baccata* and *T. brevifolia* under different media conditions and explant types. By exploring alternative methods for paclitaxel production, we can help protect endangered species like the yew tree and ensure that the production of this valuable drug is sustainable in the long run.

Materials and Methods

Explant preparation and treatment

The present study was conducted in the tissue culture laboratory of the Department of Horticultural Sciences, Gorgan University of Agricultural Sciences and Natural Resources. The young, non-polluting stems of *T. baccata* and *T. brevifolia* were collected from botany garden of university and the national botanic garden of Noshahr, respectively. Around 5 cm terminal section of suitable stems were separated from the original sample and rinsed in water containing a few drops of dishwashing liquid for 10 minutes. In order to completely remove the residue, several washes were performed in running water and the samples were immediately transferred under a laminar flow hood. The plant materials were treated with 70% alcohol for 15 seconds. Immediately, the samples were disinfected with sodium hypochlorite containing 5% activated chlorine and a drop of dishwashing liquid for 25 minutes. Finally, one-centimeter explants were prepared from disinfected plant organs. Explant were cultured in the B5 media containing 6 levels of a combination of 2,4-D and Kin including concentrations of 1, 2 and 3 mg/l 2.4-D and 0.2 and 0.5 mg/l Kin in 3 replications. The cultures were kept in growth room with the temperature and day light of 24 to 26°C and 16:8 hours of light and dark conditions, respectively. Four weeks after culture, subculture was performed. The resulting callus samples were then used to measure morphological traits such as color intensity, tissue thickness, and growth rate and biochemical characters such as total phenol, total alkaloids and the amount of paclitaxel.

Total phenol measurement

The total phenolic content of the calli was measured using the Slinkard and Singleton (1977) method to assess the potential bioactive properties of the calli. Phenolic compounds are known for their antioxidant and antimicrobial activities, as well as their potential in treating various diseases. Moreover, previous studies have reported that callus cultures of *Taxus* species have high levels of phenolic compounds, including flavonoids and tannins, which have been associated with the biosynthesis of paclitaxel, a well-known anticancer drug. Therefore, measuring the total phenolic content of the calli in this study can provide insight into the potential of these cultures as a source of bioactive compounds and anticancer agents. The obtained data were reported as mg/g of fresh weight, which enables comparison with other

studies and facilitates the identification of callus lines with higher phenolic contents for further investigation.

Total alkaloid measurement

To measure the total alkaloids, three grams of callus were extracted using a modified method based on Zarezadeh et al. (2000). First, the calli were mixed with 15-20 ml of ammonia and left for 20 minutes. Then, 30 ml of chloroform was added and the solution was mixed on a shaker at a rate of 50-60 rpm for 2.5 hours. The samples were filtered using filter paper under vacuum conditions in a chemical hood. After filtration, the solution volume was reduced to 15 ml using a 50-60°C rotary evaporator for 20 minutes. If necessary, chloroform was added to adjust the volume. Next, 15 ml of 2% tartaric acid was added to the mixture and the aqueous solution was immediately separated. The pH of the aqueous solution was then adjusted to 9 using 25% ammonia. This was followed by the addition of 20 ml of chloroform twice and 10 ml of glacial acetic acid to each sample. After 10 minutes and until the alkaloids were completely dissolved, 3 drops of crystal violet reagent were added to each sample. Finally, the resulting compound was titrated with 1N perchloric acid. At the end of the titration, the color of the crystal violet changed from purple to blue to green.

Extraction and measurement of paclitaxel

Paclitaxel is a well-known anticancer drug that is mainly extracted from the bark of the Pacific yew tree (*Taxus brevifolia*). However, callus cultures of *Taxus* species have been reported to produce paclitaxel, making them a potential alternative source of this important drug. Therefore, measuring the amount of paclitaxel in the calli of this study can provide insight into the potential of these cultures as a source of paclitaxel and contribute to the development of a sustainable and cost-effective production method. The obtained data will be reported as the amount of paclitaxel per gram of fresh weight and can be compared with other studies to identify callus lines with higher paclitaxel contents for further investigation. To extract and determine the amount of paclitaxel, two grams of three-month-old callus were crushed and soaked in 100 ml of methanol for 16-24 hours at room temperature on a shaker to ensure complete solvent permeability. To improve extraction efficiency, the sample was then subjected to ultrasonication for 10 minutes at 25°C. The resulting mixture was filtered using filter paper and equal volume of water was added. To further purify the sample, it was rinsed twice with a solvent (20-30 ml per step) and repeated three times with dichloromethane (Ghassempour et al., 2009). The methanolic extract was then subjected to rotary evaporation to remove dichloromethane, resulting in a dry matter that was dissolved in 5 ml of high-purity acetonitrile and stored at -20°C until analysis (Malik et al., 2011).

Determination of the of paclitaxel using HPLC

The amount of paclitaxel in the stem and leaf of *T. bacata* and *T. brevifolia*, as well as in the callus obtained from these species, was determined using high-performance liquid chromatography (HPLC) with a Merck-Hitachi system (Germany). The measurements were carried out using a C68 column with dimensions of 4.6 × 250 mm. The mobile phase consisted of methanol, acetonitrile, and water at a ratio of 40:40:20 (v/v/v), with a flow rate of 1 mL/min. The UV detection wavelength was set at 230 nm, and the injection rate for both the standards and samples was 20 µL. Prior to injection, the sample was filtered twice using a 0.42 µm filter. The amount of paclitaxel in each sample was calculated by injecting a Paclitaxel-USA standard. The resulting data were reported as mg paclitaxel/g fresh weight of callus. HPLC is a widely used analytical technique for the analysis of phytochemicals, including paclitaxel. The use of HPLC in this study enables the accurate and precise quantification of paclitaxel in the samples from *T. bacata* and *T. brevifolia*, as well as in the callus cultures derived from these species. The obtained data provide insight into the potential of these cultures as a sustainable and cost-effective source of paclitaxel for pharmaceutical applications.

Data analysis

The study was conducted based on a completely randomized design with a factorial arrangement of 6 treatments and 3 replications. The treatments included three levels of 2,4-D (1, 2, and 3 mg/L) and two levels of Kin (0.2 and 0.5 mg/L), as well as two

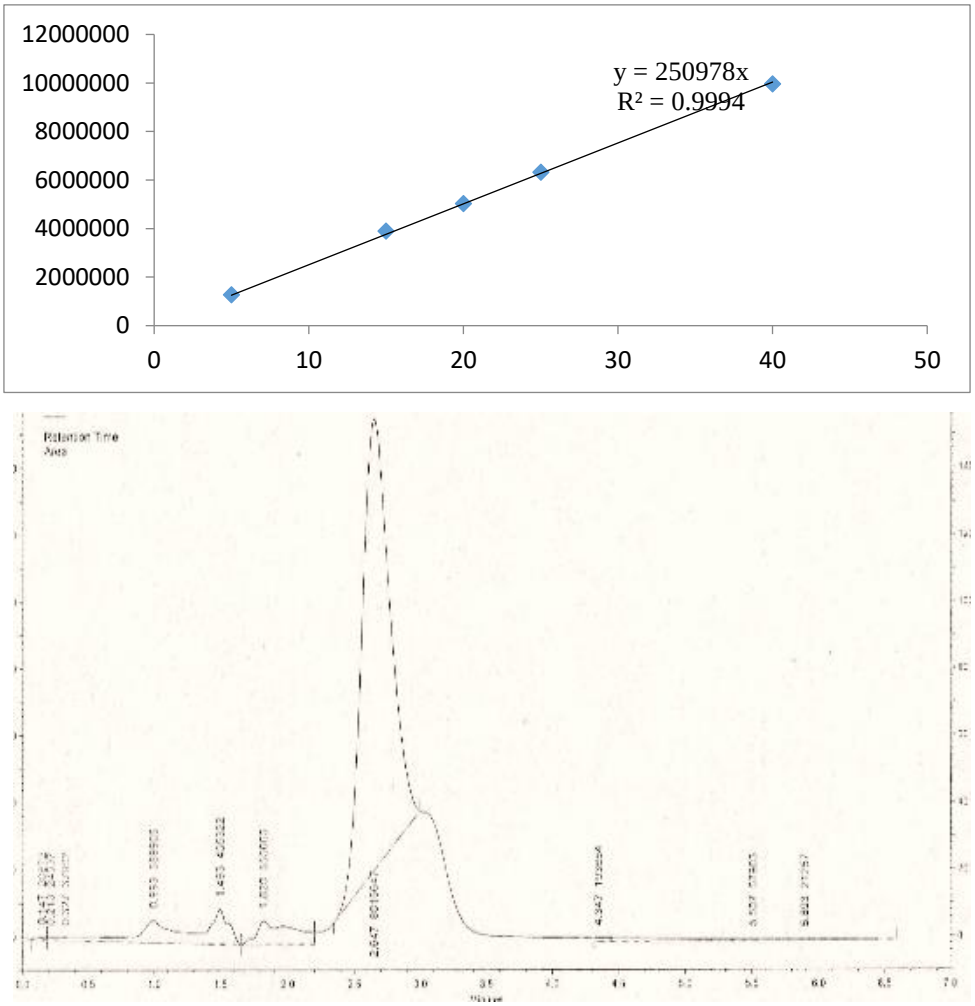

Figure 1. Calibration curve (A) and chromatogram (B) of Paclitaxel.

species of *T. baccata* and *T. brevifolia* and two organ types (stems and leaves). The data were analyzed using SPSS software version 16, and the mean values were compared using the least significant difference (LSD) test at a 5% probability level. Graphs were generated using Microsoft Excel version 2010. The use of a factorial design allows for the investigation of the effects of multiple factors and their interactions on the response variable. In this study, the aim was to optimize the production of callus cultures from *T. baccata* and *T. brevifolia* through the manipulation of growth regulators. The statistical analysis and LSD test enable the determination of significant differences between treatments, while the use of graphs facilitates the visualization of the results. Overall, the experimental design and statistical analysis used in this study enhance the rigor and reproducibility of the results.

Results

Explant treatments

In the present study, due to the high levels of surface contamination observed, different methods were used for surface disinfection of plant organs. As shown in Table 1, for both species of woody perennials tested, treating the stem samples with ethanol 70% for 15 seconds and then sodium hypochlorite (5% active chlorine) for 25 minutes was found to be the best method of surface disinfection. For leaves, 70% ethanol for 5 seconds and sodium hypochlorite (5% active chlorine) for 20

minutes resulted in the best surface sterilization (Table 1).

Effect 2,4-D and Kin on morphological characteristics

While callus formation from stem explants in both species began 15 days after culture initiation, callus formation from leaf explants in *T. baccata* began after only 12 days. In the leaf explant of *T. brevifolia*, callus formation occurred slightly later than in *T. baccata* (Figure 2). Callus formation in *T. baccata* leaves occurred throughout the explant, whereas in *T. brevifolia*, callus formation was observed only at the base of the petiole.

The results showed that among the used treatments, the highest rate of callus growth was observed in stem explants, followed by petioles and leaves (Figure 2). The callus color remained green for the first four weeks after formation. To prevent color change and promote continued growth, the calli were subcultured after 4 weeks in media with the same nutrient and hormonal composition, but with the addition of 200 mg/L of activated charcoal to refresh the media. The color of the subcultured calli returned to orang-brown. The resulting calli had a soft and friable texture at all levels of treatments.

The effect of 2,4-D and Kinetin on callus fresh weight

In this study, we investigated the effect of two plant growth regulators, 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin, on callus formation in stem and leaf explants of two species of the genus Taxus, *T. baccata* and *T. brevifolia*.

Callus formation is a crucial step in plant tissue culture and can be used for the mass propagation of plants, the production of secondary metabolites, and the study of plant cell growth and differentiation. The fresh weight of callus was used as a measure of callus formation after four weeks of culture on Murashige and Skoog (MS) medium supplemented with different concentrations of 2,4-D and kinetin. Our results showed that the combination of 2,4-D and kinetin had a significant effect on callus fresh weight in both species.

Table 1. Effect of surface sterilization on microbial contamination and burning of stem and leaf explant of two Yew species in invitro conditions

Plant species	Treatment	Burning%	Contamination%
T.baccata			
Stem	30 s ethanol 70% + 20 m sodium hypochlorite 5%	35.5	86.1
	50 s ethanol 70% + 25 m sodium hypochlorite 5%	62	5.5
	15 s ethanol 70% + 25 m sodium hypochlorite 5%	4.7	0
Leaf	20 s ethanol 70% + 10 m sodium hypochlorite 5%	0	100
	20 s ethanol 70% + 15 m sodium hypochlorite 5%	60	61.1
	5 s ethanol 70% + 20 m sodium hypochlorite 5%	10	0
T.brevifolia			
Stem	30 s ethanol 70% + 20 m sodium hypochlorite 5%	0	100
	50 s ethanol 70% + 25 m sodium hypochlorite 5%	66.6	11.1
	15 s ethanol 70% + 25 m sodium hypochlorite 5%	11.6	11.1
Leaf	20 s ethanol 70% + 10 m sodium hypochlorite 5%	0	100
	20 s ethanol 70% + 15 m sodium hypochlorite 5%	36.11	100
	5 s ethanol 70% + 20 m sodium hypochlorite 5%	0	44.4

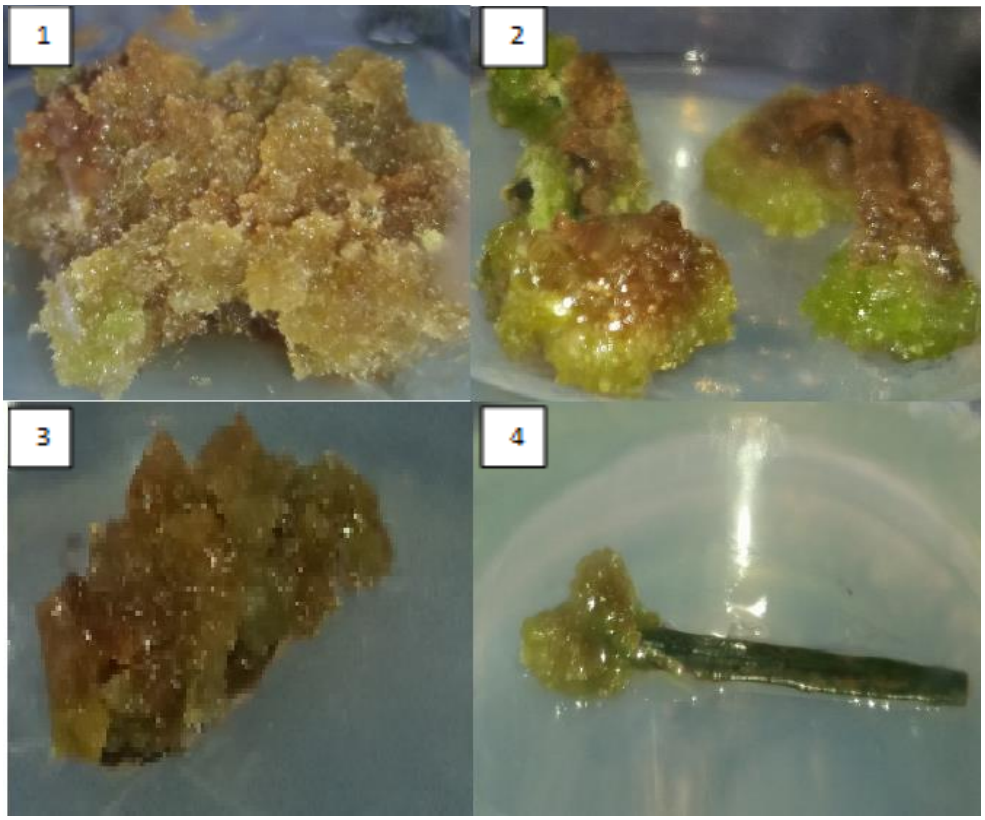

Figure 2. Callus formation in *T. baccata* and *T. brevifolia* stem and leaf explants(1) *T. baccata* stem, (2) *T. baccata* leaf, (3) *T. brevifolia* stem, (4) *T. brevifolia* leaf.

Table 2. Analysis of variance of the effect of 2,4-D, Kin and their interaction on phenol, total alkaloids and total fresh weight of callus.

Source of variation	df	Total phenol	Total alkaloid	Total fresh total sugar weight	
2,4-D	3	50.119**	13.74**	12.318**	2.845^{ns}
Kin	2	0.001^{ns}	13.74**	36.739*	0.319^{ns}
Species	1	58.75**	8.15**	20.119**	32.86**
2,4-D×kin	6	8.261**	9.275**	3.996*	0.102^{ns}
Kin× Species	2	164.905**	26.494**	24.307**	94.293**
2,4-D×Species	3	4.142**	8.13**	5.685**	0.168 ^{ns}
Species×2,4-D× Kin	6	4.078**	3.289*	0.991^{ns}	1.047^{ns}
Erro	42	0.001	0.003	0.058	0.002

ns, *, ** : non-significance and significance at the level of 5% and 1%, respectively.

Specifically, it has been found that the optimal combination of 2,4-D and kinetin for stem explants of *T. baccata* was 2 mg/L 2,4-D and 0.1 mg/L kinetin, while the optimal combination for leaf explants of *T. brevifolia* was 1 mg/L 2,4-D and 0.1 mg/L kinetin. These findings suggest that the use of appropriate concentrations of 2,4-D and kinetin can enhance callus formation in these species, which may have important implications for their propagation and the production of secondary metabolites.

The results of the analysis of variance showed that the effect of 2,4-D and kinetin individually, as well as their interaction, on callus fresh weight was significant, as shown in Table 2. Increasing the concentration of both hormonal agents individually resulted in an increase in callus fresh weight, as shown in Figures 3(b) and 3(a). The interaction effect of treatments was also found to be significant, as shown in Figure 3(d). Although increasing the concentration of kinetin did not have a significant effect on callus growth compared to increasing the concentration of 2.4-D, under the hormonal combination of 3 mg/L 2.4-D and 0.5 mg/L kin, and 2 mg/L 2.4-D and 0.5 mg/L kin, no significant difference was observed, as shown in Figure 3(d).

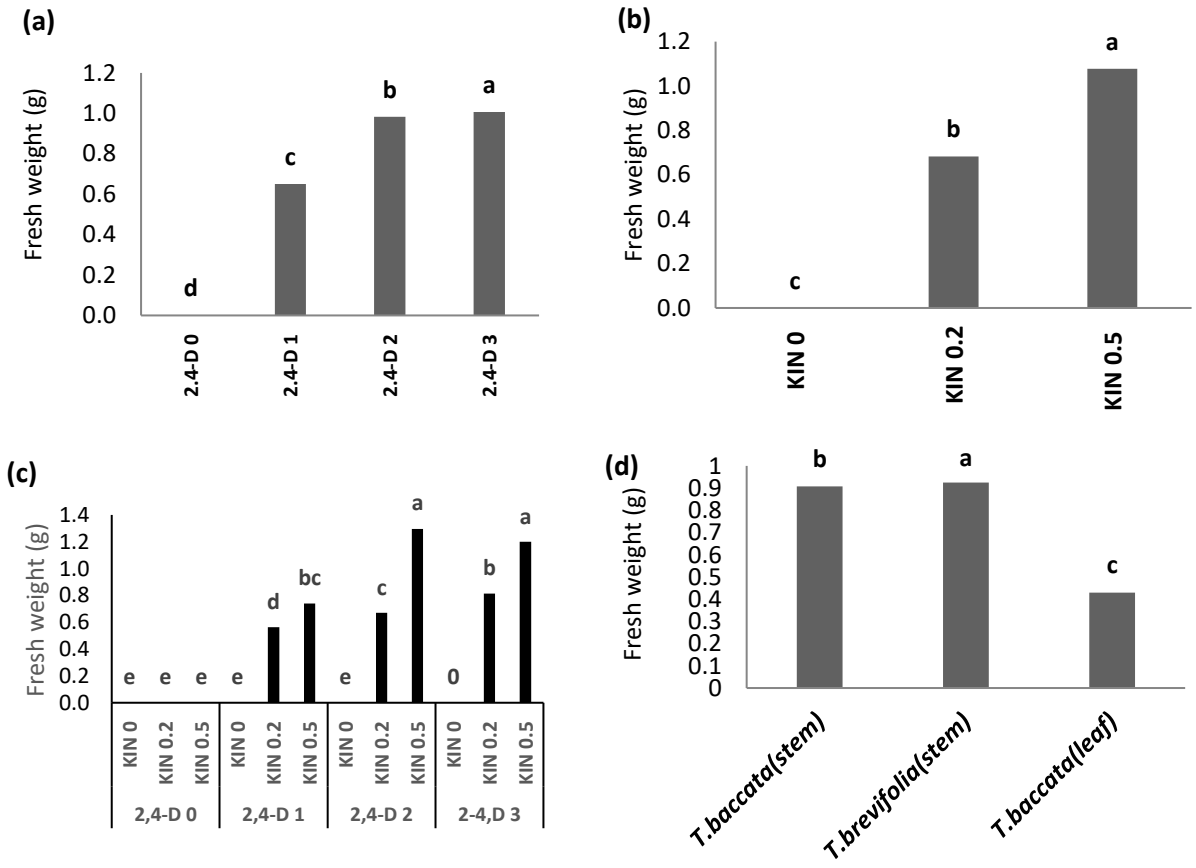

Figure 3. Effect of kin on yew callus FW (a), effect of 2,4-D on yew callus FW (b), callus FW of different yew species (c), interaction effect of treatments on callus FW(d).

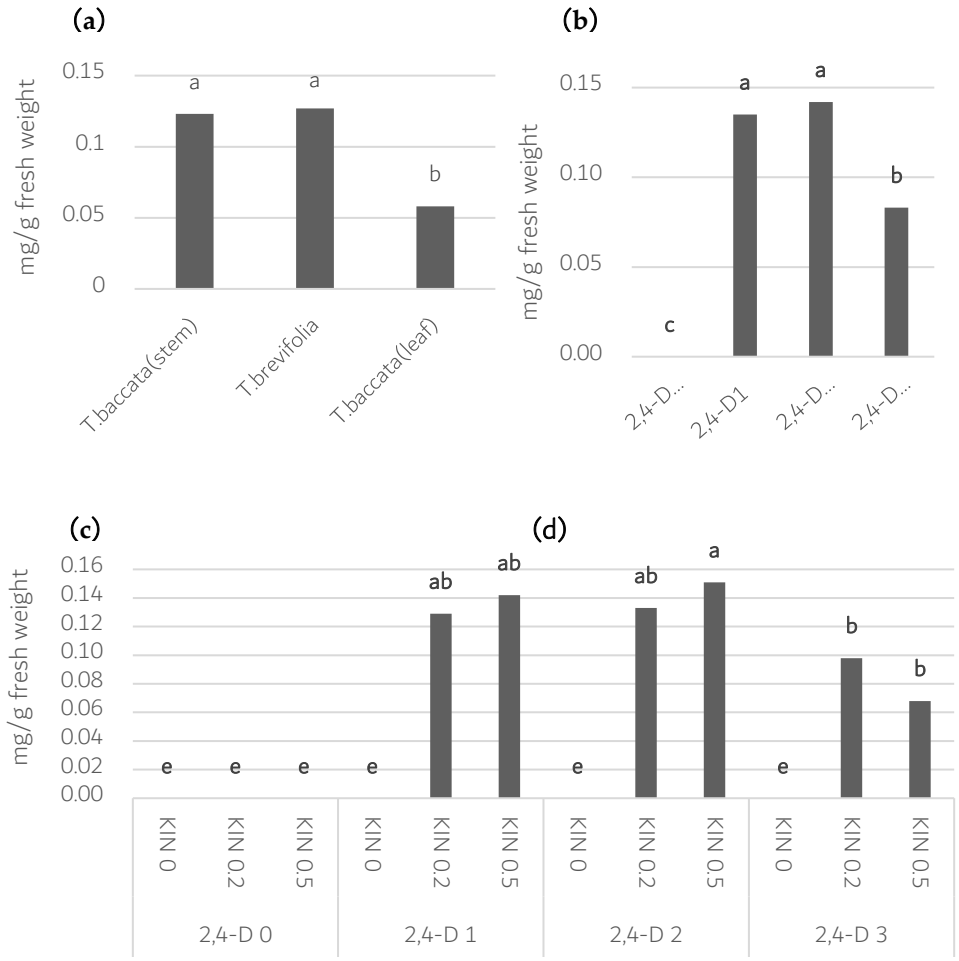

Figure 4. Effect of plant species on phenol content of callus (a), Effect of 2,4-D on phenol content of callus(b), Interaction effect of 2,4-D and kinetin on the phenol content of yew callus(c).

The effect of different levels of 2,4-D and Kin on the amount of total phenol of yew callus in two different species

In this study, the effect of different levels of 2,4-D and kinetin on the total phenol content of callus cultures from two yew species, *Taxus baccata* and *Taxus brevifolia* was investigated. The results showed that the effect of 2,4-D, the interaction effect of 2,4-D × kinetin, and the triple effect of 2,4-D × kinetin × species was significant on the amount of total phenol of callus in both species (Table 2).However, total phenol decreased with increasing 2,4-D concentration (Figure 4(b)). Phenol accumulation of leaf explants was found to be less than stems explants in both species. However, there was no significant difference between the phenol content of calli produced on stem explants (Figure 4(a)). Under the interaction of 2,4-D and kinetin, the amount of phenol decreased with increasing the concentration of treatment (Figure 4(c)). On the other hand, between the treatment of 2 mg/l of 2,4-D and 0.5 mg kinetin and 2 mg/l 2,4-D and 0.2 mg/l kinetin, no significant difference was observed. A similar trend was observed between 3 mg/l 2,4-D and 0.2 mg/l kinetin, with 3 mg/l 2,4-D and 0.5 mg/l kinetin. Unlike 2,4-D, kinetin does not appear to play an effective role in tissue phenolic compounds accumulation.

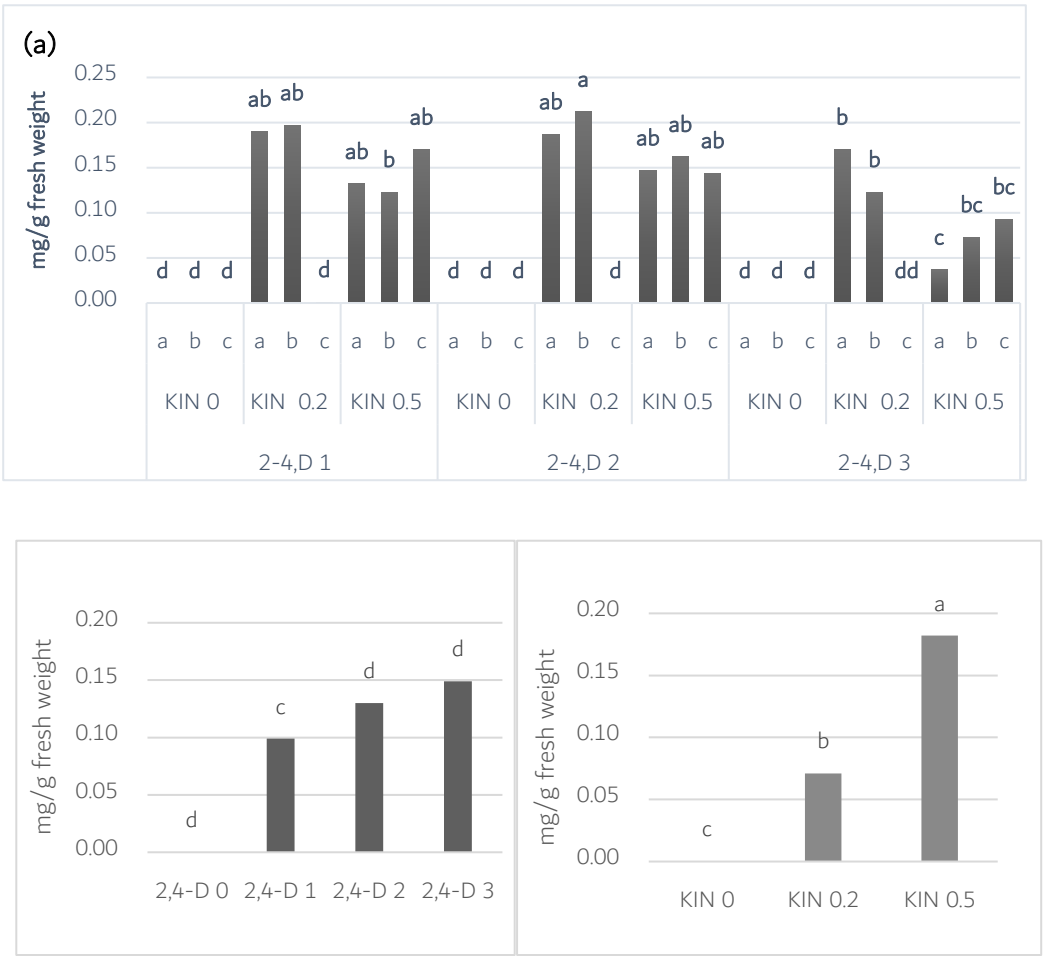

Figure 5. Interaction effect of 2, 4-D × Kin × species on the phenol content of yew callus (a = Baccata stem explant, B = *T. baccata* leaf petiole explant. C = *T. brevifolia* stem explant) (a), Effect of kinetin concentration on the total alkaloid (b), Effect of 2,4-D on the total alkaloid(c).

The effect of different levels of 2,4-D and Kin on the total alkaloid content of two species of yew

The analysis of variance in Table 3 showed that the total alkaloid content was significantly affected in both *Taxus baccata* and *Taxus brevifolia* when 2,4-D and kinetin were used alone or together. With increasing 2,4-D and kinetin levels, total alkaloid accumulation increased. However, the increase in alkaloid accumulation of stem explants of both species was higher than *T. baccata* leaves (Figure 5). In the interaction effect of 2,4-D×kinetin (Figure 6(a)), the amount of total alkaloids increased by increasing the kinetin concentration from 0.2 to 0.5 mg/l, regardless of the concentration of 2,4-D. It seems that the role of kinetin was more effective than that of 2,4-D in total alkaloids accumulation. Surprisingly, in contrast to total phenolic accumulation, no significant change was observed in the amount of total alkaloids when 2,4-D concentration increased. These findings suggest that the use of appropriate concentrations of 2,4-D and kinetin can enhance the production of alkaloids in yew callus cultures, but the effect of kinetin may be more pronounced than that of 2,4-D. Further studies are needed to investigate the specific alkaloids produced by these cultures and their potential pharmacological properties. Figure 6 shows that under the interaction of 2,4-D, kinetin, and species, the total alkaloid content of calli obtained from *T. baccata* leaf and stem explants was significantly different from that of callus obtained from *T. brevifolia* stem explant.

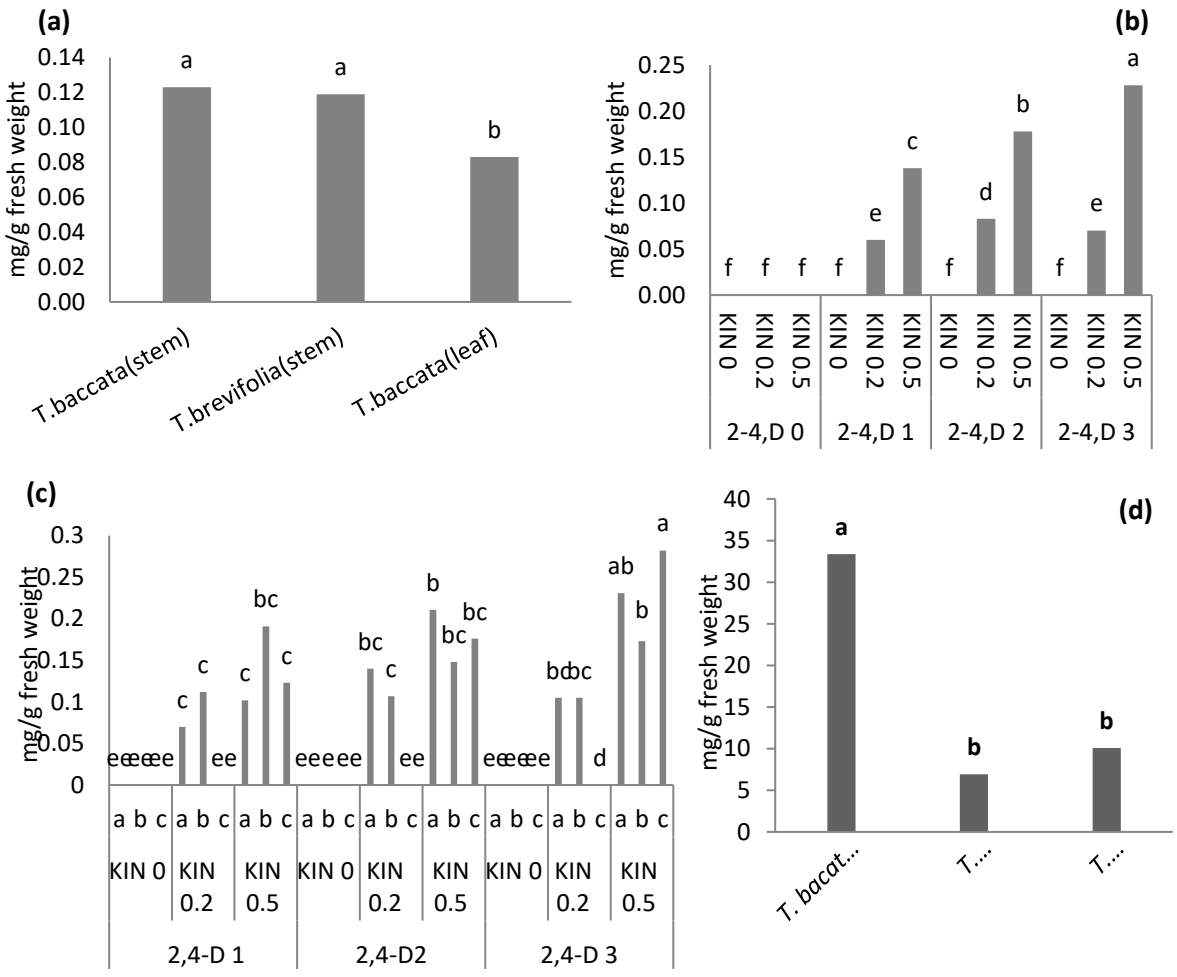

Figure 6. Interaction effect of kinetin×2,4-D on the total alkaloid (a), effect of species on the total alkaloid (b**),** total alkaloid content affected by complex effect of treatments (a =stem callus of *T. baccata*, b =leaf callus of *T. baccata*, c =stem callus of *T. brevifolia*) (c), mean value of paclitaxel affected by plant species(d).

On the other hand, in medium containing 2 mg/l 2,4-D and 0.5 mg/l kinetin, no significant difference was observed between *T. brevifolia* and *T. baccata*. However, there was a significant difference between *T. baccata* explants.

Effect of treatments on the paclitaxel content of sample

Results of this study showed that the amount of paclitaxel in *T. baccata* stem callus was significantly higher (33.36 µg/g) than that of *T. brevifolia* stems (26.45 µg/g) and leaves (23.28 µg/g) (Figure 6(d)). However, no significant difference was observed between the paclitaxel content of calli derived from *T. brevifolia* stem and leaf explants.

Discussion

Surface microbial contamination is one of the most important barriers in in-vitro culture of woody perennials, especially when explants are directly collected from the wild. The first step in tissue culture is the establishment of culture, which means the elimination of surface sterilization of tissue against fungal and bacterial contamination. Plants often contain different sources of microbial contamination, such as microorganisms present on

the surface, in small gaps, emerging leaves, and buds. Therefore, proper control of these microbial contaminants is an important and necessary step to reduce the contamination of explants used in tissue culture (Nikvash et al., 2006; Sarmast, 2018). In both stem and leaf explants, increasing the time of sterilization with ethanol resulted in a temporary reduction in the percentage of contamination, but also reduced the viability of explants. It appears that ethanol enhances the penetration of the main disinfectant solution into the tissue by removing the cuticle layer from the tissue surface. However, ethanol should be used for a short time due to its antimicrobial properties and potential to cause plant toxicity (Asareh et al., 2007). Studies have shown that in *Juniperus communis*, fungal contamination can be controlled in most cases by applying ethanol for a short duration and using an appropriate concentration of sodium hypochlorite (Sarmast, 2018).

Ashrafi et al. (2010) reported that longitudinal sections of the stem show better cell proliferation in tissue culture. They suggested that callus formation from the cambium and the outer parenchymal tissues of the stem is likely due to the larger area provided for the absorption of central components. Compared to the stem, the callus formed in leaf explants was smaller and weaker, probably due to the smaller amount of meristematic tissue in the leaf. Based on the type of explant, stem explants were found to be more effective than leaf explants for callus formation in both species, which is consistent with findings reported by other researchers (Ashrafi et al., 2010). The culture media and various growth regulators can affect callus quality, which depends on the type and amount of elements present in the culture medium (macro and micronutrients, vitamins, etc.). It has been reported that culture media containing low concentrations of kinetin (Kin) and high concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) can promote the formation of callus with a rapid growth rate and a soft and loose structure (Davarpanah et al., 2015), which was also observed in the results of the present study. Overall, the callus formation potential of *T. baccata* was found to be greater than that of *T. brevifolia* for both types of explants (Figure 2). This may be due to differences in the environmental and geographical conditions of the habitats of the two species. It should be noted that callus production of different species and even subspecies of the same species might have different responses to culture media and hormonal composition (Bruňáková et al., 2004).

The lowest callus fresh weight was observed in the control treatment. Our observations showed that explants cultured in media containing no hormonal compounds did not exhibit either callogenesis or organogenesis, indicating that the presence of growth regulators is essential for the formation of callus (Brukhin et al., 1996; Bagheri and Saffari, 2011).

We found that media containing 2.4-D at 3 mg/L was recommended for callus production from both stem and leaf explants of *T. baccata*. Karimian et al. (2014) also reported that the role of 2.4-D in callus production of *T. brevifolia* stem explant was stronger than that of kinetin. The results of the mean comparison (Figure 3(c)) showed that the fresh callus weight of *T. baccata* stem and leaf was lower than that of *T. brevifolia* stem explants. However, the callus formation of *T. baccata* leaf samples was less than that of the stem (Figure 3(c)). These results are consistent with those of Razavi et al. (2017) and Ghafoori et al. (2012), who reported that callus formation in *T. baccata* stem explants is significantly higher than that in explants prepared from the leaf.

Application of appropriate concentrations of 2,4-D was found to enhance the production of phenolic compounds in yew callus cultures, which may have important implications for the production of bioactive compounds from yew tissue cultures. However, browning is one of the most important barriers to in vitro culture, which may affect the production of phenolic compounds. Tissues cultured under in vitro conditions may become brown for various reasons, including wounds caused by cutting explants, contamination with pathogens, oxidation of phenolic compounds and tannins, high concentration of sterilizers agents, pH adjustment, ambient heat, etc. (Chee, 1995). Therefore, strategies to reduce browning in tissue cultures should be considered to improve the production of phenolic compounds.

In general, the findings of this study suggest that the manipulation of plant growth regulators can have a significant impact on the production of phenolic compounds in yew callus cultures. Further studies

are needed to investigate the specific phenolic compounds produced by these cultures and their potential pharmacological properties.

These results suggest that the genetic differences between the two yew species may play a role in the production of alkaloids in callus cultures. Therefore, optimization of growth conditions and plant growth regulators should be tailored to the specific species being studied. Khataee and Karimi (2010) showed that the simultaneous application of BA (benzyladenine) at 0.5 and 1 mg/l, with different concentrations of NAA (naphthalene acetic acid), significantly increased the alkaloid content of callus compared to the control. These findings suggest that the use of appropriate combinations of plant growth regulators can enhance the production of alkaloids in yew callus cultures. Further studies are needed to investigate the specific alkaloids produced by these cultures and their potential pharmacological properties.

The production of alkaloid tropane in tissue culture is indeed highly dependent on the composition of the culture medium. Previous studies have shown that various factors, such as nutrient sources, growth regulators, and different growth conditions, can significantly affect the production of tropane alkaloids in tissue culture (Iranbakhsh et al., 2007). The type and concentration of growth regulators have been found to be important factors that strongly influence the accumulation of these metabolites. To optimize the production of tropane alkaloids in tissue culture, it is crucial to carefully select and adjust the growth conditions and growth regulators used. The use of appropriate combinations of growth regulators, such as auxins and cytokinins, has been shown to enhance the production of tropane alkaloids in tissue culture . In addition, the use of elicitors, such as methyl jasmonate and salicylic acid, can also stimulate the production of tropane alkaloids in tissue culture .

Overall, understanding the factors that affect the production of tropane alkaloids in tissue culture is essential for the development of efficient and sustainable methods for the production of these important metabolites. Further studies are needed to elucidate the specific mechanisms underlying the regulation of tropane alkaloid biosynthesis in tissue culture and to optimize the production of specific alkaloids with potential pharmacological properties.

Despite the fact that paclitaxel was first identified in *T. brevifolia*, studies have shown that the amount of this compound and its derivatives in some other species of this genus, such as *T. baccata*, is higher than that of *T. brevifolia*. However, it should be noted that the rate of paclitaxel varies from species to species and even from organ to organ, and can vary between 0 and 500 µg/g dry weight (Vidensek et al., 1990). Previous studies by other researchers (Ahadi et al., 2013) have reported that, in general and under natural conditions, the potential of paclitaxel production is higher in *T. baccata* compared to *T. brevifolia*. This tendency was also observed in the in vitro studies of the present study (Figure 6(d)). These findings suggest that tissue culture techniques can be used to produce paclitaxel and other important metabolites in a controlled and sustainable manner. Further studies are needed to optimize the production of specific metabolites with potential pharmacological properties and to elucidate the underlying mechanisms of their biosynthesis in tissue culture.

Conclusion

The results of the study showed that the disinfection method used for tissue culture depends not only on the type and duration of disinfectant but also on the different plant species and organs being used. Therefore, it is recommended to establish a reliable disinfection method for the specific plant species and organs before conducting the main experiment. The best hormonal treatment for callus induction in yew trees was found to be 3 mg/l of 2,4-D in combination with 0.2 mg/l of kinetin, regardless of the type of treatment. The fresh weight of *T. brevifolia* callus was higher than that of *T. baccata* under equal conditions. However, the callus from *T. baccata* stems had a higher weight than that from its leaves. The study also found that an increase in the concentration of 2,4-D and kinetin in the medium led to an increase in the total alkaloid content of the callus. However, the alkaloid content of the callus induced from the stem of both species was higher than that obtained from the leaves of *T. baccata*. In contrast, an increase in the concentration of 2,4-D led to a decrease in the total phenol content of the callus. Furthermore, the phenolization of leaf

explants was found to be less than that of stem explants in both species. However, no significant difference was observed between the stem phenolization rates of the studied species. It is suggested that in future studies, the effect of different concentrations of hormones be evaluated in more detail, and the metabolic concentration in the culture medium should also be investigated to optimize the production of specific metabolites with potential pharmacological properties.

Supplementary Materials

No supplementary material is available for this article.

Author Contributions

Laboratory analysis, data curation, writing- original draft preparation A.J.; Idea, supervision methodology, A.Gh.; Advisor, M.K.S., K.R. Authors have read and agreed to the published version of the manuscript.

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Conflict of Interest Statement

The authors declare no conflict of interest.

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مقاله پژوهشی

القا و رشد کالوس، و همچنین تغییرات متابولیت، دو گونه Taxus تحت شرایط درون شیشه‌ای

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چکیده: مطالعه حاضر به بررسی اثر تنظیم‌کننده‌های رشد بر کمیت و کیفیت کالوس دو گونه Taxus می‌پردازد. آزمایشی به صورت فاکتوریل در قالب طرح کاملاً تصادفی با استفاده از 2,4-D و کاینتین بر ریزنمونه‌های برگ و ساقه گونه‌های *T. baccata* و *T. brevifolia* انجام شد. صفاتی چون وزن تر، فنل کل، آلکالوئید کل و محتوای پاکلی‌تاکسل مورد بررسی قرار گرفت. اثر ساده تیمارهای هورمونی بر میزان فنل کل، آلکالوئید کل و وزن تر کالوس معنی‌دار بود. محتوای فنل کل و وزن تر تحت تأثیر اثر متقابل تیمارهای هورمونی قرار نگرفت، در حالی که محتوای آلکالوئید کل تحت تأثیر تیمارها قرار داشت. محتوای پاکلی‌تاکسل تحت تاثیر نوع ریزنمونه قرار نداشت. بیشترین مقدار پاکلی تاکسل در ریزنمونه برگ *T .baccata* با ۳۰ میکروگرم بر گرم در مقایسه با ۵ میکروگرم بر گرم و ۱۰ میکروگرم در گرم به ترتیب در ساقه *T .baccata*، ساقه *T. brevifolia* و برگ *T. brevifolia* مشاهده شد. وزن تر و محتوای آلکالوئید کل کالوس ساقه هر دو گونه بیشتر از برگ بود. سرخدار یک گیاه دارویی ارزشمند در حال انقراض بوده که به خوبی به تیمار آزمایشگاهی پاسخ می‌دهد. با توجه به امکان تولید متابولیت‌های ارزشمند این گیاه در شرایط آزمایشگاهی، تحقیقات بیشتر درخصوص تولید پاکلی‌تاکسل درگونه T. baccata در شرایط درون شیشه‌ای توصیه می‌شود.

کلمات کلیدی: آلکالوئید، متابولیت، پاکلیتاکسل، تاکسول، کشت بافت.

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Phylogenetic analysis of *Solanum macrocarpon*: the evolutionary relationships and species diversification

Original paper

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Abstract: *Solanum macrocarpon* L., commonly known as African eggplant, originates from Africa and is consumed worldwide for medicinal and culinary values. Despite its immense value, there is limited research about eggplant species diversity and evolutionary relationships. This study sought to investigate the evolutionary relationships and species diversification using molecular phylogenetic data of DNA sequences obtained from the rbcL gene of the plant species. Maximum Likelihood and Neighbour-Joining tree methods were employed to infer phylogeny using MEGA11 software. Results obtained inferred using branch lengths revealed the consensus; *S. macrocarpon* was slightly diverse than another identical species *MH722376.1_Solanum macrocarpon*; the only sample of *rbcL* region found on the NCBI database. The results also showed that OM965625.1_*Solanum violaceum*_subsp._*multiflorum*, OM965624.1_*Solanum violaceum* subsp._*violaceum*, and MK122638.1_*Solanum_pubescens* had relatively closer evolutionary relationship with *S. Macrocarpon.* This result provides information and resources about the evolutionary relationships and species diversity of *S. macrocarpon* which can be used for plant breeding, conservation biology, and biosystematics.

Keywords: Phylogenetic analysis, DNA barcoding, solanum, RBCL.

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Introduction

Solanum macrocarpon L. commonly known as the local garden egg, African eggplant is a plant of the Solanaceae family and the genus *Solanum*. *Solanum* is the largest genus in the family Solanaceae, comprising about 2000 species distributed in the subtropical and tropical regions of Africa, Australia, and parts of Asia, e.g., China, India, and Japan (Kaunda and Zhang, 2019). In Africa and adjacent islands, it is represented by at least 100 indigenous species; about 20 of these are recent introductions. *Solanum macrocarpon* has an African ancestry. Spiny wild forms are found throughout the tropical non-arid parts of Africa. the eggplant fruits still being gathered occasionally as a vegetable, constitute an important fruit and leaf vegetable, grown for the market and in home gardens. *S. macrocarpon* is a tropical perennial plant that is closely related to the eggplant (Oboh et al., 2005). *Solanum macrocarpon* is consumed in various regions around the world. The parts of the plant that are consumed are the fruits and its young leaves. While taste of both the leaves and the fruit is very bitter, they have a high nutrient yield. Eggplant has a rich content of phytochemicals, namely, saponins, alkaloids, and flavonoids as well as minerals, bioactive components that are associated with promoting health (Usunomena and Chinwe, 2016) Plant genomes hold the key to understanding the evolutionary history of plants. This phylogeny is both a record of present and past life serving as a powerful predictive tool for both basic and applied plant science (Soltis and Soltis, 2021). Phylogenies can guide our efforts to improve crop plants, discover new medicines, and develop effective conservation strategies.

Over the years, DNA markers have been employed to trace plant phylogeny, primarily derived not from the large and complex nuclear genome, but from the plastid genome (Soltis and Soltis, 2021). The renowned study of by Chase et al. (1993) on the phylogeny of seed plants demonstrated the value of the plastid *rbcL* (ribulose-1,5-bisphosphate carboxylase/oxygenase large) gene (which encodes the large subunit of the most abundant enzyme on earth, ribulose 1,5-bisphosphate carboxylase/oxygenase, RuBisCO) in plant phylogenetics. The plastid genome, beginning with *rbcL*-based studies and analyses of complete plastid genomes has provided a compelling view of plant phylogeny (Gitzendanner et al., 2018).

DNA barcoding is a species identification tool that uses a short section of the genome that provides a genetic signature of the species. The main advantage of this novel technique is that it requires a small sample of tissue (Trujillo-Argueta et al., 2022). In most animal groups, this technique is very effective. However, in plants, the recommended standard markers, such as *rbcL*a (ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit A gene) may not always work, and their efficacy remains to be tested in many plant groups (Trujillo-Argueta et al., 2022). Of the possible plant markers, *rbcL*a appears to be one of the best plant barcodes, because of its successful amplification and sequencing. Although far from perfect, the resolution of *rbcL*a was shown to be better than other markers such as *mat*K when tested both barcodes in wild arid plants in the United Arab Emirates (Maloukh *et al*., 2017) and when tested alone, in plants of Saudi Arabia (Bafeel *et al*., 2012). Also, *rbcL*a can be a valuable tool to identify species in conditions in which other methods are impractical. For instance, this marker was successfully used to study root diversity patterns in old-field communities in Ontario, Canada (Kesanakurti *et al*., 2011).

The use of molecular markers and DNA barcoding in eggplant diversity studies has thrown light into the taxonomic darkness of the plant (Aguoru et al., 2015). According to the limited published research, this study aims to assess the genetic diversity and examine the phylogeny of *Solanum macrocarpon* (garden eggplant) in African locales using *rbcL* markers.

Materials and Methods

Materials collection

Two accessions of African eggplant NGB00247 and NG/MR/MAY/09/007 were used in the study. The seeds were collected from the National Center for Genetic Resources and Biotechnology (NACGRAB), Ibadan. The seeds were planted with each accession planted in three replicates.

DNA extraction

Young leaf samples of *S. macrocarpon* were used. A Qiagen DNeasy Plant Mini Kit was used for DNA isolation. Amplification and sequencing assays were carried out for the extracted DNA. The experiments were carried out in the Molecular Laboratory of the Department of Plant Biology, University of Ilorin, Ilorin, Kwara State.

Amplification and sequencing of RBCL

PCR amplification was performed with the primer as shown in Supplementary Table 1. The PCR was carried out with a total reaction volume of 30µl in a thermocycler (Eppendorf, Germany). Template preparation and optimum concentration for cycle sequencing reactions are shown in Supplementary Table 2. The reaction mixture consisted of 20-50 ng of genomic DNA, 10 X PCR buffer, 2.5 µM dNTPs, 5 pmol primer and 1 unit of Taq DNA polymerase (Genet.Bio. Korea). The thermocycler condition included an initial denaturation of 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 30secs. Annealing was at 55 °C for 30s and extension was at 72 °C for 1 min. The final extension was at 72 °C for 5mins (Supplementary Table 3). The amplicon was purified with EZ-10 Spin Column PCR product purification Kit (Bio Basic Inc. Ontario, Canada). The PCR setup for cycle sequencing reaction was done in a total reaction volume of 10µl and PCR condition included an initial denaturation of 96 °C for 1 min in 1 cycle, denaturation at 96 °C for 10s, and 30 cycles of annealing at 50 °C for 5s. Extension for the reaction was at 60 °C for 4 min and the final extension was done at 60 °C for 7 mins (Supplementary Table 4). The DNA sample to be sequenced was combined with rbcl primer, DNA polymerase, and DNA nucleotides (dATP, dTTP,dGTP, and dCTP). The four dye-labeled,chain-terminating dideoxynucleotides were added as well. The mixture was first heated to denature the template DNA (separate the strands) for 1 min, then cooled so the primers could bind to the single-stranded template (Abdulkareem et al., 2023). When the primer bounded, the temperature was raised again, allowing DNA polymerase to synthesize new DNA starting from the primer. No further nucleotides can be added, the strand ended with the dideoxy. This process was repeated in several cycles. The dideoxynucleotide was incorporated at every single position of the target DNA in at least one reaction (Abdulkareem et al., 2023). The various alleles were sequenced using 3130xl Genetic analyser (Applied Biosystems, CA, USA). The obtained sequences were edited by Sequence Scanner software v1.0 Applied Biosystems, CA, USA), and the full-length sequences were assembled using a local alignment algorithm CodonCode Aligner version 4.24 (Codon Code Corporation).

Data analysis

The obtained forward and reverse sequences were grouped using the SeqTrace 9.0 tool and were submitted to the NCBI (National Center for Biotechnology Information) for BLAST (Basic Local Alignment Search Tool) analysis. The query cover of the sequences was identified considering E value a<10 and maximum hits (99 or 100%) with species in the reference database of the NCBI. Evolutionary analysis was conducted in MEGA11 (Tamura et al., 2021). In the phylogenetic analysis, DNA sequences were aligned using the MUSCLE (MUltiple Sequence Comparison by Log-Expectation) algorithm for sequence alignment, The alignment process considered gaps and mismatches to maximize sequence similarity (Edgar, 2004). Genetic distances were calculated using the Tamura-Nei model, which accounts for different rates of nucleotide substitution and varying GC content among sequences.(Tamura and Nei, 1993). Phylogenetic trees were constructed using Maximum Likelihood (Kumar et al., 2018) and Neighbor-Joining (Saitou and Nei, 1987) methods. Bootstrap analysis was conducted with 1000 replicates to assess the reliability of the phylogenetic trees (Felsenstein, 1985). Cophenetic matrix and mantel tests were carried out to determine the best-fit tree using RStudio software version 4.4.1.

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei, 1993). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value.

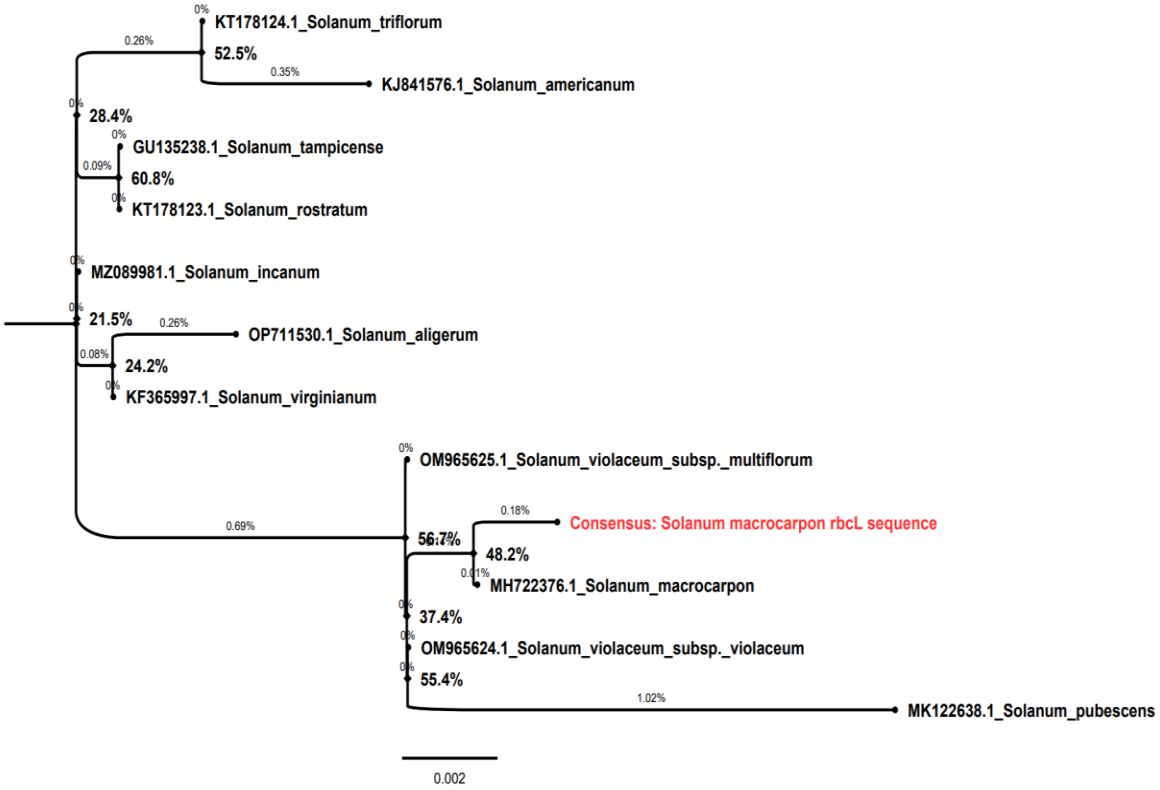

Figure 1. Phylogenetic analysis of *Solanum macrocarpon rbcL* sequences by Maximum Likelihood method.

Results

rbcL gene isolation

The DNA which was extracted from *Solanum macrocarpon* was sequenced for the *rbcL* (Ribulose 1,5 biphosphate carboxylase/oxygenase large subunit) gene. The length of the consensus sequence was 569 bp. The DNA sequences are provided in the supplementary material (see Supplementary Figure 1).

Evolutionary analysis

In this study Maximum Likelihood and Neighbour Joining trees were initially constructed, However, cophenetic matrices for each tree and Mantel tests were conducted to compare these matrices. The Mantel test results indicated that the ML and NJ trees had a very high correlation (Mantel statistic r = 0.78, p = 0.001), suggesting that these methods provided the most consistent representation of the genetic distances among represented taxa. Therefore, the ML tree was selected as the best-fit tree for our analysis due to its statistical value (Figure 1).

The phylogenetic analysis revealed that the Consensus: *Solanum macrocarpon* rbcL sequence clustered with *MH722376.1_Solanum macrocarpon*; an identical species as the consensus, forming a monophyletic group with a bootstrap support value of 48.2%. however, the branch length of Consensus: *Solanum macrocarpon* rbcL sequence is relatively longer than that of *MH722376.1_Solanum macrocarpon* with branch length values of 0.18% and 0.01% respectively thus inferring some degree of evolutionary divergence between the identical species. Also, Consensus: *Solanum macrocarpon* rbcL sequence belongs to a clade attached to a node with bootstrap support value of 56.7% containing four other members belonging to the Solanum genus including OM965625.1_*Solanum violaceum_subsp._multiflorum*, OM965624.1_*Solanum violaceum_ subsp._violaceum*, *and*

MK122638.1_Solanum_pubescens. Concerning the branch lengths within the clade containing the consensus species, the results revealed that *MK122638.1_Solanum_pubescens* is the most genetically evolved species from the last most recent common ancestor (MRCA) in the group with branch length value of 1.02%; a value relatively higher than that of the consensus. Results also revealed that there was no genetic divergence between OM965625.1_*Solanum violaceum_subsp._multiflorum* and OM965624.1_*Solanum violaceum_ subsp._violaceum* since their last divergence from a most recent common ancestor (MRCA).

Furthermore, it is evident from the phylogenetic results that Consensus: *Solanum macrocarpon* rbcL sequence is in polyphyletic relationship with two other clades with bootstrap support values of 21.5% and 28.4% respectively with both clades having members of genus solanum including *KF365997.1_Solanum virginianum, OP711530.1_Solanum aligerum, MZ089981.1_Solanum incanum and KT178123.1_Solanum rostratum, GU135238.1_Solanum tampicense, KJ841576.1_Solanum americanum, KT178124.1_Solanum triflorum* respectively. Regarding the evolutionary divergence of the major clades from their MRCA, the clade containing the consensus has a relatively higher branch length of 0.69% signifying a higher evolutionary divergence while other clades with bootstrap support values 24.2%, 60.8% and 52.5% had branch lengths of 0.08%, 0.09% and 0.26% respectively.

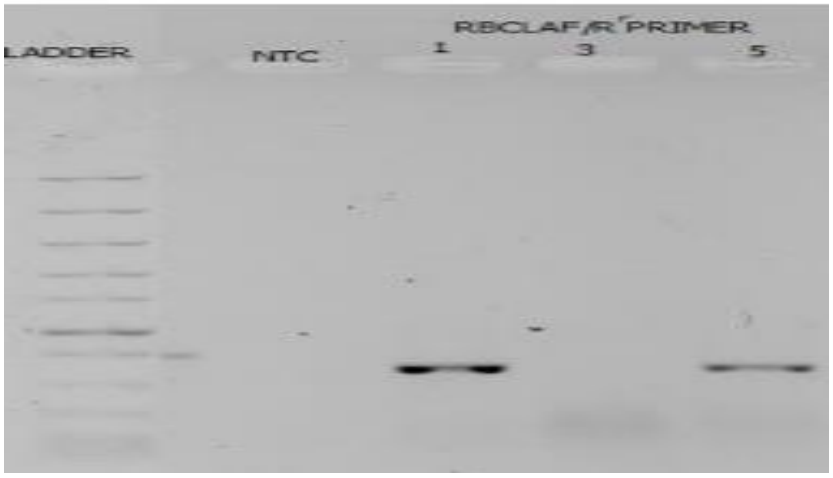

Figure 2. Gel image of amplicons of *S. macrocarpon* using RbcL primer.

Discussion

Evolutionary relationship was inferred using the Maximum Likelihood (ML) method between the consensus: *Solanum macrocarpon* rbcL, other isolates of *Solanum macrocarpon* and other related species. The choice of adopting the ML method was inferred by the strong positive correlation of cophenetic matrix and significance of mantel test (Mantel statistic r = 0.78, p = 0.001), suggesting that these methods provided the most consistent representation of the genetic distances among Solanum sequences used. Also, the likelihood of a phylogenetic tree is proportional to the probability of observing the comparative data (such as aligned DNA sequences) conditional on the tree and the tree that maximizes the likelihood can be chosen as the best estimate of phylogeny; this is the method of maximum likelihood (Huelsenbeck, 2019). Maximum Likelihood estimation conducted for this study revealed that the Consensus: *Solanum macrocarpon* rbcL sequence clustered with *MH722376.1_Solanum macrocarpon*; an identical

species thus inferring the accuracy of rbcL primers in identifying *Solanum macrocarpon* to the species level. This finding is consistent with the work of Ralte and Singh (2021), where 15 Solanaceae species were successfully identified using rbcL DNA regions. However, the branch length values of the former and later inferred evolutionary divergence between the two where Consensus: *Solanum macrocarpon* rbcL sequence had a relatively longer branch length with a difference of 0.17% signifying a slightly higher genetic diversity than *MH722376.1_Solanum macrocarpon.* Branch lengths have been considered an indicator of feature diversity on the assumption that a longer branch represents more opportunity for evolutionary change and the development of unique characteristics (Faith, 2018 as cited in Ritchie *et al*., 2020). This difference might be due to environmental factors or the distal isolation of the 2 species as *MH722376.1_Solanum macrocarpon* with 708bp was isolated in Thailand according to (Prommanee *et al*., 2018 as cited in NCBI, 2024) while the consensus with 569bp was isolated in Nigeria with a bootstrap support value of 48.2 at their node. According to Liu *et al*. (2023), the isolation-by-distance (IBD) reveals that genetic differentiation among populations increases with geographic distance, while the isolation-by-environment (IBE) assumes a linear relationship between genetic variation and environmental differences among populations.

The phylogenetic analysis placed members in the genus Solanum; OM965625.1_*Solanum violaceum_subsp._multiflorum*, OM965624.1_*Solanum violaceum* subsp. *violaceum, and MK122638.1_Solanum pubescens* in the same clade with the consensus indicating that they all share the same most recent common ancestor (MRCA) at the node with bootstrap support value of 56.7%. However, in terms of evolutionary divergence as regards this clade, *MK122638.1_Solanum pubescens* was revealed to be the most diverse amongst members of the clade with the highest branch length of 1.02% and this placed it in a paraphyletic relationship with the consensus. It is noteworthy that in this clade, 2 subspecies of *Solanum violaceum* namely *Solanum violaceum* subsp. multiflorum and *Solanum violaceum* subps violaceum with accession numbers OM965625.1 and OM965624.1 respectively are the least divergent and closest to the MRCA and could be inferred as an ancestor or close relative of an ancestor of other members of the clade as they were observed to have no branch length from the most recent common ancestor.

With respect to the origin node, with 21.5% bootstrap support value, the consensus *Solanum macrocarpon* formed a polyphyletic relationship with three other clades in the tree with members viz; *KF365997.1_Solanum virginianum, OP711530.1_Solanum aligerum, KT178123.1_Solanum rostratum, GU135238.1_Solanum tampicense, KJ841576.1_Solanum americanum,* and *KT178124.1_Solanum triflorum.* It should be noted that *MZ089981.1_Solanum incanum* was the closest to the node with no branch length and not clustering to any clade signified a close relationship with the ancestor present at the node and it could be possible that *Solanum incanum* is an ancestor or close relative to an ancestor in which some other members evolved from.

Conclusion

This study utilized DNA barcoding of the *rbcL* region to conduct a phylogenetic analysis of *S. macrocarpon*, unveiling crucial evidence about its evolutionary relationships and species diversification. The consensus sequence derived in this study was found to be more evolutionarily advanced than the only *S. macrocarpon* rbcL sequence available in the NCBI database. This research provides a more evolutionarily advanced isolate of *S. macrocarpon*, which could be valuable for crop improvement.

Supplementary Materials

The supplementary material for this article can be found online at: https://www.jpmb-gabit.ir/article_712459.html.

Supplementary Figure 1. The sequence of rbcL gene from Solanum macrocarpon.

Supplementary Table 1. Forward and reverse sequences of primer used.

Supplementary Table 2. Preparation of PCR mixture.

Supplementary Table 3. Thermo-cycler PCR condition for barcode amplification.

Supplementary Table 4. Thermo-cycler PCR condition for cycle sequencing reaction.

Author Contributions

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

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Conflict of Interest Statement

The authors declare no conflict of interest.

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مقاله پژوهشی

تجزیه و تحلیل فیلوژنتیکی گیاه *Solanum macrocarpon*: روابط تکاملی و تنوع گونه‌ها

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چکیده: بادمجان آفریقایی با نام علمی *Solanum macrocarpon* L. در سراسر جهان به منظور کاربردهای دارویی و آشپزی مصرف می‌شود. علیرغم ارزش بسیار زیاد آن، تحقیقات اندکی در مورد تنوع گونه‌های بادمجان و روابط تکاملی وجود دارد. این مطالعه به دنبال بررسی روابط تکاملی و تنوع گونه‌ها با استفاده از داده‌های فیلوژنتیک مولکولی، توالی‌های DNA به‌دست‌آمده از ژن *rbcL* گونه‌های گیاهی مورد بررسی و مقایسه قرار گرفت. برای ترسم درخت فیلوژنی در نرم‌افزار MEGA11 و با استفاده از الگوریتم‌های Maximum Likelihood و Neighbour-Joining صورت گرفت. مقایسه توالی ژن *rbcL* شناسایی شده در این تحقیق با توالی ژنی NCBI نشان داد توالی بدست آمده از تفاوت اندکی با توالی ثبت شده با کد دسترسی *MH722376.1* برخوردار بود. نتایج همولوژی بر مبنای این ژن نشان داد که گونه‌های *Solanum violaceum* subsp.*multiflorum*، *Solanum violaceum* subsp.violaceum و *Solanum pubescens* از رابطه تکاملی نسبتا نزدیکتری با *S. Macrocarpon* برخوردار بودند. نتایج این تحقیق اطلاعات ارزشمندی در مورد روابط تکاملی و تنوع گونه‌های *S. macrocarpon* فراهم می‌نماید که می‌تواند در برنامه‌های اصلاحی گیاهان، افزایش عملکرد و بیوسیستماتیک استفاده شود.

کلمات کلیدی: آنالیز فیلوژنتیک، DNA بارکدینگ، بادمجان، *RBCL*.

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Tolerance of grass pea (*Lathyrus sativus* L.) genotypes to osmotic stress under in vitro conditions

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Original paper

Abstract: Osmotic stress resulting from cell dehydration is caused by water deficit, and leads to the disruption of many cellular functions. The effect of osmotic stress was assessed on seedling and callus parameters of six grass pea (*Lathyrus sativus*) genotypes under *in vitro* conditions. Osmotic stress was induced in the agar media by adding sucrose concentrations corresponding to –4.5 and –8 bar. The growth and morphological traits of *in vitro* seedlings and calli were evaluated 12 and 30 days after exposure to osmotic stress, respectively. In seedlings, genotypes differed significantly for most traits, while the osmotic potential significantly influenced on the dry weight of the rootlet, as well as the fresh weight of the plumule and seedling, and the dry weight of plumule. Rootlet, plumule, and seedling dry matter decreased under osmotic stress. In turn, the higher dose of sucrose led to a reduction in seedling growth of the genotypes. In callus, the genotypes varied significantly for callus fresh and dry weights, as well as final callus size. The effect of osmotic potential was significant on callus fresh weight and relative water contents of the calli. Based on the tolerance indices, ICARDA-I can be recommended as a osmotic stress-tolerant genotype.

Keywords: Callus; dry matter; drought; mesocotyl; seedling.

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Introduction

Grass pea (*Lathyrus sativus* L., Fabaceae), is a legume crop that is used as food and feed. It is a diploid species with chromosome number 2n=2x=14 (Arzani, 2006). The grass pea is a legume that grows in poor fertile and rain-fed soils. Among the various abiotic stresses, drought is one of the major reasons for losses in crop productivity. Against rapid progress in new research strategies for developing crop varieties having low water requirements are few (Huang et al., 2012). Grass pea is a crop highly tolerant to the stress caused by different abiotic stresses (Patto et al., 2006). The grass pea in comparison with other legumes has some morphological traits related to drought tolerance such as low width of leaves and the developed roots. However, research about the mechanisms and rate of drought tolerance in this crop is limited (Anderson et al., 2004).

Environmental factors, such as drought, impose stress on plants and leas to significant crop losses worldwide and a situation that will be exacerbated by climate change. Drought stress reduces leaf size, stem development, and root multiplication, thus reducing water-use efficiency (Flexas and Medrano, 2002). Drought is the most important environmental stressor, especially in arid and semiarid regions, where it impacts affect the growth and development of plants (Lonbani and Arzani, 2011). Plants have developed various mechanisms to tolerate drought stress, including physiological, biochemical, and molecular processes. These mechanisms involve osmotic adjustment and the maintenance of the plant's water status through efficient water use (Hoseini and Arzani, 2023). Osmotic adjustment is widely recognized as a key adaptive strategy for coping with drought stress, enhancing plant productivity (Yu et al., 2024). Osmotic stress conducts to the generation of reactive oxygen species (ROS) which prevent the normal functioning of cells by inactivating or degrading proteins, lipids, and DNA (Blokhina et al., 2003). The application of plant cell and tissue culture is an effective methodology to shorten the plant breeding programs for achieving high yield cultivars resistant to environmental stresses (George et al., 2008).

In this study, tolerance of several high-yielding grass pea genotypes to drought stress was investigated. The objective was to evaluate the grass pea genotypes for drought stress under in vitro conditions, specifically focusing on osmotic-stressed seedlings and calli.

Materials and Methods

This research was carried out at the Laboratory of Plant Production and Breeding Department, Faculty of Agriculture, University of Maragheh, East Azerbaijan Province, Iran. Six grass pea genotypes screened for drought tolerance under field conditions were used in this study. These genotypes originated from the International Center for Agricultural Research in the Dry Areas (ICARDA) were used in this study. Based on the field primary screening on 26 genotypes (data not shown), six tolerant grass pea genotypes (including a Local local check and five ICARDA genotypes namely ICARDA-I, ICARDA-II, ICARDA-III, ICARDA-IV, ICARDA-V) were exposed to the osmotic stress under *in vitro* conditions. The genotypes were exposed to osmotic stress -4.5 and -8 bar that were determined by vant Hoff equation (Piwowarczyk et al., 2014) induce by sucrose supplemented media. The experiment was carried out as a factorial arrangement based on a completely randomized design with four replications. Teratments were six genotypes and two osmotic levels. The experiment was conducted in two experimental groups; a) applying osmotic stress to seedlings *in vitro* conditions and b) applying osmotic stress to the calli of mesocotyl explants under *in vitro*.

The basal medium used in this experiment was the Murashige and Skoog (1962). In this experiment, proline (0.3 g/l), casein (0.3 g/l), and yeast extract (0.1 g/l) were used as growth stimulants to improve the culture status. Ascorbic acid (vitamin C, 50 mg/l) and citric acid (75 mg/l) were added to the culture medium for preventing explants necrosis. In addition to organic supplements, auxin growth regulators (2,4-D and NAA), cytokinin (BAP and Kin) were also used in the medium.

First, the ½ MS medium was prepared by the addition of BAP 10 ppm. After sterilization with autoclave, The sterilized seeds were planted in the media using forceps. The media were transferred to

a germination device at 26+ 2° C with a light intensity of 0.750 lux. After 7-10 days, sterile grown seedlings were ready for explants preparation. Ten seeds of six grass pea genotypes were sown on media containing different sucrose concentrations and cultivated for 14 days in controlled conditions in terms of light, humidity, temperature.

Explant culture for callus induction and evaluation of osmotic tolerance threshold under stress conditions

MS medium supplemented with organic supplements was distributed among sterile disposable Petri dishes. After 24 hours, mesocotyls were obtained from sterile seedlings and placed on the medium. To retain moisture and to control the contamination of the medium and explants, the Petri dishes were tightened with parafilm. They were stored in a dark room at a temperature of 26+2 °C. After the induction of callus subcultures were performed after 30 days for all samples.

After a preliminary experiment and analyzing the results related to the osmotic stress threshold using probit analysis, the osmotic potential (–8 bar) induced with 6% sucrose was determined as the suitable osmotic stress treatment for screening genotypes (data not shown). While, the –4.5 bar osmotic potential was found as the treshhold of osmotic stress and hence used as the control in this study. Then, The research then continued with two experimental groups; imposition of osmotic stress on seedlings under in vitro conditions and on calli.

Osmotic stress imposition on seedlings under in vitro

The seeds were cultured in glass bottles containing ½ MS medium without hormones and placed in a germinator for three days. Seedlings were transferred to a new medium for screening a stress threshold. The new medium was Full-MS with organic supplements as well as 10 mg/l BAP, in two osmotic potentials of -4.5 bar (control) and -8 bar (dry threshold concentration). After transferring the seedlings to a new medium, they were placed in a germinator at a temperature of 25+2 °C under the light.

For seedlings traits assay and, after each culture, the bottles were transferred again to a germinator at 26°C and 1200 lux light intensity. Finally, 7 days after culture, seedlings were evaluated in terms of fresh and dry (24 h at 72 °C), weight of seedling, rootlet and plumule numbers, number of nodes, length of rootlet, plumule, and seedling length, the diameter of rootlet and plumule, number of nodes and the number of leaves.

Osmotic stress imposition on callus samples

The culture medium for this experiment was prepared as full-MS with the addition of organic supplements and growth regulators (2 mg/l 2.4-D, 2 mg/l NAA). It was then divided into two parts, including 3% sucrose (-4.5 bar) and 6% sucrose (-8 bar. The calli derived were transferred to the medium under sterile conditions and grown inside the germinator under darkness conditions. A piece of callus from each genotype was cut into five sections and cultured on the MS medium. After culture, they were closed with parafilm to prevent contamination and moisture loss.

For assaying the *in vitro* traits; Petri dishes were visited every few days for any type of contamination. Then, the explants were evaluated once every five days for callus size and callogenesis index (explants produced callus per total number of explants). After 30 days, fresh and dry (48 hours at 72ºC) callus weight (mg), length and diameter of rootlet and plumule (mm), number of nodes and shoots, the relative growth rate of callus (RGR), and the relative water content (RWC, %) were evaluated.

Osmotic stress tolerance-related indices were calculated using seedling and callus dry weight to select the most tolerant genotypes. Tolerance index (TOL) [Rosielle and Hamblin, 1981], Mean productivity (MP) (Rosielle and Hamblin, 1981), Stress tolerance index (STI) (Fernandez George, 1992), Geometric mean productivity (GMP) (Fernandez George, 1992), Harmonic mean (HARM) (Fernandez George, 1992), Stress susceptibility index (SSI) (Fischer and Maurer, 1978), Relative decrease index (RDI) (Bidinger et al., 1987), Stress susceptibility percentage index (SSPI) (Mousavi et al., 2008), Stress non-stress production index (SNPI) (Mousavi et al., 2008) and Index of tolerance based on RGR (INTOL) (Soheilikhah et al., 2013) were evaluated as well.

Data Analysis

Analysis of variance and mean comparisons (Duncan's multiple range test) were performed. For

classifying genotypes, cluster analysis by Ward's algorithm and Euclidean distance coefficient was employed. Statistical analysis was done by SPSS and NTSYS softwares.

Results and Discussion

Seedling

Analysis of Variance

Genotypes varied significantly for the number of plumules, radicle diameter, seedling length, rootlet length, seedling length, rootlet length, dry weight of rootlet, fresh weight of plumule, fresh weight of seedling, dry weight of plumule, and dry weight of rootlet. Furthermore, the effect of osmotic potential on the dry weight of rootlet, fresh weight of plumule, fresh weight of seedling, and dry weight of plumule was significant. Genotype×osmotic potential interaction was non-significant on all traits except the number of nodes (Table 1). Fallahi et al. (2015) by applying osmotic potential using PEG on grass pea found that seed germination rate, stem length and root length decreased with increasing osmotic potentials. França et al. (2000) reported that drought stress reduced stem length in *Phaseolus vulgaris* cultivars. Drought stress reduced germination percentage and seedling growth parameters in black gram (Murillo - Amador et al., 2002; Pratap and Kumar Sharma, 2010). furthermore, reported that PEG-induced drought stress reduced the percentage and rate of germination and emergence of cowpea seedlings and also reduced the initial seedling growth.

Mean comparisons (Table 2) showed that genotype ICARDA-I had the highest recorded data for seedling length, rootlet diameter, number of plumules, number of leaves, fresh weight of plumule, fresh weight of seedling, dry weight of plumule, and dry weight of seedling. The highest rootlet length and dry weight of rootlet belonged to genotype ICARDA-V. The interaction effect of genotype×osmotic stress was significant only in shoot fresh weight. Genotype ICARDA-III × osmotic stress -8 bar had the highest shoot fresh weight.

Osmotic stress tolerance indices based on plumule dry weight

The estimation of osmotic stress tolerance indices based on plumule dry weight for six grass pea genotypes (the table is not inserted), the MP index, ICARDA-I has been defined as the most tolerant genotype due to its highest value and high yield in both stress and non-stress conditions. This index decreases in other genotypes, and ICARDA-III was more sensitive to stress. Rosielle and Hamblin (1981) showed that in most yield experiments, the correlation between MP and YS as well as MP and YP were positive. So, selection based on the MP index generally increases the yield of genotypes under both stress and non-stress conditions. GMP and STI indices had the highest value for ICARDA-I genotype. Considering, ICARDA-I was selected as the most tolerant genotype. Also, in other genotypes, these two indices decreased, and finally, ICARDA-III had the lowest value for both indices. Fernandez George (1992) used harmonic mean index (HARM) for the selection under stress conditions. Based on this index, ICARDA-I is selected as a tolerant genotype. The harmonic index data was decreased in other genotypes. In SNPI index (Mousavi et al., 2008), genotypes with higher values and in the SSPI index, genotypes with lower values are more tolerant against stress. So, according to SNPI, ICARDA-I was the most tolerant genotype and ICARDA-III was the drought-sensitive genotype. If the value of the RDI index Tsakiris and Vangelis (2004) is greater than one; the genotype is somewhat tolerant, and for the values less than one, the genotype is sensitive to drought stress. According to this index, the RDI of ICARDA-I, ICARDA-IV, and ICARDA-V genotypes was greater than one and are known as tolerant genotypes. This trait was higher in ICARDA-I than other genotypes. Other genotypes had less than one, which are known as drought-sensitive genotypes. Stress sensitivity index (SSI) is based on the performance of individual genotypes in both stress and non-stress environments, as well as the average performance of all genotypes in these two environments (Fischer and Maurer, 1978). High values of SSI indicate the sensitivity of genotypes to osmotic stress. Genotypes with low SSI had less yield changes and high stability under stress conditions than in non-stress environments. So, according to the SSI index; ICARDA-I was the most tolerant and ICARDA-III was considered the most sensitive genotype.

Table 1. Analysis of variance for the seedling traits of grass pea genotypes under in vitro osmotic stress conditions.

Source of Variation	df	MS							
		Number of rootlets	Number of nodes	Number of plumules	Radicle diameter	Plumule diameter	Seedling length	Plumule length	Rootlet length
Genotype (G)	5	34.88	0.50	38.04*	0.58**	0.22	7.34*	1.16	7.74**
Osmotic Potential (OP)	1	3.06	0.16	3.37	0.05	0.00	0.03	0.84	0.09
G*OP	5	6.37	1.96*	26.17	0.12	0.05	4.71	0.24	2.32
Error	12	15.88	0.83	12.04	0.11	0.17	3.16	1.14	1.56
CV (%)		12.05	24.3	3.1	17.6	10.01	15.6	29.5	27.1

* and ** are significant at 0.01 and 0.05 levels, respectively

Table 1 (continued)

Source of Variation	df	Dry weight of Seedling	Fresh weight of plumule	Fresh weight of Rootlet	Fresh weight of seedling	Dry weight of plumule	Dry weight of rootlet
Genotype (G)	5	243.34**	7555.00*	10.29.58	22473.57*	128.64**	71.46**
Osmotic Potential (OP)	1	237.37**	35420.17**	2115.93	42168.17*	315.37**	2.82
G*OP	5	27.27	4038.57	489.76	5015.37	20.17	6.93
Error	12	36.37	3546.33	7823.54	12650.25	12.20	9.68
CV (%)		15.1	27	30.1	21.1	14.1	19.6

Table 2. Mean comparison for the effects of osmotic stress on some seedling traits of grass pea genotypes.

Genotype	Rootlet length (cm)	Seedling length (cm)	Rootlet diameter (mm)	Number of plumule	Fresh weight of plumule (mg)	Fresh weight of seedling (mg)	Dry weight of plumule (mg)	Dry weight of rootlet (mg)	Dry weight of seedling (mg)
Local check	2.65b	6.5b	1.79b	15.5a	241.25a	367.75ab	28.25a	18.25ab	46.5a
ICARDA-I	2.9b	7.27ab	2.6a	14.25ab	257.5a	423.25ab	30.25a	13.75bc	44a
ICARDA-II	5.87a	9.62a	1.72b	9.75bc	236.5a	371.5ab	26.5ab	15.25bc	41.75a
ICARDA-III	5a	6.62a	1.44b	7.75c	136.75b	238b	14.75c	14bc	22.25b
ICARDA-IV	4.5ab	7.62ab	1.99b	9.75bc	237.25a	321.25ab	27.25ab	10.5c	37.75a
ICARDA-V	5.75a	9.37ab	1.72b	9.25bc	210.75ab	446.75a	21.75b	22.75a	44.5a

Same letter in Duncan grouping are not significantly difference (p≤0.01)

The lower values of the TOL index (Rosielle and Hamblin, 1981) indicate more tolerance of the genotype to stress. According to the TOL index, ICARDA-I was selected as the most tolerant genotype, whose performance in a stressful environment had low reductions than in a stress-free environment, i.e., the mentioned genotype had a reasonable yield stability. Also, the TOL index increased in other genotypes.

Callus

The genotypic effects on callus dry weight and fresh weight was significant. The osmotic potential effect was significant on callus fresh weight and RWC of callus. The genotype×osmotic potential interaction effect was significant on final callus size and callus growth rate (Table 3). A mean comparison showed that ICARDA-I had the highest callus dry weight (Table 4). Figure 1 shows the calli of a grass pea genotype after 14 days culturing mesocotyl explants under.in vitro osmotic potentials of – 4.5 and – 8 bar.

Osmotic stress tolerance indices for calli dry weight

In MP, GMP, STI, and HARM indices, the higher values show more resistance of genotype to osmotic stress. Accordingly, ICARDA-I had the highest value in the above indices which was considered as the most tolerant genotype to osmotic stress. Also, in other genotypes, the values of these indices decreased and finally, the control genotype was the most sensitive to osmotic stress. In the RDI index, the values greater than one are resistant and smaller than one is sensitive to osmotic stress. Again, ICARDA-I was chosen as the most tolerant genotype. The lower values of SSPI and TOL indices indicate resistance to stress. Based on these two indices, ICARDA-I genotype was characterized as stress-tolerant as well (Table 5). The first and most important effect of osmotic stress is impaired germination and poor seedling establishment (Harris et al., 2002). In a study on five chickpea cultivars, drought stress impaired germination and early growth of seedlings (Okçu et al., 2005). In addition, in alfalfa, germination potential, hypocotyl length, plumule fresh weight, rootlet fresh weight, plumule dry weight and rootlet dry weight decreased due to water deficiency (PEG), while rootlet length increased (Zeid and Shedeed, 2006).

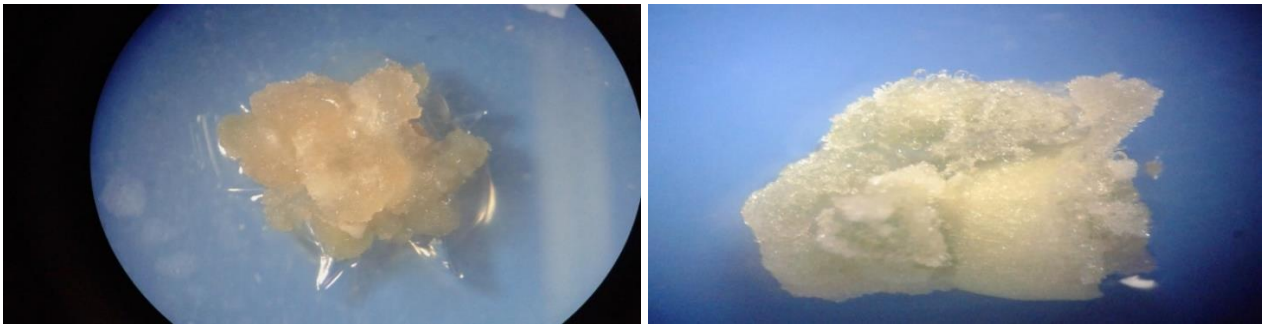

Figure 1. Callus of a grass pea genotype after 14 days of culture on media containing -4.5 and -8 bar sucrose.

Table 3. Analysis of variance for the *in vitro* traits of callus for the grass pea genotypes exposed to osmotic stress.

Source of Variation	df	MS				
		Calli fresh weight	Calli dry weight	Calli RWC	Final callus size	Callus growth rate
Genotype (G)	5	484.46**	15.74**	0.002	1.74**	0.0013
Osmotic Potential (OP)	1	286.73*	3.12	0.0086**	0.22	0.00006
G×OP	5	74.41	2.96	0.0006	1.84**	0.0025*
Error	24	62.73	2.86	0.0013	0.41	0.0008
CV (%)		24.22	47.70	4.09	6.54	35.74

Growth occurs through cell division, cell enlargement, and differentiation, involving genetic, physiological, environmental, and morphological events and their interactions. The quality and quantity of plant growth depend on the events that are affected by water deficit under drought stress (Taiz and Zeiger, 2006). Any disturbance in cell division, elongation, and development due to drought stress reduce plant height, leaf area, and overall plant growth (Kaya et al., 2006; Hoseini and Arzani, 2023). *In vitro* culture is commonly used to select drought-tolerant plants considering the differences between the response of plants to *in vivo* and *in vitro* conditions (Mohamed et al., 2000). Plant dry weight is one of the realistic criteria in determining the response of plants to various stresses such as drought, salinity, and metal toxicity (Talukdar, 2013). In bean plants subjected to drought stress using PEG, germination and seedling growth, were strongly affected and, it was concluded that inhibition of germination is mainly due to the osmotic effect of PEG (Garg, 2010). Drought stress in eight alfalfa reduced total plant growth and increased the ratio of dry weight that led to a significant reduction in the relative amount of leaf water potential (Lonbani and Arzani, 2011). Grass pea was subjected to drought stress using PEG, which showed that the plant height, stem fresh weight, root fresh weight, and leaf area decreased compared to the control (Jiang et al., 2013). In bean plants that assayed under drought stress and non-stress conditions; stem dry weight, root dry weight, and nodal dry weight were decreased under water deficit compared to the control conditions (Kabbadj et al., 2017).

Table 4. Mean comparison of callus traits between different grass pea genotypes under osmotic potential.

Genotype	Callus fresh weight (mg)	Callus dry weight (mg)	Final callus size
Local check	21.63c	1.9b	9.23b
ICARDA-I	45.83a	6.5a	10.40a
ICARDA-II	30.60bc	2.8b	10.16a
ICARDA-III	36.37ab	3.4b	9.35b
ICARDA-IV	24.37c	2.63b	10.17a
ICARDA-V	37.33ab	4.07b	9.24b

The different letters in each column indicate significant differences ($p \leq 0.01$) using Duncan's Multiple Range Test

Table 5. Osmotic stress tolerance indices of grass pea genotypes based on callus dry weight.

Genotype	TOL	SSI	SSPI	RDI	SNPI	HARM	STI	GMP	MP
Local check	0.06	-0.2	0.92	0.82	7.53	0.95	0.34	1.9	1.9
ICARDA-I	-3.27	3.73	-50.15	1.41	-10.87	3.05	3.75	6.29	6.5
ICARDA-II	0.66	1.18	10.15	0.67	5.45	1.38	0.73	2.8	2.8
ICARDA-III	-0.26	0.46	-4.15	0.91	-10.07	1.70	1.09	3.4	3.4
ICARDA-IV	-0.87	2.17	-13.23	1.18	-5.01	1.28	0.64	2.6	2.6
ICARDA-V	0.13	-0.17	2	0.82	16.05	20.3	1.56	4.1	4

Drought stress reduces cell elongation more than cell division rate and hence reduces plant growth by affecting various physiological and chemical processes such as photosynthesis, respiration, transport, and uptake of ions, as well as by interference in the metabolism of hormones (Jaleel et al., 2008).

Piwowarczyk et al. (2014) reported that with in vitro seed culture and stem propagation with BAP and PEG application; the number of branches per plant was significantly reduced, and drought conditions strongly affected the regeneration potential compared to the controls.

Cluster and principal components analysis

Figure 2 shows the grouping of studied grass pea genotypes based on seedling and callus traits. According to analysis, grass pea genotypes are divided into two separate groups. The first group included local and ICARDA-I genotype. The second group consisted of ICARDA-II, ICARDA-III, ICARDA-IV, and ICARDA-V genotypes. In other words, cluster analysis could separate the tolerant ICARDA-I genotype from others. Genotypes that located in the same group, are almost similar. In other words, they do not differ much in terms of the measured traits.

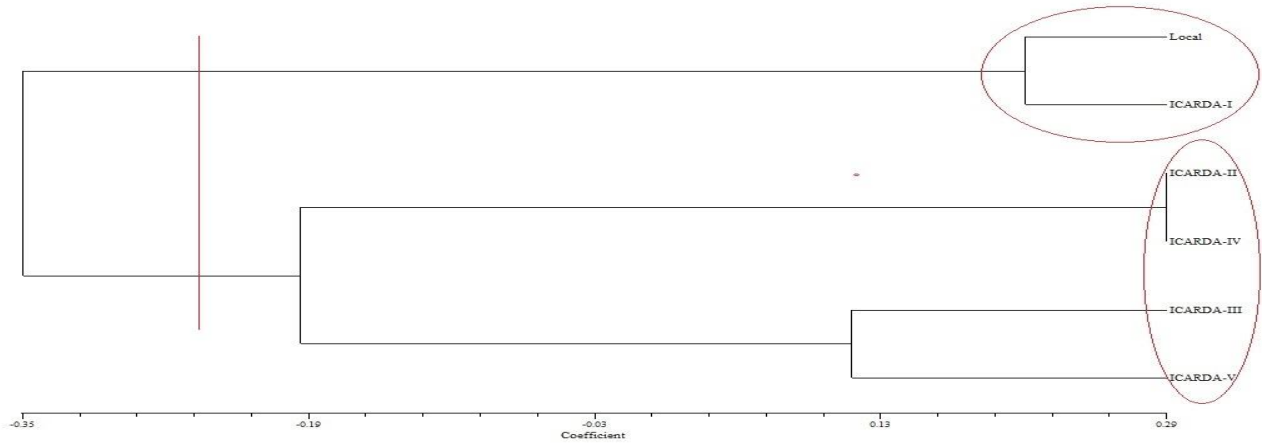

Figure 2. Cluster analysis of the six grass pea genotypes exposed to drought stress in in vitro conditions.

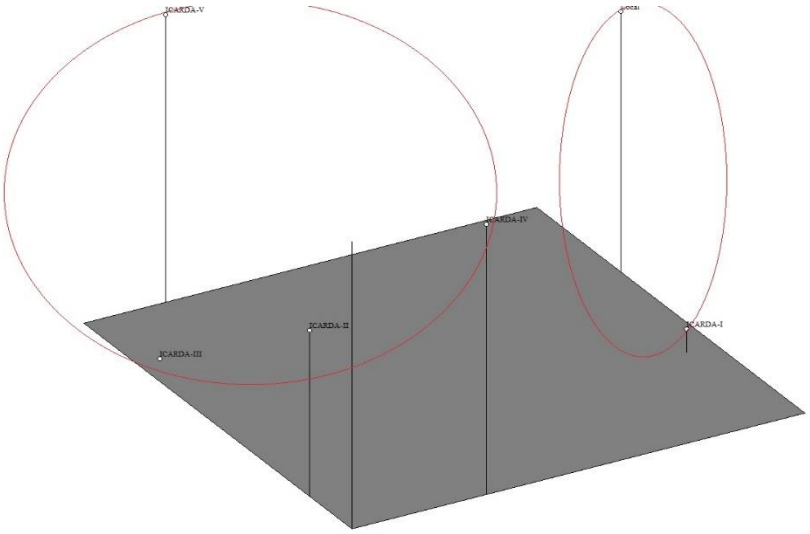

Figure 3. Three-dimensional scatterplot of grass pea genotypes (PCA) subjected to osmotic stress.

Principal components analysis for the grass pea genotypes showed that the first two components explained approximately 63.38% of variations. For medium explanation, these amount indicates relative efficiency of analysis. PCAs two and three-dimensional scatter plots of grass pea genotypes subjected to osmotic stress confirmed the results of cluster analysis (Figure 2) and genotypes were located in two groups as well. In a research by Soofinia et al. (2024), the first three main components of principal components analysis explained 82.08% of the total variation.

Conclusion

The dry weight of shoots was affected by osmotic stress but not the dry weight of the rootlet. Plant organs differed in their sensitivity to osmotic stress; shoots seem to be more sensitive than roots. The seedling indices; MP, GMP, STI, HARM, RDI, SNPI, SSPI, SSI, and TOL introduced ICARDA-I genotype as tolerant and ICARDA-III genotype as a sensitive one. Consistent with the finding of the seedling experimental group, callus indices including GMP, STI, HARM, and RDI identified ICARDA-I as the most tolerant genotype. Therefore, genotype ICARDA-I can be introduced as tolerant to osmotic stress under in vitro conditions. A remarkable reduction in the seedling dry weight was registered, which suggests a high impacts of osmotic stress on the grass pea genotypes. Likewise, evaluation of the osmotic stress tolerance indices resulted in a similar results; that, genotype ICARDA-I attained the higher tolerance related indices under osmotic stress. The osmotic stress tolerance screening criteria related to the callus and seedlings confirmed that ICARDA-I was the most osmotic stress-tolerant genotype under in vitro conditions. Thus, this genotype could be suggested for further studies in the genetic transformation and breeding programs.

Supplementary Materials

There is no supplementary material available for this article.

Author Contributions

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

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Conflict of Interest Statement

The authors declare no conflict of interest.

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مقاله پژوهشی

تحمل ژنوتیپ‌های خلر (*Lathyrus sativus* L.) به تنش اسمزی در شرایط درون شیشه‌ای

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چکیده: اثر تنش اسمزی بر پارامترهای گیاهچه و کالوس شش ژنوتیپ خلر در شرایط درون شیشه‌ای مورد ارزیابی قرار گرفت. تنش اسمزی در محیط آگار با افزودن ساکارز ۴/۵- و ۸- بار القا گردید. ۱۲ و ۳۰ روز پس از اعمال تنش خشکی، صفات مورفولوژیکی گیاهچه‌ای در شرایط درون‌شیشه‌ای و کالوس به‌ترتیب مورد ارزیابی قرار گرفتند. در گیاهچه‌ها، اثر ژنوتیپ بر اکثر صفات و اثر تنش اسمزی تنها بر وزن خشک ریشه‌چه، وزن تر ساقه‌چه و گیاهچه و وزن خشک ساقه‌چه معنی‌دار بود. ماده خشک ریشه‌چه، ساقه‌چه و گیاهچه تحت تنش اسمزی کاهش یافت. بالاترین غلظت ساکارز منجر به کاهش رشد گیاهچه‌ای ژنوتیپ‌ها شد. در کالوس اثر ژنوتیپ بر وزن خشک و تر کالوس و اندازه نهایی کالوس معنی‌دار بود. اثر پتانسیل اسمزی بر وزن تر کالوس و RWC کالوس معنی‌دار بود. به طور کلی، قرار گرفتن در معرض تنش اسمزی باعث کاهش معنی‌دار طول ریشه‌چه و ساقه‌چه گیاهچه خلر نشد، اما بر وزن تر ساقه‌چه و گیاهچه تأثیر گذاشت. با توجه به شاخص های تحمل به خشکی، ژنوتیپ ICARDA-I به‌عنوان ژنوتیپ متحمل می‌تواند توصیه شود.

کلمات کلیدی: تنش اسمزی، کالوس، گیاهچه، ماده خشک، ، مزوکوتیل.

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Original paper

Bioactive compounds and microbial evaluation of African walnuts (*Tetracarpidium conophorum* (Mull. Arg.)) Hutch & Dalziel retailed in Ilorin Metropolis

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Abstract: The African walnut (*Tetracarpidium conophorum*) is a nutrient-dense tropical fruit that provides numerous health benefits and offers a wealth of nutritional value. This study investigated the bioactive compounds and microbes associated with African walnuts retailed in the Ilorin Metropolis. After preparing stock solutions from the obtained nuts, microbial isolations were carried out on Potatoes Dextrose Agar (fungi), Nutrient Agar, and Eosin Methylene Blue Agar (bacteria) using the pour plate method. Sixteen bioactive compounds of very significant therapeutic values were identified in the raw nuts using Gas Chromatography-Mass Spectrometry (GC-MS). Also, molecular identification of the fungal species and biochemical characterization of the bacterial species isolated from the nuts revealed the presence of four fungal species, namely *Aspergillus niger*, *Penicillium rolfsii*, *Rhizopus delemar* and *Rhizopus arrhizus*, as well as three bacterial species, namely *Bacillus cereus*, *Staphylococcus aureus*, and *Escherichia coli*. The GC-MS results revealed the nutritional and medicinal benefits of consuming cooked African walnuts; however, the microbial screening serves as a precaution for those consuming cooked African walnuts retailed in the Ilorin metropolis.

Keywords: GC-MS, microbial screening, molecular identification, African walnuts, antimicrobial.

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Introduction

African walnut (*Tetracarpidium conophorum* (Mull. Arg.) Hutch & Dalziel) is a climbing plant belonging to the Euphorbiaceae family. It is native to the humid regions of Eastern and Western Nigeria, as well as, West Africa as a whole. In Eastern Nigeria, it is called Ukpa in Igbo, while in Western Nigeria, it is referred to as "Awusa" or "Asala" in Yoruba and "Okhue" or "Okwe" in Edo (Asuzu-Samuel and Nnamdi, 2023). They are characterized by their glossy, dark green leaves and small, round fruits, each containing two to four seeds or nuts. The nuts themselves are encased in a tough, woody black shell (Ayodeji and Aliyu, 2018).

Beyond their culinary appeal, African walnuts are revered for their nutritional richness, serving as a concentrated source of essential nutrients (Ayodeji and Aliyu, 2018). Each nut is packed with proteins, healthy fats, dietary fiber, vitamins (such as vitamin E, vitamin B6, and folate), and minerals (including calcium, magnesium, and potassium) (Salem et al., 2022). This nutrient profile not only makes African walnuts a valuable component of a nutritious diet but also confers numerous health benefits, ranging from cardiovascular support to cognitive enhancement on its consumption.

Moreover, African walnuts are a veritable treasure trove of bioactive compounds such as phenolic compounds, flavonoids, alkaloids, and terpenoids (Adetunji et al., 2021). These phytochemicals are endowed with potent antioxidant, antimicrobial, anti-inflammatory and anticancer properties, which contribute to the nut's therapeutic potential. The antioxidant activity of these compounds helps combat oxidative stress and free radical damage, thereby reducing the risk of chronic diseases such as heart disease, diabetes, and cancer (Mukarram et al., 2024).

The nuts of *Tetracarpidium conophorum* are used in Nigeria to increase sperm counts in men (Alenazy, 2023). It was proved that the extract of *Tetracarpidium conophorum* nut increases the viability and sperm output of male albino rats and this suggests that the nuts could be included in the formulation of male fertility drugs (Ikpeme et al., 2014). The nuts of *Tetracarpidium conophorum* have the potential to reduce hyperglycaemia; researchers also reported that the nuts increased the haemoglobin level and decreased urine output in the test group when compared with controls and could prevent diabetes associated with renal damage. In addition to its nutritional and medicinal attributes, African walnuts hold cultural and socioeconomic significance within West African communities (Amusa et al., 2016; Paulussen et al., 2017). Traditionally, the nuts are not only consumed as a snack but also incorporated into various dishes, desserts, and beverages. Furthermore, African walnut cultivation and retailing provide livelihood for many farmers and vendors across the region, contributing to the local economy and fostering social cohesion (Shigaeva and Darr, 2020). Despite its nutritional and medicinal significance, African walnuts are susceptible to microbial contamination during storage, leading to spoilage and potential health hazards (Edeh et al., 2024). In light of these factors, this study aimed to reveal the bioactive compounds present in African walnuts and identify the microorganisms responsible for their potential microbial spoilage

Materials and Methods

Sample collection

Fresh, uncooked walnuts (40 nuts) were obtained from Oja-Oba Market in Ilorin, where they were kept in open baskets placed in shaded areas to protect them from direct sunlight and moisture. After purchasing, the walnuts were transferred to Ziploc bags and brought to the laboratory for microbiological and phytochemical analyses. In the lab, they were stored at 4°C to ensure freshness and inhibit microbial growth until the analyses took place. The forty African walnut nuts were categorized into two groups of twenty nuts each; one for GC-MS analysis and the other groups for microbial assessment. The two groups of African walnut nuts were stored at room temperature prior to the analyses.

Sample preparation for GC-MS

The raw nuts in group one were shelled, sliced, dried, and milled, after which oil was extracted from them.

Extraction of oil from African walnuts

This was conducted using a Soxhlet apparatus. Approximately 40 grams of the pulp (from the

milling exercise) were extracted individually with n-hexane in the Soxhlet apparatus. The solvent was then eliminated to obtain the oil. Any residual solvent in the oil was evaporated gently in a beaker at room temperature. The oil was subsequently refrigerated at 4°C until required, and when needed, it was brought back to room temperature before analysis by taking the oil out of the refrigerator and placed in a clean, dry environment at room temperature. The volatile compounds were then identified using Gas Chromatography-Mass Spectroscopy (GC-MS).

Phytochemical screening of the extracted oil

This was conducted using an Agilent Technologies 8890 Series GC System coupled with an Agilent 5977B Mass Selective Detector with Electron Impact Ionization (GC-MS). A HP-5 capillary column measuring 30 meters in length, with an internal diameter of 0.32 mm and a film thickness of 0.25 µm, was utilized, packed with 5% phenyl methyl siloxane. The following conditions were maintained: injection port temperature set at 250°C (split-less, pressure at 8.35 psi, purge flow of 30 ml/min, purge time of 1.0 min, total flow of 35.35 ml/min); the GC-MS operated in the chosen ion-monitoring mode.

The column oven initiated at 90°C for 3 minutes, followed by a 15-minute programming period at a rate of 30°C/minute (from 90 to 200°C), 5 minutes at a constant temperature (200 to 265°C), and a subsequent 15-minute period at a rate of 3°C/min (from 265 to 276°C). The separated components exited the column and entered the Mass Spectrometer (MS) for analysis. Compound identification was achieved by comparing the extract's retention indices and mass spectra fragmentation patterns with those in published literature. Additionally, the National Institute of Standards and Technology library sources were consulted to match the identified compounds from the walnut (Oladimeji and Adebo, 2023).

Microbiological analyses

Isolation and Identification of Bacterial Species -In the second group, nuts were carefully chosen, cracked open, and weighed using a precise electronic balance. These nuts were then transferred into a sterilized mechanical blender, where a sterile water solution was added in a specific ratio (1 part of walnut to 9 parts of sterile water) before blending. This blending process produced a concentrated solution known as the stock solution. From this stock solution, further dilutions were made up to the third level/category, each being ten times less concentrated than the previous one, with the most diluted solution being labeled as 10^{-3}. A milliliter of inoculum from the 10^{-3} dilution was carefully transferred into pre-labeled 3 sterile Petri dishes. Molten agar media made up of equal volumes of sterilized Nutrient Agar (NA) and Eosin Methylene Blue (EMB) were respectively poured into the Petri dishes. The agar plates were then left to solidify and were subsequently incubated at 37°C for 24 to 48 hours to allow bacterial growth. Following the incubation period, the bacterial isolates present on the agar plates were subjected to a series of biochemical tests to characterize their properties following the procedures of Afolabi et al. (2020). These tests included the catalase test, oxidase test, sugar fermentation test, citrate utilization test, nitrate reduction, casein hydrolysis, urea hydrolysis, and motility test.

Isolation and Identification of Fungal Species- From each of the prepared stock solutions, the pour plate method was utilized to isolate fungi on potato dextrose Agar (PDA), which were then incubated at a temperature of 28°C for 3 to 5 days for fungal growth. Thereafter, pure isolates of the fungal cultures were identified molecularly following a series of stepwise procedures. Firstly, total genomic DNA was extracted from each of the pure fungal isolates using a Zymo Research DNA kit, following a detailed protocol provided by the manufacturer. The Internal Transcribed Spacer (ITS) region of each fungal isolate's DNA was then amplified using a specific primer combination (ITS1 and ITS4), as described by Liu and Chen (2007). This amplification process was carried out using thermal cycler PCR equipment. Subsequently, the amplified DNA sequences were subjected to sequencing at Inqaba Biotech facility in Ibadan. Using the methods outlined by Stucky (2012), a consensus DNA sequence was generated from both the forward and reverse sequences obtained. This consensus sequence served as a unique genetic fingerprint for each fungal isolate.

Table 1. Bioactive compounds in African walnuts.

Peak no.	Name	R.T	Peak area (%)	MF	MW (g)	Compound Nature	Biological activity
1	Nonadecane	11.916	0.88	$C_{19}H_{40}$	268.5	Straight chain alkane	Antioxidant
2	Hexadecanoic acid, methyl ester	13.026	2.35	$C_{17}H_{34}O_2$	270.4	Fatty acids	Antimicrobial, Anti-inflammatory
3	n-Hexadecanoic acid	13.547	11.49	$C_{16}H_{32}O_2$	256.42	Saturated Fatty acids	Antioxidant, Antimicrobial
4	Eicosane	13.598	1.74	$C_{20}H_{42}$	282.5	Unsaturated fatty acid	Antioxidants, anti-inflammatory and cardiovascular protective properties
5	9,12-Octadecadienoic acids, methyl ester,(E,E)-	14.394	2.59	$C_{18}H_{32}O_2$	280.4	Fatty acid methyl ester	Anti-inflammatory
6	9-Octadecenoic acid, methyl ester	14.440	6.04	$C_{19}H_{34}O_2$	294.5	Fatty acids methyl ester	Antioxidant, anti-inflammatory nematicide and anticancer
7	Methyl stearate	14.623	1.23	$C_{19}H_{38}O_2$	298.5	Fatty acids methyl ester	Antimicrobial,antioxidant,Gastrin inhibitor
8	trans-13- Octadecenoic acid	14.937	18.87	$C_{18}H_{34}O_2$	282.5	Long chain fatty acid	Antioxidant
9	9,12,15-Octadecatrien-1-ol, (Z,Z,Z)-	14.966	5.39	$C_{18}H_{32}O$	264.4	Unsaturated fatty acid	Anti-inflammatory, antioxidant
10	Octadecanoic acid	15.069	4.68	C18H36O2	284.5	Saturated fatty acid	Anti-inflammatory ,anticancer, antimicrobial
11	1,15-Pentadecanedioic acid	15.944	1.68	C15H28O4	272.38	Saturated fatty acid	Antimicrobial ,antioxidant ,anti-inflammatory
12	9-Octadecenamide, (Z)-	16.694	2.24	C18H35NO	281.5g	Fatty acid	Anti-inflammatory , neuro protective potential
13	9-Octadecenal,(Z)-	17.747	6.01	C18H34O	266.5g	Fatty aldehyde	Antibacterial, antimicrobial
14	9,12,15-Octadecatrien-1-ol, (Z,Z,Z)-	17.816	4.15	C20H34O2	306.5	Fatty acid ester	Antibacterial , anti-inflammatory, neurological function
15	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	18.348	4.51	C19H38O4	330.5	Carboxylic acid	Antioxidants, nematicide and pesticide , anti-inflammatory
16	Bis(2-ethylhexyl) phthalate	18.714	4.67	C24H38O4	390.6	Phthalate esters	Oral toxicity during pregnancy

The generated ITS DNA sequences were compared with sequences available in the National Centre for Biotechnology Information's (NCBI) GenBank database. This comparison was facilitated using the Basic Local Alignment Search Tool for Nucleotides (BLASTN) Sequences, accessible through the GenBank repository web page. (https://www.ncbi.nlm.nih.gov/). By matching the nucleotide sequences of the fungal isolates with those in the database, their respective species were determined.

Results

GC-MS results

The gas chromatogram of the sample revealed the presence of 16 compounds with different retention times as presented in Figure 1. These compounds were identified through mass spectrometry attached with GC. Table 1 contains the retention times (R.T.s), molecular formulae, molecular weights, compounds nature, peak areas, biological activities and names of the 16 bioactive compounds identified in this study. Nonadecane, Hexadecanoic acid, methyl ester, 9,12-Octadecadienoic acids, methyl ester,(E,E)-, Eicosane, n-Hexadecanoic acid, trans-13-Octadecenoic acid, Methyl stearate, 9-Octadecenoic acid, methyl ester, 1,15-Pentadecanedioic acid, 9,12,15-Octadecatrien-1-ol, (Z,Z,Z)-, Octadecanoic acid, 9-Octadecenal,(Z)-, 9-Octadecenamide, (Z)-, Bis(2-ethylhexyl) phthalate, Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester, 9,12,15-Octadecatrien-1-ol, (Z,Z,Z)-, and 9,12,15-octadecatrienoic acid, (Z, Z,Z)-.

Microbiological analyses

The microbial counts on the unprocessed African walnuts were 7.4× 10^3 and 1.5×10^3, for fungi and bacteria respectively, providing insights into the microbial population present in the nuts (Table 2). Results of all the biochemical tests conducted revealed the identities of 3 bacterial species isolated from the nuts (Table 3), while Table 4 presents the unique NCBI accession numbers assigned to each of the fungal species isolated from the same nuts.

Table 2. Microbial load of raw African walnuts.

Isolates	Counts (×10^3) (cfu/g)
Bacteria	1.5
Fungi	7.4

Table 3. Results of biochemical tests conducted on the bacterial isolates.

Selective biochemical reaction	*Bacillus cereus*	*Staphylococcus aureus*	*Escherichia coli*
Gram reaction	+	+	-
Citrate test	+	+	-
Oxidase test	-	-	-
Motility	Non-Motile	Non-Motile	Motile
Catalase test	+	+	+
Sugar fermentation	+	+	+
Urea hydrolysis	+	+	+
Nitrate reduction	+	+	+
Casein hydrolysis	-	+	+

Table 3. NCBI accession numbers and matched fungal species.

NCBI No.	ID (%)	QC (%)	Matched organism
PP756477	99	99	Rhizopusdelemar
PP756479	99	98	Rhizopusarrhizus
PP756478	100	100	Aspergillusniger
PP756481	99	99	Penicilliumrolfsii

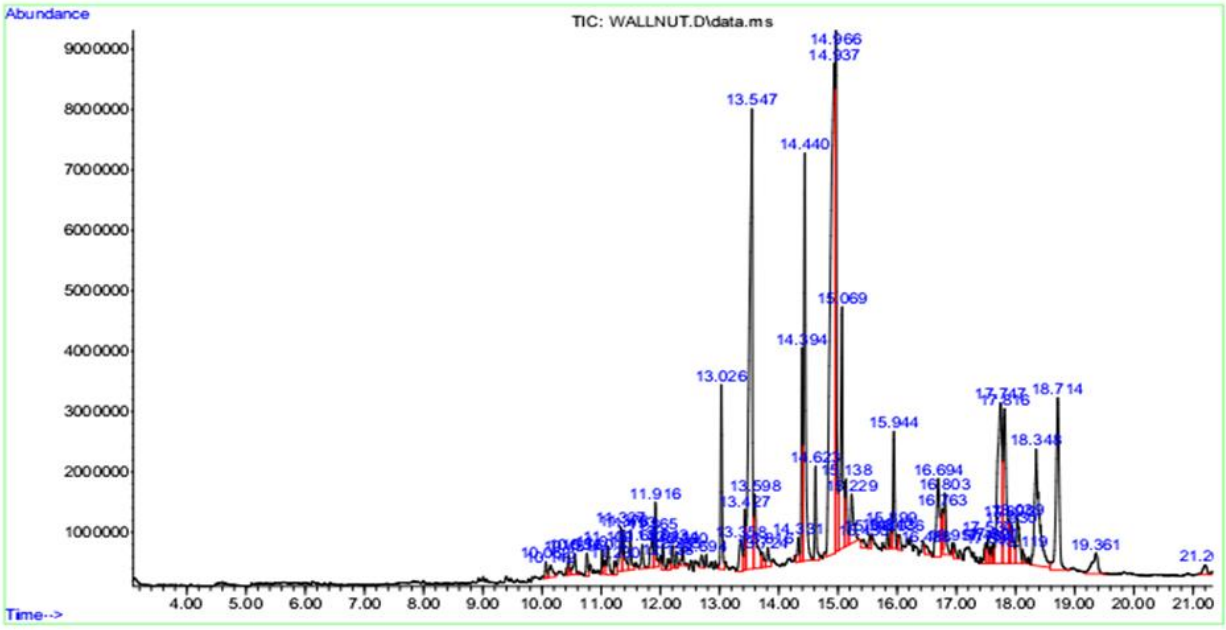

Figure 1. GC-MS chromatogram of African walnuts.

Discussion

In this study, the GC-MS analysis conducted on the oil extract of raw African walnuts revealed the presence of 16 bioactive compounds. Notably, the compound with the highest peak value was trans-13-Octadecenoic acid (68.87%). Krishnamoorthy and Subramaniam (2014) reported the anti-inflammatory and cancer-preventive properties of this compound which they got from *Solena amplexicaulis*. Nonadecane which has antioxidant, antibacterial, antimicrobial, antitoxic and antimalarial effects, according to Aziz et al. (2022) and El-Shahir et al. (2022) was also present in the oil extracted from African walnuts in this study. Hexadecanoic acid, methyl ester with hepatoprotective, antibacterial, and anti-inflammatory activities, according to Shaaban et al. (2021) and Gupta et al. (2021) were also reported in this study. 9,12-Octadecadienoic acid, methyl ester, present in the extracted oil has earlier been reported to have antimicrobial properties by Muzahid et al. (2023). Eicosane, another compound reported in this study has antifungal and antibacterial properties (Bhat et al., 2024). Semwal et al. (2018) had earlier reported that n-Hexadecanoic acid, found in the African walnuts in this study has antioxidant, anti-inflammatory, hypocholesterolemic, and cancer properties. Methyl stearate present in the African

walnuts in this study also has antibacterial properties (Nakaziba et al., 2022).

The CG-MS analysis of oil extracted from African walnuts in this study contained 1,15-Pentadecanedioic acid which Alenazy (2023) had earlier reported to have anti-inflammatory and antimicrobial activities. Similarly, 9-Octadecenoic acid, a methyl ester reported to have an antidiarrheal effect by Zahara et al. (2022) was found in the oil extracted from African walnuts. 9,12,15-octadecatrien-1-ol, (Z,Z,Z)-, an antioxidant and antibacterial according to Olivia et al. (2021) 9-Octadecenal, (Z)- and 9-Octadecenamide, (Z)- with have anti-inflammatory and flavoring roles in food respectively, according to Hatami et al. (2016) and Muzahid et al. (2023) were found in the oil extracted from African walnuts. Bis(2-ethylhexyl) phthalate with antibacterial and larvacidal properties (Javed et al., 2022) was found in the oil extracted from African walnuts; while 9,12,15-octadecatrienoic acid, (Z, Z,Z) and 2-hydroxy-1-(hydroxymethyl)ethyl ester having antimicrobial and anti-inflammatory properties. Salem et al. (2022) were also present in the oil extracted from African walnuts.

According to Matotoka et al. (2023), the effectiveness of any medicinal plant is assessed by linking its phytochemical compound constituents with significant biological functions in the human body. Given the significance of each of the compounds discovered from the oil extracted from African walnut nuts in this study, using GC–MS, walnut nuts can be classified as being medicinal.

This study identified three bacterial species: *Bacillus cereus*, *Staphylococcus aureus* and *Escherichia coli* based on outcomes of the biochemical tests conducted on each of them. These bacteria species have earlier been reported to be present in natural settings such as fruits, soil, and water (Kowalska et al., 2020; Ryu et al., 2021). *Bacillus cereus*, for instance, occurs naturally in fruit flora and it is linked to their spoilage (Garuba et al., 2024). The detection of *Escherichia coli* and *Staphylococcus aureus* suggests the usage of contaminated water in washing the walnuts or handling of the nuts with contaminated hands. *Staphylococcus aureus*, typically found in the nasal cavity and on human skin, can cause various illnesses in humans (Laux et al., 2019). In a prior investigation, Fakile et al. (2023) also identified *Bacillus subtilis* and *Staphylococcus aureus* as bacterial species associated with the spoilage of African walnut, underscoring the importance of adopting appropriate handling and storage procedures for walnut fruits.

The fungal species isolated from the examined walnuts in this study were molecularly identified as *Rhizopus delemar*, *R. arrhizus*, *Aspergillus niger*, and *Penicillium rolfsii*. Each species exhibited a query cover and percentage identity exceeding 98 % and 97 %, respectively, in the analysis, confirming their accurate identification. According to Raja et al. (2017), reliable molecular identification typically requires achieving a query cover of at least 80 % and a percentage identity of at least 97 % when comparing fungal species sequences to those in the NCBI database using BLASTN. The PCR technique was used in the current study to diagnose fungi isolated from nuts, as indicated by Freeman Weiss et al. (2021) that the traditional methods adopted in the diagnosis of fungi and based on the identification of phenotypic criteria are no longer sufficient due to the overlap of these criteria with other species in addition to the genetic difference between them. Their growth and phenotypic characteristics do not necessarily imply the difference in genotype, especially between isolates of the same genus and species.

The above fungal species align with those documented by Nooralden (2022) in their study on nut samples including walnut in the city of Kirkuk where fungi of both genus *Penicillium* and *Aspergillus* were isolated. *Aspergillus* is frequently found in various nuts because it produces a wide array of enzymes that allow it to thrive on diverse nutrients (Corbu et al., 2023). Additionally, it can adapt well to challenging environmental conditions and generate numerous spores that withstand unfavorable conditions (Paulussen et al., 2017). This aligns with Musangi et al. (2024), who found *Aspergillus* to be a common contaminant in nuts. The detection of *R. arrhizus* and *R. delemar* in this study corroborates earlier research identifying *Rhizopus* species as contaminants in nut samples. However, Eze et al. (2019) only isolated *Penicillium* sp. and *Fusarium* sp. in their investigation of raw African walnuts from the Eke Akpara market in Abia State.

Conclusion

Sixteen bioactive compounds were identified in the oil extracted from African walnuts, showing the nutritional and therapeutic significance of consuming these nuts. Also, three bacterial and four fungal species were isolated from the nuts, all of them are pathogenic.

Supplementary Materials

There is no supplementary material available for this article.

Author Contributions

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

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Conflict of Interest Statement

The authors declare no conflict of interest.

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مقاله پژوهشی

ترکیبات زیست فعال و ارزیابی میکروبی گردوی آفریقایی (*Tetracarpidium conophorum*) (Mull. Arg.) Hutch & Dalziel در ایلورین

گانیو اولاهان، ابراهیم اجادی* و ولیات سلیمان

گروه زیست شناسی گیاهی، دانشکده علوم زیستی، دانشگاه ایلورین، ایلورین، نیجریه.

چکیده: گردوی آفریقایی (*Tetracarpidium conophorum*) یک میوه استوایی مغذی است که ارزش غذایی بالایی داشته و برای سلامت انسان‌ها مفید می‌باشد. در این مطالعه ترکیبات زیست فعال و میکروب‌های مرتبط با گردوی آفریقایی موجود در کلان‌شهر ایلورین بررسی شد. پس از تهیه محلول‌های ذخیره از آجیل‌های نمونه‌گیری شده، جداسازی‌های میکروبی روی محیط PDA (قارچ)، نوترینت آگار و ائوزین متیلن بلو آگار (باکتری) به روش پورپلیت انجام شد. ۱۶ ترکیب فعال زیستی با ارزش های درمانی بسیار قابل توجه در آجیل خام با استفاده از کروماتوگرافی گازی – طیف سنجی جرمی (GC-MS) شناسایی شد. همچنین شناسایی مولکولی گونه‌های قارچی و شناسایی بیوشیمیایی گونه‌های باکتریایی جدا شده از آجیل، حضور چهار گونه قارچی به نام‌های *Aspergillus niger*، *Penicillium rolfsii*، *Rhizopus delemar* و *Rhizopus arrhizus* و همچنین سه گونه باکتری به نام‌های *Bacillus cereus*، *Staphylococcus aureus* و *Escherichia coli* را نشان داد. اگرچه نتایج GC-MS می‌تواند بیانگر برخی مزایای تغذیه ای و دارویی گردوی آفریقایی بوداده شده باشد، ولی مخاطرات آلودگی میکروبی گردوی آفریقایی برشته شده در بازارهای ایلورین را نیز باید مدنظر قرار داد.

کلمات کلیدی: GC-MS، غربالگری میکروبی، شناسایی مولکولی، گردوی آفریقایی، آنتی‌میکروبی.

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Microsatellite-based heterotic grouping of temperate maize (*Zea mays* L.) inbred lines

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Abstract: Maize (*Zea mays* L.) is an essential cereal crop globally, with breeding efforts aiming to develop high-yielding hybrids through heterotic patterns. This study assessed the feasibility of classifying 51 maize inbred lines into the heterotic groups using 30 Simple Sequence Repeat (SSR) markers. Out of the 30 marker pairs tested, 28 displayed polymorphism, producing a total of 68 alleles, ranging from 2 to 4 alleles per locus, with an average of 2.43 alleles per locus. The primers umc2152 and Bnlg1194 exhibited the highest number of alleles, while the marker mmc0481 had the highest allele frequency. Polymorphic Information Content (PIC) values ranged from 0.08 to 0.93, with an average value of 0.56. The highest ΔK value resulted in the classification of the inbred lines into five distinct heterotic groups. The findings suggest that SSR markers effectively reveal significant genetic diversity, making them valuable tools for the classification of maize inbred lines. This categorization can assist in identifying heterotic patterns and predicting heterosis for future hybrid production.

Keywords: Maize, heterotic group, genetic diversity, simple sequence repeats.

Introduction

Maize (*Z. mays* L.), a diploid (2n=2x=20) monoecious crop belonging to the family of Gramineae, is currently the second most widely grown cereal after wheat in terms of grain and forage production [1]. Its grain is generally used for food, feed, and industrial products including ethanol, biodegradable foams, plastics, and adhesives. Its forage, on the other hand, is generally utilized to feed ruminant livestock, biofuel production, and chemical production (Scott and Emery, 2016).

Development of hybrid maize varieties, by crossing two different parental inbred lines, has played a crucial role in improving the performance and productivity of maize. This breeding technique has been instrumental in supporting farmers' livelihoods and reducing risks. By effectively harnessing and utilizing heterosis, the superior performance of hybrids compared to their parents, the yield of F_1 maize cultivars has witnessed a significant increase of 50-100% compared to the traditional open-pollinated varieties [3]. Evaluating and adopting the proper parental lines, which combine well together, is the initial step towards exploiting heterosis. However, the process of testing inbred lines for their combining ability, which involves a large number of line-to-line tests to determine hybrid performance, is considered the most challenging and time-consuming aspect of F_1 hybrid breeding (Rudolf-Pilih et al., 2019). To overcome this obstacle and maximize the potential of heterosis from the available gene pools, plant breeders suggest identifying heterotic groups and patterns. This approach helps in efficiently identifying combinations of parental lines that exhibit strong hybrid vigor.

Heterotic groups are referred to as "groups of related or unrelated genotypes from the same or different populations, which display similar combining ability and heterotic response when crossed with genotypes from other genetically distinct germplasm groups" (Melchinger and Gumber, 1998). By comparison, the term “heterotic pattern” refers to a specific pair of two heterotic groups expressing the highest heterosis and consequently the highest hybrid performance in their crosses (Melchinger and Gumber, 1998). In addition to improving the accuracy of hybrid prediction, determining heterotic groups also aids in the incorporation of cytoplasmic male sterility (CMS) in the female pool and restorer gene(s) exclusively in the male pool for commercial hybrid production (Hussain et al., 2022). This approach not only enhances the efficiency of hybrid breeding but also allows for the controlled production of hybrids by manipulating the reproductive traits of the parent lines. Despite numerous studies, the precise physiological, biochemical, and molecular mechanisms underlying this phenomenon remain largely elusive (Virmani et al., 2004). and they have not been able to accurately predict heterotic combinations.

Duly assignment of inbred lines into heterotic groups is a prerequisite for attaining functional heterotic patterns. This assignment can be carried out *via* different approaches such as pedigree analysis, combining ability analysis, phenotypic and molecular marker-assisted clustering. Acquiring information on the origin of inbred lines necessary for pedigree analysis can be challenging and sometimes unattainable. Furthermore, the classification of breeding materials based on mating designs and phenotypic methods can be misleading due to unknown genetic mechanisms and environmental cues (Oyetunde et al., 2020). Providing a more stable and consistent approach for trait identification across different growing conditions, molecular markers are widely employed to assess the genetic distance between different maize genetic materials and assign inbred lines to specific heterotic groups. These markers can be incorporated into selected parental lines to develop heterotic hybrids that benefit from mechanisms not typically observed in the individual inbred lines (Virmani et al., 2004; Suwarno, 2014). Among the various molecular approaches, Simple Sequence Repeat (SSR, or microsatellites) and Single-Nucleotide Polymorphism (SNP) markers are commonly used to predict heterotic groups and patterns. The current research aims to investigate genetic diversity and determine heterotic groups in 51 maize inbred lines to maximize the exploitation of heterosis and select testers within the heterotic group for future breeding programs.

Materials and Methods

Plant materials

In this study, 51 maize inbred lines (42 early and 9 late maturing) were used for determining heterotic groups at Seed and Plant Improvement Institute (SPII), Karaj, Iran (Table 1).

DNA extraction

Seeds were sown in trays containing peat and perlite (1:1; v:v) with 8 replications in the laboratory of Maize and Forage Crops Research at the Seed and Plant Improvement Institute. Leaf sampling was conducted after the seedlings reached 2-3 leaf stage . The genomic DNA of young fresh leaves was extracted using the CTAB (cetyl trimethyl ammonium bromide) method. The concentration of the extracted DNA was measured by the NanoDrop 2000 spectrophotometer. The quality of the DNA was verified by running electrophoresis on a 1% agarose gel. In this study, a total of thirty SSR markers were selected from the entire maize genome. The information regarding these SSR markers was obtained from the Maize Genetics and Genomic Database (www.maizegbd.org) (Table 2).

Polymerase chain reaction (PCR)

The PCR was performed according to the method previously described by Shahata [10]. Each 15 µl PCR reaction consisted of 7.5 µl PCR master mix from DNA Biotech, 3.5 µl deionized H_2O, 1 µl reverse and forward primer, and 1 µl DNA (50 ng/µl). The PCR reaction involved an initial denaturation at 94°C for 3 minutes, followed by denaturation at 94°C for 30 seconds, primer annealing at 54-62°C (as specified in Table 2) for 30 seconds, and extension at 72°C for 30 seconds. This cycle was repeated 35 times, followed by a final extension at 72°C for 5 minutes, and the reaction was then stopped at 10°C. The PCR products were separated into 8% polyacrylamide: bisacrylamide (29:1) gels using vertical electrophoresis. The gels were subsequently stained with 50 µl of safe stain for one hour. After staining with a safe stain, the DNA was observed under UV light and photographed using the Gel-doc device from the American BIO-RAD company.

Statistical analysis

The Popgen32 software was used to calculate genetic diversity indicators such as the observed number of alleles (Na), the effective number of alleles (Ne) (Kimura and Crow, 1964), polymorphic information content (PIC), Shannon's information index (I) (Lewontin, 2014), and Nei's index (Nei, 1973). PIC was calculated based on the number of identified alleles and their frequency in the population according to the following formula:

PIC = 1 - ∑pi^2

(where Pi is the frequency of allele i among all alleles produced by the genotypes used (Anderson et al., 1992). The marker index (MI) is derived from the number of polymorphic alleles for each marker. The MI value for each marker is calculated using the formula: MI = PIC × EMR. The Effective Multiplex Ratio (EMR) is obtained by calculating the ratio of polymorphic markers to the total number of markers. The population structure analysis was carried out using the Bayesian Markov Chain Monte Carlo model (MCMC) implemented in STRUCTURE v2 software (Pritchard et al., 2000). The Bayesian-based model included in the Structure program was utilized for the genetic structure analysis of molecular data in the maize-inbred lines investigated.

The precise genotypic classification (K values) into suitable subpopulations based on genetic structure was confirmed using the Bayesian model-based method and Structure software. The optimal value of K, representing the number of subpopulations, was determined considering indices such as LnP(D) and ΔK (Zhou et al., 2018). The parameters used in this model included K (ranging from 4 to 9 for microsatellite data), and 10 repetitions for each K. The number of runs and the MCMC repetitions were set to 10000 to achieve the maximum likelihood curve (Anderson et al., 1992).

Results and Discussion

Genetic diversity of maize inbred lines

The genetic diversity analysis of maize inbred lines in this study was conducted using divergent reproducible amplification produced by 30 SSR primers tested (Figure 1; Table 3).

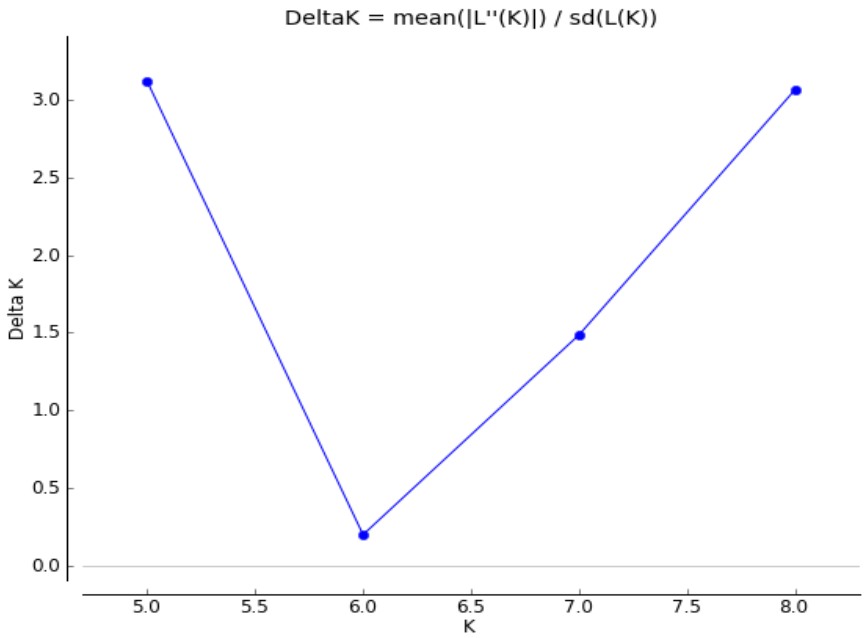

Figure 1. ΔK curve for determining the optimal value of K.

Results revealed 68 alleles distributed across 28 marker loci, with an average of 2.43 alleles per locus. Previous studies indicated varying allele numbers ranging from 1 to 8, depending on the number of SSR primers used (Nikhou et al., 2013; Nikolić et al., 2015; Synrem et al., 2017; Aung et al., 2023). Expectedly, small variations in the number of alleles between studies may primarily be attributed to the size of samples investigated, their pedigrees, homogeneity, and most importantly the repetition type of SSR primers employed. Genetic diversity levels were found to vary from 0.04 (umc1648) to 0.67 (bnlg1194), with an average value of 0.4. The SSR primers in this investigation displayed significant values of gene diversity together with allelic variations.

The PIC value is considered a critical metric used to assess the effectiveness of a marker in estimating genetic variation among genotypes. In this study, the PIC value ranged from 0.11 (umc1648) to 0.93 (umc1525) with an average value of 0.57 (Table 3). Based on the highest PIC values, the primers phi2513 (0.93), phi041 (0.78), bnlg1194 (0.74), bmc1979 (0.72), umc1795 (0.71), and bnlg244 (0.7) were found to be the most potent markers for assessing genetic diversity among the inbred lines. The average PIC value observed in this research was greater than the values documented by Shiri et al. (2014) (ranging from 0.23 to 0.79 with an average of (0.53) and Aung et al. (2023) ranging from 0.01 to 0.76 with an average of 0.48). The mean PIC value in our study, however, turned out to be inferior to the PIC value observed by Vathana et al. (2019) which ranged from 0.311 to 0.903 with a mean value of 0.743.

Strong molecular markers have the potential to be a useful tool for classifying genotypes into different heterotic groupings (Xia et al., 2004). Information on the degree of polymorphisms among genotypes is greatly aided by research on genetic variation and PIC values in plant breeding programs (Eltaher et al., 2018). Low polymorphism is indicated by a PIC value of less than 0.25, moderate polymorphism is shown by a value between 0.25 and 0.5, and a highly polymorphic locus is indicated by a value more than 0.5 [16]. With the use of 28 SSR markers, the current study offers insightful data on the genetic makeup of 51 genotypes of maize inbred lines. PIC values ranged from 0.11 to 0.28 for 10.7% of markers had low polymorphism, 0.28 to 0.47 for 14.3% of the

markers had average polymorphism, and 0.53 to 0.93 for 75% of the markers had high polymorphism.

Population structure analysis and genetic relationship

The Bayesian-based model included in the Structure program was utilized for the genetic structure analysis of molecular data in the maize inbred lines investigated. First presented by Pritchard et al. (2000), the Structure program is a clustering technique based on an independent marker system and a genetic model. Before experimentation or observation, Bayesian approaches assign probabilities or distributions to the data based on optimal assumptions; then, they repeatedly modify these assumptions following the experiment (Kumar et al., 2017).

In the present study, the ideal number of K was found to range from 4 to 9 groups with the largest ΔK value (Figure 1) at K=5, signifying the division of the lines into 5 groups. The matrix of membership proportions of individuals in each cluster was calculated, and K=5 was shown to be the ideal K for predicting the structure of these inbred lines (Figure 1 and Table 3). The vertical color column identified each line and its matching number in a biplot (Figure 2) based on the findings of the molecular data analysis performed with the Structure software. Each line's presence of color diversity reflects the genetic mixing of that line and shows how genetically similar the lines are to one another. To put it more clearly, an individual 'n' might have inherited some DNA from its population K ancestors. In this instance, the person is a member of the group in that cluster with the broadest color range.

The 51 inbred lines investigated in this study were divided into five groups based on the aforementioned information (Figure 2). There are 12 genotypes in the red group (23.53%), 9 genotypes in the green group (17.65%), 11 genotypes in the blue group (21.57%), 10 genotypes in the yellow group (19.61%), and 9 genotypes in the pink group (17.675%). The red group includes inbred lines 4, 5, 7, 13, 14, 15, 25, 26, 28, 30, 31, and 40, all of which belonged to the early maturing corn lines. The green group included inbred lines 1, 2, 20, 23, 24, 38, 39, 42, and 50 all, except for numbers 42 and 50 were early corns. Likely, one of the parents of inbred lines 42 and 50 had already been selected from the early maturing types. In the blue group, the most of inbred lines, except for lines numbers 6, 22, and 41, were considered to be late-maturing types.

Table 1. Names and codes of the maize inbred lines investigated.

No.	Early inbred lines	No.	Early inbred lines	No.	Early inbred lines	No.	Early inbred lines	No.	Late inbred lines
1	K1263/17	12	KE78011/6112	23	KE80001/72111	33	KE81027/3-1-1	42	K3653/2
2	K1263/2-1	13	KE78015/111	24	KE81015/211	34	KE81027/2-3-1-1	43	MO17
3	R319	14	KE78005/511	25	KE81015/521	35	KE81027/4-2-1	44	B73
4	R59	15	KE78027/1113	26	KE83008/2211	36	KE83001/4111	45	K3547/4
5	KE72012/12	16	KE78008/212	27	KE81009/311	37	K2331	46	K3640/3
6	KE75016/232	17	KE78011/6121	28	KE81009/511	38	K615/1	47	K47/2-2-1-3-3-1-1-1
7	KE75006/212	18	KE78010/421	29	KE81010/521	39	K722	48	K74/1
8	KE77008/1	19	KE78011/61231	30	KE81015/1-1-1	40	OH43/1-42	49	K18
9	KE77005/3	20	KE78011/61232	31	KE81012/3-1-1	41	K1264/1	50	K222
10	KE77008/2	21	KE79017/3211	32	KE81027/4-4-3			51	KE81018/611
11	KE78016/212	22	KE80001/5212	32	KE81027/4-4-3				

Table 2. Characteristics of the applied SSR markers.

Marker code	Forward and reverse sequence	Annealing Tm(⁰C)	Bin	Marker code	Forward and reverse sequence	Annealing Tm(⁰C)	Bin
phi056	ACTTGCTTGCCTGCCGTTAC(F) CGCACACCACTTCCCAGAA(R)	57	1.00	umc1406	AGAGGAGACAGGAGGTCGGTAGTT(F) TGTGGTGTGGTCTTCTCTCTTCTG(R)	62	7.05
umc2124	ATGCGGAGGGGTCTACTACACATA(F) CTGTGTCTCACTGGAAATGACGAT(R)	61	1.00	bnlg1194	GCGTTATTAAGGCAAGCTGC (F) ACGTGAAGCAGAGGATCCAT(R)	54	8.02
phi002	CATGCAATCAATAACGATGGCGAGT(F) TTAGCGTAACCCTTCTCCAGTCAGC(R)	62	1/08	umc1858	GTTGTTCTCCTTGCTGACCAGTTT(F) ATCAGCAAATTAAAGCAAAGGCAG(R)	57	8.04
umc1542	TAAAGCTATGATGGCACTTGCAGA(F) CATATTTGCCTTTGCCCTTTTGTA(R)	60	2/02	umc1957	CATGATCGCCGGGATTAATACTAC (F) GTCCAAGGACGACGATTACGAC (R)	61	9
umc1326	GACCAAAGAATTCCCTCCCCTA(F) TACCTAGTACTCGGCCAGTTCCTC(R)	61	2/04	umc1982	TTCATCTTCCTAGTCTCGTCTCCG(F) AATCGTACTTGGAGGAGGCGTT(R)	59	9.08
phi2513	CCAGTCCAATGGAGAGGG(F) GAGATTCCCCTGCAGGACT(R)	57	2.07	uhi041	TTGGCTCCCAGCGCCGCAAA(F) GATCCAGAGCGATTTGACGGCA(R)	61	10
umc1394	CCCGAGTCAGAAAAACATTCACTT(F) CCTAACCTGAAGAAGGGAGGTCAT(R)	55	3.01	bnlg1360	TTGGCTCCCAGCGCCGCAAA(F) GATCCAGAGCGATTTGACGGCA(R)	55	10
umc1135	TTTTTAACCTCACGAGCATCGTCT(F) CGCTAGCTTAGCTCCATCGTTTTA(R)	60	3.07	bnlg244	GATGCTACTACTGGTCTAGTCCAGA(F) CTCCTCCACTCATCAGCCTTGA(R)	60	9.02
umc2152	TAGCTTCACCTGATGATCTTGCAC(F) CCTTTGTCTTCCGCTATCTTCCTT(R)	62	3.09	umc1648	CTGCAGTACGTGAGCCTGTACG(F) GCTTGAGCTGTGAGGAAGTTTTG(R)	61	10.04
umc1008	TCTAGCTTGTGGTGGTGGTTGA(F) ACATGAGCACAAAGACTGACGC(R)	59	4	umc1792	CATGGGACAGCAAGAGACACAG(F) ACCTTCATCACCTGCAACTACGAC(R)		5.08
umc2285	GAAGAAGAGGGAAAGGAAGGGAG(F) AAGTAGCTGGGCTTGGAGGG(R)	60	4.08	umc1979	AATTTCGGGAAACAGGCCAT(F) GAGTCCCCGAAACTGAACACC(R)	54	6.04
phi006	AGGCGGCGTGCTGAACACCT(F) CGCTTCATCTCCCGTGACAATG(R)	61	4.11	umc1883	GAATAATCAATCCATCGATCTCGC(F) AACTGCTGTGGATGAAAGAGGAAG(R)	58	6
mmc0481	TGTTTGAGCCGTTCTAGACT(F) GCACCTGCGAGACTAGG(R)	52	5.06	umc1795	CCCTCTCTTCCTAGGTTCATCGTT(F) CAGCGGCGTCTTGAAGAGTAG(R)	60	6.05
mmc0483	CTTTCCTCTCTGGAGCGTGTATTG(F) ATATGTTGCAGAACCATCCAGGTC(R)	60	6.02	umc1301	CATCCATAAGCTGAAGGAGTGAGG(F) AACAGTCAAGCTCACTTTCCGC(R)	60	7.03
mmc1270	ACAAGGCAGGCAGACTACTTCTTG(F) CCTAAGAAGGTGGCAACCCC(R)	59	7.01	umc1260	CTTAAGCAGAGCTCAAAAACTGCC(F) TAAATTGTCAAGCGAGGTTTGGAT(R)	58	5.00

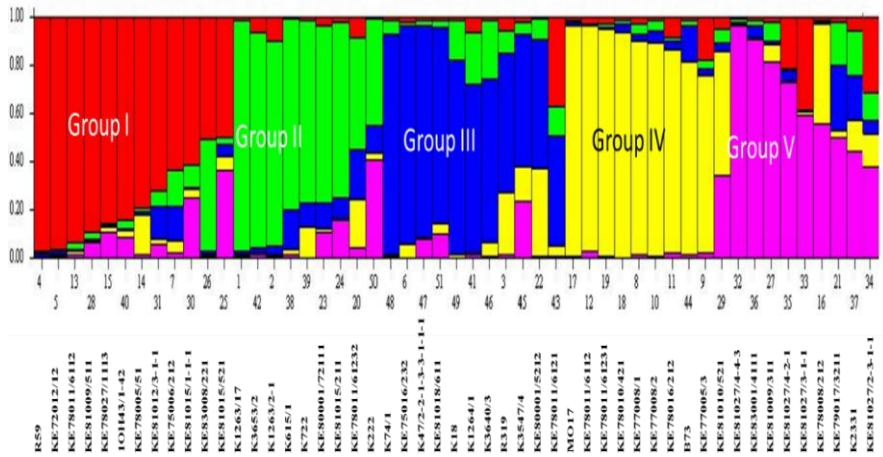

Figure 2. Population structure analysis of 51 maize lines using a model-based approach with SSR markers.

Table 3. Information of 28 SSRs, including number of alleles, Na, PIC, Ne, Nei, I, MI and RP across 51 maize inbred lines.

Number	Marker code	Na	A. frequency	PIC	Ne	Nei	I	MI	RP
1	phi056	2	0.76	0.62	1.8	0.44	0.63	1.24	1.18
2	umc2124	3	0.88	0.68	2.59	0.62	1.02	2.05	1.76
3	phi002	2	0.88	0.47	1.47	0.32	0.5	0.94	0.94
4	umc1542	3	0.88	0.63	2.08	0.52	0.86	1.88	1.57
5	umc1326	2	0.7	0.58	1.18	0.15	0.28	1.15	0.82
6	phi2513	2	0.27	0.93	1.15	0.13	0.26	1.87	0.55
7	umc1394	2	0.94	0.28	1.18	0.15	0.28	0.56	0.51
8	umc1135	2	0.88	0.53	1.64	0.39	0.58	1.05	1.18
9	umc2152	4	0.76	0.79	2.8	0.64	1.14	3.16	1.53
10	umc1008	3	0.9	0.35	1.3	0.23	0.48	1.04	0.59
11	uhi006	2	0.82	0.61	1.74	0.42	0.62	1.22	1.37
12	mmc0481	3	1	0.65	2.87	0.65	1.07	1.96	2.00
13	umc0483	2	0.96	0.18	1.13	0.11	0.23	0.37	0.31
14	umc1406	2	0.9	0.37	1.29	0.59	0.38	0.74	0.67
15	bnlg1194	4	0.92	0.74	3.08	0.67	1.2	2.94	1.84
16	umc1858	3	0.96	0.56	2.1	0.61	0.87	1.68	1.57
17	umc1957	2	0.67	0.62	1.25	0.2	0.35	1.25	0.94
18	umc1982	3	0.74	0.64	1.54	0.35	0.66	1.92	1.14
19	phi041	2	0.5	0.78	1.16	0.14	0.27	1.55	1.02
20	bnlg1360	3	0.96	0.64	2.55	0.61	1.02	1.91	1.88
21	bnlg244	3	0.9	0.7	2.76	0.64	1.05	2.09	1.80
22	umc1648	2	0.96	0.11	1.04	0.04	0.1	0.23	0.16
23	umc1792	2	0.7	0.66	1.45	0.32	0.49	1.31	1.14
24	umc1979	2	0.74	0.72	1.97	0.5	0.69	1.44	1.49
25	umc1883	2	0.94	0.54	1.94	0.49	0.68	1.09	1.69
26	umc1795	2	0.74	0.71	1.9	0.47	0.67	1.42	1.49
27	umc1301	2	0.94	0.54	1.94	0.49	0.68	1.09	1.69
28	umc1260	2	0.96	0.47	1.74	0.42	0.62	0.94	1.25
total		68	23.16	16.1	50.64	11.31	17.68	40.09	34.08
mean		2.423	0.83	0.575	1.81	0.4	0.63	1.43	1.22

By crossing the inbred line K722 (paternal parent from the green group) and the inbred line 1264/1 (paternal parent from the blue group) with the inbred line B73 (from the yellow group) the single crosses KSC-604 and KSC-647 were developed, respectively (Botstein et al., 1980). Likewise, by crossing the inbred lines K3640/3 and K3547/4 (both as the maternal parent from the blue group) with MO17 (paternal parent from the yellow group), the single crosses KSC-705 (Dehghanpour et al., 2018) and KSC-706 (Dehghanpour et al., 2018) were developed, respectively. When the inbred line K615/1 (maternal parent from the green group) was crossed with K1264/1 (paternal parent from the yellow group), the single cross K260 was developed. Dehghanpour et al. (2018) and Choukan et al. (2013) collectively pointing out to the validity of the present clustering.

Conclusion

In the current study, 28 out of 30 SSR primers proved to be polymorphic. In total, 68 alleles with a range of two to four alleles (and an average of 2.43 alleles per locus) were amplified. Out of the 30 SSR markers examined, markers phi2513, umc2152, bnlg1194, phi041, bnlg244, umc1979, and umc1795 exhibited the highest power for assessing genetic diversity among the inbred lines based on their PIC values. These robust molecular markers can be effectively utilized for genotype identification,

allowing for the classification of genotypes into distinct heterotic groups. Population structure analysis categorized 51 inbred lines into five distinct clusters. The highest and lowest MI values were found in the markers umc2152 (3.16) and umc1648 (0.23), respectively. The highest value of the Nei's index was observed in the bnlg1194 marker (3.08), and the lowest Nei's index was obtained in the umc1648 marker (1.04). Moreover, the highest Shannon's index value was found in the bnlg1194 marker (2.10). In conclusion, the use of microsatellite markers provides valuable insights into the genetic diversity of maize inbred lines, offering a robust tool for various applications in genetic studies and breeding programs.

Supplementary Materials

There is no supplementary material available for this article.

Author Contributions

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

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Conflict of Interest Statement

The authors have no relevant financial or non-financial interests to disclose.

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مقاله پژوهشی

گروه‌بندی هتروتیکی با استفاده از نشانگرهای ریزماهواره در اینبرد لاین‌های ذرت (*Zea mays* L.) مناطق معتدله

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چکیده: ذرت (*Zea mays* L.) یکی از غلات مهم در جهان محسوب می‌شود که اصلاح آن به منظور توسعه هیبریدهای با عملکرد بالا از طریق الگوهای هتروتیک انجام می‌گردد. در این تحقیق، امکان گروه‌بندی ۵۱ لاین خالص ذرت به گروه‌های هتروتیک را با استفاده از ۳۰ نشانگر توالی تکراری ساده (SSR) مورد ارزیابی قرار گرفت. از ۳۰ جفت نشانگر آزمایش شده، ۲۸ نشانگر چند شکلی را نشان دادند و مجموعاً ۶۸ آلل تولید کردند که از ۲ تا ۴ آلل ، با میانگین ۲.۴۳ آلل در هر لوکوس متغیر بود. آغازگرهای umc2152 و Bnlg1194 بیشترین تعداد آلل‌ها را نشان دادند، در حالی که نشانگر mmc0481 بالاترین فراوانی آللی را دارا بود. مقادیر محتوای اطلاعات پلی‌مرفیسم (PIC) از ۰.۰۸ تا ۰.۹۳ متغیر و میانگین آن ۰.۵۶ بود. بالاترین مقدار ΔK نیز منجر به طبقه‌بندی لاین‌های خالص به پنج گروه هتروتیک متمایز گردید. یافته‌های این تحقیق نشان می‌دهد که نشانگرهای SSR می‌توانند به‌طور مؤثری تنوع ژنتیکی را آشکار نمایند، و این موضوع آن‌ها را به ابزاری با ارزش برای گروهبندی لاین‌های خالص ذرت تبدیل می‌کند. این گروه‌بندی می‌تواند در شناسایی الگوهای هتروتیک و پیش‌بینی هتروزیس برای تولید هیبریدها در برنامه های آینده کمک نماید.

کلمات کلیدی: ذرت، گروه هتروتیک، تنوع ژنتیکی، توالی تکراری ساده.

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Original paper

Evaluation of chili (*Capsicum annuum* L.) genotypes for nutritional phytochemicals and mineral content

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Abstract: Chili (*Capsicum annuum* L.) is a worldwide important crop known for its nutritional and phytochemical properties. This study aimed to identify suitable candidates for breeding programs by evaluating the phytochemical and mineral content of 28 chili genotypes. The capsaicin concentration varied from 0.08% (G20) to 0.44% (G28). The maximum ascorbic acid concentration was recorded in G7 (125.56 mg/100 g), whilst the minimum was in G20 (19.45 mg/100 g). β-carotene concentration ranged from 0.29 mg/100 g FW in G12 to 0.13 mg/100 g in G13 and G26. The chlorophyll content (a and b) reached its zenith in G24 (0.30 and 0.32 mg/g FW, respectively), whereas the minimal amounts were seen in G15 and G18. The anthocyanin concentration varied from 4.18 µg/g FW in G18 to 0.48 µg/g FW in G11. Genotype G16 demonstrated the highest overall phenolic and antioxidant levels, whereas G25 exhibited the highest flavonoid concentration. Mineral analysis indicated that G4 and G26 contained the highest sodium (0.39%), G21 had the highest potassium (2.07%), and G10 exhibited the highest calcium (1.65%) and magnesium (0.63%). Cluster IV had high levels of ascorbic acid and anthocyanin, while cluster I had low levels, according to the heatmap analysis of the genotypes. Significant connections were discovered among ascorbic acid, anthocyanin, potassium, and calcium concentration. In breeding initiatives for nutritionally improved chili varieties, genotypes G2, G7, G12, G16, G17, G18, and G25 stand out with their excellent phytochemical and mineral profiles. This study gives important information about the genetic diversity of chili genotypes in Bangladesh.

Keywords: Pepper, total phenol, flavonoids, antioxidant activity, anthocyanin content.

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Introduction

Chili (*Capsicum annuum* L.) is referred to by various names globally, including red pepper, bell pepper, pod pepper, hot pepper, paprika, cayenne pepper, and pimento. It is closely associated with tomato, eggplant, potato, and tobacco (Faustino et al., 2007). The domestication of chili initially took place in central America, predominantly in Mexico, with additional concentrations in Guatemala and Bulgaria (Salvador, 2002). The principal chili-producing nations include India, Mexico, Japan, Ethiopia, Uganda, Nigeria, Thailand, Turkey, Indonesia, China, and Pakistan. It is also cultivated to considerable degree in Italy, Spain, and the United States. India is the leading producer and consumer of chili globally (Salvador, 2002). The chili fruits are utilized to provide pungency in both their immature and mature stages. The fruit ranges in size from 1 to 20 cm in length, exhibiting forms from slender elongated to conical and robust blocky shapes. The popularity of chile stems from its diverse shapes, sizes, and sensory characteristics, including color, pungency, and piquancy, which enhance the flavor of otherwise bland staple foods like grains and vegetables. In the food and beverage industries, chili is utilized as oleoresin, facilitating enhanced distribution of color and flavor in products. Pungency results from the presence of capsaicin (Parthasarathy, 2008).

Capsaicin is an alkaloid found in the fruit is placenta that may directly neutralize various free radicals (Bhattacharya et al., 2010) and has extensive applications in the food, medical, and pharmaceutical sectors. It has been utilized as a topical analgesic for arthritis pain and inflammation (Deal et al., 1991). Capsaicin interacts with the same category of nociceptors that elicit sensations of pain from heat and acid (Julius and Basbaum, 2001), and it alleviates pain and inflammation by depleting the neurotransmitters that convey pain signals. Capsaicin exhibits anti-mutagenic properties (Ramirez-Victoria et al., 2001; Morré and Morré, 2003) and possesses significant antioxidant activity (Lee et al., 1995; Chakrabarty and Islam, 2017). It is utilized in balm formulation, while color extracts (carotenoid pigments) serve as color additives in the food and prawn feed industries. The primary functional attributes of chili are pungency, vitamin C, natural colors, and several minerals such as sodium, potassium, calcium, magnesium, iron, and zinc (Starykh and Nosova, 1982). Green chilies are abundant in Vitamin A and Vitamin E. Chili is extensively utilized in curry powder, curry paste, various pickles, sauces, and soups. The quality of dried chili is measured by several different parameters such as color, hotness, ascorbic acid content, and volatile compounds (Kim et al., 2006; Wang et al., 2009; Yaldiz et al., 2010). Ribes - Moya et al. (2020) characterized 14 genotypes of Capsicum based on phytochemicals present in pepper fruits harvested at various maturity stages. Paredes Andrade et al. (2020) characterized 198 genotypes of Capsicum collected from 21 different countries. They found a strong correlation between polyphenols and flavonoids, while weak correlation was observed between polyphenol and antioxidants.

The development of high-yielding cultivars necessitates an understanding of the existing genetic variation and the degree of connection between yield-contributing traits. The observed variability represents a composite assessment of genetic and environmental factors, of which only the genetic component is heritable. Nevertheless, the heritability estimate alone does not indicate the anticipated gain in the subsequent generation; it must be evaluated alongside genetic development. Correlation and path analysis will determine the degree of link between yield and its components, elucidating the relative significance of their direct and indirect impacts, so providing a thorough comprehension of their relationship with yield (Vijaya et al., 2014). This study was initiated to assess the phytochemical contents of 28 chili genotypes available at Bangabandhu Sheikh Mujibur Rahman Agricultural University.

Materials and Methods

Plant materials and field experiments

The field experiment was conducted at the experimental farm of the Department of Genetics and Plant Breeding, Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU), Gazipur. All chemical analyses were carried out at the laboratory of the Department of Genetics and Plant Breeding and the Department of Crop Botany,

BSMRAU. Seeds of 28 genotypes of chili were sown in the pots to raise seedlings as it ensures uniform growth which facilitates better plant establishment after transplanting. The seedlings were ready to transplant in the main field 45 days after sowing (DAS).

A population of twelve plants was maintained per replication per genotype by planting a single plant per hill. Gap filling was done after one weak whenever the death of a previously transplanted seedling occurred. One seedling was transplanted per hill and light irrigation was given immediately after transplanting. The spacing was maintained at 45 cm between rows and 45 cm between plants within a row for all the genotypes. One-meter distance was kept between blocks of the replication. Soil fertility was ensured by applying additional quantities of Urea, triple superphospaht (TSP), muriate of potash (MOP), Gypsum, Zinc Sulphate و and Boric Acid in amounts of 270-170-100-150-115-88 kg/ha, respectively(Anonymous, 1999) . Total TSP, MP, Gypsum, zinc sulphate, and boric acid were applied during final land preparation. Cowdung was also applied @ 10/ha during final land preparation. Total urea was applied in three installments, at 15, 30, and 50 days after transplanting (DAT). Recommended inter-cultural operations were done to raise the healthy chili crop. Five plants were selected randomly from each replication and selected plants were marked by labeling for recording data. Data on the following qualitative characters were recorded after the harvest of mature green chili. The solution preparation protocols used in the analysis of different chilli's phytochemicals are presented in Supplementary Table 1.

Capsaicin content

The capsaicin concentration in fruits was assessed using the colorimetric technique outlined by Bajaj (1980). 0.5 g of dry chili powder was measured into a glass-stoppered test tube; 10 ml of dry acetone (prepared by adding 25 g of anhydrous sodium sulfate to 500 ml of acetone at least one day prior) was added into the test tube and allowed to extract overnight. The following day, samples were centrifuged at 10,000 rpm for 10 minutes to get a clear supernatant. 1 cc of the supernatant was transferred to a test tube and evaporated to dryness using a hot water bath. The residue was subsequently dissolved in 5 ml of 0.4% NaOH solution, followed by the addition of 3 ml of 3% phosphomolybdic acid. The contents were agitated and then allowed to remain undisturbed for one hour. After one hour, the solution was promptly filtered into centrifuge tubes to eliminate any suspended material and subsequently centrifuged at 5000 rpm for 15 minutes. The transparent blue solution was directly put into the cuvette, and absorbance was measured at 650 nm alongside a reagent blank. A standard graph was constructed with 0-200 µg of pure capsaicin. Simultaneously, 0.2, 0.4, 0.6, 0.8, and 1 ml of the working standard solution were aliquoted into new test tubes. The stock standard capsaicin solution was prepared by dissolving 50 mg of capsaicin in 50 ml of 0.4% NaOH solution (1000 µg/ml). The working standard solution was prepared by diluting 10 ml of the stock standard to 50 ml with 0.4% NaOH solution (200 µg/ml) and processed as previously described.

Simultaneously 0.2, 0.4, 0.6, 0.8, and 1 ml of working standard solution (stock standard capsaicin solution was prepared by dissolving 50 mg capsaicin in 50 ml of 0.4% NaOH solution (1000 µg/ml) and working standard solution prepared by diluting the 10 ml of the stock standard to 50 ml with 0.4% NaOH solution (200 µg/ml)) was taken into new test tubes and proceeded as mentioned above.

Calculation: Percent capsaicin calculated using the formula mentioned below-

Capsaicin content (%) = (µg capsaicin × 100 × 100) / (1000 × 1000 × 1 × 0.5)

Total ascorbic acid content

The ascorbic acid content was determined as per the procedure described by Pleshkov (1976). 5 ml extract solution was taken in a 50 ml conical flask. Then 5 ml of 5% KI, 2 ml of 100% glacial acetic acid, and 2 ml of 2% starch solution were added to it respectively. Total ascorbic acid was estimated by titrating that aqueous extract solution against 0.001 N of KIO_3 solution. The total ascorbic acid content was quantified by using the formula:

Ascorbic acid (mg/100g) = (T×F×V×100) / (v × W)

Here,

T = Titrated value of KIO_3 ml

F = 0.088 mg of ascorbic acid per ml of 0.001 N of KIO_3

V = total volume of the sample extracted (ml)

v = volume of the extract (ml) taken

W = weight of the sample taken (g)

Total antioxidant content

Total antioxidant content was determined by the procedure described by Abdul-Hafeez et al. (2014) with slight modification. The 1.0 ml plant extracts and standards (Butylated hydroxyl toluene standards) were taken in the test tubes and 1.0 ml methanol (instead of plant extract) was taken in another test tube which served as the control. Then 3 ml 0.2 mM of DPPH solution was added to each test tube and incubated the reaction mixture at 25°C for 5 minutes. After incubation, the absorbance was measured at λ = 517 nm and methanol was used as the blank. DPPH radical scavenging activity of each plant extract and standards were calculated as the percentage inhibition.

% Inhibition DPPH radical activity = ($A_{control} - A_{sample}$) × 100 / $A_{control}$

Then a calibration curve from the standards was prepared and expressed the antioxidant capacity (µg/g fresh weight) as BHT equivalent.

Total β -carotene content

Total β – carotene content was determined by the procedure described by Nagata and Yamashita (1992). A 1.0 g fresh sample of chili was taken into a mortar and homogenized with 10 ml acetone: hexane (4:6) solution. This sample was centrifuged and the optical density of the supernatant was measured by spectrophotometer (Model 200-20, Hitachi, Japan) at 663 nm, 645 nm, 505 nm, and 453 nm.

Calculation was done by the following formula:

β- Carotene (mg/100g) = 0.26 (**OD_{663}**) + 0.452 (**OD_{453}**) **– 1.22(OD_{645}) - 304 (OD_{505})**

Where the bold figure indicates optimal density.

Chlorophyll and carotenoid content

A 100 mg of fresh chili sample was taken in a glass vial. Then 5 ml of 80% (v/v) acetone was added and made the vial air tight. After that the vial was kept at 4°C in the dark for 24 hrs. 1 ml supernatant was taken in a 1 ml glass cuvette and the absorbance was read at 663 nm, 646 nm, and 470 nm, corresponding to chl a, chl b and carotenoids respectively. 80% acetone was used as a blank (Porra et al., 1989). Quantification of chl a, chl b, and carotenoids was done as described by Lichtenthaler and Wellburn (1983).

Chl a (µg/ml) = 12.21 (A_{663}) – 2.81(A_{646})

Chl b (µg/ml) = 20.13 (A_{646}) – 5.03(A_{663})

Carotenoids (µg/ml) = {1000 (A_{470}) – 3.27 (Chl a) – 104 (Chl b)} / 229

Expressed the amount as mg /g = (µg ml^{-1} × V) / (1000 ×W)

Where,

V = Volume of acetone used (ml)

W = Weight of fruit sample (g)

Total anthocyanin content

1.0 g tissue was taken in an ice-cold glass vial. Then 5 ml of extraction solution was added and make the vial airtight. After that, the vials were kept at 4°C in the dark for 24 hrs. 2ml solution was taken from the vial in a centrifuge tube and added 2 ml distilled water. After that 2 ml chloroform was added to separate anthocyanin (insoluble in chloroform) from the chlorophylls. Then the mixture was centrifuged for 15 minutes at 5000 rpm at 4°C. After that 3 ml of the top layer (containing anthocyanin) was taken in a glass cuvette and the absorbance was read at 530 nm. The extraction solution was used as blank (Hughes and Smith, 2007). Total anthocyanin content was calculated (as cyaniding-3-glucoside equivalent) using the absorbance and a molar extinction coefficient for anthocyanin at 530 nm of 30000 $L^{-1}M^{-1}cm^{-1}$ (Murray and Hackett, 1991). Anthocyanin (µg/g FW) = (Abs × MW × V × DF × 1000) / (30000 × w)

Where,

A = absorbance at 530 nm

MW = Molecular weight of cyaniding-3-glucoside (449.22g mol^{-1})

V = Volume of extraction solution used (ml)

DF = Dilution factor

w = Used sample weight

FW = Fresh weight

Total phenolic content

Total phenolic content was determined spectrophotometrically according to the Folin-Ciocalteu's method described by Abdul-Hafeez et al. (2014) with slight modification. 1.0 ml plant extracts and standards (gallic acid standards) were

taken into test tubes and 1.0 ml methanol (instead of plant extract) was taken in another test tube which served as the control. Then 0.5 ml 10% (0.2 N) Folin-Ciocalteus reagent was added to each test tube. Then the test tubes were shaken for 10 seconds, covered, and incubated the reaction mixture for 15 minutes at room temperature. After incubation, 2.5 ml 700 mM Na_2CO_3 aqueous solution was added and mixture was again shaken, covered incubated the reaction mixture for 2 hrs. The absorbance was measured at λ = 765 nm using methanol as the blank. A calibration curve from the standards was prepared and expressed the phenolic content (µg/g fresh weight) as gallic acid equivalent.

Total flavonoid content

The total flavonoid concentration was quantified spectrophotometrically using the aluminum chloride colorimetric assay (Zhishen et al., 1999) with minor modifications. 1.0 ml of plant extracts and quercetin standards were placed in test tubes, whereas 1.0 ml of methanol was used in a separate test tube as the control. Subsequently, 0.4 ml of a 5% $NaNO_2$ solution was included into the mixture. After 5 minutes, 0.6 ml of 10% $AlCl_3 \cdot 6\ H_2O$ was introduced, and at after 6 minute, 2 ml of 1M NaOH was added to the mixture. The mixture was agitated for thorough homogenization, and the absorbance was recorded at λ = 510 nm, with methanol as the blank. A calibration curve derived from the standards was established, quantifying the flavonoid concentration (µg/g fresh weight) as quercetin equivalent.

Total sodium, calcium, magnesium and potassium content

To calculate the total content of sodium, calcium, magnesium, and potassium in chili, ripe fruits were subjected to air-drying at ambient temperature for a duration of 3 days. The air-dried samples were subjected to oven drying at 70°C, after which 0.5 gr of the ground oven-dry samples were digested using a nitric acid (HNO_3) and perchloric acid ($HClO_4$) mixture in a 5:1 ratio for 2 hours. The digests were employed to ascertain the amounts of sodium, calcium, magnesium, and potassium utilizing the methodologies outlined by Piper (1947) with an atomic absorption spectrophotometer (Model 200-30, Hitachi, Japan). Statistical analysis was conducted with the software STAR (statistical tools for agricultural research). The data underwent one-way analysis of variance for mean comparison, and significant differences were determined using Fisher's LSD test. The data were presented as the mean. Differences with a p-value of less than 0.05 were deemed statistically significant.

Results

Analysis of nutritional phytochemicals

Analysis of variance (Table 1) revealed highly significant differences among the genotypes for five quality traits such as ascorbic acid content, total phenolic content, total flavonoids content, total antioxidant content, and anthocyanin content indicating the presence of variability in the materials and considerable scope for their further improvement. Capsaicin content of fruits of 28 chili genotypes was assessed. The solution preparation protocol is provided in Supplementary Table 1, and the results are presented in Supplementary Table 2. The results showed that the capsaicin content of fruits ranged from 0.08 % to 0.44 %. The highest amount of capsaicin content was observed in genotypes G20 followed by G27 (0.39 %) and G25 (0.33 %). The lowest amount of capsaicin content was observed in the genotypes G28 followed by G2 (0.16 %) and G13 (0.17 %). Capsaicin content showed an 8.85 % coefficient of variation.

Ascorbic acid content in fruits ranged from 19.45 and 125.56 (mg/100g) (Supplementary Table 2). The highest value of ascorbic acid content was observed in the fruits of genotype G7 (125.56 mg/100g) followed by G17 (113.26 mg/100g), G3 (109.33 mg/100g), G10 (102.53 mg/100g) and G14 (101.16 mg/100g). The lowest amount of ascorbic acid content was observed in the genotypes G20 (19.45 mg/100g) followed by G19 (19.46 mg/100g) and G1 (40.83mg/100g). The ascorbic acid content of fruits showed a 12.04% coefficient of variation. β - carotene content showed low variability among the 28 genotypes of chili and it ranged from 0.13 to 0.29 (mg/100g) (Supplementary Table 2). The highest amount of β - carotene content was observed in genotype G12 (0.29 mg/100g) whereas the lowest was in genotype G9 and G26 (0.13 mg/100g). The mean value of G2, G20 and G24 were same which followed by G3, G16, G28, G4, G11, G15, G22.

Chlorophyll a content of the genotypes varied between 0.05 mg/g and 0.30 mg/g, the maximum being in G24 followed by G25 (0.28mg/g), G6 (0.24mg/g) and G9 (0.22 mg/g) in Supplementary Table 2. The minimum chlorophyll content was observed in the genotypes G15 followed by G3 (0.07 mg/g) and G8 (0.07 mg/g). The chlorophyll content showed an 11.23 % coefficient of variation. Chlorophyll b content (mg/g) ranged from 0.04 to 0.32 mg/g and maximum being in G24 followed by G9 (0.24 mg/g) and G13 (0.23 mg/g) in Supplementary Table 2. The minimum chlorophyll b content observed in the genotypes G18 followed by G3 (0.06 mg/g), G8 (0.06 mg/g), G15 (0.07 mg/g) and G19 (0.07 mg/g). The chlorophyll b content showed 13.53% coefficient of variation. Wide range of variation was observed among 28 genotypes of chili for anthocyanin content of fruit extract (Supplementary Table 2). The highest anthocyanin content was found in the genotype G18 (4.18 µg/g FW) followed by G23 (3.26 µg/g FW) and G24 (2.83 µg/g FW). The lowest anthocyanin content was observed in genotype G11 (0.48 µg/g FW) followed by G7 (0.74 µg/g FW), G27 (0.74 µg/g FW) and G21 (0.84 µg/g FW). Total carotenoid content ranged from 0.04 to 0.47 (mg/g) among 28 genotypes of chili (Supplementary Table 2). The highest amount of total carotenoid content was observed in the genotype G14 followed by G10 (0.14 mg/g), G19 (0.14 mg/g), and G6 (0.13 mg/g). The lowest amount of carotenoid content was observed in the genotype G17 followed by G21 (0.05 mg/g), G13 (0.06 mg/g), and G20 (0.06 mg/g). Variation in the total phenol content of 28 genotypes of chili is presented in Figure 1.

Table 1. Analysis of variance (ANOVA) for nutritional phytochemicals and mineral contents in chili.

Source of variation	df	Mean squares (MS)												
		CAP	AAC	BBC	TPC	TFC	TAO	Chl a	Chl b	TCC	ANC	Na	K	Ca	Mg
Replication	2	0.00	143.2	0.001	592.56	1127.55	229.18	0.003	0.002	0.04	0.01	0.02	0.00	0.00	0.00
Genotype	27	0.02^{ns}	2129.84**	0.005^{ns}	334905.59**	540124.88**	2124.62**	0.013^{ns}	0.012^{ns}	0.02^{ns}	1.94*	0.02^{ns}	0.022^{ns}	0.15^{ns}	0.01^{ns}
CV (%)	-	8.85	12.04	8.38	13.45	11.53	14.23	11.23	13.53	11.41	4.66	6.85	2.02	7.53	3.78
Error	54	0.00	77.46	0.00	218.83	505.7	43.39	0.00	0.00	0.01	0.01	0.02	0.001	0.01	0.00

*, ** and ns indicate significance at 5% and 1% levels and non-significance, respectively, df – Degrees of freedom, CAP- Capsaicin content (%), AAC - Ascorbic acid content (mg/100g), BCC - β - carotene content (mg/100g), TPC - Total phenolic content (µg/g FW), TFC - Total flavonoids content (µg/g FW), TAO - Total antioxidant content (µg/g FW), Chl a - Chlorophyll a content (mg/g), Chl b - Chlorophyll b content (mg/g), TCC - Total carotenoid content (mg/g), ANC - Anthocyanin content (µg/g FW), Na - Sodium content (%), K - Potassium content (%), Ca - Calcium content(%), Magnesium content (%).

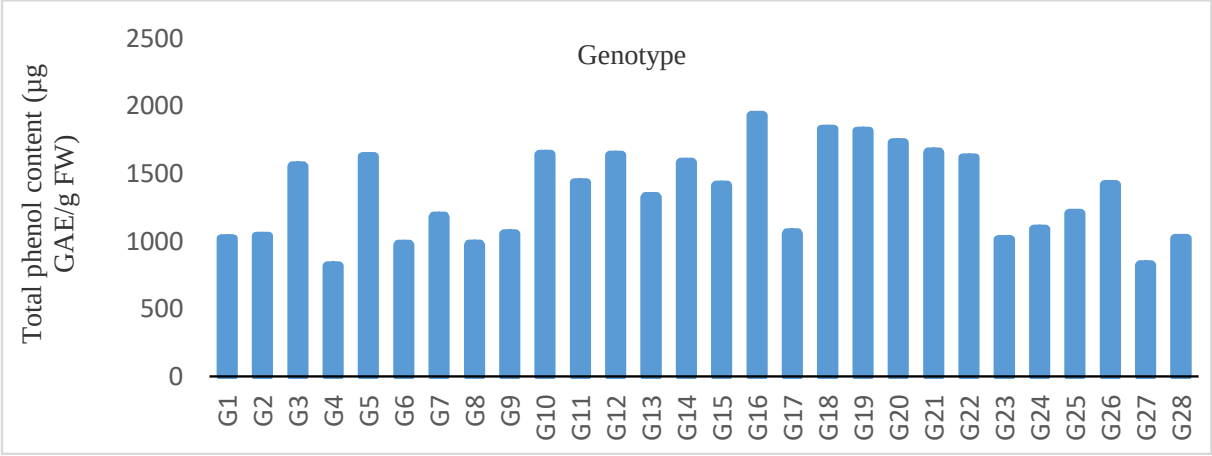

Figure 1. Variation in total phenol content in the fruit extract of 28 genotypes of chili, (GAE= Gallic Acid Equivalent).

Table 2. Means of nutritional phytochemicals and mineral contents of fruits of 28 chili genotypes arranged in four clusters. Abbreviations are as defined in Table 1.

Cluster	Counts	CAP	AAC	BBC	Chl a	Chl b	TCC	ANC	Na	K	Ca	Mg
1	2	0.37	19.46	0.22	0.14	0.09	0.10	1.74	0.10	1.94	0.85	0.56
2	10	0.23	53.99	0.18	0.17	0.18	0.09	1.53	0.18	1.93	0.99	0.53
3	10	0.21	81.56	0.22	0.12	0.10	0.09	1.45	0.12	1.89	1.06	0.53
4	6	0.25	108.60	0.21	0.12	0.13	0.16	1.83	0.14	1.93	1.15	0.53

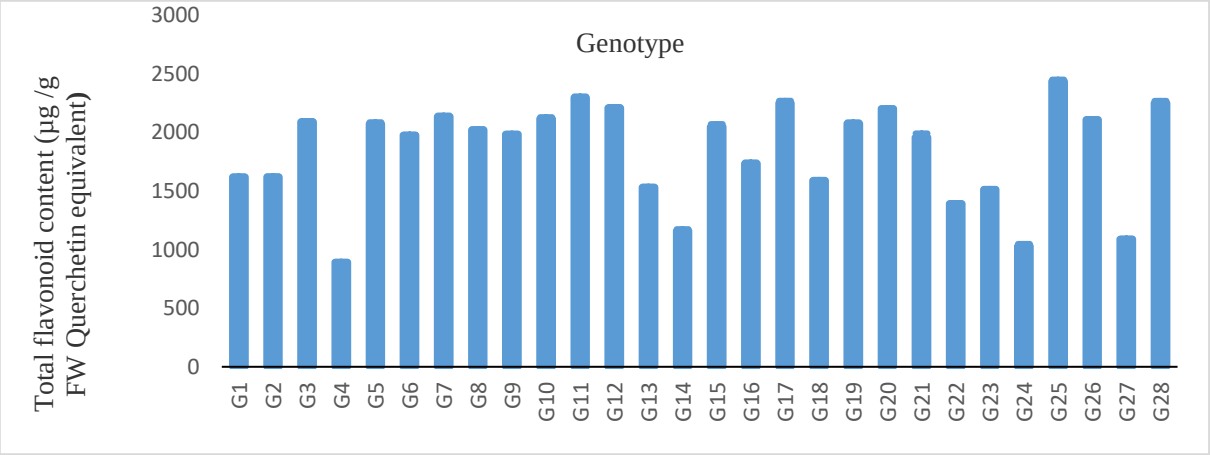

Figure 2. Variation in total flavonoid content in the fruit extract of 28 genotypes of chili.

Among the genotypes, total phenol content was high in G16 followed by G18, G19, G20, G21, G10, G12, G5, G22, G14, G3, G11, G26, G15 and G13. The lowest amount of total phenol content was observed in the genotype G4 followed by G27, G6, G8, G23, G1, G28, G2, G17, G9, G24, G7, and G25. Variation in total flavonoid content of chili fruit extract of 28 genotypes is presented in Figure 2. Total phenol content was high in G25 followed by G11, G17, G28, G12, G20, G7, G10, G26, G3, G19, G5, G15, G8, G9, G6, G21, G23, G13. The lowest amount of total phenol content was observed in the genotype G4 followed by G24, G27, G14, G22, G13, G18, G1, G2, and G16. The total antioxidant content of 28 genotypes of chili is presented in Figure 3. Among the genotypes, total antioxidant content was high in G2 and G16. The lowest amount of total antioxidant content was observed in the genotype G20 followed by G22, G5, G27, and G17.

Mineral composition of fruit extract of chili

Among the 28 genotypes of chili, sodium content (%) showed low variability and ranged from 0.06% to 0.39 %. The highest amount of sodium was observed in genotypes G4 and G26 (0.39 %) followed by G10 (0.27 %) and G13 (0.23%). The lowest amount of sodium was observed in the genotype G8 (0.06%) followed by G5, G7, and G19 (0.08 %). The mean sodium content in 28 genotypes was 0.15% and six genotypes showed above-average value (Supplementary Table 3). Fruit extract of 28 genotypes of chili was investigated for potassium content (%) and found pronounced variability which ranged from 1.71% to 2.07% (Supplementary Table 3). The highest amount of potassium content was observed in the fruit extract of the genotype G21 followed by G17 (2.01 %) and G22 (2.01 %).

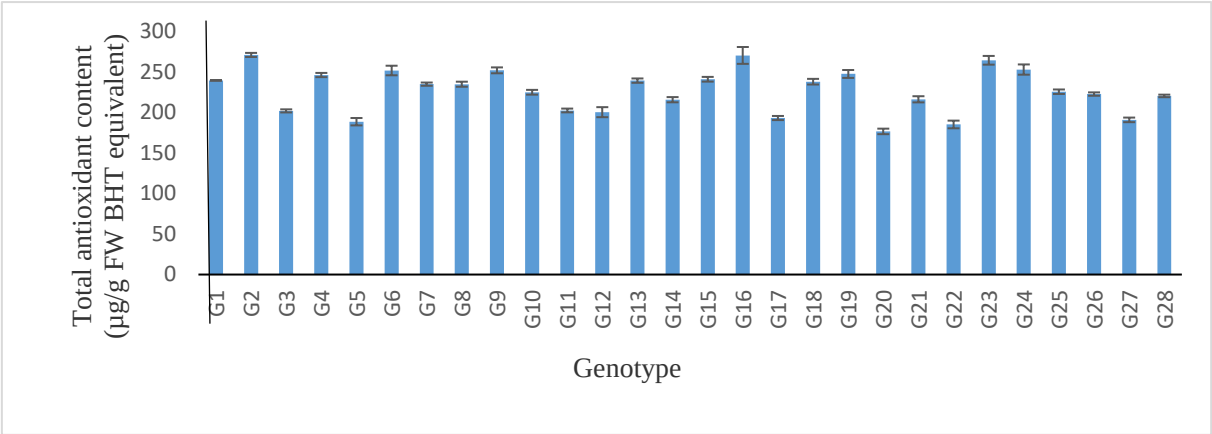

Figure 3. Variation in total antioxidant content in the fruit extract of 28 genotypes of chili, (BHT = Butylated Hydroxy Toluene).

The lowest amount of potassium content was observed in the fruit extract of the genotype G25 (1.71%) followed by G23 (1.79%) and G26 (1.8 %). The mean of potassium content in the fruit extract of 28 genotypes was 1.92% and among them 15 genotypes showed above average mean value. Calcium content (%) of the fruit extract of the chili genotypes under investigation noticed wide range of variation and was ranged between 0.72% and 1.65% (Supplementary Table 3). The highest amount of calcium content was observed in the fruit extract of the genotype G10 followed by G11 (1.48 %) and G15 (1.41 %). The lowest amount of calcium content was observed in the fruit extract of the genotypes G9 (0.72%) and G19 (0.72%) followed by G13 (0.75%) and G8 (0.83 %). The mean of calcium content in 28 genotypes was 1.04% and 12 genotypes showed above-average calcium content in the fruit extract. Magnesium content (%) showed low variability in the fruit extract of 28 genotypes of chili and ranged from 0.40% to 0.63 % (Supplementary Table 3). The highest amount of magnesium was observed in the fruit extract of G10 followed by G12 (0.62 %), G21 (0.62 %) and G27 (0.62 %). The lowest amount of magnesium was observed in the fruit extract of the genotype G13 (0.4 %) followed by G3 (0.44 %) and G14 (0.45 %). The mean of magnesium content in 28 genotypes was 0.54 %.

Heatmap and correlations among traits

By using 2-way clustering and heatmap, a dendrogram was constructed based on nutritional phytochemicals and mineral contents of fruits of 28 chili genotypes which clustered the genotypes into 4 clusters (Figure 4). The trait variables visualized as co-cluster heatmap were classified into 3 groups. The chili genotypes grouped into row cluster showed high similarity while the genotypes in column cluster showed strong association. Among the 4 clusters, the cluster I contains minimum number of genotypes (2: G19, G20), remaining 3 clusters contains 10 (cluster II), 10 (cluster III) and 6 (cluster IV) genotypes (Figure 4). The results of cluster mean showed that genotypes of cluster I had maximum values for capsaicin content (0.37%), β - carotene content (0.22 mg/100g), potassium content (1.94 %) and magnesium content (0.56 %). Genotypes of cluster II had maximum mean values for Chl a content (0.17 mg/g), Chl b content (0.18 mg/g) and sodium content (0.18 %). Cluster mean of cluster II depicted the highest mean values only for β - carotene content (0.22 mg/100g) along with genotypes of cluster I (Supplementary Table 2, Figure 4). The genotypes of cluster IV had maximum mean values for ascorbic acid content (108.60 mg/100g), total carotenoid content (0.16 mg/g), ANC - anthocyanin content (1.83 µg/g FW) and calcium content (1.15 %). Heatmap shows the genotypes of cluster IV was rich in ascorbic acid content (ACC) and anthocyanin content (ANC), on the other hand, the genotypes of cluster I was poor for ascorbic acid content. The other variables had poor associations with these four and among each other.

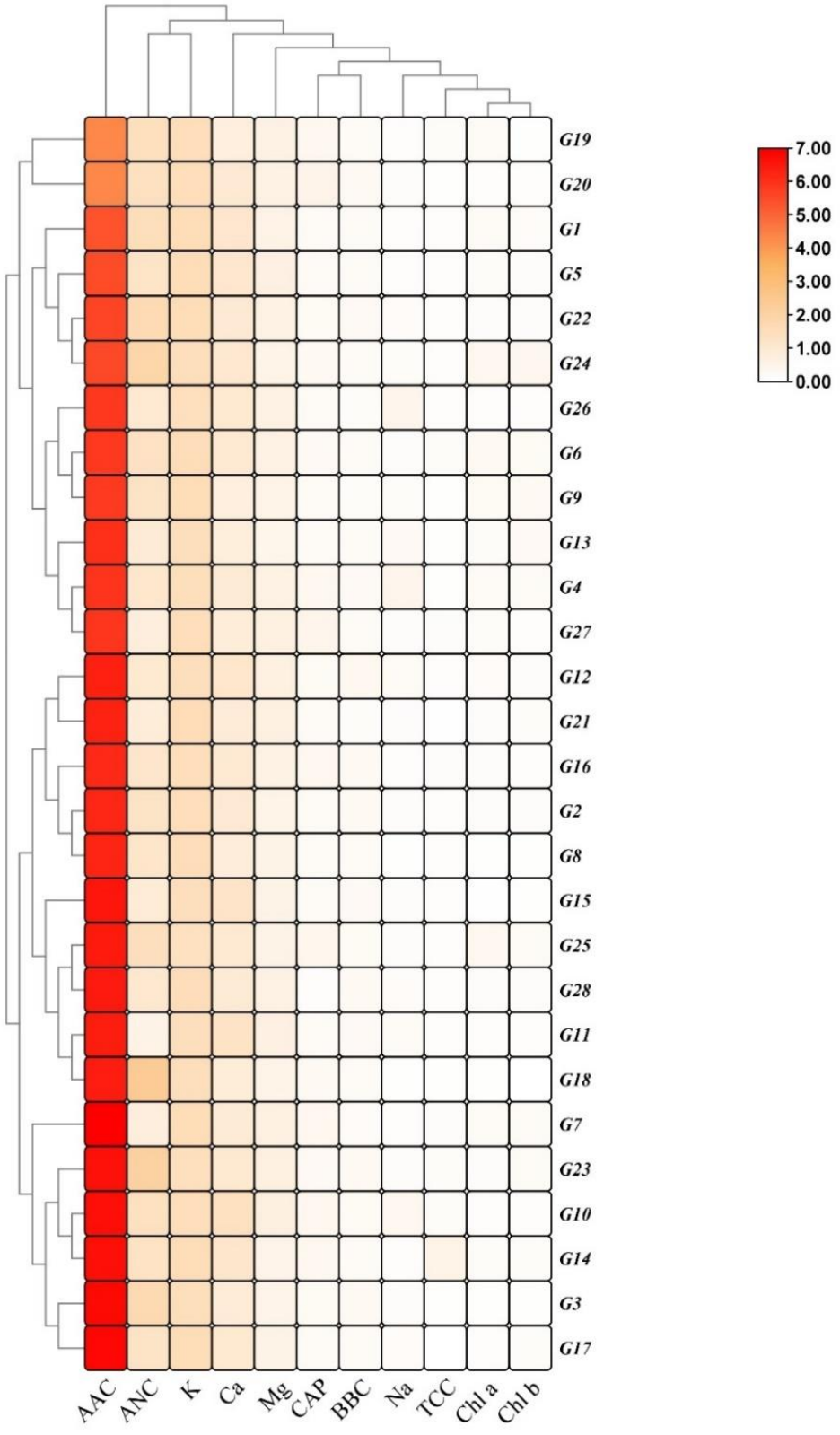

Figure 4. Cluster heatmap showing visual representation of 28 genotypes of chili in different groups based on similarity and correlations among traits. Abbreviations are as defined in Table 1.

Discussion

The evaluation of the extent of genetic variation within species has become now an essential tool in breeding program for their improvement (Dubey et al., 2015). The degree of genetic diversity of genotypes and association of yield and quality traits are the important determinant for any breeding program of the crops (Shrestha, 2023). Genotype screening for yield and quality traits are the established techniques for selecting and managing genetic resources of any crops. The present research was conducted to evaluate chili genotypes for their nutritional phytochemicals and mineral content. A significant variation was observed for ascorbic acid content, total phenolic content, total flavonoids content, total antioxidant content, and anthocyanin content of fruits of 28 chili genotypes. The results of the current study are in accordance with the findings of Guzman et al. (2021) who evaluated genotypes of capsicum for bioactive compounds. Other traits under this study showed no variation among the genotypes for these traits and further improvement would not be possible by selection. Variations in other biochemical traits were also noted by González-López et al. (2021).

The greater amount of capsaicin content was observed in genotypes G20, G27 and G25 from 28 chili genotypes. The comparable genotypic differences were also reported by Bhagawati and Saikia (2015) for capsaicin content in different chili cultivar. Constantino et al. (2020) also confirmed the findings of previous studies. The genotypes with high capsaicin content could be utilized for the development of highly pungent variety and will serve as potential sources in the local and global markets. The capsaicin content in chili observed to be an effective antioxidant (Olatunji and Afolayan, 2018). The ascorbic acid content showed a significant variation among the 28 chili genotypes and produces the highest amount of 125.56 mg/100g and the lowest amount of 19.45 mg/100g. Similar results were reported by Pradhan et al. (2018). Janaki et al. (2015) found that the ascorbic acid content of chili fruits ranged from 43.99 to 223.22 mg/100g. The highest amount of ascorbic acid content was observed in the fruits of genotype G7 (125.56 mg/100g) and G17 (113.26 mg/100g) could be useful in the development of nutritionally rich varieties.

β - carotene content showed low variability among the 28 genotypes of chili and the highest amount of β - carotene content was observed in genotype G12 (0.29 mg/100g) whereas the lowest was in genotype G9 and G26 (0.13 mg/100g. Sarker et al. (2012) reported the highest beta carotene content (0.39 mg/100g) in chili. A wide range of variation was observed for anthocyanin content in chili genotypes. The highest anthocyanin content of fruit extract was found in the genotype G18 (4.18 µg/g FW) and the lowest in the genotype G11 (0.48 µg/g FW). The anthocyanin content showed a 4.66 % coefficient of variation. Arnnok et al. (2012), and Adhikari and Pradhan (2014) reported that anthocyanin content ranged from 0.796 to 4.70 mg CGE kg^{-1} fresh weight.

Campos et al. (2013) showed total carotenoid content ranged from 1.00 to 1.26 mg/100 g sample of chili. The genotypes identified based on bioactive compounds could be helpful in future breeding programs for cultivar improvement (Karim et al., 2021). Chili contains a large number of phenolic compounds or flavonoids called quercetin, luteolin, and capsaicinoids (Hasler, 1998; Nahak et al., 2017). The consumption of these bioactive compounds provides beneficial effects on human health due to their antioxidant properties, which protect against damage to cells and thus prevent the development of common degenerative diseases such as cancer, cardiovascular diseases, cataracts, and diabetes (Blanco-Ríos et al., 2013). These chemical compounds also prevent the oxidation of essential fats within the cells of the brain that are considered necessary for its optimal functioning (Oboh and Rocha, 2007). Total phenolic content varies from cultivar to cultivar (Hamed et al., 2019). Dubey et al. (2015) reported the existence of variations in the concentration of different polyphenols and flavonoids in chili accessions. The presence of phenolic compounds in Capsicum fruits were reported by Sukrasno and Yeoman (1993). The presence of various individual phenols and flavonoids were also reported in chili by Materska et al. (2003) .

Fruit extract of 28 genotypes of chili was investigated for potassium, calcium and magnesium content (%) and found noticeable

variability. The highest amount of potassium content was observed in the fruit extract of the genotype G21. The highest amount of calcium and magnesium content was observed in the fruit extract of the genotype G10 (1.65% and 0.63%, respectively). Potassium, calcium and magnesium are very important nutrient elements for the body. Potassium prevents high blood pressure, plays a role in neurotransmission and helpful for synthesis of protein and amino acids (Khan et al., 2019). Calcium is important for muscle movement and it helps to carry message between brain and other body parts. Magnesium plays important role in the release of parathyroid hormone which is important for kidney function, backbone activities, and also acts as catalyst for converting Vitamin D (Khan et al., 2019) . The amount of these minerals in the chili fruits depends on their genotypes as well as fertility status of the soil and farming practices (Emmanuel-Ikpeme et al., 2014). The fruit extracts of chili genotypes are an excellent source of health-related phytochemicals and minerals such as ascorbic acid (Vitamin C), carotenoids (pro-Vitamin A), flavonoids, capsaicin, potassium, calcium, and magnesium. These elements are very much essential for preventing several chronic diseases such as cancer, asthma, sore throats, diabetes, cardiovascular disease (Shrestha, 2023).

Cluster analysis represents to define the pairwise differences between genotypes. Two-way clustering partitioned data in two directions, it clustered the genotypes together and also clustered the variables at the same time (Hageman et al., 2012). The heatmap classified chilli genotypes into 4 groups and variables (phytochemicals and nutrient contents) into 3 groups. Heatmap shows the genotypes of cluster IV was rich in ascorbic acid content (ACC) and anthocyanin content (ANC), on the other hand, the genotypes of cluster I was poor for ascorbic acid content. So, genotypes from cluster IV can be considered as parent for future breeding program specially G7 and G17 which contain high amount of ascorbic acid in fruits (Supplementary Table 2). The variables ascorbic acid, anthocyanin, K, and Ca content correlated strongly. These associations can help to improve these traits together in chilli. The other variables had poor associations with these four and among each other and couldn't considered together to improve phytochemical content in chili through any breeding program.

Conclusion

The present research through evaluation of nutritional phytochemical and mineral content provides an understanding of cultivated and available chili genotypes in Bangladesh. For capsaicin, ascorbic acid, and β-carotene content the genotypes G20, G7, and G12 were found to be superior than other genotypes. The highest amount of anthocyanin, carotenoid, total phenol, total flavonoid, and total antioxidant content was found in the genotypes G18, G14, G16, G25, and G2, respectively. The genotypes G4 and G26 contained a higher amount of Na and G21 had a higher amount of K. The highest amount of Ca and Mg were observed in the genotype G10. In case of heatmap analysis based on nutritional phytochemicals and minerals content, 28 chili genotypes were divided into 4 distinct clusters and traits into 3 clusters. Heatmap showed the genotypes of cluster IV was rich in ascorbic acid and anthocyanin content, and genotypes of cluster I was poor. The traits ascorbic acid, anthocyanin K and Ca content revealed strong association among them. Based on above findings the genotypes G2, G7, G12, G16, G17, G18 and G25 could be selected as parents for further improvement of chili through breeding program.

Supplementary Materials:

The supplementary material for this article can be found online at: https://www.jpmb-gabit.ir/article_717989.html

Supplementary Table 1. Solution preparation protocol used in the analysis of different phytochemicals in chili.

Supplementary Table 2. Mean performance of fruits of 28 chili genotypes for seven quality traits.

Supplementary Table 3. Mean performance of 28 chili genotypes mineral content of fruit extract of chili.

Author Contributions

Conceptualization, A. K. M. A. I. and P. H. C.; methodology, P. H. C., M. M. U. and M. M. H. S.; writing—original draft preparation, P. H. C.; writing—review and editing, A. K. M. A. I and P. H.

C. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest Statement

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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مقاله پژوهشی

ارزیابی ژنوتیپ‌های فلفل قرمز (*Capsicum annuum* L.) از نظر خصوصیات فیتوشیمیایی و مواد مغذی

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چکیده: فلفل قرمز (*Capsicum annuum* L.) یک محصول مهم در سراسر جهان بوده که به دلیل خواص تغذیه‌ای و فیتوشیمیایی آن شناخته شده می‌باشد. این مطالعه با هدف شناسایی ژنوتیپ‌های مناسب در برنامه‌های اصلاحی، با ارزیابی محتوای فیتوشیمیایی و معدنی ۲۸ ژنوتیپ فلفل قرمز انجام شد. غلظت کپسایسین از ۰/۰۸درصد (G20) الی ۰/۴۴ درصد (G28) متغیر بود. حداکثر غلظت اسید اسکوربیک در G7 (۱۲۵/۵۶ میلی‌گرم در ۱۰۰ گرم)، در حالی‌که حداقل آن در G20 (۱۹/۴۵ میلی‌گرم در ۱۰۰ گرم) تعیین شد. غلظت β-کاروتن از ۰/۲۹ میلی‌گرم در ۱۰۰ گرم FW در G12 تا ۰/۱۳ میلی‌گرم در ۱۰۰ گرم در G13 و G26 متغیر بود. حداکثر محتوای کلروفیل (a و b) در G24 (به ترتیب ۰/۳ و ۰/۳۲ میلی‌گرم بر گرم وزن تر) مشاهده شد، در حالی‌که حداقل مقادیر در ژنوتیپ‌های G15 و G18 ثبت گردید. غلظت آنتوسیانین از ۴/۱۸ میکروگرم بر گرم FW در G18 تا ۰/۴۸ میکروگرم بر گرم FW در G11 متغیر بود. ژنوتیپ G16 بالاترین سطح فنول کل و آنتی اکسیدان را نشان داد، در حالی‌که G25 بالاترین غلظت فلاونوئید را نشان داد. تجزیه و تحلیل مواد معدنی نشان داد که G4 و G26 حاوی بالاترین سدیم (۰/۳۹ درصد)، G21 دارای بالاترین پتاسیم (۲/۰۷ درصد)، و G10 دارای بالاترین کلسیم (۱/۶۵ درصد) و منیزیم (۰/۶۳ درصد) بودند. طبق تجزیه و تحلیل نقشه حرارتی ژنوتیپ‌ها، خوشه IV دارای سطوح بالایی از اسید اسکوربیک و آنتوسیانین بوده، در حالی که خوشه I از سطوح پایینی برخوردار بود. ارتباط قابل توجهی بین غلظت اسید اسکوربیک، آنتوسیانین، پتاسیم و کلسیم شناسایی شد. در برنامه‌های اصلاحی خصوصیات تغذیه‌ای واریته‌های فلفل، ژنوتیپ‌های G2، G7، G12، G16، G17، G18 و G25 با پروفایل‌های فیتوشیمیایی و معدنی قابل توجه خود حایز اهمیت می‌باشند. این مطالعه اطلاعات مهمی در مورد تنوع ژنتیکی ژنوتیپ‌های فلفل قرمز در بنگلادش می‌دهد.

کلمات کلیدی: فلفل، فنول کل، فلاونوئیدها، فعالیت آنتی‌اکسیدانی و محتوای آنتوسیانین.

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Original paper

The effects of Trichoderma fungi symbiosis and nitrogen on essential oil and leaf pigments in green cumin (*Cuminum cyminum L.*) under weed competition

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Abstract: Green cumin is one of the most widely utilized medicinal plants both in Iran and globally, which is mainly exploited for the purpose of using its essential oil. It seems necessary to conduct an experiment in this direction to pay attention to sustainable agriculture, which can be achieved by using biofertilizers along with chemical inputs such as nitrogen. This research was carried out in Maraveh-Tappeh county and in two distinct regions (Qarah-Dam and Shalami) during the 2023 crop year, as a split factorial experiment based on randomized complete block design with three replications. The experimental treatments were nitrogen fertilizer levels (control, 50 and 100 % recommended nitrogen from urea) as the main plot, biofertilizer treatments (control, seed inoculation with *Trichoderma longibrachiatum*, *Trichoderma atroviride*, and co-inoculation (*Trichoderma longibrachiatum* and *Trichoderma atroviride*) and weeding management (no weeding and weeding) as sub-plots. The results showed that the highest essential oil content and yield in both regions were achieved under the no weeding condition with 100 % recommended nitrogen and co-inoculation treatments. Also, the use of Trichoderma fungi and nitrogen had a positive effect on the content of chlorophyll a, chlorophyll b and total chlorophyll.

Keywords: Anthocyanin, biofertilizer, chlorophyll content, essential oil content, nitrogen.

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Introduction

Medicinal plants play a vital role in preserving biodiversity, providing food and medicine, improving soil and water quality, and reducing pollution. Additionally, they strengthen ecosystems and aid in their sustainability (Agbo et al., 2023; Ahmadinia and Heidari, 2023; Hosseinpour Azad, 2023). Green cumin (*Cuminum cyminum* L.) is one of the most important medicinal plants known in Iran and the worldwide, valued for the properties of its seeds (Hadi et al., 2018). These seeds are widely used in the pharmaceutical, culinary and traditional medicine industries due to their unique taste and numerous health benefits (Karik et al., 2021). Cumin seeds are rich in fiber, which naturally stimulates the digestive system and improves digestion. In addition, due to the abundant presence of bioactive compounds, green cumin has anti-cancer, anti-bloating, anti-spasmodic, antioxidant, digestive system strengthening, anti-fungal, anti-microbial and anti-inflammatory properties (Mohammed et al., 2024).

The green cumin plant is cultivated in different regions around the world (Hadi et al., 2018). However, green cumin cultivation is fraught with challenges, especially weed competition, which threatens agricultural sustainability and crop productivity. Weeds compete with crops for light, water, and nutrients. Also, studies have shown that weed competition can reduce crop yield by 20% to more than 80%, depending on the severity of the infestation and the time of weed emergence. To combat this yield reduction, farmers have traditionally relied on chemical herbicides (Scavo and Mauromicale, 2020). However, growing concerns about environmental sustainability and human health have stimulated interest in alternative and environmentally friendly weed management strategies (Kohestani et al., 2023).

In this context, the role of beneficial microorganisms, especially *Trichoderma* fungus, has attracted much attention in agricultural research. These fungi are known for their high ability to increase plant resilience through various mechanisms, including enhancing nutrient availability, improving root growth, and providing biocontrol against soil-borne pathogens (Rostaminia et al., 2021; Kulbat-Warycha et al., 2024). In addition, *Trichoderma* species can establish symbiotic relationships with plants, which leads to increased stress tolerance and growth even in competitive conditions (Guo et al., 2020). In fact, the use of innovative biological formulations containing microbes, by improving the yield and quality of medicinal plants, provides an opportunity to develop agricultural systems with the least environmental effects in the agricultural production process (Comite et al., 2021).

In addition, nitrogen plays an important role in plant growth, affecting growth rate, biomass accumulation, and yield (Mahmud et al., 2020). Nitrogen plays an important role in the composition of proteins, enzymes, chlorophyll, vitamins, and alkaloids (Mu and Chen, 2021). Nitrogen deficiency in plants causes the growth of vegetative organs to stop and the leaves take on a yellowish-green color, in addition, it causes disruption in the synthesis of chlorophyll and enzymes (Anas et al., 2020; Adl et al., 2023). By providing nitrogen in the soil, the absorption of nutrients and the production of metabolites in the plant increases. Therefore, the use of nitrogen fertilizers improves crop nutrition, which is especially important in nutrient-deficient soils (Hao et al., 2023). However, the interaction between nitrogen application and microbial inoculations such as *Trichoderma* in reducing weed competition, especially in the field of green cumin, has not been widely studied.

Also, some researchers have reported the positive effects of *Trichoderma* fungi on the quantitative and qualitative traits of medicinal plants. For example, the application of *Trichoderma* fungi positively affected the chlorophyll and anthocyanin content in the medicinal plant (*Hibiscus sabdariffa* L.) (Rostaminia et al., 2021), and the increase of the chlorophyll index in the black cumin plant (*Nigella sativa* L.)(Nassif et al., 2023). In another experiment, the use of *Trichoderma* spores added to the soil led to an increase in flavonoid and terpenoid content were found in the leaves of *thyme* species (Kulbat-Warycha et al., 2024). In addition, the results of the effect of *Trichoderma* fungi on yield, photosynthetic activity and production of secondary metabolites of basil showed that photosynthetic efficiency, phenolic acids and rosmarinic acid content increased (Comite et al., 2021).

This study aimed to evaluate the response of essential oil content and yield, representing overall phytochemicals, as well as photosynthetic and non-photosynthetic pigments of green cumin medicinal plant to symbiosis with *Trichoderma* fungus and nitrogen application under weed competition.

Materials and Methods

Field experiment locations

This research was carried out in Maraveh-Tappeh county and in two different regions (Qarah-Dam and Shalami) in 2023 crop year as a split factorial experiment based on randomized complete block design with three replications.

The treatments of the experiment

The experimental treatments included nitrogen fertilizer (control, 50 and 100% recommended nitrogen from urea fertilizer source) as the main factor, biofertilizer (control, seed inoculation with *Trichoderma longibrachiatum*, *Trichoderma atroviride*, and co-inoculation (*Trichoderma longibrachiatum* + *Trichoderma atroviride*)) and weeding management (no weeding and weeding) are sub-factors. The fungi used in this research were obtained from the Environmental Stress Laboratory located at Sari Agricultural Sciences and Natural Resources University.

Field growth conditions

Before planting, in order to determine the characteristics of the soil, sampling was done from 0-30 cm of the research site (three mixed samples from different parts of the field), and its physical and chemical characteristics were evaluated (Table S1). The characteristics of the meteorological data were shown in Table S2. To experiment, land preparation was completed on February 9, 2023. Planting commenced following the land's leveling on February 14, 2023. The main plots were 21 × 3 m with a distance of one meter from each other and the sub-plots were 3 × 2 m with a distance of 60 cm from each other. In each experimental plot, rows were spaced 20 cm apart, with a plant spacing of 4 cm within each row. As a result, the target density of 125 plants per square meter was effectively achieved. In this experiment, a local variety seeds were used. Before planting, the seeds were pre-treated with fungi. Based on the soil test results, the chemical fertilizers (e.g., nitrogen (urea source; 150 kg ha^{-1}), triple superphosphate (100 kg ha^{-1}) and potassium sulfate (100 kg ha^{-1}) were applied to the soil. Regarding the nitrogen application, 50 kg ha^{-1} was applied before planting and the remainder was applied in two stages: 30 and 60 days after planting. No herbicides, fungicides, or chemical pesticides were used during the growth period. Weed control was carried out manually using mechanical method.

Measurement of characteristics

After removing a half-meter border around the plot, the plants were harvested from the remaining area, and the seed yield was evaluated per square meter. The seeds were dried in a shaded area to preserve the quality of the seeds and essential oils.

Measurement of essential oil content and yield

For essential oil extraction, 30 grams of cumin seeds were used. The essential oil was extracted using a Clevenger apparatus. Then the essential oil was dehumidified by sodium sulfate and the amount of essential oil was weighed with a precise balance. The yield of essential oil was calculated by equation (1).

Equation (1) $Essential\ oil\ yield\ (\frac{kg}{ha}) = (Essential\ oil\ content \times seed\ yield)$

Measurement of chlorophyll and anthocyanin

Sampling for physiological characteristics was conducted using young leaves at the flowering stage. The leaf samples were immediately transferred to the freezer at -80 °C. At this stage, characteristics such as chlorophyll *a*, chlorophyll *b*, total chlorophyll, carotenoid Lichtenthaler (1987), and anthocyanin Krizek et al. (1993) were measured by spectrophotometry.

Data analysis

In order to analyze the data, the homogeneity of the variances of the two research sites was tested using Bartlett's test. The data obtained in the experiments were analyzed by SAS software version 9.1. Least significant difference (LSD) method was used to compare the means. The graphs were drawn using Excel software.

Results

Phytochemical and physiological characteristics of at Qarah-Dam samples region

Essential oil content and yield

The results of the analysis of variance showed that the essential oil content was significantly ($P \leq 0.01$) affected by nitrogen, biofertilizer and weed treatments (Table S3). Also, two-way and three-way interaction for essential oil content were significant ($P \leq 0.01$) (Table S3).

Under no nitrogen application, the highest content of essential oil was related to the treatment of simultaneous inoculation of two fungi + no weeding (0.77 %), compared to the treatments of simultaneous inoculation of two fungi + weeding (0.72 %) and *T. atroviride* inoculation + no weeding (0.68%) had no statistically significant difference, and the lowest content of essential oil was observed in the treatment of no inoculation + weeding (0.61 %) (Figure 1). In the conditions of application of 50 % recommended nitrogen fertilizer, treatments of T. atroviride inoculation + no weeding (0.83 %) and simultaneous inoculation of two fungi + no weeding (0.83 %) had the highest content of essential oil and there was no statistically significant difference with simultaneous inoculation of two fungi + weeding (0.75 %), T. *atroviride* inoculation + weeding (0.72 %) and *T. longibrachiatum* inoculation + no weeding (0.78 %) treatments. On the other hand, the lowest content of essential oil in the conditions of application of 50 % recommended nitrogen fertilizer was related to the treatment of no inoculation + weeding (0.62 %). In application of 100 % recommended nitrogen fertilizer, the highest essential oil content was recorded with simultaneous inoculation of two fungi + no weeding (1.30 %). In contrast, the lowest amount of essential oil content was observed under conditions of no inoculation + weeding (0.72 %) without statistically significant difference with no inoculation + no weeding (0.84 %), *T. longibrachiatum* inoculation + weeding (0.84 %) and *T. atroviride* inoculation + weeding (0.86 %) treatments (Figure 1).

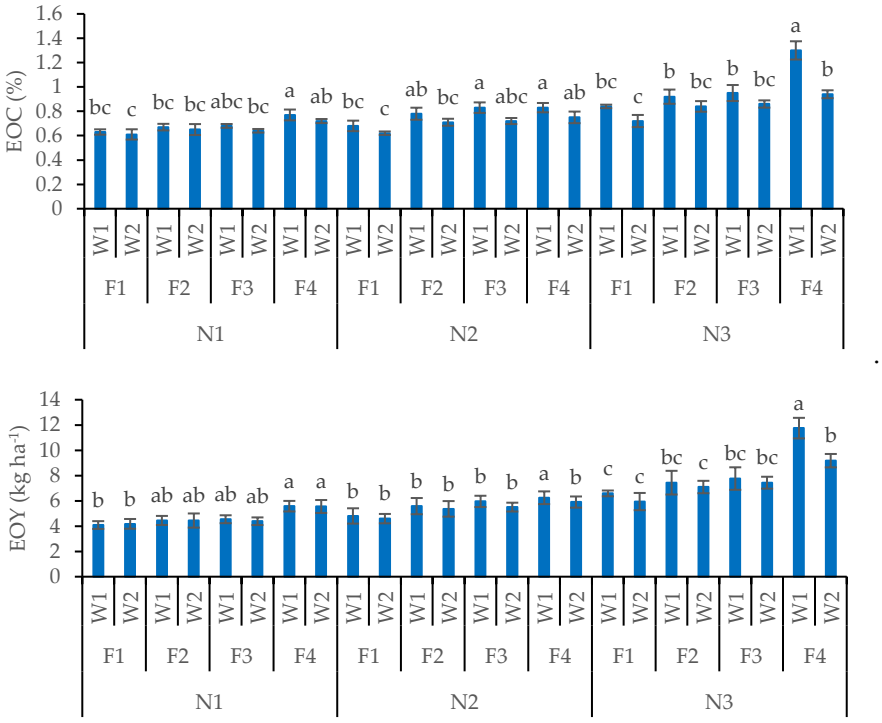

Figure 1. Influence of nitrogen, biofertilizer and weeding on essential oil content (EOC) and essential oil yield (EOY) characteristics in green cumin plants cultivated in Qarah-Dam region (W1: no weeding, W2: weeding, F1: no inoculation, F2: *T. longibrachiatum* inoculation, F3: *T. atroviride* inoculation, F4: simultaneous inoculation of *T. longibrachiatum* and *T. atroviride*, N1: no nitrogen application, N2: application of 50 % of recommended nitrogen, N3: application of 100 % of recommended nitrogen). Means followed by different letters in the same column for the same factor are significantly different ($P \leq 0.05$) according to the LSD test. Mean comparisons were shown as mean ± standard error (SE).

The results of the analysis of variance showed that the essential oil yield was significantly ($P \leq 0.01$) affected by nitrogen, biofertilizer, and weed treatments (Table S3). Also, two-way and three-way interactions for essential oil yield were significant ($P \leq 0.01$). Based on the results, under no nitrogen application, it was observed that the treatments of *T. atroviride* inoculation + no weeding (5.58 kg ha^{-1}) and simultaneous inoculation of two fungi + weeding (5.55 kg ha^{-1}) had the highest amount of essential oil yield without statistically significant difference with *T. longibrachiatum* inoculation + no weeding (4.45 kg ha^{-1}), *T. longibrachiatum* inoculation + weeding (4.4 kg ha^{-1}), *T. atroviride* inoculation + no weeding (4.55 kg ha^{-1}) and *T. atroviride* inoculation + weeding (4.39 kg ha^{-1}) treatments (Figure 1). On the other hand, in the conditions of no nitrogen application, the lowest amount of essential oil yield was related to the treatments of no inoculation + no weeding (4.09 kg ha^{-1}) and no inoculation + weeding (4.18 kg ha^{-1}). In the conditions of application of 50 % of recommended nitrogen, the treatment of simultaneous inoculation of two fungi + no weeding (6.24 kg ha^{-1}) had the highest amount of essential oil yield, while the other treatments had the lowest essential oil yield (Figure 1). In the conditions of 100% recommended nitrogen, the simultaneous inoculation of two fungi + no weeding (11.75 kg ha^{-1}) had the highest amount of essential oil yield; If the treatments of no inoculation + no weeding (6.59 kg ha^{-1}), no inoculation + weeding (5.94 kg ha^{-1}) and *T. longibrachiatum* inoculation + weeding (7.09 kg ha^{-1}) had the lowest essential oil yield and had no statistically significant difference with *T. longibrachiatum* inoculation + no weeding (7.44 kg ha^{-1}), *T. atroviride* inoculation + no weeding (7.76 kg ha^{-1}) and *T. atroviride* inoculation + weeding (7.44 kg ha^{-1}) treatments (Figure 1).

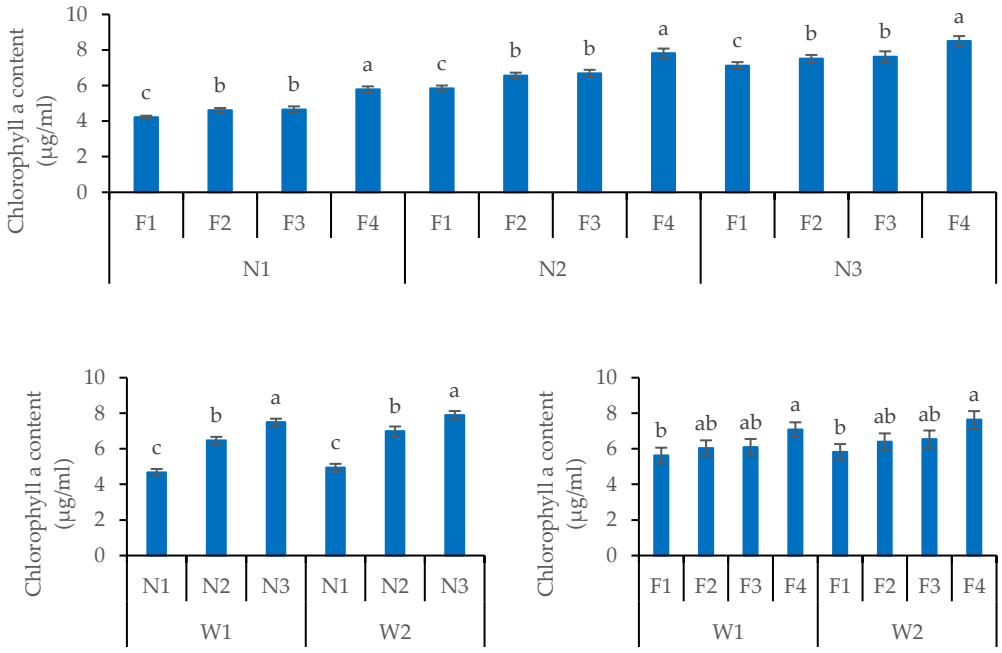

Figure 2. Influence of nitrogen, biofertilizer and weeding on chlorophyll a content characteristic in green cumin plants cultivated in Qarah-Dam region (W1: no weeding, W2: weeding, F1: no inoculation, F2: *T. longibrachiatum* inoculation, F3: *T. atroviride* inoculation, F4: simultaneous inoculation of *T. longibrachiatum* and *T. atroviride*, N1: no nitrogen application, N2: application of 50 % of recommended nitrogen, N3: application of 100 % of recommended nitrogen). Means followed by different letters in the same column for the same factor are significantly different ($P \leq 0.05$) according to the LSD test. Mean comparisons were shown as mean ± standard error (SE).

Chlorophyll a content

The chlorophyll *a* was significantly ($P \leq 0.01$) affected by the main effects of nitrogen, biofertilizer and weeding. Also, nitrogen × biofertilizer interaction ($P \leq 0.01$) and nitrogen × weeding and biofertilizer × weeding interactions ($P \leq 0.05$) were significant for chlorophyll *a* (Table S3). The results showed that in all nitrogen levels, the use of biofertilizer increased the content of chlorophyll *a* compared to the treatment without the use of biofertilizer. Under conditions of no application of nitrogen, the highest amount of chlorophyll *a* content was observed in the treatment of simultaneous inoculation of two fungi (5.78 µg/ml), which showed a 37 % increase compared to the treatment of no inoculation of fungi (Figure 2). Under the conditions of application of 50 % recommended nitrogen, treatments of simultaneous inoculation of two fungi and no inoculation of fungi had the highest and lowest amounts of chlorophyll *a* content with 7.82 and 5.85 µg/ml, respectively. The results showed that the application of simultaneous inoculation of two fungi caused a 33 % increase in the content of chlorophyll *a* compared to the treatment of no inoculation of fungi under the conditions of application of 50 % recommended nitrogen (Figure 2).

In addition, the results showed that under the conditions of 100 % recommended nitrogen, the treatment of simultaneous inoculation of two fungi (8.5 µg/ml) had the highest amount of chlorophyll *a* content, while the treatment of no inoculation of fungi had the lowest amount of chlorophyll *a* content (7.13 µg/ml) (Figure 2). Under the conditions of 100 % recommended nitrogen, the application of the simultaneous inoculation of two fungi increased the content of chlorophyll *a* by 19 % compared to the treatment of no inoculation of fungi.

The results of interaction between weeding and nitrogen revealed that under no-weeding conditions, the application of nitrogen at 50 and 100 % recommended levels, increases the content of chlorophyll *a* by 38 and 60 % respectively, compared to the treatment of no application of nitrogen. On the other hand, under weeding conditions, the application of nitrogen at the recommended levels of 50 and 100 % increased the content of chlorophyll *a* by 41 and 59 %, respectively, compared to the no nitrogen application treatment. Under no weeding conditions, the highest amount of chlorophyll *a* content was observed in the treatment of the application of 100 % recommended nitrogen (7.49 µg/ml). In contrast the lowest amount of chlorophyll *a* was recorded in the treatment of without nitrogen application (4.68 µg/ml). Also, under weeding conditions, treatments with 100 % recommended and no nitrogen application had the highest and lowest chlorophyll *a* content by 7.89 and 4.94 µg/ml, respectively (Figure 2).

The results of the interaction of biofertilizer × weeding showed that in the condition of no weeding, the application of simultaneous inoculation of two fungi, inoculation of *T. atroviride* and inoculation of *T. longibrachiatum* caused an increase in the content of chlorophyll *a* by 26, 9 and 7 %, respectively compared to no inoculating of fungi treatment. On the other hand, in weeding conditions, the application of simultaneous inoculation of two fungi, inoculation of *T. atroviride* and inoculation of *T. longibrachiatum* increased the content of chlorophyll *a* by 31, 12 and 8 %, respectively, compared to the treatment of no inoculation of fungi (Figure 2).

Under no weeding conditions, the highest amount of chlorophyll *a* content was observed in the treatment of simultaneous inoculation of two fungi (7.08 µg/ml), which had no statistically significant difference with *T. atroviride* inoculation (6.10 µg/ml) and *T. longibrachiatum* inoculation (6.05 µg/ml) treatments. In contrast, the lowest amount of chlorophyll *a* content was observed in no inoculation treatment (5.62 µg/ml). Under weeding conditions, the highest content of chlorophyll *a* was related to the treatment of simultaneous inoculation of two fungi (7.64 µg/ml), which had no statistically significant difference with *T. atroviride* inoculation (6.54 µg/ml) and *T. longibrachiatum* inoculation (6.40 µg/ml). On the other hand, the lowest amount of chlorophyll *a* content was related to the treatment of no inoculation of fungi (5.83 µg/ml) (Figure 2).

Chlorophyll b content

The results showed that the chlorophyll *b* was significantly ($P \leq 0.01$) affected by the main effects of nitrogen, biofertilizer and weeding (Table S3). Also, the interactions of nitrogen × biofertilizer ($P \leq 0.01$),

nitrogen × weeding (P ≤ 0.05) and biofertilizer × weeding interactions (P ≤ 0.01) were significant for chlorophyll *b*. Chlorophyll *b* was significantly affected by the three-way interaction of nitrogen × biofertilizer × weeding (P ≤ 0.01).

The interaction of nitrogen × biofertilizer × weeding showed that under no nitrogen application, the simultaneous inoculation of two fungi combined with weeding (1.83 µg/ml) had the highest amount of chlorophyll *b* content. This was not statistically different from treatments involving simultaneous inoculation of two fungi + no weeding (1.75 µg/ml), inoculation of *T. atroviride* + weeding (1.69 µg/ml), inoculation of *T. longibrachiatum* + weeding (1.74 µg/ml) and *T. longibrachiatum* inoculation + no weeding (1.61 µg/ml). On the other hand, the lowest amount of chlorophyll *b* content was recorded in the treatment without inoculation of fungi combined with no weeding (1.35 µg/ml) (Figure 3). In the conditions of application of 50 % of the recommended nitrogen, the simultaneous inoculation of two fungi + weeding (2.12 µg/ml) had the highest amount of chlorophyll *b* content, which had no statistically significant difference with simultaneous inoculation of two fungi + no weeding (1.98 µg/ml), inoculation of *T. atroviride* + weeding (1.86 µg/ml) and inoculation of *T. longibrachiatum* + weeding (1.83 µg/ml) treatments; whereas the lowest amount of chlorophyll *b* content was related to the no inoculation + no weeding (1.63 µg/ml) treatment.

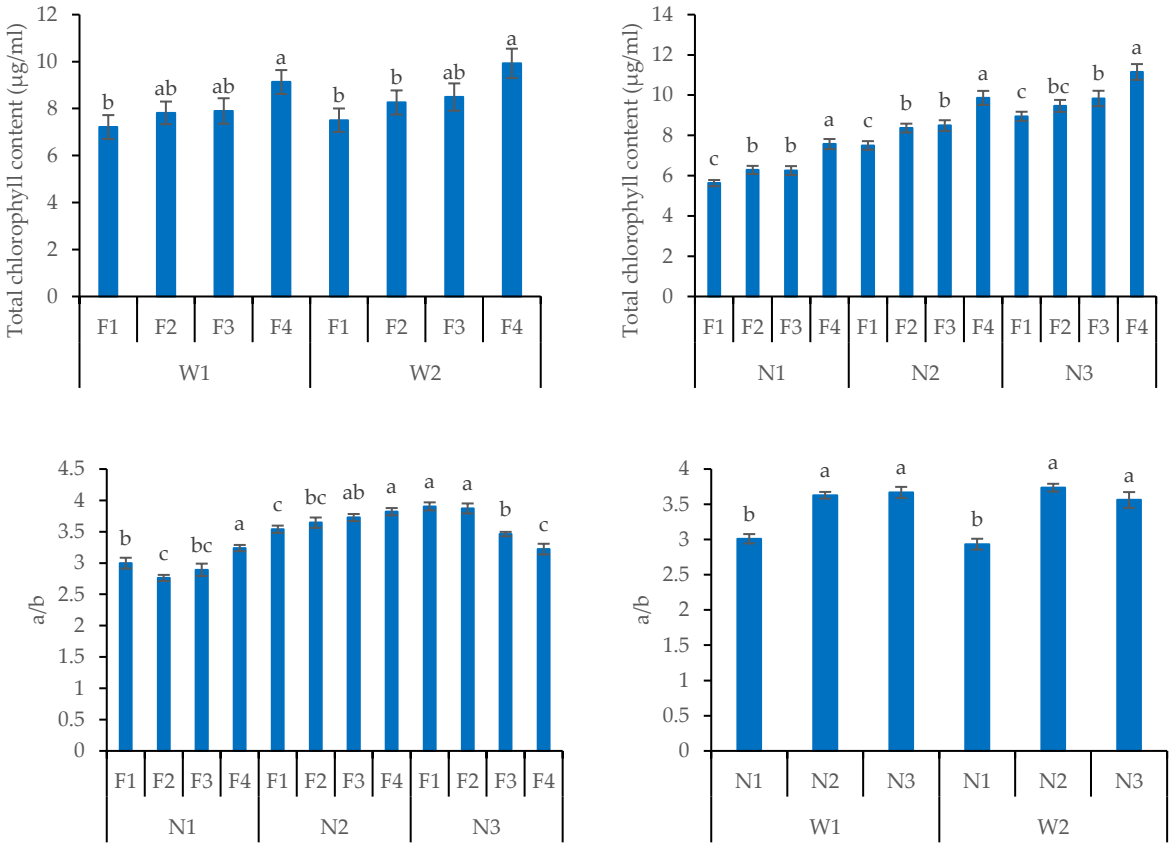

Figure 3. Influence of nitrogen, biofertilizer and weeding on chlorophyll b content, total chlorophyll content and ratio of chlorophyll a to b characteristics in green cumin plants cultivated in Qarah-Dam region (W1: no weeding, W2: weeding, F1: no inoculation, F2: *T. longibrachiatum* inoculation, F3: *T. atroviride* inoculation, F4: simultaneous inoculation of *T. longibrachiatum* and *T. atroviride*, N1: no nitrogen application, N2: application of 50 % of recommended nitrogen, N3: application of 100 % of recommended nitrogen). Means followed by different letters in the same column for the same factor are significantly different ($P \leq 0.05$) according to the LSD test. Mean comparisons were shown as mean ± standard error (SE).

With applying 100 % of the recommended nitrogen, the treatment of simultaneous inoculation of two fungi + weeding (2.89 µg/ml) had the highest amount of chlorophyll *b* content. While the lowest amount of chlorophyll *b* content was related to the treatments of no inoculation + no weeding (1.79 µg/ml), no inoculation + weeding (1.86 µg/ml) and *T. longibrachiatum* inoculation + no weeding (1.91 µg/ml) which had no statistically significant difference with *T. longibrachiatum* inoculation + weeding (1.99 µg/ml) and *T. atroviride* inoculation + no weeding (2.12 µg/ml) (Figure 3).

Total chlorophyll content

Based on the results, the total chlorophyll content was significantly affected by the main effects of nitrogen, biofertilizer, and weeding ($P \leq 0.01$). The interactions of nitrogen × biofertilizer ($P \leq 0.05$) and biofertilizer × weeding ($P \leq 0.01$) were significant for the total chlorophyll trait (Table S3).

Based on the interaction of biofertilizer and weeding, under no weeding conditions, the simultaneous inoculation of two fungi (9.13 µg/ml) resulted in the highest amount of total chlorophyll content, with no statistically significant difference observed between *T. atroviride* inoculation (7.89 µg/ml) and *T. longibrachiatum* inoculation (7.81 µg/ml). On the other hand, the lowest amount of total chlorophyll content was observed in the treatment of no inoculation of fungi (7.21 µg/ml) (Figure 3). In the condition of no weeding, the simultaneous inoculation of two fungi, inoculation of *T. atroviride* and inoculation of *T. longibrachiatum* increased the total chlorophyll content by 26, 9 and 8 %, respectively, compared to the no inoculation of fungi (Figure 3).

In addition, the results showed that in weeding conditions, the simultaneous inoculation of two fungi (9.92 µg/ml) had the highest total chlorophyll content, which had no statistically significant difference with *T. atroviride* inoculation (8.49 µg/ml); However, the no inoculation (7.51 µg/ml) and *T. longibrachiatum* inoculation (8.26 µg/ml) treatments had the lowest total chlorophyll content (Figure 3). The results in weeding conditions showed that the application of simultaneous inoculation of two fungi and inoculation of *T. atroviride* inoculation increased the total chlorophyll content by 32 and 13 %, respectively, compared to the no inoculation of fungi treatment (Figure 3).

The results of the interaction of nitrogen and biofertilizer showed that in the condition of no nitrogen application, the simultaneous inoculation of two fungi (7.57 µg/ml) had the highest amount of total chlorophyll content and the lowest amount of this trait was observed in the no inoculation treatment (5.63 µg/ml) (Figure 3). In the conditions of no nitrogen application, the treatment of simultaneous inoculation of two fungi increased the total chlorophyll content by 34 % compared to the treatment of no inoculation of fungi. In the conditions of application of 50 % of the recommended nitrogen, the simultaneous inoculation of two fungi (9.87 µg/ml) had the highest amount of total chlorophyll content and was able to increase the total chlorophyll content by 32 % compared to the no inoculation of fungi (Figure 3).

In the condition of applying 100 % of the recommended nitrogen, the highest amount of total chlorophyll content was observed in the treatment of simultaneous inoculation of two fungi (11.15 µg/ml); while the lowest amount of total chlorophyll content was observed in the treatment of no inoculation of fungi (8.95 µg/ml) and had no statistically significant difference with *T. longibrachiatum* inoculation (9.47 µg/ml) (Figure 3). In the condition of applying 100 % of the recommended nitrogen, the treatment of simultaneous inoculation of two fungi increased the total chlorophyll content by 24 % compared to the treatment of no inoculation of fungi (Figure 3).

Ratio of chlorophyll a to b

The results showed that among the main effects, only the main effect of nitrogen was significant ($P \leq 0.01$) for the ratio of chlorophyll *a* to *b*. Also, the interactions of nitrogen × biofertilizer ($P \leq 0.01$) and nitrogen × weeding ($P \leq 0.05$) were significant for the ratio of chlorophyll *a* to *b* (Table S3).

The results of the interaction of nitrogen and biofertilizer showed that in the condition of no application of nitrogen, the simultaneous inoculation of two fungi (3.24) had the highest value of the ratio of chlorophyll *a* to *b*; but on the other hand, the lowest amount of the ratio of chlorophyll *a* to *b* was observed in the treatment of *T. longibrachiatum* inoculation (2.76), which had no

statistically significant difference with *T. atroviride* inoculation (2.89) (Figure 3). In addition, in the condition of applying 50 % of the recommended nitrogen, the treatment of simultaneous inoculation of two fungi (3.82) had the highest ratio of chlorophyll *a* to *b*, which had no statistically significant difference with *T. atroviride* treatment (3.73); on the other hand, the lowest amount of the ratio of chlorophyll *a* to *b* was observed in the treatment of no inoculation of fungi (3.54). In the conditions of application of 100 % of recommended nitrogen, the treatments of no inoculation of fungi (3.9) and inoculation of *T. longibrachiatum* (3.87) had the highest amount of the ratio of chlorophyll *a* to *b*, and the treatment of simultaneous inoculation of two fungi (3.22) had the lowest amount of chlorophyll *a* to *b* ratio (Figure 3).

The interaction of nitrogen and weeding showed that in the condition of no weeding, the use of recommended 50 and 100 % nitrogen treatments could increase the ratio of chlorophyll *a* to *b* by 20 and 22 %, respectively, compared to the treatment of no nitrogen application. In addition, in weeding conditions, the use of recommended 50 and 100 % nitrogen treatments could increase the ratio of chlorophyll *a* to *b* by 27 and 21%, respectively, compared to the no nitrogen application treatment (Figure 3).

Carotenoid content

The results of analysis of variance showed that the carotenoid content was significantly affected by the main effects of nitrogen, biofertilizer and weeding ($P \leq 0.01$) (Table S3). The two-way and three-way interactions for carotenoid content were not significant. The highest amount of carotenoid content was observed in the treatment of simultaneous inoculation of two fungi (2.13 µg/ml), which showed an increase of 18 % compared to the no inoculation of fungi treatment. There was no significant difference between the *T. longibrachiatum* inoculation and *T. atroviride* inoculation in terms of carotenoid content (Figure 4).

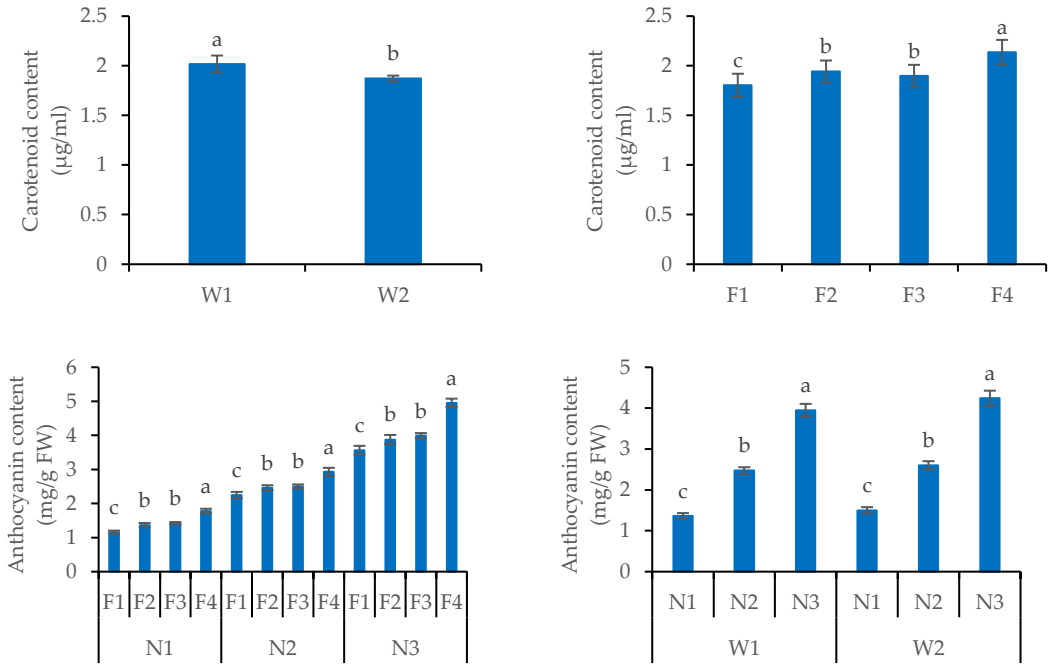

Figure 4. Influence of nitrogen, biofertilizer and weeding on carotenoid and anthocyanin content characteristics in green cumin plants cultivated in Qarah-Dam region (W1: no weeding, W2: weeding, F1: no inoculation, F2: *T. longibrachiatum* inoculation, F3: *T. atroviride* inoculation, F4: simultaneous inoculation of *T. longibrachiatum* and *T. atroviride*, N1: no nitrogen application, N2: application of 50 % of recommended nitrogen, N3: application of 100 % of recommended nitrogen). Means followed by different letters in the same column for the same factor are significantly different ($P \leq 0.05$) according to the LSD test. Mean comparisons were shown as mean ± standard error (SE).

Anthocyanin content

Based on the results, the main effects of nitrogen, biofertilizer, and weeding were significant for the anthocyanin content ($P \leq 0.01$). Also, the content of anthocyanin was significantly affected by the interactions of nitrogen × biofertilizer and nitrogen × weeding ($P \leq 0.01$) (Table S3). The results of the weeding treatment showed that the amount of carotenoid content was reduced by 7 % compared to the no-weeding treatment (Figure 4). The interaction of nitrogen and biofertilizer showed that in the condition of no application of nitrogen, the simultaneous inoculation of two fungi (1.78 mg/g FW) had the highest amount of anthocyanin content and the lowest amount of anthocyanin content was observed in the no inoculation of fungi (1.16 mg/g FW) treatment (Figure 4).

Also, at the no application of nitrogen level, the treatments of *T. longibrachiatum* inoculation, *T. atroviride* inoculation, and simultaneous inoculation of two fungi increased anthocyanin content by 18, 23 and 53 %, respectively, compared to the no inoculation of fungi treatment (Figure 4). Under conditions of application of 50 % recommended nitrogen, treatments of simultaneous inoculation of two fungi (2.93 mg/g FW) and no inoculation of fungi (2.25 mg/g FW) had the highest and lowest amount of anthocyanin content respectively (Figure 4). In the conditions of application of 50 % of recommended nitrogen, application of *T. longibrachiatum* inoculation, *T. atroviride* inoculation, and simultaneous inoculation of two fungi increased anthocyanin content by 10, 11 and 30 %, respectively, compared to the no inoculation of fungi (Figure 4).

In addition, under the condition of applying 100 % of the recommended nitrogen, the highest amount of anthocyanin content was observed in the simultaneous inoculation of two fungi (4.96 mg/g FW). The lowest amount of anthocyanin content was related to the no inoculation of fungi (3.57 mg/g FW) (Figure 4). At the condition of applying 100 % of the recommended nitrogen, *T. longibrachiatum* inoculation, *T. atroviride* inoculation, and simultaneous inoculation of two fungi increased the amount of anthocyanin content by 9, 12 and 39 %, respectively, compared to no inoculation of fungi (Figure 4). The interaction of nitrogen and weeding showed that in the condition of no weeding, the use of recommended 50 and 100 % nitrogen treatments could increase anthocyanin content compared to the treatment of no nitrogen application; the highest and lowest amounts of anthocyanin content were related to the 100 % recommended nitrogen treatments (3.94 mg/g FW) and no nitrogen application (1.36 mg/g FW), respectively (Figure 4). In addition, in weeding conditions, the application of nitrogen had a positive effect on anthocyanin synthesis; So that the highest amount of anthocyanin content was observed in the treatment of application of 100 % of recommended nitrogen (4.24 mg/g FW) and the lowest amount of anthocyanin content was related to the treatment of no nitrogen application (1.5 mg/g FW) (Figure 4).

Phytochemical and physiological characteristics at Shalami region

Essential oil content and yield

The results showed that the essential oil content and yield were significantly affected by the main effects of nitrogen, biofertilizer, and weeding ($P \leq 0.01$). Also, two-way and three-way interactions for these traits were significant ($P \leq 0.01$) (Table S4). Based on the results obtained from the interaction of weeding, nitrogen fertilizer and biofertilizer, it was observed that in the conditions of no nitrogen application, the simultaneous inoculation of two fungi + no weeding (1.06 %) had the highest content of essential oil; while the lowest amount of essential oil content was observed in the treatment of no inoculation of fungi + weeding (0.72 %), which had no statistically significant difference with *T. longibrachiatum* inoculation + weeding (0.76 %) (Figure 5). In the conditions of application of 50 % of the recommended nitrogen, the treatment of simultaneous inoculation of two fungi + no weeding (1.12 %) had the highest content of essential oil. In comparison, the lowest content of essential oil was observed in the treatment of no inoculation of fungi + weeding (0.73 %) (Figure 5). In addition, the results showed that the treatment of simultaneous inoculation of two fungi + no weeding (1.71 %) had the highest content of essential oil under the conditions of 100 % recommended nitrogen; while the treatments of no inoculation of fungi + weeding (0.91 %), *T. longibrachiatum* inoculation + weeding (0.93 %) and *T. atroviride* inoculation + weeding

(0.96 %) had the lowest content of essential oil (Figure 5). The results of weeding, nitrogen fertilizer and biofertilizer interactions showed that in the conditions of no nitrogen application, the treatment of simultaneous inoculation of two fungi + no weeding (6.04 kg/ha) had the highest amount of essential oil yield. The lowest amount of essential oil yield was observed in the treatments of no inoculation of fungi + no weeding (4.11 kg/ha), no inoculation of fungi + weeding (3.79 kg/ha), *T. longibrachiatum* inoculation + weeding (4.01 kg/ha) and *T. atroviride* inoculation + weeding (4.07 kg/ha) (Figure 5).

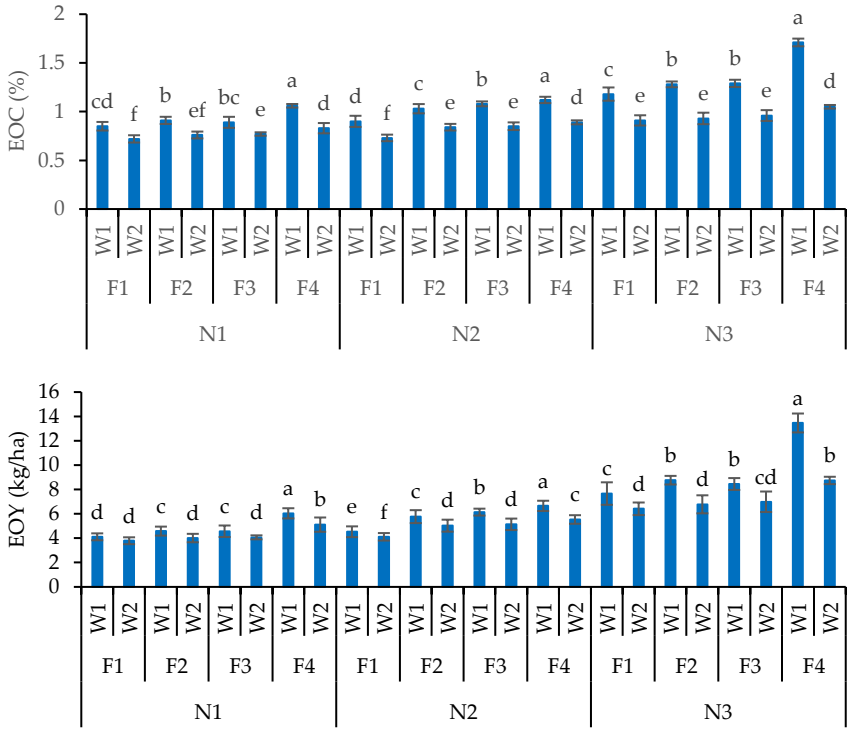

Figure 5. Influence of nitrogen, biofertilizer and weeding on essential oil content (EOC) and essential oil yield (EOY) characteristics in green cumin plants cultivated in Shalami region (W1: no weeding, W2: weeding, F1: no inoculation, F2: *T. longibrachiatum* inoculation, F3: *T. atroviride* inoculation, F4: simultaneous inoculation of *T. longibrachiatum* and *T. atroviride*, N1: no nitrogen application, N2: application of 50 % of recommended nitrogen, N3: application of 100 % of recommended nitrogen). Means followed by different letters in the same column for the same factor are significantly different ($P \leq 0.05$) according to LSD test. Mean comparison were shown as mean ± standard error (SE).

In addition, under the conditions of application of 50 % of recommended nitrogen, the treatment of simultaneous inoculation of two fungi + no weeding (6.65 kg/ha) had the highest amount of essential oil yield. On the other hand, the lowest amount of essential oil yield was observed in the treatment of no inoculation of fungi + weeding (4.11 kg/ha) (Figure 5).

Also, under the conditions of 100 % recommended nitrogen, the results showed that the treatment of simultaneous inoculation of two fungi + no weeding (13.46 kg/ha) had the highest amount of essential oil yield; In the event that the lowest amount of essential oil yield was observed in the treatments of no inoculation of fungi + weeding (6.41 kg/ha) and *T. longibrachiatum* inoculation + weeding (6.78 kg/ha) which had no statistically significant difference with *T. atroviride* inoculation + weeding (6.99 kg/ha) treatment (Figure 5).

Chlorophyll a content

The results of the analysis of variance showed that the main effects of nitrogen, biofertilizer, and weeding were significant for chlorophyll *a*, chlorophyll *b* and total chlorophyll content ($P \leq 0.01$)

(Table S4). Also, two-way and three-way interactions for chlorophyll *a*, chlorophyll *b*, and total chlorophyll content were significant ($P \leq 0.01$). The main effects of nitrogen ($P \leq 0.01$), biofertilizer ($P \leq 0.01$) and weeding ($P \leq 0.05$) were significant for the ratio of chlorophyll *a* to *b*. Also, two-way and three-way interactions were significant ($P \leq 0.01$) for the ratio of chlorophyll *a* to *b* (Table S4).

Based on the results of weeding, nitrogen fertilizer and biofertilizer interactions, it was observed that in the conditions of no nitrogen application, the simultaneous inoculation of two fungi + weeding (5.82 µg/ml) had the highest amount of chlorophyll *a* content, and the lowest amount of chlorophyll *a* content was observed in the treatment of no inoculation of fungi + no weeding (3.47 µg/ml), which had no statistically significant difference with no inoculation of fungi + weeding (3.62 µg/ml) (Figure 6). The results in conditions of no nitrogen application showed that the treatment of simultaneous inoculation of two fungi + weeding caused a 68 % increase in chlorophyll *a* content, compared to the treatment of no inoculation of fungi + no weeding (Figure 6). In addition, in the condition of applying 50 % of the recommended nitrogen, the treatment of simultaneous inoculation of two fungi + weeding (7.63 µg/ml) had the highest amount of chlorophyll *a* content; In the case, the lowest amount of chlorophyll *a* content was observed in the treatment of no inoculation of fungi + no weeding (5.11 µg/ml) (Figure 6). The results under the conditions of 50 % recommended nitrogen showed that the treatment of simultaneous inoculation of two fungi + weeding caused a 49 % increase in chlorophyll *a* content compared with no inoculation of fungi + no weeding (Figure 6).

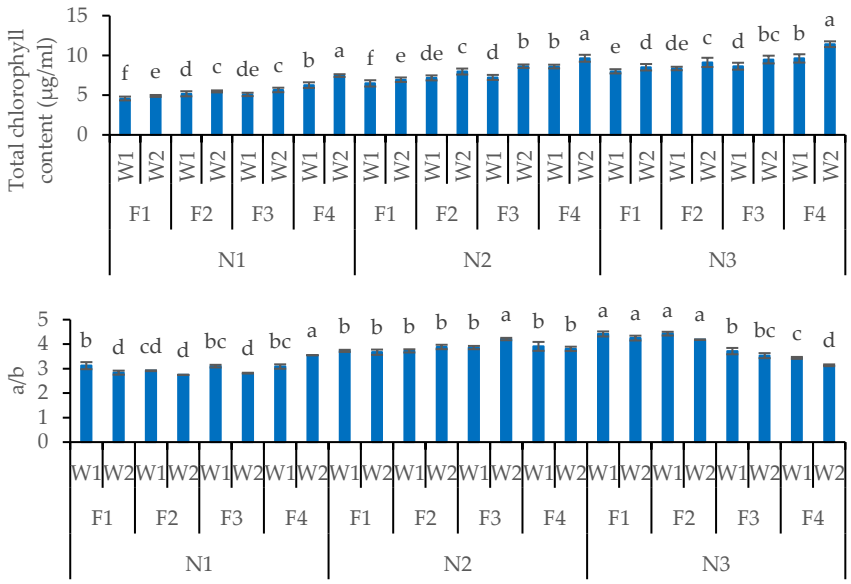

Figure 6. Influence of nitrogen, biofertilizer and weeding on chlorophyll a content, chlorophyll b content, total chlorophyll content and ratio of chlorophyll a to b characteristics in green cumin plants cultivated in Shalami region (W1: no weeding, W2: weeding, F1: no inoculation, F2: T. longibrachiatum inoculation, F3: T. atroviride inoculation, F4: simultaneous inoculation of T. longibrachiatum and T. atroviride, N1: no nitrogen application, N2: application of 50 % of recommended nitrogen, N3: application of 100 % of recommended nitrogen). Means followed by different letters in the same column for the same factor are significantly different ($P \leq 0.05$) according to the LSD test. Mean comparisons were shown as mean ± standard error (SE).

Under the conditions of 100 % recommended nitrogen, the simultaneous inoculation of two fungi + weeding (8.65 µg/ml) had the highest amount of chlorophyll *a* content, and the lowest amount of chlorophyll *a* was observed in the treatment of no inoculation of fungi + no weeding (6.52 µg/ml)

which had no statistically significant difference with *T. longibrachiatum* inoculation + no weeding (6.81 µg/ml) and *T. atroviride* inoculation + no weeding (6.81 µg/ml) (Figure 6). The results of applying 100 % recommended nitrogen showed that the treatment of simultaneous inoculation of two fungi + weeding treatment caused a 33 % increase in chlorophyll *a* content compared to the treatment of no inoculation of fungi + no weeding (Figure 6).

Chlorophyll b content

The results of the interaction of weeding, nitrogen fertilizer and biofertilizer showed that in the condition of no nitrogen application, the simultaneous inoculation of two fungi + weeding (1.64 µg/ml) had the highest amount of chlorophyll *b* content; However, the lowest amount of chlorophyll *b* content was observed in the treatment of no inoculation of fungi + no weeding (1.11 µg/ml) (Figure 6). In conditions of no nitrogen application, the results showed that simultaneous inoculation of two fungi + weeding caused a 48 % increase in chlorophyll *b* content compared to the treatment of no inoculation of fungi + no weeding (Figure 6). In the conditions of application of 50 % of the recommended nitrogen, the results showed that the treatment of simultaneous inoculation of two fungi + weeding (2.01 µg/ml) had the highest content of chlorophyll *b* and the lowest amount of chlorophyll *b* content was observed in the no inoculation of fungi + no weeding (1.37 µg/ml) (Figure 6). Under the conditions of 50 % nitrogen, the results showed that the application of the simultaneous inoculation of two fungi + weeding caused a 47 % increase in chlorophyll *b* content compared to the treatment of no inoculation of fungi + no weeding (Figure 6).

On the other hand, under the conditions of 100 % recommended nitrogen, the results showed that the treatment of simultaneous inoculation of two fungi + weeding (2.76 µg/ml) had the highest amount of chlorophyll *b* content; If the treatment of no inoculation of fungi + no weeding (1.48 µg/ml) had the lowest amount of chlorophyll *b* content, which had no statistically significant difference with *T. longibrachiatum* inoculation + no weeding (1.54 µg/ml) (Figure 6). Under the conditions of 100 % recommended nitrogen, the results showed that the application of the simultaneous inoculation of two fungi + weeding resulted in an 86 % increase in chlorophyll *b* content compared to the treatment of no inoculation of fungi + no weeding (Figure 6).

Total chlorophyll content

The results of the interaction of weed, nitrogen fertilizer and biofertilizer showed that in the condition of no nitrogen application, the simultaneous inoculation of two fungi + weeding (7.46 µg/ml) had the highest amount of total chlorophyll content and the lowest amount of total chlorophyll content was observed in the treatment of no inoculation of fungi + no weeding (4.58 µg/ml) (Figure 6). Based on the results obtained in the conditions of no nitrogen application, the use of simultaneous inoculation of two fungi + weeding, increased the total chlorophyll content by 63 % compared to the treatment of no inoculation of fungi + no weeding (Figure 6).

The results of application of 50 % of the recommended nitrogen showed that the treatments of simultaneous inoculation of two fungi + weeding (9.64 µg/ml) and no inoculation of fungi + no weeding (6.48 µg/ml) had the highest and lowest content of total chlorophyll, respectively (Figure 6). Under the conditions of 50 % recommended nitrogen application, the results showed that the use of simultaneous inoculation of two fungi + weeding, caused a 46 % increase in total chlorophyll content compared to the treatment of no inoculation of fungi + no weeding (Figure 6). In addition, under the conditions of 100 % recommended nitrogen, the results showed that the treatment of simultaneous inoculation of two fungi + weeding (11.41 µg/ml) had the highest amount of total chlorophyll content; If the treatment of no inoculation of fungi + no weeding (8 µg/ml) had the lowest amount of total chlorophyll content, which had no statistically significant difference with *T. longibrachiatum* inoculation + no weeding (8.35 µg/ml) (Figure 6). Under the conditions of 100 % recommended nitrogen, the results showed that the use of simultaneous inoculation of two fungi + weeding, resulted in a 43 % increase in total chlorophyll content compared to the treatment of no inoculation of fungi + no weeding (Figure 6).

Ratio of chlorophyll a to b

The results of the interactions of the weeding, nitrogen fertilizer and biofertilizer showed that in the condition of no nitrogen application, the

simultaneous inoculation of two fungi + weeding (3.55) had the highest ratio of chlorophyll *a* to *b*; In contrast, the lowest amount of chlorophyll *a* to *b* was observed in no inoculation of fungi + weeding (2.84), *T. longibrachiatum* inoculation + weeding (2.75) and *T. atroviride* inoculation + weeding (2.82) treatments, which had no statistically significant difference with *T. longibrachiatum* inoculation + no weeding (2.91) (Figure 6). In addition, under the conditions of application of 50 % of the recommended nitrogen, the inoculation of *T. atroviride* + weeding (4.21) had the highest amount of the ratio of chlorophyll *a* to *b*, and other treatments at this level of nitrogen had the lowest amount of the chlorophyll *a* to *b* (Figure 6). Under the conditions of 100 % recommended nitrogen, the results showed that the treatments of no inoculation of fungi + no weeding (4.42), no inoculation of fungi + weeding (4.25), *T. longibrachiatum* inoculation + no weeding (4.43) and *T. longibrachiatum* inoculation + weeding (4.18) had the highest amount of chlorophyll *a* to *b* ratio, and on the other hand, the lowest amount of chlorophyll *a* to *b* ratio was observed in the treatment of simultaneous inoculation of two fungi + weeding (3.14) (Figure 6).

Carotenoid content

The results of the analysis of variance showed that the main effects of nitrogen, biofertilizer and weeding as well as the two-way and three-way interactions of these factors were significant for the carotenoid content ($P \leq 0.01$) (Table S4). The results of weed, nitrogen fertilizer and biological fertilizer interactions showed that in the condition of no nitrogen application, the highest amount of carotenoid content was observed in the simultaneous inoculation of two fungi + no weeding (1.89 µg/ml); in contrast, the lowest amount of carotenoid content was observed in no inoculation of fungi + weeding (1.28 µg/ml) (Figure 7). In the conditions of application of 50 % of the recommended nitrogen, the highest and lowest amount of carotenoid content were observed in simultaneous inoculation of two fungi + weeding (2.41 µg/ml) and no inoculation of fungi + weeding (1.83 µg/ml) treatments, respectively. On the other hand, under the conditions of 100 % recommended nitrogen, the highest and lowest amount of carotenoid content were observed in simultaneous inoculation of two fungi + no weeding (3.06 µg/ml) and no inoculation of fungi + weeding (2.37 µg/ml), respectively (Figure 7).

Anthocyanin content

Based on the results, the main effects of nitrogen, biofertilizer and weeding were significant for the anthocyanin content ($P \leq 0.01$) (Table S4). Also. the interactions of nitrogen × biofertilizer and nitrogen × weeding were significant for anthocyanin content ($P \leq 0.01$). The results of the nitrogen and biofertilizer interaction showed that in the condition of no nitrogen application, the highest amount of anthocyanin content was observed in the treatment of simultaneous inoculation of two fungi (2.02 mg/g FW) and the lowest amount of anthocyanin content was observed in the treatment of no inoculation of fungi (1.37 mg/g FW) (Figure 7). In the conditions of no nitrogen application, the use of *T. longibrachiatum* inoculation, *T. atroviride* inoculation and simultaneous inoculation of two fungi increased the anthocyanin content by 17, 22 and 47 %, respectively, compared to the treatment of no inoculation of fungi (Figure 7). Under the conditions of application of 50 % of the recommended nitrogen, the highest amount of anthocyanin content was observed in the treatment of simultaneous inoculation of two fungi (3.28 mg/g FW) and the lowest amount of anthocyanin content was observed in the treatment of no inoculation of fungi (2.51 mg/g FW) which had no statistically significant difference with *T. longibrachiatum* inoculation (2.71 mg/g FW) (Figure 7). In the conditions of 50 % recommended nitrogen, the use of *T. longibrachiatum* inoculation, *T. atroviride* inoculation and simultaneous inoculation of two fungi increased the anthocyanin content by 8, 9 and 31 %, respectively, compared to the no inoculation of fungi (Figure 7). Under the conditions of 100 % recommended nitrogen, the highest amount of anthocyanin content was observed in the treatment of simultaneous inoculation of two fungi (5.21 mg/g FW) and the lowest amount of anthocyanin content was observed in the treatment of no inoculation of fungi (3.81 mg/g FW) (Figure 7). In the conditions of 100 % recommended nitrogen, the use of *T. longibrachiatum* inoculation, *T. atroviride* inoculation and simultaneous inoculation of two fungi increased the anthocyanin content by 9, 13 and 37 %,

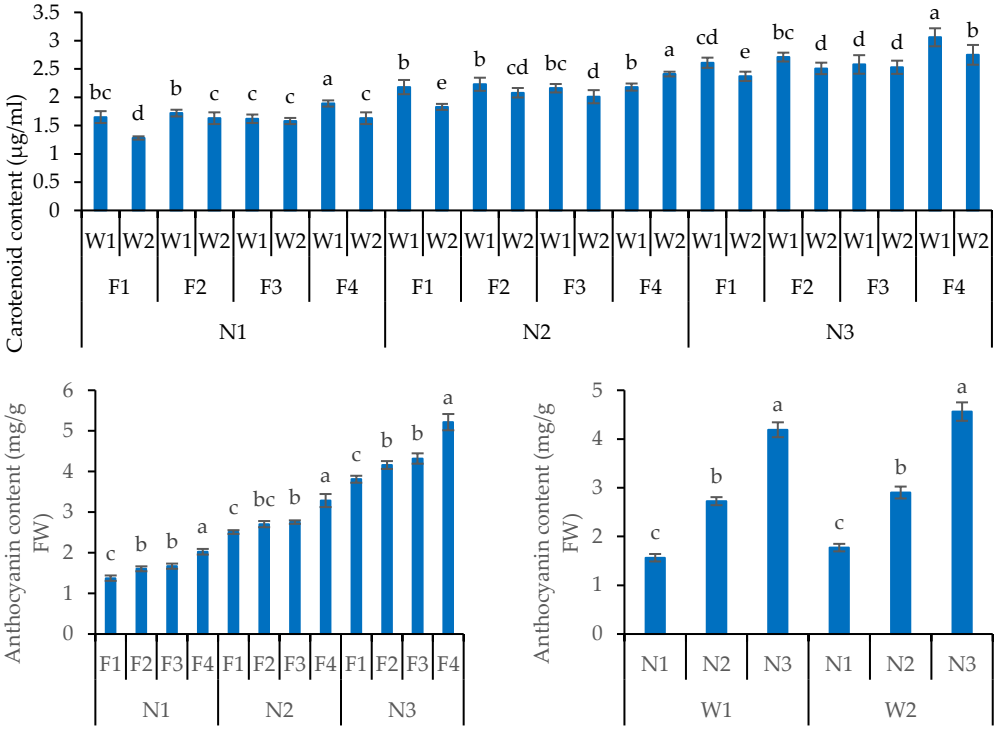

Figure 7. Influence of nitrogen, biofertilizer and weeding on carotenoid and anthocyanin content characteristics in green cumin plants cultivated in Shalami region (W1: no weeding, W2: weeding, F1: no inoculation, F2: *T. longibrachiatum* inoculation, F3: *T. atroviride* inoculation, F4: simultaneous inoculation of *T. longibrachiatum* and *T. atroviride*, N1: no nitrogen application, N2: application of 50 % of recommended nitrogen, N3: application of 100 % of recommended nitrogen). Means followed by different letters in the same column for the same factor are significantly different (P ≤ 0.05) according to the LSD test. Mean comparisons were shown as mean ± standard error (SE).

respectively, compared to the no inoculation treatment (Figure 7). The results of the nitrogen and weeding interaction showed that in the condition of no weeding, the treatment with 100 % recommended nitrogen (4.19 mg/g FW) had the highest amount of anthocyanin content and the lowest amount of anthocyanin content was observed in no nitrogen application (1.56 mg/g FW). In the condition of weeding, the treatment with 100 % recommended nitrogen (4.56 mg/g FW) had the highest amount of anthocyanin content, while the lowest amount of anthocyanin content was observed in the treatment of no nitrogen application (1.77 mg/g FW)

Discussion

Photosynthetic pigments are considered as an important source of production of photosynthetic products in plants (Silva et al., 2020). On the other hand, the efficiency and preservation of the function of the photosynthetic apparatus in the face of plants with different environmental and soil conditions is one of the important topics of plant physiology and can play a role as an important criterion in determining the proper growth of plants (Manzoor et al., 2021). The results of the present study showed that the simultaneous application of nitrogen and inoculation with *Trichoderma longibrachiatum* and *Trichoderma atroviride* could significantly increase the amount of chlorophyll *a*, chlorophyll b and total chlorophyll content compared to the treatment of not using them. Several researchers stated that nitrogen application and inoculation with *Trichoderma* fungi increase the chlorophyll content in plant leaves, which is consistent with our results ((Ji et al., 2020; Liang et al., 2020; Vukelić et al., 2021). Chlorophyll synthesis is influenced by several factors, such as the physicochemical properties of

the soil, nutrients, temperature, water balance in plants, etc. (Li et al., 2024). It seems that the application of *Trichoderma* fungi has improved the physicochemical properties of the soil and thereby increased the synthesis of chlorophyll, which is consistent with the results of Guo et al. (2020) in the peppermint plant.

Also, inoculation with *Trichoderma* fungi increases the absorption of nutrients in the plant, and for this reason, the synthesis of chlorophyll content is affected (Andrzejak and Janowska, 2022). On the other hand, *Trichoderma* fungi by preserving water in the environment around the root causes the plant to obtain more water and protects the plant from the damage of temporary moisture stress, therefore, increasing the amount of chlorophyll content (Khoshmanzar et al., 2020).

Unfavorable conditions such as insufficient absorption of nutrients by the plant, lack of sufficient water in the surrounding environment of the roots increase the activity of the chlorophyllase enzyme and as a result reduce the synthesis of chlorophyll *a*, chlorophyll *b*, and carotenoid as well as decrease protein bands which decrease (Siddiqui et al., 2022). A number of researchers showed that the chlorophyllase enzyme causes the degradation of chlorophyll under the influence of increasing the amount of ethylene and abscisic acid hormones (Bai et al., 2021). Other reasons for the reduction of plant chlorophyll include the activation of the chlorophyll catabolism pathway or lack of chlorophyll synthesis (Hu et al., 2021). The simultaneous use of fungus and nitrogen prevents unfavorable conditions for plant growth, and as a result, the amount of chlorophyllase enzyme decreases, as a result, the plant will be in a favorable condition in terms of photosynthetic pigments, which is consistent with the results of our experiment. Inoculation with fungi has many benefits for plant growth, one of which is the increase of photosynthetic pigments (Liang et al., 2020). Inoculation with fungi increases the root growth in the plant and in this way improves the absorption of nutrients and water by the plants, as a result of the absorption of sufficient nutrients and water by the plants, adverse conditions will be lighter and as a result induces the plant growth (Khoshmanzar et al., 2020).

Alipour et al. (2021) showed that the inoculation of the seeds of the fennel (*Foeniculum vulgare*) with *mycorrhizal* fungi increased plant pigments and thus improved plant growth. Since the fungus depends on the cells of the host plant to grow and complete its life cycle, it seems that under the conditions of nitrogen application, the amount of chlorophyll synthesis in the plant increases. As a result, more photosynthetic pigments are produced by the plant and greater amount of assimilate have been provided to the fungus. With the increase in the activity of the fungus, the absorption of nutrients and water by the plant has increased, and for this reason, it has had a positive effect on increasing the amount of chlorophyll content and the synthesis of secondary metabolites (Han et al., 2020).

The researchers stated that by applying different treatments due to the decrease in the function of chlorophyll *a*, the role of chlorophyll *b* and carotenoid as auxiliary pigments to transfer the received energy to chlorophyll *a* and also to compensate for the deficiencies related to chlorophyll *a* had great importance. (Hosseini et al., 2021). Several researchers pointed to the role of chlorophyll *b* and carotenoids as auxiliary photosynthetic pigments and showed that auxiliary pigments can play an effective role in the performance of the photosynthetic apparatus (Simkin et al., 2022; Zhao et al., 2022; Cheaib et al., 2023).

Based on the results of the present experiment, the application of nitrogen and inoculation with fungi, due to providing favorable conditions for plant growth, were able to increase the seed yield compared to control (without nitrogen and inoculation with fungi). During an experiment on wheat plants, the researchers showed that the simultaneous application of nitrogen and inoculation with fungi increased seed yield Meena et al. (2017), which is consistent with the results of the present experiment. It appears that in our experiment, the use of nitrogen and inoculation with fungi has increased the synthesis of secondary metabolites compared to the control treatment. This can be attributed to the positive effect on plant growth, increased absorption of nutrients, increased production of sugar and chlorophyll, and photosynthetic activity. Since the synthesis of phytochemicals requires energy and the factors that boost plant energy can lead to an increase in these bioactive compounds (Ghasemy-Piranloo et al.,

2022). The researchers showed that with the simultaneous application of nitrogen and inoculation with fungi, the growth rate of the product and the leaf area index had a significant superiority over the control treatment in wheat plants (Mohammed et al., 2024). Greenness index with simultaneous application of nitrogen and inoculation with fungus showed a significant increase compared to control treatment in soybean plant (Spagnoletti et al., 2020). The inoculation of mycorrhizal fungi and the application of nitrogen improve the absorption of nutrients, increase the amount of chlorophyll, strengthen the photosynthetic activity, increase the amount of carbohydrates and amino acids in the leaves of the plant, and in this way provide the necessary energy to increase the synthesis of essential oil content (Xie et al., 2022).

The effect of environmental factors such as light, water, nutrients, soil micro-organisms, andagronomic practices on the synthesis of plant secondary metabolites varies (Khalvandi et al., 2019; Hosseini et al., 2021; Soleimani et al., 2022). It can be stated that the treatments used in this experiment positively contributed to the increase in the content of secondary metabolites. The pathways of the synthesis of secondary metabolites are extremely complex and are affected by environmental factors, and the amount of synthesis of secondary metabolites may decrease or increase with the availability of favorable conditions (Mansinhos et al., 2024). In an experiment, researchers showed that the amount of essential oil production in peppermint plant increased by inoculation with *Trichoderma* fungus, which is consistent with the results of the present experiment (Guo et al., 2020). Also, Alhasan and Hussein (2022)showed during an experiment that the application of nitrogen fertilizer increased the synthesis of essential oil in *Mentha spicata* L., which alligns with the results of our experiment. On the other hand, in the present experiment, the competition of weeds with cumin plants led to the reduction of photosynthetic pigments. It seems that the competition with weeds reduced the absorption of nutrients, water, and light by the cumin plant, and as a result, the synthesis of photosynthetic pigments decreased. Our finding is consistent with the results of Fawad and Khan (2022) in the tomato plant. On the other hand, the amount of carotenoid increased in the conditions of competition with weeds; In this regard, it can be stated that plants increase the amount of carotenoid pigment when faced with stress to increase energy production by absorbing more light and making it available to chlorophyll *a* in the conditions of competition with weeds (Abdelaal et al., 2022). In the present experiment, competition with weeds increased the content of essential oil in some treatments. This weed competition, considered as stress, appears to stimulate an increase in synthesis rate of essential oil. In this regard, the researchers stated that the increase in the synthesis of bioactive compounds in the face of plants with stress has a defensive aspect and increases the tolerance of plants against adverse conditions (Amini et al., 2020; Soleimani et al., 2022).

Conclusion

The results of this study showed that the application of nitrogen and inoculation with *T. longibrachiatum* and *T. atroviride* fungi improved the essential oil content and yield of green cumin plants. The simultaneous use of nitrogen and inoculation with fungi led to an increase in chlorophyll *a*, chlorophyll *b*, and total chlorophyll pigments in the cumin plant, which increased energy production, essential oil content, and essential oil yield with the production of more photosynthetic assimilates. The co-inoculation of *T. longibrachiatum* and *T. atroviride* fungi had a better effect compared to their separate use. Also, the highest amount of synthesis of photosynthetic pigments was obtained when 100% recommended nitrogen was applied. Competition with weeds decreased the content of photosynthetic pigments and in some cases, carotenoid synthesis increased. Competition with weeds increased the essential oil content in some treatments. Finally, it can be stated that the use of nitrogen treatments, along with inoculation of *T. longibrachiatum* and *T. atroviride* fungi can be recommended as an effective strategy for promoting sustainable and organic agriculture.

Supplementary Materials

The supplementary material for this article can be found online at: https://www.jpmb-gabit.ir/article_719402.html.

Supplementary Table 1. Physiochemical properties of soil related to Qarah-Dam and Shalami regions.
Supplementary Table 2. Meteorological characteristics of the Qarah-Dam region.
Supplementary Table 3. Analysis of variance of different characteristics related to Qarah-Dam region.
Supplementary Table 4. Analysis of variance of different characteristics related to Shalami region.

Author Contributions

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

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Conflict of Interest Statement

The authors declare no conflict of interest.

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مقاله پژوهشی

اثر همزیستی قارچ تریکودرما و کاربرد نیتروژن بر خصوصیات اسانس و رنگـدانه‌های برگ در زیـره سبـز (*Cuminum cyminum* L.) تحت رقابت با علف‌های هرز

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چکیده: زیره سبز یکی از پرمصرف‌ترین گیاهان دارویی در ایران و جهان است که عمدتاً برای استفاده از اسانس آن مورد بهره‌برداری قرار می‌گیرد. در راستای کشاورزی پایدار، طراحی آزمایشی بمنظور بررسی کارکرد کودهای زیستی در کنار نهاده‌های شیمیایی نظیر نیتروژن ضروری به نظر می‌رسد. این تحقیق در سال زراعی ۱۴۰۳-۱۴۰۲ در شهرستان مراوه تپه و در دو منطقه مجزا (قره دام و شلمی) به صورت آزمایش اسپلیت فاکتوریل در قالب طرح بلوک‌های کامل تصادفی با سه تکرار انجام شد. تیمارهای آزمایشی شامل سطوح کود نیتروژن (شاهد، ۵۰ و ۱۰۰ درصد نیـتروژن توصیه شده از منبع اوره) به عنوان عامل اصلـی، تیـمارهای کود زیستی (شاهـد، تلقیـح بذر با *Trichoderma longibrachiatum*، *Trichoderma atroviride*، و تلقیح همزمان دو قارچ) و مدیریت علف‌های هرز (وجین و عدم وجین) از عوامل فرعی هستند. نتایج نشان داد که بیشترین مقدار و عملکرد اسانس در هر دو منطقه مربوط به تیمار بدون وجین + ۱۰۰ درصد نیتروژن توصیه شده + استفاده همزمان از دو قارچ بود. همچنین استفاده از قارچ تریکودرما و نیتروژن تأثیر مثبتی بر محتوای کلروفیل a، کلروفیل b و کلروفیل کل داشت.

کلمات کلیدی: آنتوسیانین، کود زیستی، محتوای اسانس، محتوای کلروفیل، نیتروژن.

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Molecular marker utilization in oilseed crop breeding: a review

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Review paper

Abstract: It has been observed that molecular markers are one of the most important tools in the advancement of oilseed crops and in developing varieties capable of withstanding drought, salinity, and high-temperature stresses. The present review focuses on the use of DNA-based molecular markers such as SSRs and SNPs in oilseed crop breeding. These markers, in addition to being able to identify and locate QTLs associated with stress tolerance, by using Marker-Assisted Selection (MAS) will aid in accelerating selection. Although the oilseed crops, including canola, soybean, and sunflower, have achieved certain levels of environmental stress tolerance, their inability to overcome abiotic stresses such as drought, salinity, and temperature extremes further dampens growth and productivity to contribute to the existing yield gap. However, the integration of molecular markers into breeding programs saves not only genetic diversity but also contributes to the quicker release of high-yielding varieties under extreme conditions. This review, therefore, enumerates examples of successful applications and emphasizes the future research needs of using molecular markers in breeding these under-investigated oilseed crops to build more robust world food systems resilient to global warming.

Keywords: Genetic variability, global warming, marker-assisted selection, quantitative trait loci.

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Introduction

With time, molecular markers have developed as one of the important tools for improvement in oilseed crops and can develop tolerant varieties against adverse environmental conditions like drought, salinity, and high temperatures. These markers allow for the identification of specific genetic traits linked to stress tolerance, enabling breeders to select and multiply only those plants which are most capable of surviving harsh climatic conditions.

Genetic mapping and marker-assisted selection (MAS)

Molecular markers are segments of DNA that may be associated with a specific plant trait. Genetic mapping requires them to locate the genes that control desirable traits such as drought tolerance and heat resistance. MAS uses these markers, therefore enabling its use to accelerate breeding processes and hence allowing new varieties of oilseeds to be developed more rapidly than would be possible by the use of traditional methods alone. This approach is crucial for crops like soybean, canola, and sunflower, which, in these years, have increasingly been cultivated at the mercy of climate change and attendant stresses.

Improvement of stress tolerance

Tolerance against abiotic stresses, such as drought or salinity, is one of the keys for preserving crop yields against unpredictable environmental changes (Lesk et al., 2022). Research has shown that molecular markers offer a shortcut to the identification of genes which may be involved in the stress response mechanism such as maintenance of osmotic balance and ion transport in plants (Teixido et al., 2021). Some of these genes are associated with water use efficiency and root depth, both being very important for drought tolerance (Kühn et al., 2022). In addition, identification of the genetic mechanisms underlying salinity resistance allows breeders to generate cultivars that can be cultivated in saline soils, which are increasingly becoming more common due to irrigation practices and climate change (Holsman, 2023).

Challenges and future directions

These advantages of molecular markers in developing resilient oilseed crops are encompassed by a number of challenges. Most of the resilient-related traits, for example, heat tolerance, generally result from a large number of genes interacting together. Such polygenic traits are challenging to identify and manipulate through conventional breeding approaches. Additionally, the complexity involved in isolating, cloning, and transforming quantitative traits, along with the public concerns and strict regulatory hurdles associated with GMOs, make developing crops with tolerance to abiotic stresses especially challenging (Lynas et al., 2022). Overcoming these challenges, research studies have continuously strived to integrate molecular markers into genomic technologies, such as CRISPR and other gene-editing tools (Segelbacher et al., 2022). These developments hold bright prospects for improving precision in breeding programs and for making rapid strides toward the development of oilseed varieties (Sinha et al., 2023). In other words, molecular markers, a revolutionary methodology, are now contributing to changing the face of oilseed crop breeding through the facilitation of developing varieties that are tolerant to drought, salinity, and high temperature (Soltabayeva et al., 2021). As this research goes on, this tool will undoubtedly contribute to developing more robust agricultural systems against a shifting climate (Lawrence et al., 2023). Application of different DNA-based molecular markers in the improvement of oilseed crops has resulted in a sea change in the breeding methodology to produce varieties with enhanced environmental stress tolerance and more suitable agricultural requirements. Among these, SSRs, RAPD, SNPs, and other types of molecular markers carry out genetic diversity enhancement, identification of desirable features within the crop, and speed up the breeding process (Malgaonkar et al., 2020; Adje et al., 2023).

Overview of DNA-based molecular markers

Simple sequence repeats (SSRs)

SSRs or microsatellites are small fragments of DNA that form tandem repeats and are highly polymorphic. Due to their abundance and stability over generations, they find wide applications in genetic mapping and diversity studies (Sunde et al., 2020; Baba Nitsa et al., 2023).

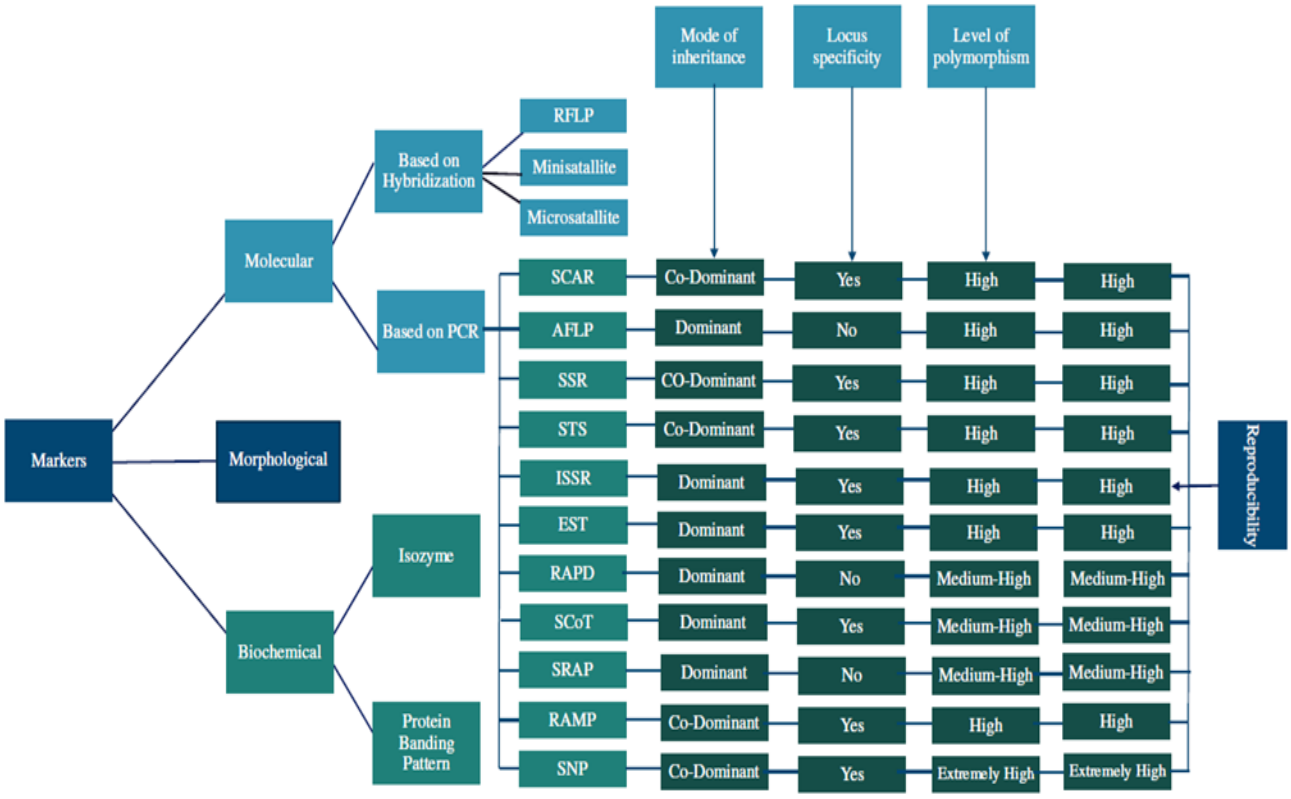

Figure 1. A flowchart that classifies various genetic markers based on their distinct features. Abbreviations represent RFLP: Restriction Fragment Length Polymorphism; SCAR: Sequence Characterized Amplified Region;AFLP: Amplified Fragment Length Polymorphism; SSR: Simple Sequence Repeat; STS: Sequence Tag Site; ISSR: Inter-Simple Sequence Repeat; RAPD: Random Amplified Polymorphic DNA;SCOT: Start Codon Targeted Polymorphism;SRAP: Sequence Related Amplified Polymorphism;RAMP: Random Amplified Microsatellite Polymorphism;SNP: Single Nucleotide Polymorphism.

In oilseed crops, SSRs allow the identification of genetic variation among cultivars, thereby enabling breeders to choose the best parental lines for hybridization (Heidari et al., 2023; Kour et al., 2023). Their codominant inheritance allows detailed insight into the genetic constitution of populations, which is a prerequisite for effective breeding strategies (Martin-Gutierrez et al., 2022).

Random amplified polymorphic DNA

In RAPD markers, random segments of the genomic DNA are amplified using short primers (usually 10 bases). The technique has the advantage of speed and reasonable low cost. As no prior information is required on the genome sequence, genetic diversity of improvement can easily be estimated using this marker. In oilseed crop improvement, RAPD has been utilized for detecting variation within and among cultivars that enable breeders to select the most promising lines in breeding programs (O'Brown et al., 2019; Hosseinpour Azad, 2023).

Single nucleotide polymorphisms

SNPs are the most common form of genetic variation among individuals in a population, where the variation involves a change in only one nucleotide (Walker et al., 2021). Because of their high density across the genome, they have been very important in studies of complex traits. They have widely been used in fine-mapping QTLs related to important agronomic traits such as yield and stress tolerance (Yang et al., 2021). High-throughput SNP genotyping enables the testing of very large populations with high efficiency and contributes to the acceleration of crop improvement (Allen et al., 2017). Figure 1 and Table 1 categorize different types of molecular markers along with their applications and characteristics.

Amplified fragment length polymorphism

Amplified fragment length polymorphism (AFLP) markers have been employed in oilseed research covering analysis of genetic diversity, identification of cultivars and mapping genes linked with tolerance to abiotic stress (Čurn et al., 2002). Oilseed rape has also been assessed for suitability as a subject of fluorescence-based AFLPs as molecular markers (Sobotka et al., 2004). Such technique was also compared with other marker types including, isozyme, RAPD and SSR markers in determining genetic similarity among oilseed lines (Havlíčková et al., 2014).

Random amplified microsatellite polymorphism

Random amplified microsatellite polymorphism (RAMP) markers are often employed in oilseeds research regarding the study of genetic diversity, identification of the traits associated with drought tolerance. For instance, RAMP markers have been successfully applied to studying genetic diversity among oilseed sunflower under conditions of different irrigations (Akbari and Darvishzadeh, 2024). Furthermore, RAMP markers are based on PCR that may detect various types of induced genetic variations and mechanisms similar to SSRs (Hasan et al., 2021).

NGS: a game changer in marker development

Next-Generation Sequencing (NGS) is a swift, economical sequencing method that facilitates the concurrent sequencing of millions of DNA and RNA fragments, transforming genomics through the efficient analysis of intricate genomes (Hashemi-Petroudi et al., 2022; Satam et al., 2023). The history of NGS commenced in the early 2000s with the launch of 454 Life Sciences' GS20 in 2005, succeeded by Illumina's sequencing technology, which gained prominence owing to its precision and scalability (Akacin et al., 2022). Innovative NGS techniques like Ion Torrent and PacBio offer distinct advantages for read length, accuracy, and versatility, with several platforms demonstrating particular attributes (Hu et al., 2021). Various types of NGS platforms are presently accessible, each possessing unique characteristics:

- *Illumina Sequencing:* Illumina sequencing, recognized for its high throughput and precision, is extensively utilized in genomics and transcriptomics. It utilizes a sequencing-by-synthesis technique, wherein fluorescently labeled nucleotides are integrated into elongating DNA strands (Abdi et al., 2024)
- *Ion Torrent Sequencing:* Ion Torrent Sequencing use semiconductor technology to monitor pH variations as nucleotides are incorporated into a developing DNA strand. It provides expedited sequencing at reduced costs, rendering it appropriate for many applications (Kumar et al., 2024).
- *PacBio Sequencing:* PacBio's Single Molecule Real-Time (SMRT) sequencing offers extended read lengths, advantageous for elucidating intricate genomic areas and structural changes (Zhou et al., 2022b).

Genetic Markers: MAS, QTL mapping, and transferability

Marker-assisted selection (MAS)

A biotechnological approach known as Marker-Assisted Selection (MAS) utilizes molecular markers to select for desired traits in breeding programs. The application of molecular markers in MAS has accelerated the breeding process by allowing breeders to select individuals carrying desirable alleles linked to specific QTLs early in the breeding cycle. This method has been particularly beneficial in oilseed crops, where traits such as oil yield and disease resistance can be efficiently selected based on marker data (Teixido et al., 2021). At the outset of the breeding cycle, it is possible to enhance decision-making by pinpointing specific markers linked to desired traits, thereby significantly boosting the efficiency and accuracy of crop development (Mahjoob et al., 2016). With the incorporation of molecular markers into plant breeding program during the late 1980s and early 1990s, the concept of MAS emerged. Methods such as SSR and RFLP enable the mapping of Quantitative Trait Loci associated with important agronomic traits (Wenzl et al., 2006). Progress in genomics and the emergence of NGS in recent years have significantly enhanced the applicability of MAS, facilitating more precise marker identification and selection (Pandey et al., 2016).

Quantitative Trait Loci (QTL)

Quantitative Trait Loci (QTL) are specific regions of the genome that correlate with variation in a quantitative trait (Aguet et al., 2023). The

identification and mapping of QTLs are crucial for breeding programs as they provide insight into the genetic basis of traits that can be selected for improvement. The ability to locate these loci on chromosomes using molecular markers enhances the precision of breeding efforts.

Recent studies have focused on developing chromosome-specific markers that allow for precise localization of QTLs on specific chromosomes. For instance, researchers have utilized gene-based markers such as Conserved Ortholog Set (COS) markers to map QTLs related to starch granule content in wheat, demonstrating their effectiveness in facilitating gene introgression from wild relatives into cultivated species. This approach not only aids in identifying QTLs but also enhances our understanding of chromosomal relationships among different species (Lindqvist-Kreuze et al., 2013). The integration of NGS technologies has significantly advanced the ability to identify and characterize molecular markers linked to QTLs. NGS allows for comprehensive genome-wide scans to detect SNPs associated with traits of interest, leading to more accurate QTL mapping (Yang et al., 2010). This technology enables breeders to develop high-density genetic maps that improve the resolution at which QTLs can be identified.

Transferability of markers

The transferability of markers opens up avenues for tapping into genetic diversity present in the related species, particularly for crops with low genetic variation.low genetic variation. With markers from closely related species, breeders can introduce new alleles enhancing traits of interest. Transferable markers can acceleratebreeding programs by enabling the identification of specific trait more quickly. These increases in speed are invaluable for oilseed crops, as global food security challenges demand the development of high-yielding and resilient crop varieties (Huang et al., 2016).

Applications of molecular markers in improvement of oilseed crop

This has been possible with the integration of these molecular markers into breeding strategies, where significant strides have been made in the improvement of oilseed crops (Table 1).

Genetic diversity analysis

Breeders are allowed, through molecular markers, to investigate the genetic diversity present in the oilseed germplasm collections. This becomes very important for choosing appropriate parents to hybridize and also for ensuring a broad genetic base for further breeding programs. For instance, research has shown the use of SSRs together with other markers to effectively characterize the genetic diversity among oilseed cultivars, hence informing breeding decisions.

In MAS, breeders can select plants that have the desired trait at an early growth stage. This advances the pace of breeding and helps in the more rapid development of cultivars with improved tolerance to abiotic stresses such as drought and salinity (Hasan et al., 2021). MAS enables selection with significantly greater precision than before, potentially reducing the time required to develop new varieties by several years compared to traditional methods (Table 1). QTL Mapping: QTL mapping studies allowed the identification of genomic regions responsible for relevant traits such as heat tolerance or disease resistance by molecular markers (Zhou et al., 2022a). Such information is important for targeted breeding focused on improving such traits in oilseed crops. For example, QTLs concerning drought tolerance in soybean were mapped by using SNP markers (Wang et al., 2022). SNP markers enable genomic selection methodologies to project the performance of untested individuals based on their genomic profile. This approach simplifies the selection process, and shortens the length of the breeding cycle since breeders can be extremely informed on which plants to advance.

Addressing abiotic stresses

Abiotic stresses, including drought, salinity, heat, and flooding, have wholly taken their toll on oilseed crops. Molecular marker applications helped in developing stress-resistant varieties. To cite a few examples, studies have demonstrated how SSRs and SNPs have been mapped with QTL associated with drought resistance in major oilseed crops of rapeseed and soybean (Song et al., 2021). Such efforts are being directed toward closing the gap between actual and potential yields by enhancing

Table 1. List of some studies involving molecular markers for Genetic diversity and improvement of oilseeds. Abbreviations are as defined in Figure 1.

Trait	Marker used/ linked to QTL	Crop	Reference
Oil content	SSR	Sesame	(Li et al., 2014)
Cadmium toxicity	SSRs	Soybean	(Jegadeesan et al., 2010)
Cold stress	RFLPs, AFLPs	Mustard and Canola	(Kole et al., 2002))
Cold stress	SSRs	Soybean	(Funatsuki et al., 2005; Ikeda et al., 2009; Zhang HaiYang et al., 2012)
Cold stress	ESTs	Sunflower	(Hewezi et al., 2006; Fernandez et al., 2008)
Cyst Nematodes	AFLP, RFLP	Sunflower, Soybean	(Ali et al., 2017)
Drought stress	AFLPs	Safflower	(Poodineh et al., 2021)
Drought stress	SSR	Soybean	(Chen et al., 2021)
Drought and salt stress	SNPs	Sesame	(Li et al., 2018)
Drought tolerance	SSRs	Groundnut	(Ravi et al., 2011; Gautami et al., 2012)
Drought tolerance	SSRs and ISSRs	Safflower	(Mirzahashemi et al., 2015)
Drought tolerance	SNPs	Sesame	(Dossa et al., 2019)
Drought tolerance	SSR	Soybean	(Specht et al., 2001; Bhatnagar et al., 2005; Monteros, 2006)
Drought tolerance	RFLP	Soybean	(Mian et al., 1998)
Drought stress	RFLP	Soybean	(Mian et al., 1996)
Flooding stress	SSRs	Soybean	(VanToai et al., 2001; Cornelious et al., 2005; Githiri et al., 2006; Dhungana et al., 2021)
Flooding stress	SSRs and SNPs	Soybean	(Nguyen et al., 2012)
Fungal diseases	SSRs, SNPs	Sunflower	(Dimitrijevic and Horn, 2018)
Sclerotinia stem rot	IRAP, REMAP	Canola, Soybean	(Negi et al., 2016)
Genetic diversity	EST-SSRs	Multiple oil crops	(Miladinović et al., 2019)
Genetic diversity	EST-SSRs	Cotton	(Yu et al., 2008)
Genetic diversity	EST-SSR, SCoT	Sesame	(Bhattacharjee et al., 2020)
Heat stress	AFLPs, SCARs	Rapeseed	(Zeng et al., 2014)
Manganese toxicity	SSRs	Soybean	(Kassem et al., 2004)
Oil content	EST	Soybean, Peanut, Sesame, Rapeseed	(Ke et al., 2015)
Root-knot nematodes	SSRs, SNPs	Soybean, canola	(Ragimekula et al., 2013)
Salt stress	SSRs	Soybean	(Lee et al., 2004; Chen et al., 2008; Hamwieh et al., 2011)
Tobacco streak virus	IRAP, REMAP	Canola	(Negi et al., 2016)
Viruses	SNPs, SSRs	Soybean	(Jones et al., 2021)

the capacity of crops to withstand severe abiotic pressures (Figure 2).

Improving oil content

Application of molecular markers to improve oilseed crops' oil content is extremely encouraging. Recent innovations in genomics have enhanced our knowledge of the genes involved in oil production that are targets for genetic alteration, claim Chao et al. (2023). Based on the findings of Savadi et al. (2017), candidates found through studies on such genes offer useful resources for enhancing oil content through the application of transgenic and gene editing technologies. Molecular markers associated with fatty acid components also contribute to the enhancement of overall crop quality. Molecular markers associated with fatty

acid components also contribute to the enhancement of overall crop quality. For example, several markers that would enhance the oil content and composition of seeds have been found in *Lepidium campestre* (Lodenius, 2023).

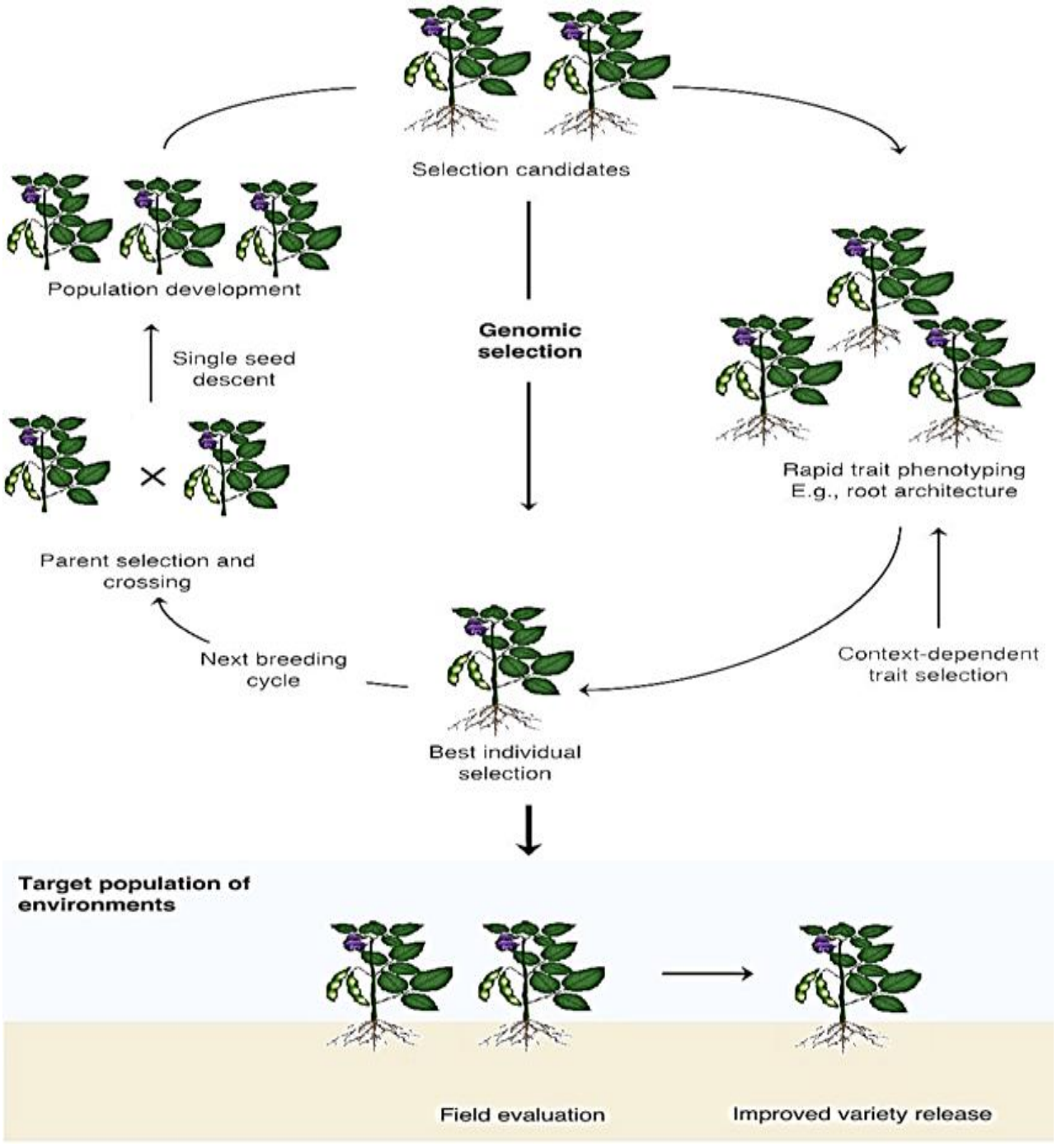

Figure 2. The connection between traditional plant breeding and molecular plant breeding in developing Soybean varieties withstand environmental stresses.

Challenges and future perspectives

In conclusion, the application of molecular markers towards the improvement in oilseed crops is doubtlessly a jump upwards in breeding methodologies. Not only does it advance the selection with increased efficiency concerning tolerance to various abiotic and biotic stresses, but the pace of breeding also accelerates, while new possibilities emerge due to enhanced usage of genetic variability and using methods of modern genetic manipulation. Therefore, such inventions are important in the development of resilient oilseed crops that will be resistant to climate change and continue to ensure food security for future generations. Further research, in fact still ongoing in the fields, promises to expand possibilities for sustainable varieties of oilseeds and contribute immensely to the resilience of global agriculture. Otherwise, apart from that, the potentials of molecular markers, coupled especially with state-of-the-art genome editing techniques such as CRISPR-Cas9, among others, cannot be left aside in ensuring additional dimensions of accuracy in plant improvement. For all these, integrated use of traditional breeding in amalgamation with progressive biotechnology is likely to effect total transformation for developing better improved varieties toward enhancement of nutritional profiles and enhanced quality of oils and a superior level of yields thereof in oilseed crops. Secondly, investments in research and development related to the technology of molecular markers and training of the next generation of plant breeders and geneticists should be done on a continuous basis. The progress needs to reach varieties, regions, economies, and farmers for truly fair agricultural development to take place. After all, the successful use of molecular markers in oilseed crop breeding contributes not only to agricultural sustainability but also plays a very important role in solving such challenges as overpopulation of the globe, changes in eating habits, and the need for more efficient land use. All the present molecular markers, along with related technologies, have considerable promise in attempting to secure the future productivity of the oilseed crops for the aspects of food security and sustainability.

Supplementary Materials

No supplementary material is available for this article.

Author Contributions

These authors contributed equally.

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Conflict of Interest Statement

The authors declare no potential conflict of interest with respect to the research, authorship, and/or publication of this article.

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مقاله مروری

استفاده از نشانگرهای مولکولی در به‌نژادی دانه‌های روغنی: مقاله مروری

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چکیده: نشانگرهای مولکولی به عنوان ابزارهای حیاتی برای بهبود محصولات دانه‌های روغنی و ایجاد ارقام با قابلیت تحمل شرایط خشکی، شوری و دماهای بالا مطرح هستند. در این این مقاله مروری بر کاربرد نشانگرهای مولکولی مبتنی بر DNA، مانند ردیف‌های تکراری ساده (SSRs) و پلی‌مورفیسم‌های نوکلئوتید منفرد (SNPs) در اصلاح گیاهان زراعی دانه‌های روغنی متمرکز است. این نشانگرها در شناسایی و مکان‌یابی ژنی کنترل کننده صفات کمی (QTL) که مرتبط با تحمل به استرس هستند، کمک نموده و فرایند انتخاب را از طریق انتخاب مبتنی بر نشانگرها (MAS) تسریع می‌بخشند. اگرچه دانه‌های روغنی مانند کلزا، سویا و آفتابگردان از تحمیل بالاتری نسبت به تنش‌های محیطی برخوردارند، ولی از دلایل اصلی شکاف عملکرد موجود ناتوانی آنها در مدیریت اثرات تنش غیرزیستی مانند خشکسالی، شوری، و دمای شدید بوده، که به طور قابل توجهی بر رشد و بهره وری تاثیر می‌گذارد. گنجاندن نشانگرهای مولکولی در برنامه‌های اصلاحی نه تنها به حفاظت تنوع ژنتیکی کمک می‌کند بلکه به توسعه سریع‌تر ارقام با عملکرد بالا که می‌توانند شرایط سخت را تحمل کنند نیز تاثیرگذار خواهد بود. در این بررسی، مثال‌هایی از کاربردهای موفق این فناوری‌ها ارائه گردیده، ضمن اینکه تحقیقات بیشتر بر روی کاربرد نشانگرهای مولکولی در اصلاح دانه‌های روغنی کمتر مطالعه شده به منظور بهبود امنیت غذایی در برابر تهدیدات گرم شدن کره زمین را ضروری می‌داند.

کلمات کلیدی: تنوع ژنتیکی، گرمایش زمین، انتخاب مبتنی بر نشانگرها، مکان‌یابی ژنی کنترل کننده صفات کمی.

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Achievements and challenges in hybrid rice breeding in Iran

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Review paper

Abstract: To ensure food security for the growing population, raising the yield potential of rice remains a priority in Iran. Based on the per capita rice consumption of 35 kg and a stable rice cropping area of 650,000 hectares, the rice yield should be increased to approximately 8,000 kg per hectare. However, achieving high rice yields has become increasingly challenging. This article examines the achievements and challenges associated with hybrid rice breeding in Iran. Its aim is to analyze the current status, identify barriers and propose solutions for improving hybrid rice breeding processes. The key factors that constrain hybrid rice development are analyzed, and possible solutions to these challenges are proposed. Modern technologies in hybrid rice breeding, including gene editing (CRISPR/Cas9 technology), transgenic technology, and artificial intelligence, significantly enhance the ability to improve desirable traits such as disease resistance and grain quality. However, the effective implementation of these technologies in Iran faces multiple challenges, including the lack of a clear regulatory framework, public and social concerns, insufficient research infrastructure, and the need for investment. Ultimately, Iran's efforts in hybrid rice breeding are crucial for enhancing agricultural productivity and ensuring food security. There is a need for continuous investment in research, farmer education, and sustainable practices to strengthen the country's capacity to produce rice despite increasing challenges.

Keywords: Hybrid rice breeding, food security, cytoplasmic male sterility (CMS), *Oryza sativa*.

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Introduction

Rice is one of the most important agricultural products in Iran, playing a vital role in ensuring food security and supporting farmers' livelihoods. Rice is primarily cultivated in the northern provinces, where the climate and soil conditions are favorable for rice growth. The provinces of Gilan and Mazandaran are the leading rice-producing regions (Gava et al., 2024), benefiting from abundant water resources and suitable growing environment (Zamanialaei et al., 2022). The production of rice in these areas not only supports local economies but also provides employment opportunities for thousands of farmers and agricultural workers. However, the rice sector faces several challenges, including water scarcity, climate change, and competition from imported rice. The huge surge of rice import in 2013 and 2023 (FAO, 2024) set the import records (Fig 1). Figure 1 provides crucial insights into the relationship between population growth and rice imports in Iran, highlighting the challenges faced by the country in achieving self-sufficiency in rice production and the urgent need for effective agricultural strategies to enhance domestic output. These factors pose significant risks to both production levels and the livelihoods of farmers (Zamanialaei et al., 2022). To address these challenges, efforts are being made to enhance rice breeding programs, improve water management practices, and promote sustainable agricultural techniques. By focusing on these areas, Iran aims to secure its rice production and ensure that this vital crop continues to support food security and the economy for years to come. Given the increasing demand for rice production due to population growth and the impacts of climate change (Ali et al., 2021a), improving and developing cultivation methods has become essential. In this context, hybrid rice breeding has emerged as an effective strategy for enhancing yield and improving the quality of rice (Chen et al., 2024b; Wang et al., 2024; Zheng et al., 2024).

In recent years, significant advancements have been made in hybrid rice breeding in Iran. These advancements include the identification of new hybrid varieties (Dorosti et al., 2006; Afkhami Ghadi, 2020); improvements in cultivation practices and management strategies (Modarresi, 2023), and increased awareness and training for farmers in the use of these varieties (Nematzadeh et al., 2003). However, several challenges remain that may affect the development and expansion of hybrid rice. These challenges include limitations in water resources, climate change impacts, and the need for greater investment in research and development (Afkhami Ghadi, 2020; Modarresi, 2023).

This study examine the achievements and challenges associated with hybrid rice breeding in Iran. The objective of this study is to analyze the current status, identify barriers and challenges, and propose solutions for enhancing hybrid rice breeding processes in the country. Given the importance of rice in feeding the population and promoting sustainable agricultural development, this topic requires careful consideration and thorough investigation to optimize production and productivity in this vital sector.

Iran's future demand for high yield and quality rice

To secure food for the expanding population, enhancing the yield potential of rice is a key priority in Iran (Keramat et al., 2021). Considering the per capita rice consumption of 35 kg and a consistent rice cultivation area of 650,000 hectares (Ministry of Jihad Agriculture (FAO, 2024), it is necessary to elevate rice yields to approximately 8,000 kg per hectare by 2030. However, increasing these yields has become progressively more difficult.

Iran's future demand for high-yield and high-quality rice is expected to rise significantly due to several factors:

Population growth

As the population of Iran continues to grow, the demand for staple foods (Elferink and Schierhorn, 2016), particularly rice, will increase. This requires higher production levels to ensure food security (Ponnuswamy et al., 2024).

Changing dietary preferences

With rising incomes and urbanization, there has been a shift towards higher consumption of rice and other staple foods. This trend further drives the need for improved rice varieties that meet quality expectations.

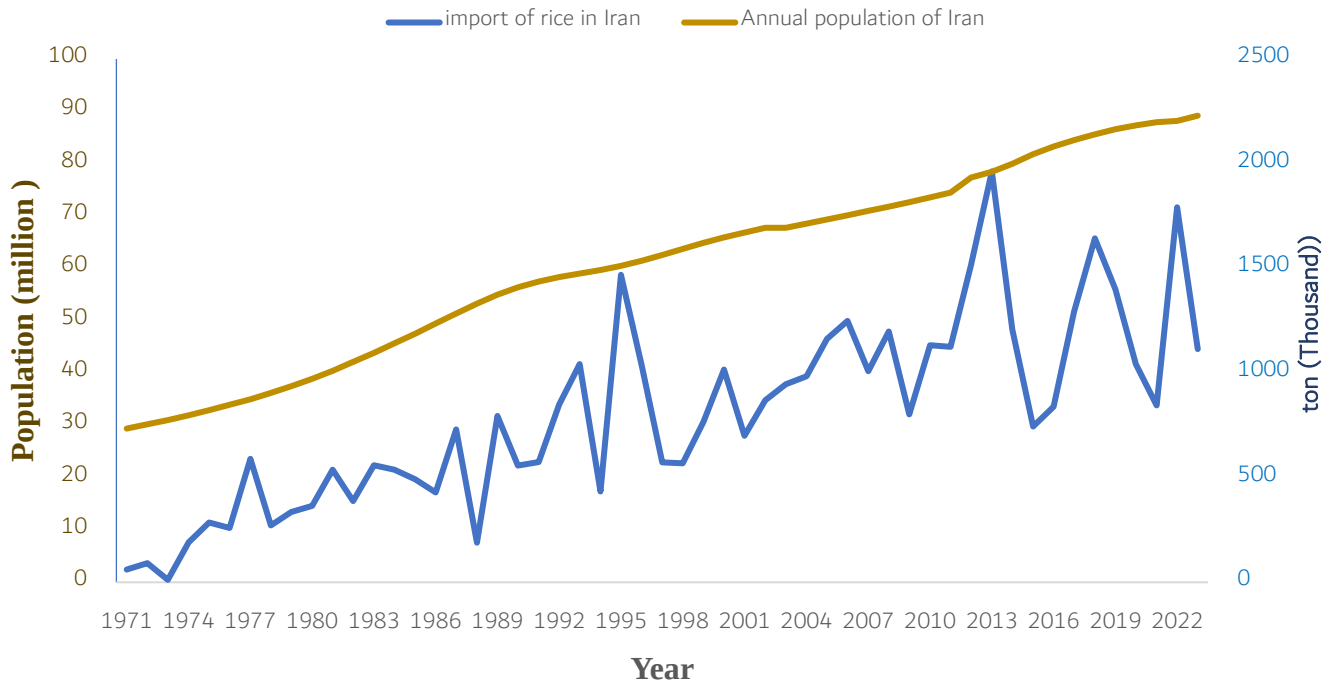

Figure 1. Variation in the population and import of rice in Iran (1971-2023).

Economic development

As the economy develops, the demand for quality food products likely increase. Consumers may seek rice varieties that offer better taste, nutritional value, and cooking qualities (Anang et al., 2011).

Climate change and environmental factors

Climate change poses challenges to traditional rice farming practices, making it essential to develop high-yield varieties that are resilient to changing environmental conditions, such as drought and temperature fluctuations (Javadi et al., 2024; Li et al., 2024).

Sustainability goals

There is a growing emphasis on sustainable agricultural practices. High-yield rice varieties can help meet food demand while minimizing the environmental impact of farming by reducing the land and water needed for cultivation (Sharma, 2024).

Government policies and investments

The Iranian government has been focusing on agricultural self-sufficiency and food security (Shayanmehr et al., 2024). Investments in research and development for high-yield rice varieties, as well as improvements in agricultural practices, will likely enhance production capabilities.

In summary, Iran's future demand for high-yield and quality rice is driven by demographic, economic, and environmental factors. Addressing this demand will require a concerted effort in agricultural innovation, investment and sustainable practices to ensure that the country can meet its food security needs effectively.

Progress in hybrid rice development in Iran

Development of CMS lines in hybrid rice breeding in Iran

Rice breeding in Iran has undergone three distinct periods, each characterized by unique strategies and methodologies aimed at enhancing rice production and quality. The first was Before 1979, only local rice varieties were used; the second was between from 1978 to 2000, when high-yielding semidwarf varieties were introduced; The third one began in 1991 with the introduction of certain cytoplasmic male sterile (CMS) lines from the International Rice Research Institute (IRRI)

(AghaAlikhani et al., 2013; Afkhami Ghadi, 2020). V20A and W32A, were the first CMS lines introduced from IRRI to Iran. The first attempts with hybrid rice in Iran were made at the Sari Science of Agriculture College and Amol Rice Research Center through the introduction of two CMS lines (V20A and W32A) from IRRI in 1987 and later in 1990 (Nematzadeh, 1977). Two other CMS lines, IR58025A and IR29298A, were introduced from IRRI and the study and breeding of new CMS lines from well-adapted and high-yielding improved varieties began in 1991. Dr. Jauhar Ali played a pivotal role in advancing rice breeding techniques through his coordination of hybrid and molecular rice breeding program as part of the International Rice Research Institute (IRRI)-Iran project from 2003 to 2009 (ICRISAT, 2014). This initiative enhanced rice production and improve the resilience of rice varieties to various environmental challenges. In this context, Dr. Ghorbanali Nematzadeh also played a vital role as the project leader for hybrid rice in Iran. His leadership and guidance of the research team significantly contributed to the development and implementation of effective strategies in hybrid rice breeding. Additionally, his efforts in foster international collaborations and facilitat the transfer of modern technologies to Iran greatly enhanced local capacities in this field. The first cytoplasmic male sterile lines were developed by Nematzadeh (1991) in Iran from indica sources of cytoplasm (IR58025A) in the genetic background of indica varieties Kazar, Nemat, Neda, Dasht, and Champa by backcrossing (Nematzadeh et al., 2003). Some appear well adapted to the tropics, possess acceptable grain quality, are good general combiners, and exhibit satisfactory outcrossing rate. Afkhami *et. al.* reported that NematA, Shastak mohammadiA, GerdeA, HasaniA and KhazarA are complete and stable sterile lines (Afkhami et al., 2013; Afkhami et al., 2015). Table 1 provides information on the cytoplasmic male sterile (CMS) lines that have been utilized for developing hybrid rice in Iran. This information reflects the diversity and efforts made to improve CMS lines and their adaptation to climatic conditions in Iran. This event marked the advent of hybrid rice technology in Iran, leading to the development of hybrid rice varieties (Nematzadeh et al., 2003).

Development of fertility restoration lines in hybrid rice breeding in Iran

The successful use of hybrids depends on effective fertility restoration mechanisms. Here, we present an overview of the process and key considerations involved in developing fertility restoration lines in hybrid rice breeding in Iran.

Restorer lines were selected from testcross nurseries based on the fertile reaction of the F_1 plants. The use of restorer lines such as IR24R, IR60969R, IR56R, and Amol 1R for Neda A, including a study of allogamy associated traits, for new improved CMS lines was carried out (Nematzadeh et al., 2003). Ahmadikhah et al. (2007) showed that lines IR28, Amol1 and Amol2 carry the *Rf4* gene linked with SSR marker RM171 on the long arm of chromosome 10, lines IR36 and IR60966 carry the *Rf3* gene linked with SSR marker RM1 on the short arm of chromosome 1, line IR62030 carries the *Rf5* gene on the short arm of chromosome 10, and finally line IR24 carries the *Rf4* gene on the long arm of chromosome 10 and an unknown *Rf* gene, respectively. Sattari et al. (2007) reported that because of their close linkage to *Rf* genes and distinct banding patterns, STS markers RG140/PvuII and S10019/BstUI are well suited for marker-aided selection, enhanced backcross procedures, and pyramiding of *Rf* genes in agronomically superior non-restorer lines. Ahmadikhah and Alavi (2009) in study of cold-inducible modifier QTL affecting fertility restoration of WA CMS in rice and reported that one major QTL (qRf-1-1) localized on the short arm of 1st chromosome near RFLP marker RG140 and the other one (qRf-1-2) localized on the same chromosome between RM7180 and RM6100d. Alavi et al. (2009) showed that *Rf3* was flanked by tow SSR markers RM1 and RM3873 at distances of 5.6 and 14 cM, respectively. Majidi et al. (2009) revealed that among candidate genes, only two genes, OsIFCD036677 and *Rf1*-B, showed differential expression among restorer and cytoplasmic male sterile lines, especially during the heading stage. As a result, these two genes were identified as the most likely candidates for fertility restoration at the *Rf4* locus within the WA CMS system.

A. Baluch-Zehi et al. (2013) investigated genetic distance among parental lines of hybrid rice using cluster analysis of morphological traits. Their

findings indicated that the lines R9, R2, IR50, and Poya were potential restorer lines. A restorability test with known wild-abortive restorer lines (viz. IR36 and IR24) showed that combination of Yosen A × IR24 could produce highly fertile F_1 hybrid (Yazdanpanah, 2009; Ahmadikhah et al., 2015). reported that two STS makers of RG140 which are linked to the *Rf3* locus on chromosome 1, and S10019 which is linked to the *Rf4* locus on chromosome 10, were used to screen a restorer line. Kiani (2018) validated SSR markers linked to restoring fertility (*Rf*) genes and genotyping rice lines at *Rf* loci reported that RM258, RM171, and RM3148 markers could be used for screening of genotypes to identify restorers and non-restorer lines in hybrid rice breeding programs Afkhami Ghadi et al. (2019) identified male sterility maintainer and fertility restorer lines from Iranian landraces and improved rice cultivars and reported that IR50 and IR67924R lines with more than 96% and 80% fertility, respectively, were strong fertility restorer lines. Mirzababapour Amiri et al. (2021) reported that three genotypes (K7, K12 and K16) were found to desirable fertility restorer lines for NedaA due to their beneficial characteristics as well as representing more than 80% fertility percentage of pollen grains and seed setting in their panicles. Mahdikhani et al. (2023) showed that IR65622-151-1-2-2-2R for NedaA and IR68078-15-2-1-2-2-R and IR86403-5-5-2-1-1-1-1-1R were suitable fertility restorer lines for JelodarA.

Photoperiod-sensitive genic male sterility in rice breeding

Two well established male sterility systems in rice are cytoplasmic genetic male sterility (CMS), a three-line system, and environmentally sensitive genic male sterility (EGMS). EGMS has two types of mechanisms: PGMS and TGMS. Sattari (2001) confirmed the induction of photoperiod-sensitive genic male sterile (PGMS) mutants of rice, and identified them as genetic male sterile mutants of the Nemat variety.

Table 1. CMS lines introduced and developed in Iran by transferring WA cytosterility into the genetic background of elite breeding lines and varieties.

CMS line	Developed at	Pedigree	Breeder
IR58025A	IRRI	IR 22/Improved Sabarmati//V20A	IRRI
IR29298A	IRRI	-	IRRI
OM 6378A	IRRI	Type3/Jasmine 85//NedaA	IRRI
IR75596A	IRRI	-	IRRI
IR70416-53-2-2A	IRRI	IR 66295-71-2/IR 66696-97-4-3-1//NedaA	IRRI
NedaA	Iran	Hasansaraiy/Sangetarom/Amol 3//IR58025A	Nematzadeh et. al. (2003)
NematA	Iran	Hasansaraiy/Sangetarom/Amol 3//IR58025A	Nematzadeh et. al. (2003)
KazarA	Iran	IR2071-625-152 /TANU7456//IR58025A	Nematzadeh et. al. (2003)
DashtA	Iran	Amol1/IR24//IR58025A	Nematzadeh et. al. (2003)
ChampaA	Iran	Champa/NedaA	Nematzadeh et. al. (2003)
ShiroudiA	Iran	Deilamani/Khazar//NedaA	Afkhami (2020)
RoshanA	Iran	Mutant of Nemat//NedaA	Afkhami (2020)
JelodarA	Iran	Tarom deylamani/Sange tarom//NedaA	Afkhami (2020)
Shastak mohammadiA	Iran	Shastak mohammadi/NedaA	Afkhami (2020)
GerdeA	Iran	Gerde/NedaA	Afkhami (2020)
OndaA	Iran	Italy germplasm/NedaA	Afkhami (2020)

Table 2. Some of promising three-line hybrid rice combinations identified in Iran with quantity and quality characteristics.

Hybrid combination	Plant height (cm)	Duration (d)	Pollen sterility	Yield (kg m 2)	Kernel length (mm)		Aroma	Amylose content (%)
					Before cooking	After cooking		
DashtA/IR 68061-27-3-	118.00	131	27.67	863.03	6.66	11.40	Intermediate	23.56
JelodarA/IR 68061-27-	115.33	126	15	884.24	6.35	10.70	None	19.90
ShastakA/IR 57301-	165.00	128	10	938.95	5.86	10.43	Intermediate	20.75
DashtA/IR 73014-59-2-	120.00	125	15	900.94	6.71	10.60	None	22.00
IR75596A/MILYANG	117.33	123	23	1159.30	5.95	10.37	Strong	25.25
DashtA/SUWEON 294	125.67	129	18	1242.30	6.46	10.50	Intermediate	23.07
IR68899A/SUWEON	125.67	130	20	1286.12	6.00	10.13	None	19.02
NematA/IR 56	125	131	9	1074.10	6.75	11.60	Intermediate	25.64
IR68280A/ IR 56	117	122	23	1022.00	6.73	11.00	None	22.63
IR68899A/ IR 56	118	131	9	1045.70	6.78	10.63	None	25.99
IR102572A/ IR 9761-	120	125	11	998.34	6.28	10.90	None	23.56
IR78369A/IR46R	122	128	9	901.00	6.89	10.97	Intermediate	20.28
IR102572A/NSIC RC	120.67	124	6	969.95	6.56	10.97	None	28.65
IR102572A/IR 85593-	127.67	124	14	1196.50	5.90	10.13	Intermediate	19.02
IR 78369A/IR8 5593-	125	122	9	1322.30	6.16	11.00	Strong	19.30

Afkhami et al. (2015) evaluated the sterility stability of several rice cytoplasmic male sterile lines and reported that the IR68899A line, which demonstrated complete sterility, was fertile under high temperature (> 24°C) and low light (< 13.75 h) conditions in the greenhouse. Therefore, it could be used for two-line hybrid programs. Siahchehreh et al. (2023) tagging of temperature-sensitive genic male sterility (TGMS) gene in rice mutant Nemat cultivar reported that RM110 and RM29 primers had a high correlation with TGMS gene (5.74 and 11.63 c M, respectively).

Yielding ability and grain quality of hybrid rice in Iran

Hybrid combinations were evaluated using four kinds of experiments: advanced yield trials (AHRT), preliminary yield trials (PHRT), observational yield trials (OHRT), and combining ability experiments (CA) (Virmani, 1997). The best hybrid combinations in each experiment are promoted to the next yield trial in the following season. The first hybrid rice to be introduced to Iran was the hybrid Dilam (Bahar 1), which achieved an average yield of 7.5 tons per hectare (Dorosti et al., 2006).

This hybrid was developed by crossing of IR58025A and IR42686R. In comparison with the control variety Khazar, this variety exhibited a standard heterosis of 57.9%. Additionally, the two parent lines had a maturity difference of 5 days. Hashemi et al. (2009)used 16 rice genotypes, including three cytoplasmic male sterile (CMS) lines, five restorer lines, and eight hybrid combinations. Out of 16 SSR markers, 10 markers showed polymorphic bands. The first hybrid rice, IRH1, was distinguished from other hybrids using SSR markers. Afkhami Ghadi (2020) evaluated the morphological and molecular characteristics of cytoplasmic male sterile maintainer lines and fertility restorer lines from various genetic sources of rice by introducing 13 rice hybrids with a yield of over 1,000 grams per square meter. They reported the hybrids IR 75596 A/MILYANG 54 and IR 78369 A/IR85593-23-2-1-3-1-2-1-1-1 as among the most aromatic hybrids, comparable to the quality of the aromatic variety Hashemi. The priorities of rice grain quality

characteristics vary from region to country. The main quality characteristics for the commercial evaluation of rice varieties include: 1) milling and head rice recovery, 2) shape and appearance of the grain, and 3) cooking and eating characteristics.

Iran has made significant strides in the development of hybrid rice varieties recently (Fig 2, Table 2). Table 2 examines various hybrid rice combinations and their quantitative and qualitative characteristics. These data demonstrate the advancements made in developing high-yield hybrids of suitable quality, assisting farmers and researchers in making better cultivation choices.

Figure 2 depicts the historical development of hybrid rice in Iran from the beginning of efforts in 1987 to 2024. Key trends, including the introduction of CMS lines and advancements in hybrid production, are illustrated in this figure. This information can help researchers understand the trajectory of hybrid rice development in Iran and the associated challenges. This progress is crucial for enhancing rice production, improving food security, and meeting the growing demand for high-quality rice. Here are some key aspects of this progress:

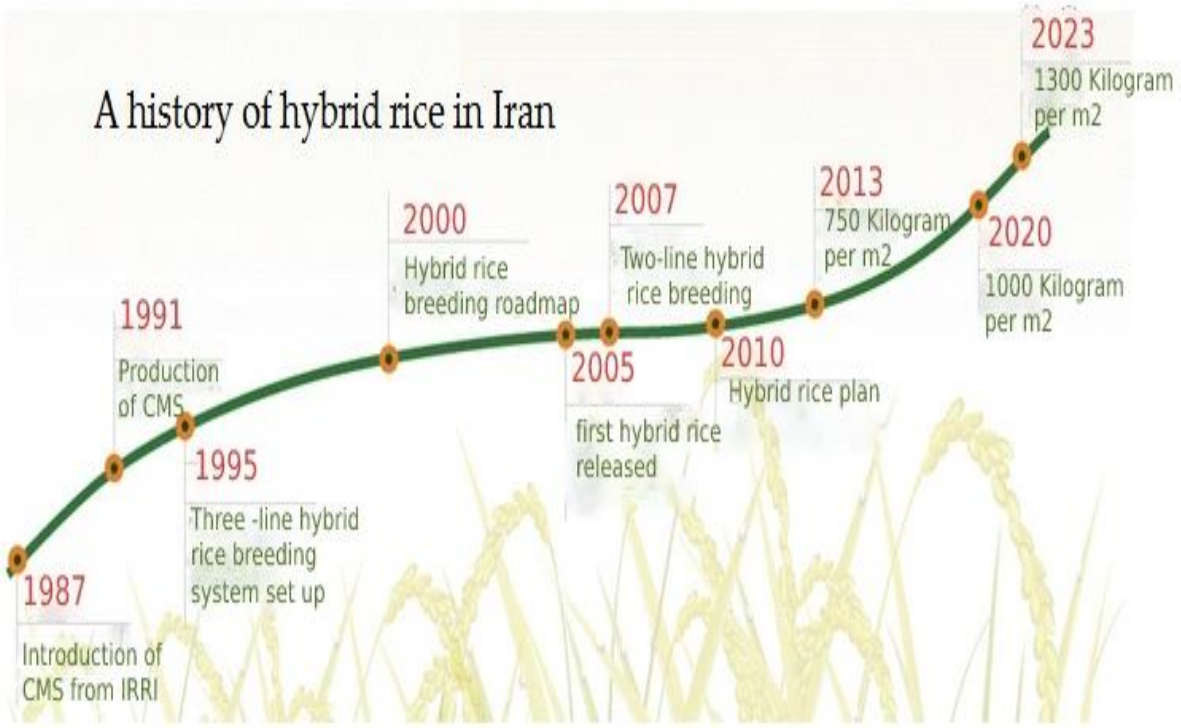

Figure 2. A shematic history of hybrid rice in Iran of 1987-2024.

Innovating hybrid rice production in Iran

Research and development

Research and development in hybrid rice production in Iran are key to improving rice production and enhancing its quality (Peykani et al., 2008). Iranian agricultural research institutions are actively engaged in developing hybrid rice varieties that are better suited to the country's climatic conditions. Collaborations with international agricultural research organizations have also facilitated knowledge transfer and access to advanced breeding techniques (Virmani et al., 2006; FAO, 2014).

High-yield varieties

Hybrid rice varieties developed in Iran have shown substantial increases in yield compared to traditional varieties. These hybrids are designed to

be more productive, with some varieties yielding up to 20-30% more than conventional rice (Afkhami Ghadi, 2020).

Resilience to environmental stress

Many new hybrid rice varieties are bred to be more resilient to environmental stresses, such as drought, salinity, and pests (Singh et al., 2024). This is particularly important given the challenges posed by climate change and water scarcity in Iran.

Government support and policies

The Iranian government recognizes the importance of hybrid rice in achieving food security and has implemented policies to support research, development (Virmani et al., 2006), and dissemination of hybrid seeds. Financial incentives and subsidies for farmers adopting hybrid rice cultivation have also been introduced.

Training and capacity building

Extension services and training programs for farmers have been established to promote the adoption of hybrid rice cultivation practices. This includes educating farmers on best practices for planting, fertilization, and pest management to maximize the benefits of hybrid varieties (Salam and Sarker, 2023; Gupta et al., 2024).

Market acceptance

As hybrid rice varieties have gained popularity, acceptance between consumers and farmers has increased. The improved yield and quality of hybrid rice help boost its market presence (Gupta et al., 2024)

Sustainability considerations

Efforts are being made to ensure sustainable hybrid rice cultivation. Research has focused on minimizing the use of chemical fertilizers and pesticides, promoting integrated pest management, and optimizing water usage.

Future prospects

The future of hybrid rice in Iran appears to promising, with ongoing research aimed at developing even more resilient and high-yield varieties. Continued investment in agricultural technology and infrastructure are essential for maintaining this progress. In conclusion, Iran's progress in hybrid rice development represent a critical step toward enhancing agricultural productivity (Peykani et al., 2008) and ensuring food security. By focusing on research, farmer education, and sustainable practices, Iran seeks to strengthen its rice production capabilities despite growing challenges.

Challenges in hybrid rice breeding

Grain quality of hybrid rice needs improvement

With the increasing living standards of rice consumers in Iran, it is necessary to improve the grain quality of rice (Butardo et al., 2019). In comparison with landrace rice, hybrid rice exhibits poorer grain quality in terms of head rice recovery and aromatic (Gong et al., 2023). The development of rice hybrids with both high yield and good grain quality remains a challenge for breeders (Zeng et al., 2019).

The sources of male sterility-inducing cytoplasm for developing better CMS lines are poor

Currently, 100% of the CMS lines used in trial production are WA types (Afkhami Ghadi, 2020). This dominant cytoplasm in the existing three line hybrid rice cultivars could lead to the development of destructive pests and diseases (Virmani et al., 2003; Faiz et al., 2007).

Screening for resistance to biotic stresses

The incorporation of resistance to the major biotic stresses of the region is necessary for the successful adoption of hybrids. Hence, promising hybrids are regularly screened for resistance to major pests and diseases (Horgan and Crisol, 2013).

Future perspectives

Develop hybrids with acceptable grain quality that meet the specific requirements of different regions. The magnitude of heterosis should be enhanced to 20% and above by developing two line and intersubspecific hybrids (Ali et al., 2021b). Enhance seed yields beyond 2.0 t ha $^{-1}$ to bring down the seed cost. In addition to the above, efforts on technology transfer have been intensified through the conduct of a large number training programs to create awareness about the benefits of hybrid rice among rice farmers and consumers across the country. Policy interventions by the government for increased support, aggressive efforts to popularize hybrids, and the assured procurement of hybrid rice produce at a minimum support price are needed at this juncture.

Modern technologies in hybrid rice

Gene editing technology

CRISPR/Cas9- This technology allows precise editing of genes, enabling the enhancement of desirable traits such as disease resistance and improved grain quality in rice (Romero and Gatica-Arias, 2019; Zafar et al., 2020; Chen et al., 2024a).

Transgenic technology

Gene transfer from other species- Transgenic methods facilitate the introduction of genes associated with traits such as abiotic stress tolerance, pest resistance, and increased photosynthetic efficiency, thus improving yield potential and resilience in rice varieties (Sabar et al., 2024).

Bioinformatics technologies

Modeling and simulation- Advanced software and algorithms are used to predict genetic traits and performance in hybrid rice (Xu, 2007; VanRaden, 2008; Xu et al., 2014; Xu et al., 2021; Fritsche-Neto et al., 2024).

The use of artificial intelligence (AI) in hybrid rice technology

By utilizing artificial intelligence systems and algorithmic processing, it has become possible to analyze large volumes of data, including genotype, phenotype, generations, and grain quality of rice (Sabouri and Sajadi, 2022; Crossa et al., 2024). This capability allows for the rapid generation of millions of potential rice combinations for hybrid rice production (Xu et al., 2022; Ashraf et al., 2024). Furthermore, in the consumer market, the development of identification and detection methods for rice seeds through artificial intelligence can significantly reduce fraud and seed mixing, ultimately ensuring seed purity (Hruthik Chandra et al., 2022; Felizardo et al., 2024).

Limitations of using these technologies in Iran

Legal and policy considerations

Lack of clear regulatory framework- The absence of well-defined laws and regulations regarding transgenic and gene-editing technologies can hinder research and commercialization efforts (Mohajer et al., 2011).

Public and social concerns

Public resistance to transgenic products- There are concerns among consumers and farmers regarding the safety and ethics of transgenic and gene-edited crops, which may hinder acceptance (Akbari et al., 2023).

Lack of awareness and education- Insufficient information and education about the benefits and safety of these technologies can lead to resistance (Akbari et al., 2023).

Technical and Economic challenges

Insufficient research infrastructure- Many research institutions and universities in Iran lack the advanced equipment and technology needed for cutting-edge research in gene editing and transgenics.

Need for investment- Research and development in these areas require significant investment, which may be challenging under current economic conditions.

High costs- The costs associated with research, development, and commercialization of new technologies can be a barrier for farmers and private companies (Spielman et al., 2012; Spielman et al., 2021).

Competition with imported products- Imported products that may utilize more advanced technologies can pressure local markets and farmers.

Environmental, cultural and social challenges

Environmental impact concerns- The use of modern technologies may raise concerns about their environmental effects, such as impacts on biodiversity and ecosystems.

Climate change- Climate change can affect the performance of modern technologies and pose challenges for agriculture (Lu, 2024).

Resistance in local communities- Some communities may resist changes due to traditional agricultural practices and cultural beliefs.

Social impacts- Changes brought about by modern technologies may affect the social and economic structures of rural communities. While modern technologies in hybrid rice hold great potential for improving production and quality, their effective

implementation in Iran faces various challenges. Addressing legal, public, technical, economic, environmental, and cultural issues is essential for maximizing the benefits of these technologies and enhancing food security in the country.

Conclusion

Rice is a crucial agricultural product in Iran. The rice sector significantly contributes to food security and farmers' livelihoods, but it faces challenges such as water scarcity, climate change, and competition from imported rice. The surge in rice imports in 2013 and 2023 highlighted the need for enhanced domestic production. To address these challenges, Iran is focusing on improving rice breeding programs, water management practices, and sustainable agricultural techniques. The future demand for high-yield and quality rice in Iran is driven by factors such as population growth, changing dietary preferences, economic development, climate change, and sustainability goals. To meet this demand, raising yield potential is essential, with projections indicating a need for an increase to 8.076 tons per hectare by 2030.

Hybrid rice breeding is a promising strategy to enhance yield and quality. Significant advancements have been made in this filed, including the introduction of new hybrid varieties and improved cultivation practices. However, challenges remain, such as limited water resources and the need for increased research and development investment. The study analyzes the current status of hybrid rice breeding in Iran, identify barriers, and propose solutions to optimize production. Iran's hybrid rice breeding has progressed through three distinct periods, beginning with local varieties, followed by the introduction of high-yielding semidwarf varieties, and a recent focus on cytoplasmic male sterile (CMS) lines. The development of fertility restoration lines and the use of environmentally sensitive genic male sterility (EGMS) are crucial for the successful implementation of hybrid rice. Despite progress, challenges such as improving grain quality, sourcing male sterility-inducing cytoplasm, and ensuring resistance to biotic stresses persist. Future efforts should focus on developing hybrids with acceptable grain quality, enhancing heterosis, and increasing seed yields.

In conclusion, Iran's progress in hybrid rice development are vital for enhancing agricultural productivity and ensuring food security. Continued investments in research, farmer education, and sustainable practices will strengthen rice production capabilities despite growing challenges. The government's support and policies play a critical role in promoting hybrid rice cultivation and ensuring its success in meeting the country's food security needs.

Supplementary Materials

There is no supplementary material for this article.

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Conflict of Interest Statement

The authors declare no conflict of interest.

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مقاله مروری

دستاوردها و چالش‌های اصلاح برنج هیبرید در ایران

عمار افخمی قادی

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چکیده: افزایش پتانسیل عملکرد برنج، جهت تضمین امنیت غذایی برای جمعیت رو به رشد، همچنان یک اولویت در ایران است. بر اساس مصرف سرانه برنج به مقدار ۳۵ کیلوگرم و سطح زیرکشت برنج پایدار ۶۵۰ هزار هکتار، عملکرد دانه باید به حدود ۸۰۰۰ کیلوگرم در هکتار افزایش یابد. این مقاله به بررسی دستاوردها و چالش‌های مرتبط با اصلاح برنج هیبرید در ایران می‌پردازد. هدف آن بررسی وضعیت فعلی، شناسایی موانع و پیشنهاد راه‌حل‌هایی برای بهبود فرآیندهای اصلاح برنج هیبرید است. عوامل کلیدی که توسعه برنج هیبرید را محدود می‌کنند، تجزیه و تحلیل شده و راه‌حل‌های ممکن برای این چالش‌ها پیشنهاد می‌شوند. فناوری‌های مدرن در اصلاح برنج هیبرید، شامل ویرایش ژن (فناوری CRISPR/Cas9)، فناوری انتقال ژن و هوش مصنوعی، به طور قابل توجهی توانایی بهبود ویژگی‌های مطلوب مانند مقاومت به بیماری و کیفیت دانه را افزایش می‌دهند. با این حال، اجرای مؤثر این فناوری‌ها در ایران با چالش‌های متعددی از جمله نبود چارچوب قانونی مشخص، نگرانی‌های عمومی و اجتماعی، کمبود زیرساخت‌های تحقیقاتی و نیاز به سرمایه‌گذاری مواجه است. در نهایت، پیشرفت‌های ایران در توسعه برنج هیبرید برای افزایش بهره‌وری کشاورزی و تضمین امنیت غذایی بسیار ضروری است. نیاز به سرمایه‌گذاری مستمر در تحقیقات، آموزش کشاورزان و شیوه‌های پایدار برای تقویت قابلیت‌های تولید برنج کشور در مواجهه با چالش‌های فزاینده وجود دارد.

کلمات کلیدی: اصلاح برنج هیبرید، امنیت غذایی، لاین‌های نرعقیم سیتوپلاسمی (CMS)، *Oryza sativa*.

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Original paper

Genetic diversity of some thornless blackberry genotypes using ISSR molecular markers

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Abstract: Genetic diversity in blackberries is crucial for improving quality and yield. This study evaluates thornless blackberry genotypes using 26 morphological traits and 19 inter simple sequence repeats (ISSR) markers to assess diversity and genetic relationships. A total of 28 blackberry genotypes, including both thorny and thornless types, were analyzed. Fourteen ISSR primers were selected from a pool of 19 based on their capacity to generate polymorphic bands. The findings highlight the efficiency of ISSR markers in distinguishing thorny and thornless blackberry genotypes at the subgenus level, effectively differentiating chimera-derived thornless samples. A total of 406 bands were produced, of which 402 were polymorphic. The average percentage of polymorphic bands for each primer in this experiment was 98.98%, and the highest polymorphic information content (PIC) was associated with ISSR 16, which had a value of 0.35. The findings highlight the efficiency of ISSR markers in distinguishing thorny and thornless blackberry genotypes at the main genotype groups: the first linked to initial thornless generations of *Rubus laciniatus*, and the second comprising crosses related to the Merton cultivar. Overall, significant genetic diversity among blackberry cultivars suggests valuable applications in breeding and improvement programs.

Keywords: *Rubus laciniatus*, merton thornless, genetic diversity, molecular marker.

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Introduction

Thornless blackberry (*Rubus* L. subgenus *Rubus* Watson) belongs to the Rosaceae family. This perennial plant typically exhibits a semi-erect growth habit, which distinguishes it from erect and trailing blackberry species. The Rosaceae family includes pome fruits, stone fruits, and aggregate fruits, with blackberries classified among the latter (Swanson et al., 2011). The domestication of blackberries began with the selection of wild samples, and by the early 20th century, genetic improvement was pursued through selected cultivars. The first thornless cultivar, "Evergreen," was derived from wild germplasm (*Rubus laciniantus*), following a mutation that led to the introduction of 'Thornless Evergreen (Abdi et al., 2021). Issues related to chimerism which easily return to thorny traits. In subsequent step through tissue culture the introduction of the 'Everthornless' cultivar. As reported by the thornless Merton from the John Innes Institute in the UK Swanson et al. (2011) developed and resulted in the creation of the Navaho, Arapaho, Apache, Ouachita, and Natchez cultivars. The trend of improving thornless continues with the introduction of new cultivars and the use of molecular markers to identify diverse sources (Coyner et al., 2005). PCR-based markers (RAPD, AFLP, SSR, ISSR, etc.) are widely used for the molecular classification of plant species in breeding programs (Adje et al., 2023; Agbo et al., 2023; Hosseinpour Azad, 2023; Kour et al., 2023). Coyner et al. (2008) investigated four sources of thornless using molecular markers (RAPD), differentiating cultivars of various origins and identifying common backgrounds among them. They also introduced the molecular markers used as tools for identifying valuable cultivars. The latest thornless cultivar, Prime-Ark Traveler, emphasizes early fruiting in the first year and superior quality, making it a suitable option for commercial transport (Clark and Salgado, 2016).

Hadadinejad and Moradi (2016) have been assessing wild blackberries with high antioxidant properties, and have established of a collection of various cultivars of blackberries, including both thorny and thornless. Initial studies indicated differences among the thornless cultivars in this collection, and these differences were confirmed by analyzing five thornless genotypes using ISSR molecular markers (Abdi et al., 2018). ISSR is recognized as an effective technique in studies of genetic diversity and phylogenetics, with advantages including low cost, easy preparation, and high accuracy in identifying cultivars, although its main drawback is the initial cost associated with designing markers (Barandalla et al., 2006; Selkoe and Toonen, 2006).

Debnath (2008) assessed genetic diversity and similarity within blackberry genotypes using ISSR markers and pedigree information. This study included nine North American blackberry cultivars (*Rubus*) and four Canadian breeding lines. Using 18 primers, the analysis produced 306 polymorphic bands. Cluster analysis results indicated a similarity range of 24% to 49% among 13 genotypes and 3% to 25% for nine genotypes.

Coyner et al. (2008) utilized RAPD markers to examine the relationship among thornless blackberries. RAPD markers have been used in several blackberry-related programs. Coyner et al. used molecular markers to investigate the genetic relationships among 11 blackberry cultivars derived from four thornless backgrounds. They used 140 random primers, and their analysis placed the cultivars into three distinct categories. They also reported that 98 primers produced 113 specific bands that could be useful for cultivar identification. Several primers, capable of distinguishing a cultivar, produced between one and 24 bands. A study estimated the genetic diversity and relationships among 74 blackberry accessions from five different cultivars using interspersed simple sequence repeat (ISSR) marker analysis and morphological characterization. Sixteen characteristics were examined, including phenological, vegetative, and reproductive traits in 57 accessions, alongside 10 ISSR primers. Findings revealed that yield had the highest genetic diversity (diversity index = 62.57), while leaf width showed the lowest (13.74), with a strong correlation (r = 0.98) between flowering and ripening dates. The primers generated 161 amplified fragments, of which 113 were polymorphic. Principal component analysis (PCA) and principal coordinate analysis (PCoA) explained 84.9% and 67.06% of the total variation, respectively. Cluster analysis categorized the populations into two to three groups based on

morphological traits and ISSR data, reflecting the influence of species and geographical origin on genotype distribution (Garazhian et al., 2022).

Sedighi and Rahimmalek (2015) examined the genetic diversity of wild blackberry species scattered around the Caspian sea using both morphological traits and ISSR molecular markers. Based on the results, they differentiated two groups of blackberries: those from the western and eastern regions of the northern strip of the country and those related to the central region. Given the high genetic diversity observed in the central region, it appears that samples from the eastern and western regions may have originated from the central region, leading to a report of relatively narrow genetic base for Caspian blackberries. Their results also indicated that of the 20 primers used, only 10 were polymorphic, and out of 204 bands produced, 157 were polymorphic. Ataei-e et al. (2015) investigated the genetic diversity of blackberries in northern Iran. A total of 60 genotypes from Gilan and Mazandaran provinces, representing seven species, were studied using SSR markers. Among the populations, high genetic diversity was observed within the species, while there was low genetic differentiation among the studied populations. Out of the 10 primers used by these investigators, six were polymorphic, yielding 32 polymorphic alleles. The average number of alleles per locus and the polymorphism information content (PIC) were 2.8 and 0.593, respectively. The highest PIC value obtained was 0.71, while the lowest was 0.50. The number of alleles at each gene locus ranged from one to five, with an average of 3.15. The species *R. sanctus* had the highest effective number of alleles at 1.7, while *R. hirtus* had the lowest at 0.1. The average expected heterozygosity across populations was 0.121, indicating low genetic differentiation within the species. Geographic separation, natural barriers, pollen transfer by insects, cross-pollination, and polyploidy were significant factors in creating and maintaining genetic diversity among blackberry species. Abdi et al. (2021) investigated the genetic diversity of thorny blackberries in the collection at the University of Agricultural Sciences and Natural Resources in Sari using ISSR molecular markers. All 10 primers used exhibited a desirable level of polymorphism in the studied population. A total of 345 bands were generated, of which 344 were polymorphic. The blackberry genotypes in the collection examined in this study are considered a genetic reservoir, which includes both an initial genetic reserve comprising thorny cultivated and thornless cultivars, as well as a secondary genetic reserve consisting of wild thorny blackberries. According to the results and the resulting dendrogram, the primers employed successfully divided the samples into three distinct groups. Based on the Dice similarity coefficient, the highest similarity was found between the thornless samples from Sari and Qaem Shahr at 73%, while the lowest similarity was between the wild samples from Qaem Shahr and Kermanshah at 7%. The imported thorny cultivars, which were completely distinguishable by ripening time and included early, mid-season, and late-maturing cultivars, were grouped separately. This study demonstrated that ISSR markers effectively distinguish genotypes and identify the existing genetic diversity.

In another study screening and evaluating blackberry cultivars and strains, 17 plant growth indices and orchard characteristics, as well as fruit nutritional traits, were measured. Twenty simple sequence repeat (SSR) markers were analyzed, and a fingerprinting of 23 blackberry cultivars and variety was developed, along with processing characteristics evaluated for 10 of the cultivars and strains assessed. The results indicated that 'Chester' and 'Shuofeng' had the highest yield per plant (6.5 kg per plant), with 'Chester' also showing the highest fruit firmness (2.78 kg/cm^2). 'Kiowa' had the highest individual fruit weight (10.43 grams). The cultivar "10-5n-2" had the highest total anthocyanin content (225.4 mg per 100 g FW) and total phenolic content (3.24 mg per gram FW), although it exhibited low plant yield. These results suggest that 'Shuofeng' and 'Chester' are the top-performing blackberry cultivars cultivated in Nanjing, exhibiting the best growth and overall quality. Additionally, a total of 119 alleles were identified, with an average of 6 alleles per locus. The polymorphism information content (PIC) ranged from 0.374 to 0.844, with an average of 0.739, indicating high genetic diversity among the 23 blackberry cultivars and strains (Zhao et al., 2023).

This study aimed to examine the genetic diversity of thornless blackberry genotypes collected from

various locations of south of the Caspian sea using ISSR molecular markers to identify the genetic relationships among the genotypes.

Materials and Methods

Investigating morphological diversity

Most of the desired morphological traits were selected based on the blackberry UPOV descriptor (Button, 2006) and measured as the average of the available repetitions. The examined traits and their measurement units were listed in Table 1. The coded data related to the color of leaves, fruits, canes, etc., were recorded according to the coding provided in the international descriptor of blackberry. For data without clear geographical origin, such as leaf blooming time, flowering time, and ripening time, we aimed to establish the lowest common range among the recorded data for a trait by selecting the appropriate origin. Continuous traits were measured using a ruler and caliper. A digital refractometer (ATAGO PR-32) was employed to measure total dissolved solids. Anthocyanin was quantified using the pH difference method (Wrolstad, 1993).

Samples from a collection of blackberries were selected, which included 28 genotypes from various geographical regions (Table 2). This collection comprises both thorny and thornless genotypes. Genomic DNA was extracted from young leaves using a modified CTAB method (Murray and Thompson, 1980). The DNA of the samples was diluted after assessing quality and quantity using a 0.8% agarose gel and a 260/280 nm absorbance ratio with a spectrophotometer (Biochrome Ltd, Cambridge, UK). Out of 19 primers, 14 ISSR primers were selected that produced the most polymorphic bands (Table 2). PCR reactions were conducted in a BioRad system. The thermal cycle included an initial denaturation step for 5 minutes at 94 °C, followed by 34 cycles of 50 seconds at 94 °C, 60 seconds at the annealing temperature (54-55 °C), and 80 seconds at 72 °C with a final extension step for 7 minutes at 72 °C. The amplified products were separated by electrophoresis in a 1.8% (w/v) agarose gel at 80 volts for 2 hours and 30 minutes in 0.5X TBE buffer, and after staining with ethidium bromide, the gel image was recorded using a Gel Doc Analyzer. The amplified bands were scored based on the presence (1) or absence (0) of bands. The Jaccard similarity coefficient, suitable for binary data and effectively indicating genetic similarity based on shared traits, along with the arithmetic mean algorithm (UPGMA), was employed to construct a cluster diagram using the weighted pair group method, as it visually displays these relationships in a clear and interpretable manner. Cluster analysis and principal coordinate analysis (PCA) were performed using NTSYSpc software, version 2.02. The polymorphic information content (PIC) was calculated using a formula for dominant markers: PICi = 2pi (1–pi), where pi is the frequency of the amplified alleles.

Table 1. Investigated traits and their measurement unit.

Raw	Traits	Unit	Raw	Traits	Unit
1	Growth habit	code	14	Flower anthesis	day
2	Spin number	number	15	Unripeand ripe friut color	code
3	Spin size	ml	16	Ripening	day
4	Spin direction	code	17	Fruit diameter	cm
5	Shoot cross sec	code	18	Fruit lenght	cm
6	Shoot diameter	cm	19	Fruit size	cm
7	Shoot number	number	20	Fresh weight	gr
8	Leaflet width	cm	21	TSS	Brix
9	Leaflet number	number	22	TA	%
10	Flower length	cm	23	TSS/TA	-
11	Flower diameter	cm	24	Seed number	number
12	Flower size	cm	25	Empty seed	number
13	Leaf anthesis	day	26	Anthocyanin	mg/100ml

Table 2. The list of genotypes were used in this study.

No.	Genotype ID	Loccation of collection	No.	Genotype ID	Loccation of collection
1	Marion	Sari University	15	cvTLNurnazari53	Mazandaran, Nur
2	Silvan	Sari University	16	cvTLSafzalian81	Mazandaran, Sari
3	cvTLSshirazi13	Mazandaran, Sari	17	cvTLAjafarzadeh35	Mazandaran,Amol
4	cvTLAdavudi18	Mazandaran,Amol	18	cvTLBaghajanzadeh49	Mazandaran,Babol
5	cvTLBizadkhast23	Mazandaran,Babol	19	cvTLQrastkhiz77	Mazandaran, Qaemshahr
6	cvTLmohammadi25	Mazandaran,Amol	20	cvTLAhoseinirad80	Mazandaran,Amol
7	cvTLSDrSadeghi6	Mazandaran, Sari	21	cvTLQrastkhiz175	Mazandaran, Qaemshahr
8	cvTLAbakhtiari224	Mazandaran,Amol	22	cvTLSmoafei69	Mazandaran, Sari
9	cvTLBrazinejad17	Mazandaran,Babol	23	cvTLBmiladi85	Mazandaran,Babol
10	cvTLSasadi26	Mazandaran, Sari	24	cvTLAjafarzadeh63	Mazandaran,Amol
11	cvTLBaghajanzadeh22	Mazandaran,Babol	25	cvTLSnurmohammadi68	Mazandaran, Sari
12	cvTLMshojaei51	Mazandaran, Miandorud	26	cvTLSDrMoradi2	Mazandaran, Sari
13	cvTLRahmadi54	Mazandaran, Ramsar	27	cvTL.mashaal66	Mazandaran, Sari
14	cvTLStaherpour38	Mazandaran, Sari	28	cvTLBkalantari92	Mazandaran,Babol

The order of blackberry genotypes on the wells of agarose gel was as follows and was also analyzed in NTSYS 2.02 software in the same way.

The marker index (MI), which indicates marker efficiency, was calculated for each primer using the following formula: PIC × percentage of polymorphism. The Shannon index (I), which indicates diversity for each primer, was determined using expected and observed heterozygosity, the number of alleles, and the number of effective alleles with GeneAlex software version 6.5. Correlation and factor analysis calculations were also conducted using SPSS software version 27.

Results

Correlation between morphological traits

Examining the correlation coefficient between the morphological traits revealed that the growth habit is inversely correlated with the number, size, and direction of spines, as well as the TSS/TA ratio, at a significance level of 1%. This shows that thornless genotypes have better growth habit compared to thorny cultivars. The growth habit also has an inverse correlation with the number of thorns (-0.54**), the size of the thorns (-0.49*), and the direction of the thorns (-0.47**) as well as with the TSS/TA ratio (-0.51**) (P≤ 0.01 level), which indicates that thornless genotypes are more vigorous than thorny cultivars. The number of thorns is also inversely related to traits such as cane diameter, flower length, flower diameter, ripening time, and TA.

In general, thornless genotypes are more late than thorny genotypes, which is consistent with the results of this study. Also, this trait, relative to the size and direction of the thorn, as well as the cross-sectional area of the cane, the fresh weight of the fruit, the TSS/TA ratio, and the seed pod, has a significant direct relationship at the probability level of one percent (Supplementary Table 1). The results of the correlation coefficient for the ripening time trait showed that this trait has a significant positive correlation with flower length (0.61**), flower diameter (0.75**), and acidity (0.55**) (P≤ 0.01 level). This means that the later the blackberry is, the higher its acidity is, and as a result, the taste index decreases. Therefore, in breeding programs, we should look for early thornless cultivars to have a better taste from a marketable point of view. Also, this trait has an inverse and significant relationship with fruit TSS, TSS/TA ratio, and soft seed, which indicates the lower quality of late cultivars compared to early cultivars.

Factor analysis

In this analysis, seven main and independent factors with eigenvalues greater than one accounted for a total of 83.12% of the overall variance (Supplementary Table 2). Based on the results, it can

be stated that the first factor includes traits such as the number, size, and orientation of thorns, cross-sectional area of the cane, the diameter of the cane in winter, flower length, flower diameter, maturity, fresh weight, acidity, and the ratio of TSS to acidity. The second factor comprised the number of leaflets, fruit color, fruit diameter, fruit length, fruit size, seed count, and growth habit. The third factor included traits such as primary canees on the plant, flower size, and fruit TSS content, while factors four, five, and six were characterized by flowering time, presence of float or hollow seeds, and bud blooming, respectively. Finally, the seventh factor included two traits: anthocyanin content and the width of the terminal leaflets (Supplementary Table 2). From the factor analysis, it can be concluded that the combination of the first and second factors explained 59.54% of the variance.

Cluster analysis based on morphological traits

The cluster analysis using the WARD method divided the genotypes into three groups. The thorny cultivars Marion and Silvan were placed in one group with a similarity of less than 20%, distinctly separated from other thornless genotypes by 100% difference. The thornless genotypes were further divided into two categories: genotypes cvTLRahmadi54, cvTLSafzalian81, cvTLSasadi26, and cvTLNurnazari53 from Ramsar and Noor in western Mazandaran and Sari formed the second group, which was distinguished from other thornless genotypes by a 40% difference. These genotypes were differentiated from others based on flower size, fruit color, flavor index, seed count, and dry seed weight. The third group comprised 22 genotypes that could not be distinguished through morphological markers due to a similarity of over 80% (Figure 1).

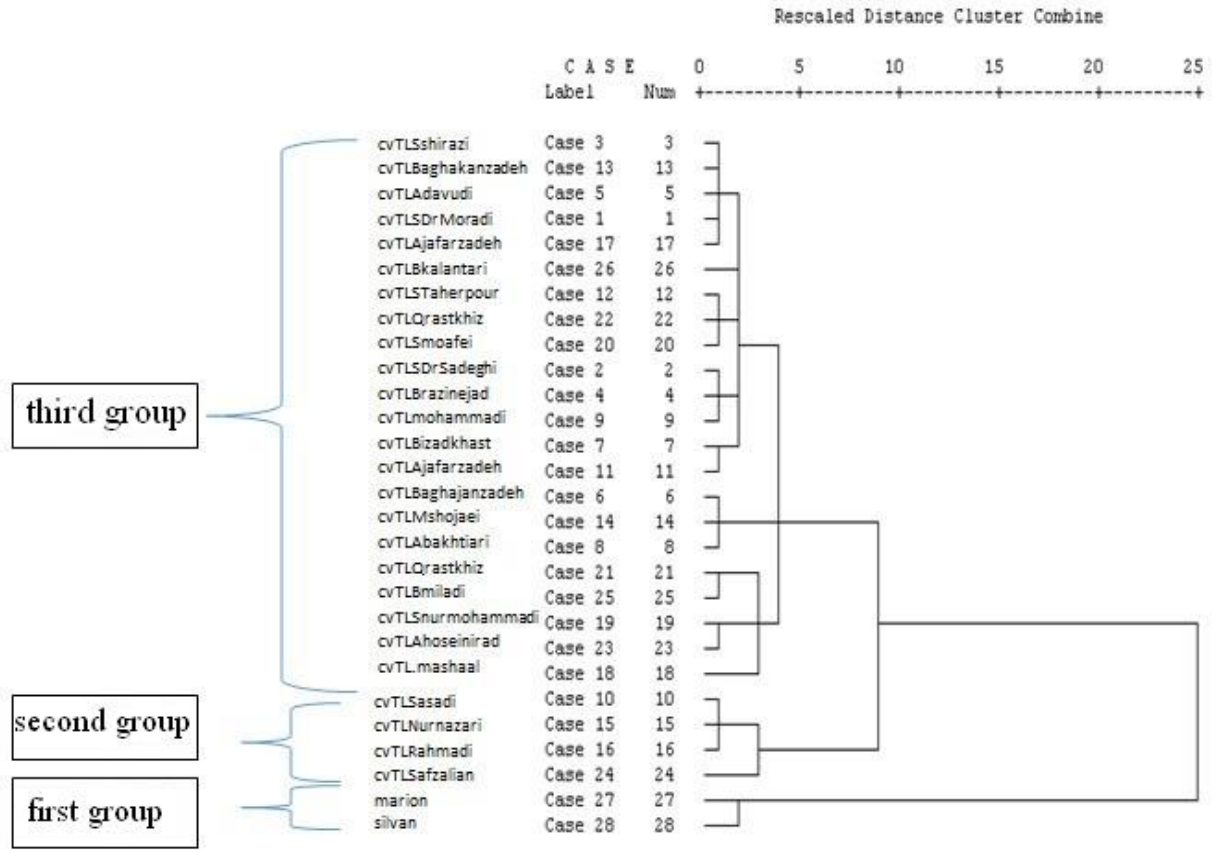

Figure 1. Cluster analysis by Ward method using morphological traits in blackberry genotypes.

Table 3. Results of statistical analyses for each ISSR primer in blackberry genotypes.

Primer Name	Total Bands	Polymorphic Bands	Polymorphic Percentage	Na[1]	Ne[2]	I[3]	Ho[4]	He[5]	PIC[6]	MI[7]
ISSR 1	25	25	100	2	1.2843	0.3315	0.19	0.19723	0.143	4.2188
ISSR 2	29	29	100	2	1.17	0.26	0.151	0.14	0.22	19.31
ISSR 3	39	39	100	2	1.23	0.29	0.23	0.16	0.26	26.21
ISSR 4	22	20	90.9	2	1.3458	0.3371	0.21	0.2115	0.108	4.4868
ISSR 5	18	18	100	2	1.2905	0.3025	0.197	0.1831	0.119	4.1546
ISSR 6	24	24	100	2	1.4783	0.4389	0.289	0.2860	0.144	6.7694
ISSR 7	36	35	97.22	2	1.2970	0.313	0.21	0.1902	0.114	3.9895
ISSR 10	40	40	100	2	1.3213	0.340	0.20	0.20788	0.132	4.5146
ISSR 11	13	13	100	2	1.35	0.38	0.24	0.23	0.30	29.32
ISSR 18	10	10	100	2	1.697	0.5760	0.277	0.3934	0.20	10.627
ISSR 15	53	53	100	2	1.22	0.28	0.17	0.16	0.25	25.11
ISSR 16	20	20	100	2	1.41	0.42	0.39	0.26	0.35	33.24
ISSR 18	34	34	100	2	1.37	0.36	0.242	0.23	0.29	28.38
ISSR 19	43	42	97.67	2	1.22	0.28	0.163	0.15	0.23	20.21
mean	29	28.71	98.98	2	1.33	0.350	0.225	0.214	0.204	16.48

1-number of different alleles, 2- number of effective alleles, 3- shannon's information index, 4- observed heterozygosity, 5- expected heterozygosity, 6- polymorphic information contents, 7- marker index

Molecular marker results

The thornless blackberry genotypes in Iran included imported cultivars which form a rich source of biodiversity, and it is essential to thoroughly identify their genetic structure for preservation and utilization. As shown in Supplementary Figure 1, the mentioned markers exhibited significant polymorphism and produced a clear and high-quality pattern, indicating the potential of these markers for further studies in blackberry genotypes. The results indicated that out of 20 primers used on five randomly selected genotypes, 14 primers generated distinct and clear bands, and the most suitable annealing temperatures for the primers during PCR reactions were 54 and 55°C. All 14 primers used exhibited desirable polymorphism in the studied population. A total of 406 bands were produced, of which 402 were polymorphic. The number of bands generated varied from 40 bands with primer number 10 to 10 with primer number 18. The average number of bands for each primer across the examined genotypes was 29. The average percentage of polymorphic bands in this experiment for each primer was 98.98%, with the lowest being 90.9% for primer number 4. Based on the obtained information, the highest polymorphic information content (PIC) was related to primer number 16, with a value of 0.35 Therefore, this primer also had the highest marker index (MI) at 33.24. Primer number 7 had the lowest PIC and MI, with values of 0.114 and 3.9, respectively (Table 3).

Cluster analysis of molecular data

The results of the comparison between the cophenetic coefficients obtained from three algorithms—UPGMA, complete linkage, and single linkage—with the Jaccard, Dice, and Simple similarity coefficients showed that the Jaccard similarity coefficient, based on the UPGMA algorithm, had the highest cophenetic correlation for grouping the blackberry genotypes (Supplementary Table 3). The value of this coefficient was 0.84, indicating a good fit; therefore, the Jaccard similarity coefficient was used for constructing the cluster diagram.

Similarity matrix

According to the results obtained from the Jaccard similarity matrix derived from the UPGMA algorithm, the highest similarity was observed between two samples collected from Sari (Sadeghi and Taherpour), which was approximately 70%. The lowest similarity was between the thorny genotype (control) Marion and cvTLBkalantari92, with a value of 0.21. Given that more distant cultivars have greater genetic differences,

performing crosses between more distant cultivars, provided they have complementary traits, can enhance hybrid productivity and the potential for heterosis. However, since only 14 markers cover a small portion of the blackberry genome, it cannot be expected that this clustering will fully differentiate the genotypes based on their origin and genetic traits (Dossett et al., 2012). At the same time, it is evident that the differentiation of genotypes based on the mentioned markers has resulted in a fairly acceptable grouping.

Principal component analysis (PCA)

The principal component analysis revealed that the first component, which is the largest, explained 24% of the total diversity. In total, the first three components accounted for 30.8% of the total variance. While this percentage may not be sufficient for drawing strong conclusions, it still encompasses meaningful patterns of genetic diversity among the blackberry genotypes studied. It is important to note that the relatively low percentage of explained variance may be attributed to the uneven distribution of markers across the genome. Therefore, future analyses might benefit from utilizing a greater variety of markers to achieve a more comprehensive understanding of the genetic diversity present in these populations (Supplementary Table 4, Figure 2).

The gathering of people in one point of the plot shows the genetic similarity of those people. In fact, the purpose of displaying two-dimensional and three-dimensional visualizations of indicator data is to reduce the amount of data in order to clarify the relationships between two or more variables and justify the changes in the original and primary data by means of a limited number of new independent variables. The name is the main coordinate.

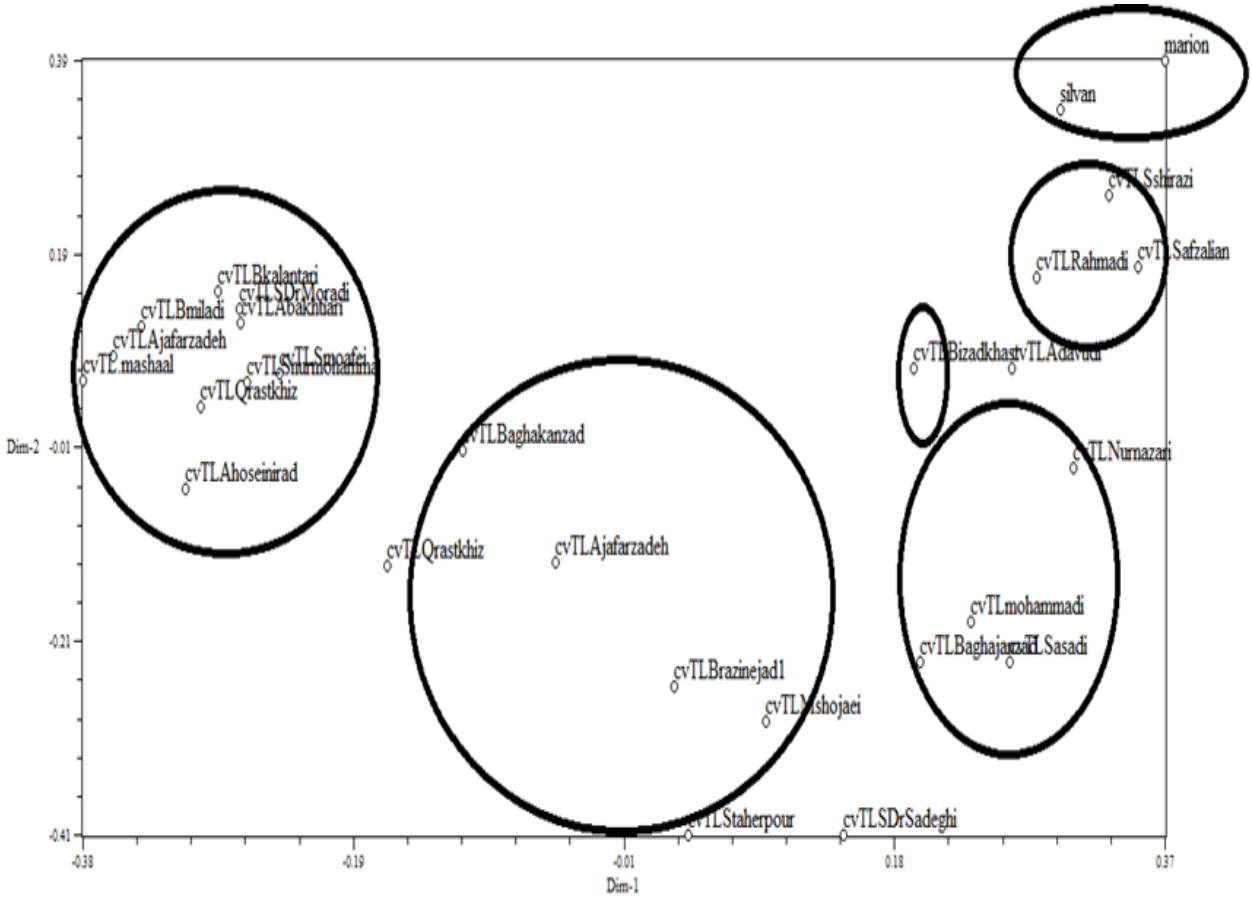

Figure 2. The two-dimensional diagram of decomposition into principal coordinates based on the Jaccard similarity matrix.

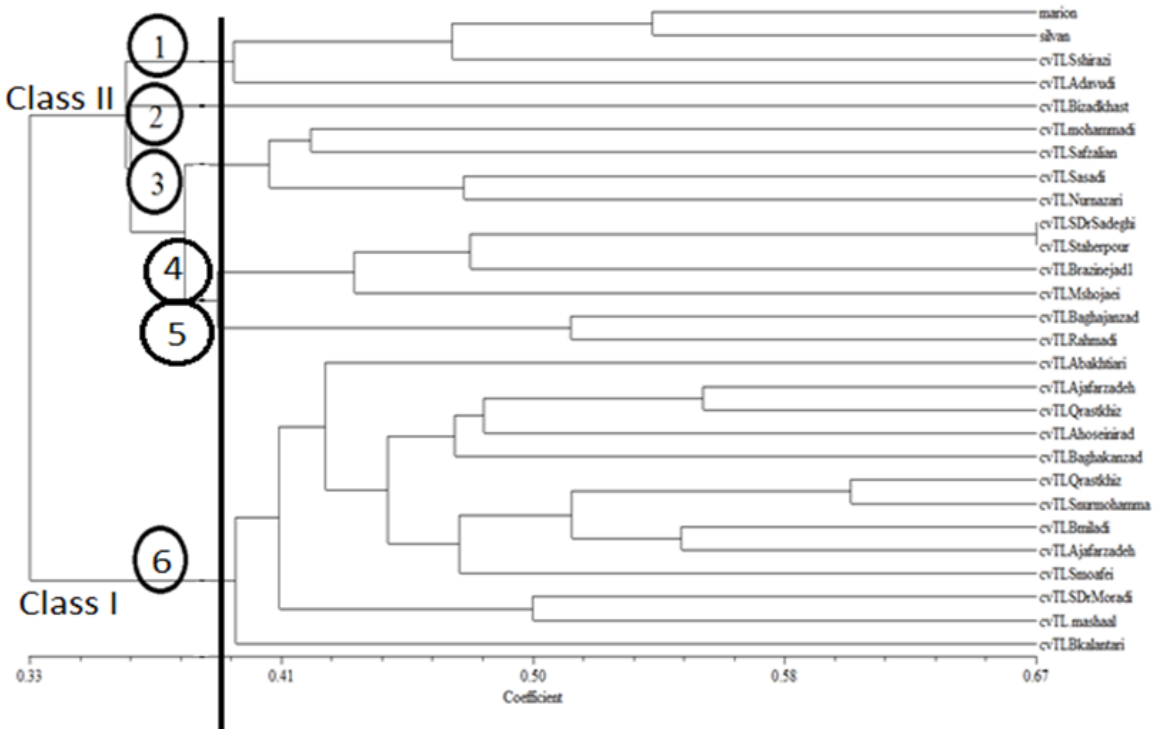

Figure 3. Dendrogram drawn based on UPGMA method and Jaccard similarity coefficient for 28 blackberry genotypes.

Cluster analysis

In the first group, the prickly cultivars Marion and Silvan along with cvTLSshirazi13 and cvTLADavudi18 were placed, and the genotype Izadkhast (cvTLBizadkhast23) alone was placed in the second group. Samples from Noor and Sari In the data related to molecular markers, the justification of the small amount of variation between the markers by the first few principal components is proof of the independence of the places and markers studied and their distribution in different regions of the genome. Therefore, if the markers are selected from different parts of the genome, the connection between them will be low, and as a result, more components are needed to justify their total changes. In examining the diversity, the best case is that the markers have a distribution of genetic DNA using data related to uniform and suitable markers in the genome and can be sampled from the entire genome. Therefore, if the markers are selected from different parts of the genome and their correlation is low, more components are needed to justify the total changes (Idrees and Irshad, 2014). The results of principal component analysis showed that the markers used in this research are scattered in different regions of the genome, and the information obtained from principal component analysis confirms that these starting compounds have a relatively good distribution at the genome level. Analysis of principal components was done in order to compare it with the results of cluster analysis. According to the two-dimensional diagram of the analysis of the main coordinates based on the Jaccard similarity matrix, the genotypes can be divided into five groups (Figure 2). formed the third group. The fourth group included Sadeghi and Taghipur cultivars (70% similarity) and Shojaei from Sari and Aghajanzadeh cultivar from Babylon. The fifth group included cultivars from Ramsar and Babylon. In the sixth group, cultivars from Amal and Babylon, Qaimshahr and Sari were also included (Figure 3).

Discussion

The results of the morphological markers indicated that the thornless genotypes were more vigorous

than the thorny cultivars, which was attributed to the differences in their semi-upright growth habit compared to the trailing growth habit of the thorny cultivars. However, this growth habit also resulted in the thornless genotypes being later-maturing than the thorny genotypes. The correlation coefficient (0.55+) suggested that as the blackberry becomes later-maturing, its acidity increases, which in turn reduces the flavor index. The results of the factor analysis indicated that all measured traits (26 traits) could be summarized into 7 factors, with the first two factors (thorn, flower, and fruit ripening) accounting for 54.59% of the variance.

Cluster analysis using the WARD method divided the genotypes into three groups. The thorny cultivars Marion and Sivan grouped with less than 20% similarity, distinctly separated from other thornless genotypes by 100% difference. The thornless genotypes were further divided into two categories. The genotypes cvTLRahmadi54, cvTLSafzalian81, cvTLSasadi26, and cvTLNurnazari53, which originated from Ramsar, Noor in western Mazandaran, and Sari, formed the second group, separated from other thornless genotypes by 40% difference. This group of genotypes differed from others in terms of flower size, fruit color, flavor index, seed number, and seed dry weight. The third group included 22 genotypes that could not be distinguished due to over 80% similarity based on the morphological markers.

The results showed that the average percentage of polymorphic bands in this experiment for each primer was 98.98%. In this context, Abdi et al. (2021) reported that the average percentage of polymorphism across various markers was 99.76%. the range of polymorphic information content (PIC) for dominant markers, such as ISSR, was from zero to 0.5, while for co-dominant markers, this range was from zero to 1. The average PIC obtained for all primers was 0.2, indicating that they contained relatively satisfactory polymorphic information. The highest PIC was associated with primer number 16, which had a value of 0.35. The level of polymorphism is one of the important indicators for comparing different markers in terms of their discriminatory power. A high value of this criterion indicates significant polymorphism at a marker locus, which plays a crucial role in differentiation and discrimination. Therefore, markers with high PIC are beneficial for distinguishing closely related genotypes (Santhosh et al., 2009). Abdi et al. (2021) reported. The Polymorphic Information Content (PIC) range for dominant markers, such as the ISSR, is 0 to 0.5, and for co-dominant markers, this range is between zero and one in blackberry. The average PIC is 0.29 and indicates adequate polymorphism. Primer 7 obtained the highest PIC (0.39). Considering that the MI marker index is a general criterion for determining the efficiency of a marker in estimating polymorphism, primer number 16, with the highest rate (33%), indicates a high level of effectiveness and better discriminatory power of this primer in determining genetic distance. This suggests that the aforementioned primer was able to better account for the genetic diversity within the population and can be recommended as a superior primer for this study.

Estimation of the cophenetic correlation coefficient is used to compare different clustering algorithms, and the method with the highest cophenetic correlation coefficient is considered the most suitable for analysis. The highest cophenetic correlation coefficient obtained in this study was 0.84, based on the Jaccard similarity coefficient and the UPGMA algorithm, indicating a good fit according to the defined range. According to the correlation coefficient, a value of r≥0.9r≥0.9 shows a very good fit, 0.9≥r≥0.80.9≥r≥0.8 indicates a good fit, 0.8≥r≥0.70.8≥r≥0.7 signifies a weak fit, and r≤0.7r≤0.7 represents a very weak fit. However, a low cophenetic correlation coefficient for molecular data does not imply inefficacy of the related algorithm; rather, it indicates the disruption caused by the presence of missing data. In fact, this coefficient reflects how much of the initial information or input matrix has been successfully transferred to the tree diagram. It essentially shows the correlation between the input and output matrices. In other words, this coefficient indicates the extent of similarity between the dendrogram and the similarity matrix. The larger the obtained rr value, the more closely aligned the dendrogram is with the similarity matrix, and they fit well with each other (Mohammadi and Prasanna, 2003).Mohammadi (2006) stated in a report analyzing molecular data from the perspective of genetic diversity that the UPGMA algorithm provides reliable results in

concordance with the phylogenetic relationships of genetic materials.

Mahjoob et al. (2015) utilized 36 genotypes of the Brassica genus and 13 ISSR markers to identify effective ISSR markers for distinguishing species within the genus. The results indicated that these markers could be used to identify inter-species and intra-species differences in phylogenetic studies.

The differences in classification resulting from cluster analysis and principal component analysis may arise from the nature of principal component analysis, in which missing data are simply replaced with the average of the corresponding variable when calculating the distance or similarity matrix. As a result, genotypes with a higher amount of missing data tend to cluster closer to the center of the relevant group. In the case of molecular data, the first two or three components account for approximately 10-20% of the variability related to the initial variability of the markers. Although these results may not be statistically suitable for PCA and graphical representation, they represent a desirable sampling of markers from the overall genome from a genetic perspective. This means that each of the markers used is derived from different parts of the genome, thereby exhibiting less correlation (Mohammadi and Prasanna, 2003). The first three components were able to account for a total of 30% of the overall variability, indicating a relatively adequate distribution of ISSR markers across the entire genome.

Cluster analysis of the blackberry genotypes divided them into six groups, with an approximate similarity of 0.38. In the first group, the thorny cultivars Marion and Silvan, along with cvTLSshirazi13 and cvTLAdavudi18, were included, suggesting that although morphological markers have less accuracy in distinguishing thorny from thornless cultivars, they are still sufficient. However, the use of molecular markers revealed the actual similarities between these two groups of blackberry cultivars, which relate to their subgenus similarity (*Rubus* sub *Rubus*). The highest similarity was found between the blackberry genotypes cvTLSDrSadeghi6 and cvTLStaherpour38, which had a similarity of 0.67, both collected from the Sari region. The lowest similarity was between the thorny genotype (control) Marion and cvTLBkalantari92, with a value of 0.21. The samples from Rashtakhiz and Dr. Moradi, which showed about 70% similarity with 10 markers in Abdiyan's work, revealed a difference of 60% in the current research using 14 markers. The similarity between a sample from Babol (cvTLBkalantari92) and a sample collected from Sari (cvTLSDrMoradi2) in Abdi et al. (2021) was 40%, which aligns with the findings of the present study. Among two samples collected from Babol, one from Amirkola (cvTLBizadkhast23) and the other from the western Bandpay area identified by Abdiyan, a 60% difference was recorded, whereas in this study, a 67% difference was observed.

Abdi et al. (2021) named the samples from Sari and Qaemshahr as Everthornless and noted the presence of genetic differences between the two samples collected from Babol due to genetic diversity in a geographic range. The results of Abdi et al. (2021) indicated that the Everthornless cultivar is approximately 60% similar to the Thornlessevergreen cultivar, while in the present study, using more markers, these two cultivars showed only 43% similarity. It seems that the good adaptability of the Everthornless cultivar to the climate of Amol has led to its highest frequency in that area and its presence in the sampling conducted. Additionally, the Thornless evergreen cultivar is also present in the same region. Amol has proven to be more suitable in terms of climate for older blackberry cultivars. Continuing the breeding efforts for thornless cultivars to overcome the dominance of the thornless trait, crosses and hybridizations between the used cultivars have been carried out to introduce the thornless Merton cultivar, which has a dominant gene for the thornless trait. Consequently, as observed in the second category, approximately 37% similarity was noted, which includes five groups. The first group contains two thorny cultivars, one being a parent and the other its offspring, which share over 50% similarity. The remaining thornless cultivars are grouped with a distance relative to these two cultivars in four additional groups. Two cultivars from Sari are the most similar. The Izadkhast cultivar from Babol (cvTLBizadkhast23) is placed alone in a separate group. Samples from Noor and Sari made up the third group, while samples from Mahmoudabad, Babol, and Sari were in the fourth group. Two samples collected from Ramsar and

Babol, which have a different fruit color from the others and are reported by farmers to be earlier and more sensitive to biotic and abiotic stresses, were placed alone in the fifth group. These two samples were called Ramezan-berry by the farmers after the worker who found them. The highest similarity, approximately 70%, was observed between the two samples collected from Sari (cvTLSDrSadeghi6 and cvTLStaherpour38).

Conclusion

The overall results of this study indicate that morphological markers perform well in distinguishing thornless cultivars from thorny ones and have some capability in classifying thornless cultivars. However, these markers were insufficient for differentiating species from one another. ISSR molecular markers successfully identified thorny and thornless genotypes at the subgenus level and were also able to separate the shimmer sample from the thornless samples that were obtained. Based on the information collected from the molecular markers, two categories of genotypes were identified. The first category includes the initial generation of thornless and belongs to the species *R. laciniatus*. The second category pertains to subsequent crosses aimed at producing the Merton cultivar. In this category, different groups were observed, either relating to various thornless Merton cultivars or reflecting the diversity that has arisen in the Mazandaran climate. Overall, these results indicate that the existing diversity in these cultivars is a result of various human and climatic factors, and they can be utilized in breeding program planning.

Supplementary Materials

The supplementary material for this article can be found online at: https://www.jpmb-gabit.ir/article_720852.html.

Supplementary Figure 1. ISSR banding pattern resulting from the multiplication of thornless blackberry genotypes using primers.

Supplementary Table 1. Correlation coefficient for studied traits.

Supplementary Table 2. Decomposition into factors.

Supplementary Table 3. Cophenetic coefficient resulting from algorithms with similarity coefficients.

Supplementary Table 4. Decomposition into PCA principal components.

Author Contributions

Conceptualization, M.H. and H.M.; methodology, K.Sh.; software K.Sh.; validation, M.H., H.M. and K.Sh.; formal analysis, K.Sh.; investigation, K.Sh.R; resources, H.M.; data curation, K.Sh.R; writing—original draft preparation, K.Sh.R; writing—review and editing, M.H. and H.M.; visualization, K.Sh.; supervision, M.H. and H.M.; project administration, K.Sh.; funding acquisition, H.M., H.M. and K.Sh.R (thesis funding).

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Conflict of Interest Statement

The author declares no conflict of interest.

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مقاله پژوهشی

بررسی تنوع ژنتیکی برخی از ژنوتیپ های تمشک سیاه بدون خار با استفاده از نشانگرهای مولکولی ISSR

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چکیده: تنوع ژنتیکی در تمشک سیاه برای بهبود کیفیت و عملکرد بسیار مهم است. این مطالعه ژنوتیپ های تمشک سیاه بدون خار را با استفاده ۲۶ صفت مورفولوژیکی و ۱۹ نشانگر تکراری بین توالی ساده (ISSR)برای ارزیابی تنوع و روابط ژنتیکی ارزیابی می‌کند. در مجموع ۲۸ ژنوتیپ تمشک سیاه شامل هر دو نوع خاردار و بدون خار مورد تجزیه و تحلیل قرار گرفت. ۱۴ آغازگر ISSR از بین ۱۹ پرایمر بر اساس ظرفیت آنها برای تولید باندهای چندشکلی انتخاب شدند. یافته‌ها کارایی نشانگرهای ISSR را در تشخیص ژنوتیپ‌های تمشک سیاه خاردار و بدون خار در سطح زیرجنس نشان داده و به طور موثر نمونه‌های بدون خار مشتق شده از ژنوتیپ‌های شیمری را متمایز می‌کنند. در مجموع ۴۰۶ باند تولید شد که ۴۰۲ باند چند شکلی بودند. میانگین درصد باندهای چندشکلی برای هر آغازگر در این آزمایش ۹۸.۹۸ درصد بود و بیشترین مقدار اطلاعات چندشکلی (PIC) مربوط به پرایمر شماره ۱۶ بود که دارای مقدار ۰/۳۵ بود. آنالیزها دو مورد را نشان داد. یافته‌ها نشانگر کارایی نشانگرهای ISSR در تشخیص ژنوتیپ‌های تمشک سیاه خاردار و بدون خار در دو گروه اصلی می‌باشد: اولی مرتبط با نسل‌های اولیه بدون خار *R. laciniatus*، و دومی شامل تلاقی‌های مربوط به رقم مرتون است. به طور کلی، تنوع ژنتیکی شناسایی شده در بین ارقام تمشک سیاه، کاربردهای ارزشمندی را در برنامه‌های اصلاحی و اصلاحی نشان می‌دهد.

کلمات کلیدی: *Rubus laciniatus*، مرتون بدون خار، تنوع ژنتیکی، نشانگر مولکولی.

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Original paper

Identification and expression analysis of *Oleosin* gene family in walnut (*Juglans regia* L.)

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Abstract: Oleosins as structural proteins play a crucial role in stabilization the oil-bodies, their size, number, and content. Here, exon/intron structure, conserved motifs/domains, and expression patterns of *Oleosin* gene family were identified in diploid crop *Juglans regia* L. This study found seven non-repetitive *JrOleosin* genes through a genome-wide analysis, displaying remarkable structural and physicochemical differences. Our phylogenetic analysis of the 46 oleosin genes revealed that how these genes are related, highlighting that they share conservations while also having unique divergences across different species. The *JrOleosin* intronless genes (IGs) might be examples of how evolution adapts to enhance the accuracy and effectiveness of gene expression. Transcriptome analysis showed that the *JrOleosin* genes were spatially and temporarily regulated with the highest transcript found in seeds and fruits. This suggests they are vital for storing lipids during these developmental stages. These results help us to better understand the features of the *JrOleosins* gene family, especially their structure and expression. Future research should concentrate on revealing how *oleosin* expression is controlled and how these proteins interact with others linked to lipid synthesis during seed development.

Keywords: Oil-body, oleosin, meta-analysis, walnut, genome-wide.

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Introduction

Walnut (*Juglans regia* L.), native to central Asia, is an old fruit tree prized economically for its output of edible seeds, vegetable oil, and industrial lumber. (Hao et al., 2024). Walnut oil seeds are rich in essential unsaturated fatty acids, including linoleic and linolenic acids, and their nutritional importance has been highlighted in the context of dried fruit (Goodarzi et al., 2023). In the majority of cultivars, walnut kernels comprise approximately 60% oil. After China and the United States, Iran ranks third in production and second in cultivated area for these trees. Consequently, Iran accounts for 7.1% of global walnut production (FAO, 2022). Studies indicate that wild populations of persian walnuts are present in the Hyrcanian woodlands of northern Iran (Shamlu et al., 2018). Given the significant genetic diversity of Persian walnuts in Iran, the core of walnut origin, breeding operations in this nation concentrate on harnessing this genetic variation. For a long time, researchers have focused on fruit characteristics like early fruit maturity, size, color, and percentage of walnut kernel oil in breeding programs, with an eye toward improving the product's quality and quantity. However, there is a lack of data on the molecular level regarding the metabolism of oil seeds and the storage processes of fatty acids.

Lipids serve as carbon stores and energy providers for plants. Seed lipids are primarily triacylglycerols, which are the primary reserves found in seeds and oil bodies (Yang and Benning, 2018). Abundant in both prokaryotic and eukaryotic cells, oil bodies—also called oleosomes—are specialized organelles that store energy in the form of neutral lipids. Oil bodies' architecture consists in an oil core and a hydrophobic exterior surrounded by a phospholipid monolayer membrane and membrane proteins, forming spherical vesicles with an approximate 0.5–2 micrometer (μm) diameter (Khor et al., 2013; Jackson, 2019). These entities are synthesized in the endoplasmic reticulum during the Initial phases of seed development and then transported to the cytoplasm via the germination process (Huang, 1996). Besides seeds, they are found in several sections of higher plants, including pollen, tapetum, leaves, fruits, and roots (Yuan et al., 2021).

Plant naturally occurring protective mechanism against lipid oxidation and severe stress circumstances is their oily bodies, which show capacity to maintain lipid stability. Along with the stability of membrane proteins and various metabolic activities, oil body shape and dimensions change constantly during seed development (Huang and Huang, 2017; Pyc et al., 2021). The size of oil bodies can fluctuate over the phase of seed development. Because of their surfactant qualities, these chemicals are environmentally beneficial and renewable. They are employed in the food, pharmaceutical, and cosmetic sectors. Recent studies demonstrate that the overexpression of some genes associated with oleosin can modulate the dimensions of oil bodies, suggesting that seed size and weight significantly influence seed oil accumulation (Chen et al., 2019). Reports indicate a negative link between the size of oily substances and the quantity of oil (Siloto et al., 2006).

Oleosin, caleosin, and streosin are the most well-known plant oil body proteins, and they all play key roles in the size, number, and stability of oil bodies, as well as lipid accumulation (Nogales-Bueno et al., 2021; Kim and Hyun, 2024). Oleosin comprises 80-90% of structural proteins among these proteins (Siloto et al., 2006; Dong et al., 2024). Relatively tiny with a molecular weight of 15–26 kilodaltons, this protein has three distinct regions and alkaline features (Tzen et al., 1997). The middle part is hydrophobic and has 72 residues. The amino terminal parts are hydrophilic and have 50 to 70 residues. The dipolar carboxyl has a length that can vary. The highly conserved non-polar middle region with beta structures in two reverse directions penetrates the lipid membrane and forms a hairpin structure to stabilize oil bodies (Roux et al., 2004; Wu et al., 2010). Two arms of this hairpin shape are protected by a proline ring containing three proline and one serine residue. Despite their dipolar alpha-helical configurations, the carboxyl and amino portions are less conserved than the hairpin area. They can bind to metabolic enzymes and regulatory proteins on the phospholipid surface and in the cytoplasm (Huang and Huang, 2017). The negative charge of these places causes steric hindrance and electrostatic repulsion, preventing the integration of stored lipids, particularly during seed drying, and thereby facilitating the movement of oil bodies

during germination (Leprince et al., 1997; Baud and Lepiniec, 2010).

Research on the oleosin gene sequence began with maize and has now expanded to other plants including sunflower, rapeseed, sesame, rice, and soybean (Vance and Huang, 1987; Keddie et al., 1992; Chen et al., 1997; Sarmiento et al., 1997; Wu et al., 1998; Alexander et al., 2002). The majority of the 17 oleosin genes found in the *Arabidopsis thaliana* genome are expressed in the seeds, according to previous studies (Siloto et al., 2006). Oleosin mutant seeds are more sensitive to freezing, implying that oleosins help plants survive in cold stress condition. (Shimada et al., 2008). Fatty acid chains undergo a conformational transition from their disordered to their regular form at low temperatures, leading to the dispersion of massive, unstable oil bodies in the cytoplasm. In this case, the fluidity, physiology, and thickness of the lipid membrane are significantly reduced. Because it interferes with the plant's physiological processes, this can severely limit its growth and development potential (Parthibane et al., 2012).

The diploid walnut seed (2n=2x=32) contains around 108 genes that are involved in lipid biosynthesis. Among these genes are 33 genes that are engaged in triacylglycerol biosynthesis, seven genes that are involved in oil bodies, and eight genes that are transcription factors (Yan et al., 2021). The function of oleosins in walnut oil seeds remains ambiguous. Thus, the present study utilized bioinformatics approaches to clarify the *JrOleosin* gene structure, protein domains, chromosomal distribution, expression patterns, and evolutionary relationships of oleosin with other plant species.

Materials and Methods

Identification of the JrOleosin gene family members

The protein sequences of the *Oleosin* gene family members from *Arabidopsis thaliana* were initially identified and downloaded from the TAIR (http://www.arabidopsis.org/) database. Genomic sequences of *A. thaliana* (TAIR10.1), *Juglans regia* (Walnut 2.0), *Elaeis guineensis* (African oil palm) and *Olea europaea* (Olive) were downloaded from public databases NCBI (https://www.ncbi.nlm.nih.gov/) and Phytozome (phytozome.jgi.doe.gov). To identify oleosin homologs, the oleosin domain profile (PF01277) was used for HMMER (v3.3, http://hmmer.org/) searches (E-value < 1E-5). Finally, the coding sequence (CDS), protein, and genomic sequences of *JrOleosin* family were identified. The presence of the conserved oleosin domain in deduced peptides was confirmed using InterProScan(https://www.ebi.ac.uk/interpro/search/sequence-search) (Jones et al., 2014), Pfam (http://pfam.xfam.org/) (Finn et al., 2016) and SMART (http://smart.embl-heidelberg.de/) (Letunic et al., 2015) protein database.

Physiochemical analysis of JrOleosin proteins

The subcellular localization of JrOleosin proteins was predicted using the WoLF PSORT algorithm (https://wolfpsort.hgc.jp/) (Horton et al., 2007). The sequences of JrOleosin were analyzed with ExPASy ProtParam (http://www.expasy.org/tools/protparam.html) to obtain the number of amino acids, theoretical isoelectric point (pI), molecular weight, GRAVY, instability index, and aliphatic index.

Exon/intron structures and motif organization

The *JrOleosin* gene'' exon/intron structures were visualized via Gene Structure Display Server 2.0 (http://gsds.cbi.pku.edu.cn) (Hu et al., 2015). The following parameters were used to find the conserved protein motifs of all JrOleosin using the MEME program (http://meme-suite.org/tools/meme/): Ten is the maximum number of motifs, and the lengths of the motifs range from six to fifty amino acid residues (Bailey et al., 2009).

Phylogenetic tree construction

Oleosin protein sequences in walnut, Arabidobsis, olive and oil palm were obtained from Phytozome database (Goodstein et al., 2012). Multiple sequence alignment of oleosin proteins in *J. regia*, Arabidopsis, olive and oil palm were performed using MUSCLE and finally the maximum likelihood (ML) phylogenetic tree were constructed using MEGA 11 (Tamura et al., 2013).

Expression profiling of JrOleosin genes based on RNA-seq data

To examine the function and expression of the *JrOLE* gene family, 210 RNA-seq samples from 13 projects were gathered from publicly accessible RNA-seq data associated with *J. regia*.

Transcriptome datasets were selected from various treatments or genetic backgrounds, their accession number were listed as follow: PRJNA232394, PRJNA235890, PRJNA237044, PRJNA609369, PRJNA622910, PRJNA642991, PRJNA643637, PRJNA673559, PRJNA688391, PRJNA734671, PRJNA776681, PRJNA794344, PRJNA806342, PRJNA862472. TPM (transcripts per million) had been utilized to measure transcript expression levels.

Results

JrOleosin gene family identification

Seven *Oleosin* non-repetitive genes were detected in *J. regia* genome based on Oleosin specific domain with the Pfam number of PF01277 (Table 1). Twelve, nine, and eighteen *Oleosin* genes were found in the genomes of Arabidopsis, African oil palm, and olive, respectively, by a hidden Markov model (HMM) tools based on the Oleosin domain (Pfam accession number PF01277). The comparative genomics analysis revealed the existence of varied numbers of *Oleosin* genes among various species, therefore offering understanding of the evolutionary divergences and functional conservation of these genes in respect to lipid storage and metabolism. In a previous report, 17 genes belonging to the *Oleosin* family were identified in the Arabidopsis (Siloto et al., 2006; Yuan et al., 2021). In the current investigation, one new *AtOleosin* gene within arabidopsis genome was identified (18 loci, 28 isoforms), which was made possible due to the well-annotated genome of *A. thaliana* (Araport11) and the high-quality genome sequencing data available. The *Oleosin* genes were named based on their positions on phylogenetic tree, and by using genus and species abbreviations for clarity. This systematic nomencalture may support upcoming research into gene functions across plant species (Table 1, Figure 1).

Table 1. Physiochemical properties of the *Oleosin* genes in *Juglans regia.*

Gene name	Gene ID	mRNA RefSeqs ID	Homolog to Arabidopsis genes		PL	MW (kDa)	pI	GRAVY	II	AI
JrOleosin01	LOC108998787	XM_018975486.2	AT4G25140.1		140	14.838	9.65	0.316	46.25	103.86
JrOleosin02	LOC108983309	XM_019000720.2	AT4G25140.1	*AtOleosin02*	139	14.691	10.14	0.308	36.55	105.97
JrOleosin03	LOC109009462	XM_018989925.2	AT4G25140.1		147	15.606	9.56	0.42	29.42	108.84
JrOleosin04	LOC109010520	XM_018991380.2	AT5G51210.1	*AtOleosin01*	156	16.776	9.03	0.435	39.78	98.27
JrOleosin05	LOC109007243	XM_018986840.2	AT3G01570.1		154	16.278	10.1	0.230	52.53	112.14
JrOleosin06	LOC108982740	XM_018954195.2	AT3G01570.1	*AtOleosin05*	155	15.896	9.65	0.290	31.56	95.23
JrOleosin07	LOC109003841	XM_018982154.2	AT3G01570.1		159	16.620	9.91	0.181	34.65	95.16

A.C.: Accession number; PL: Protein length (aa); MW: Molecular weight; pI: Isoelectric point; II: Instability index; AI: Aliphatic index.

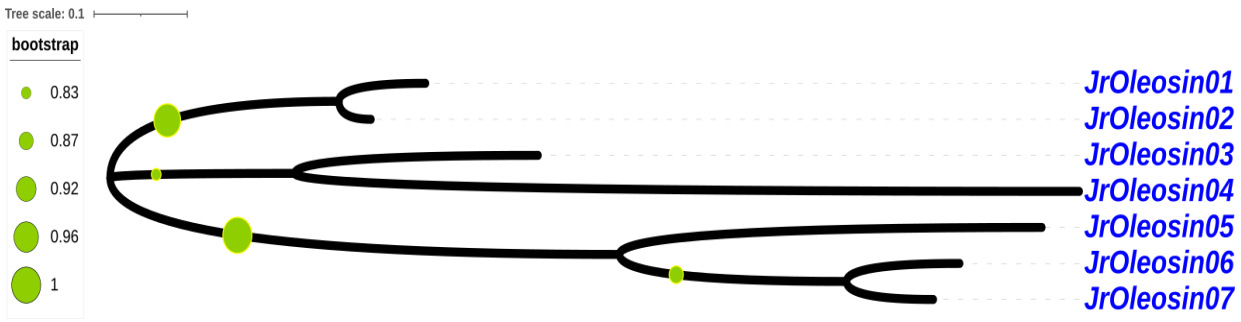

Figure 1. Phylogenetic analysis of Oleosin in *J. regia*.

Physiochemical properties of JrOleosin

Our analysis on the *JrOleosin* gene family showed significant differences in the biochemical properties of gene family members, which are listed in Table 1, and include the following critical parameters: molecular weight, theoretical isoelectric point (pI), aliphatic index, instability index, and grand average of hydropathicity (GRAVY). Each gene product was assessed for critical parameters, including amino acid (aa) length, molecular weight, theoretical isoelectric point (pI), instability index, aliphatic index, and grand average of hydropathicity (GRAVY). The average length of the JrOleosin proteins ranged from 139aa (JrOleosin02) to 159aa (JrOleosin07), with an average of roughly 151aa. The pI values also ranged from 9.03 (JrOleosin04) to 10.14 (JrOleosin02). rOleosin07 having the highest molecular weight of 16.62 kDa and the lowest JrOleosin02 having the lowest at 14.691 kDa indicate a varied functional capacity within the gene family, which may influence their molecular function and biological processes.

The increased pI values are likely connected with JrOleosins' specific features and functions, which impact their capacity to interact with other biomolecules and their overall performance. The instability indices ran from 29.42 (JrOleosin03) to 52.53 (JunrOleosin05). The higher instability index of the latter implies it could be less stable and more prone to degradation, which would affect its physiological functions under stress conditions. From 95.16 (JrOleosin07) to 112.14 (JrOleosin05), the aliphatic index—which shows protein stability at high temperatures— were ranged. Higher values show thermal stability and capacity for operation at several temperatures. Essential for their interaction with water-soluble components inside plant cells, the GRAVY values for these proteins ranged from 0.181 (JrOleosin07) to 0.435 (JrOleosin04), indicating a generally hydrophilic character for them (Table 1).

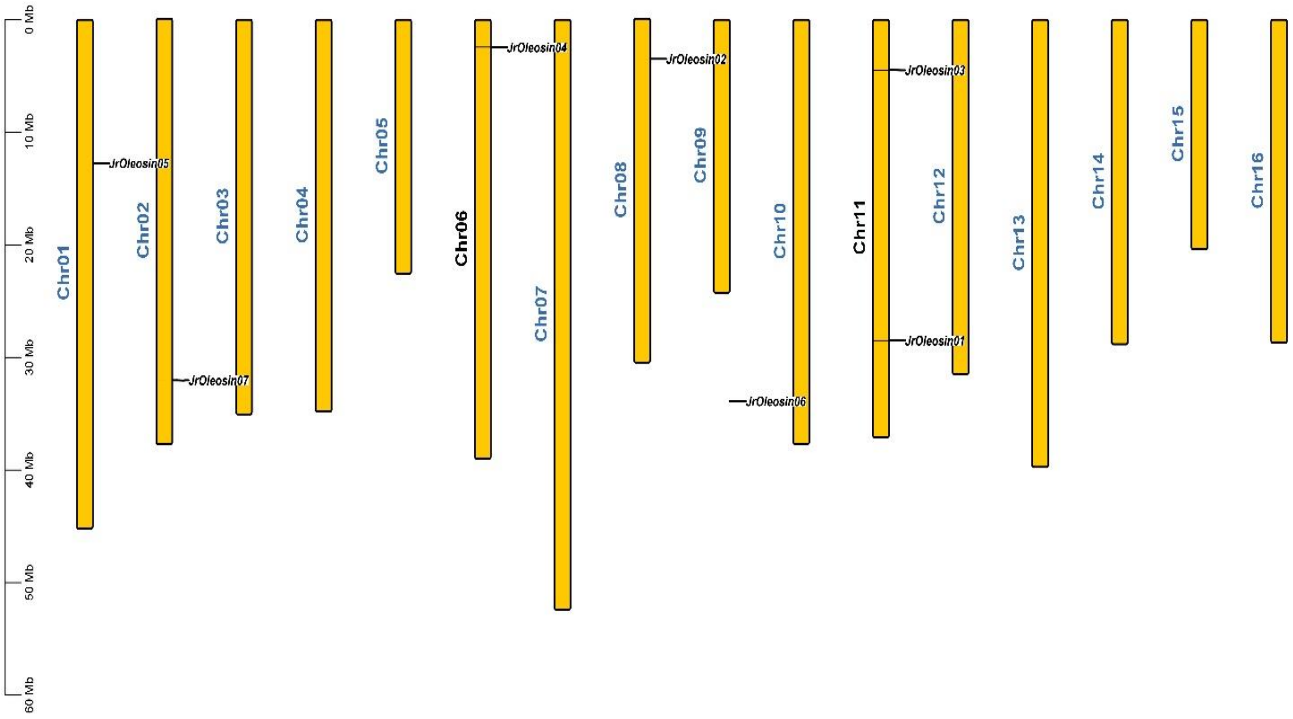

Figure 2. The chromosomal distribution of the *Oleosin* gene family in *Juglans regia*. The left side of each chromosome displays the chromosome numbers and their respective approximate sizes.

Phylogenetic analysis of JrOleosin

Figure 1 depicts the phylogenetic tree that elucidates the evolutionary relationships among the Oleosin gene family members in *J. regia*. The analysis employed bootstrap values to evaluate the confidence of the branching patterns, with circles indicating the level of support for each node. The bootstrap values span from 0.83 to 1.0, showing a significant level of confidence in the evolutionary relationships among the *JrOleosin* genes. The larger circles (bootstrap values of 0.96 and 1.0) indicate strong support for the clades established by *JrOleosin* genes, hence enhancing the credibility of the inferred evolutionary relationships.

Figure 3. Conserved structural domains (a) distribution of identified motifs (b), and motif logo sequences in the JrOleosin family. In the above forms, motifs and domains were shown with different color boxes. Scale indicates amino acid length.

The JrOleosin genes are categorized into distinct groups, with JrOleosin01, JrOleosin02, and JrOleosin03 forming a closely related cluster. JrOleosin04, exhibiting unique characteristics, is also included in this cluster, suggesting potential shared functional roles or similar evolutionary pressures. In contrast, JrOleosin05, JrOleosin06, and JrOleosin07 appear more distantly related, indicating possible divergence in function or adaptation. The phylogenetic relationships illustrated in the tree offer insights into the evolutionary background of the JrOleosin gene family. The close clustering of certain JrOleosins may indicate conserved functions associated with lipid metabolism and stress responses, which are essential for the survival of *J. regia* in diverse environmental conditions.

Chromosomal distribution of JrOleosin gene

The results of the distribution pattern of *JrOleosin* genes on 16 chromosomes showed that seven were located on six chromosomes (Chr 01, 02, 06, 08, 10 and 11). The highest numbers of genes on Chr11 were *JrOleosin01* and *JrOleosin03* genes. Other chromosomes (Ch 01, 02 , 06, 08 and 10) had one *JrOleosin* gene. Therefore, Chr01 had *JrOleosin05* gene, Chr02 had *JrOleosin07* gene, Chr06 had *JrOleosin04* gene, Chr08 had *JrOleosin* gene, and Chr10 had *JrOleosin06* gene. In addition, most of the *JrOleosin* genes are distributed near the end of each chromosome (Figure 2).

JrOleosin domain and motif organization

Seven different JrOleosin proteins were identified through the investigation of the Oleosin superfamily, and each one showed a notable degree of alignment with the Oleosin superfamily domain (Figure 3). For every protein, the data is summarized in Table 1. While the others genes had bigger domain sizes of 113 to 114 amino acids, JrOleosin01 had the shortest domain size of 87 amino acids, hence the lengths of the protein domains varied. Different activities or genetic modifications occurring in the JrOleosin proteins could be the reason of this variation in domain size. Several Oleosin superfamily members have constant domain sizes, suggesting a conserved structural characteristic that may be essential for lipid storage and oil body stabilization.

Motifs 1, 2, 3, and 4 were found in all seven JrOleosin proteins out of a total of ten, while several of them shared some motifs. For example, JrOleosin 06 and 07 had motifs 5 and 7, JrOleosin 03 and 05 had motifs 6, JrOleosin 07 and 08 had motifs 8, JrOleosin 01 and 02 had motifs 9, and JrOleosin 04 and 07 had motifs 10. Also, the highest number of motifs (eight numbers) belonged to JrOlesin07 protein, and the width of 6 and 50 residues for each conserved motif (Figure 3).

JrOleosin exon/intron structure

The study indicates that the *Oleosin* gene family possesses comparable coding sequences (CDS) and untranslated region (UTRs) patterns, implying structural conservation, functional similarity, and regulatory functions, while also featuring a distinct UTR region (Figure 4).

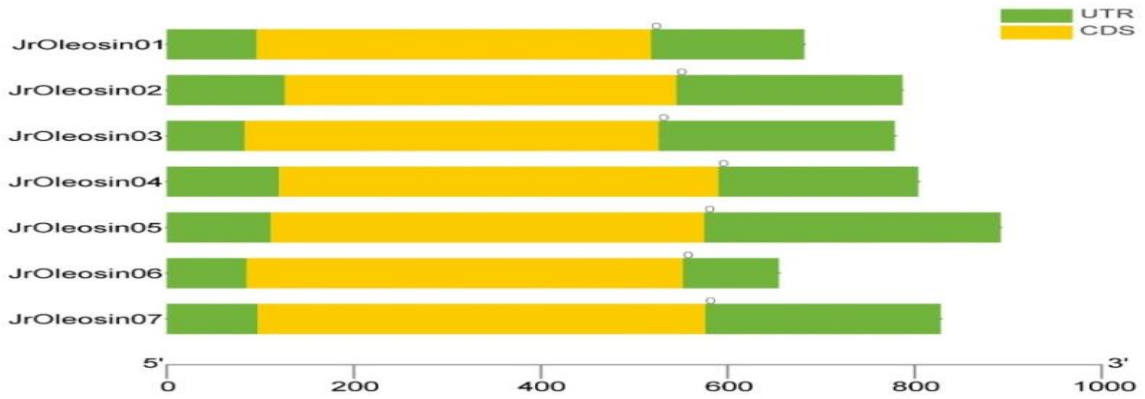

Figure 4. Analysis of *JrOleosin* gene family structures. The green and yellow boxes denote the *JrOleosin* UTR regions and CDS, respectively.

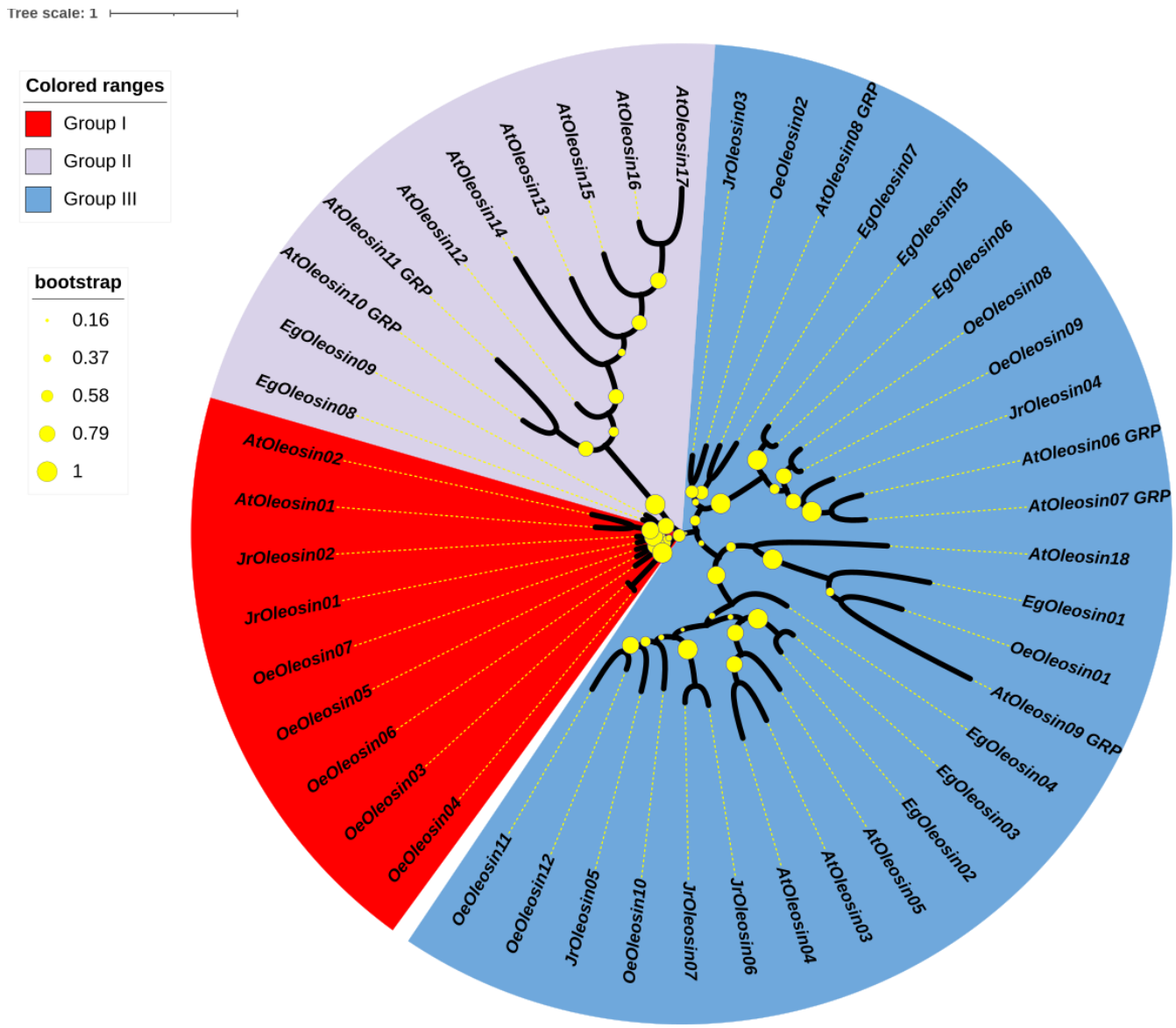

Figure 5. Phylogenetic tree of the Oleosin family based on an alignment of the proteins found in *Arabidopsis thaliana*, *Olea europaea*, *Elaeis guineensis* and *Juglans regia*. Different Oleosin groups are presented by different-colored.

Furthermore, all these genes demonstrate tissue-specific expression in seed tissue, underscoring their specialized roles in lipid metabolism during seed development. The analysis of the JrOleosin gene architecture also indicates the lack of introns in the examined genes.

Phylogenetic relationships of Oleosin gene family

The phylogenetic analysis of the *Oleosin* subfamily highlights the evolutionary dynamics within the oil body genes. The high bootstrapping score for the observed clades suggests that these relationships are well-established. Understanding evolutionary relationships, especially identification *AtOLE* ortholog in *J. regia*, will enhance our understanding of *JrOleosins'* roles in plant biology and their potential applications in stress tolerance. To analyze the evolutionary relationships of *JrOleosin* gene family members, 18 *A. thaliana*, 12 *O. europaea*, 9 *E. guineensis* oleosin proteins and 7 *J. regia* oleosin proteins were used to construct a phylogenetic tree. So JrOleosin proteins were divided into three main groups. The number of JrOleosins in groups I and III were 2 and 5, respectively. Moreover, the group II contained no JrOleosins mamber. Group III

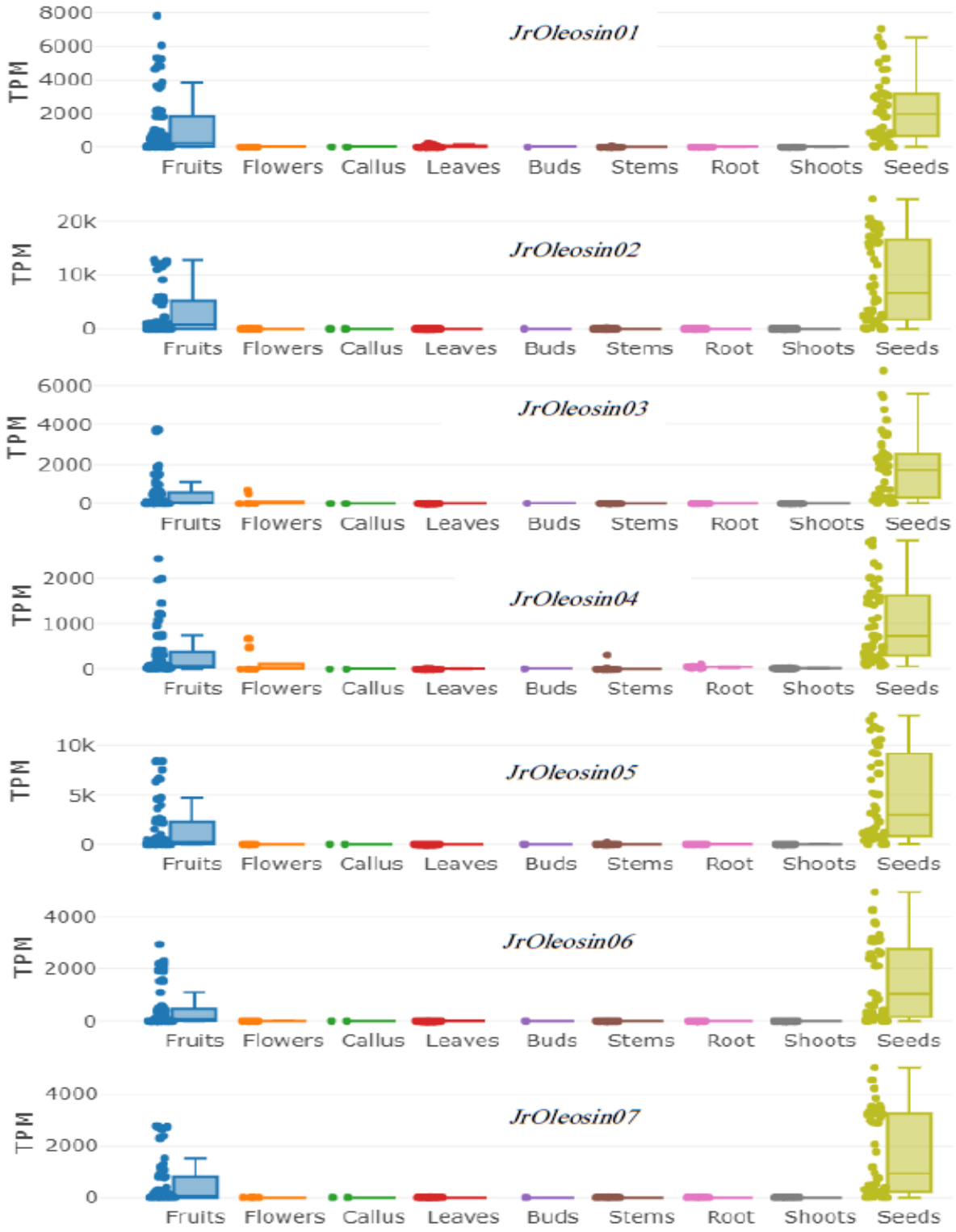

Figure 6. Meta-analysis of the *Oleosin* 's expression pattern in nine distinct tissues of *Juglans regia*. The box-plot illustrates the relative expression levels of the *JrOleosin* genes across tissues, including roots, stems, leaves, flowers, seeds, and other organs in TPM (transcripts per million) format. This analysis highlights tissue-specific expression patterns of *JrOleosin* genes in the sues of seeds and fruits.

encompass the largest number of JrOleosin genes family mambers (Figure 5). In addition, by comparing the distribution of oleosin proteins in different groups and species, it can be found that the distribution of oleosin proteins in various groups was similar, indicating that their functions may be similar and strongly conserved during evolution.

Expression profiles of JrOleosin

As presented in Figure6, the transcript levels of *JrOleosin* gene family mamber in different walnut organs including fruit, flower, callus, leaf, bud, stem, root, shoot and seed were different, and the highest level of gene expression found in seed and fruit organs (Yang et al., 2024). The lowest and highest levels of gene expression (TPM) were observed in *JrOleosin01* and *JrOleosin02* genes, respectively. Transcriptome analyses indicate that the *JrOleosin* genes were expressed spatially and temporally during seed and fruit development.

Discussion

Oleosins are well-known to play a role in lipid metabolism and oil body stabilization, both of which are necessary for seed germination and development (Shao et al., 2019). Oleosins are the most abundant protein constituents that play essential roles in the formation and stabilization of lipid droplets in seeds of oil crops (Zhi et al., 2017; Kim and Hyun, 2024). According to Huang and Huang (2017)Huang and Huang (2017)Huang and Huang (2017)Huang and Huang (2017)Huang and Huang (2017), the oleosin gene is primarily found in higher plants and green algae. Oleosins in plants can be classified into six types: primary, general, tapetum, mesocarp, and seed, with low and high molecular weights (Huang and Huang, 2015). Nine forms of this protein were found in tapetum branches, three in general branches, two in seeds with low molecular weight, and three in seeds with high molecular weight, according to research on *Arabidopsis* (Chen et al., 2019). The identified characteristics of JrOleosins convey significant insights into their potential biological functions. Due to their differing molecular weights and stability indices, these proteins may serve unique functions in lipid metabolism, seed development, or stress response pathways in *J. regia*. The preference for higher aliphatic indices and GRAVY values may indicate a specific function in preserving cellular integrity and regulating osmotic balance under environmental stresses (Hashemipetroudi et al., 2023; Mohammadi et al., 2023). Members of the JrOleosin family have comparable physicochemical characteristics to those reported in prior research, including low molecular weight, alkaline isoelectric point, and a positive GRAVY value (Zou et al., 2022; Zou et al., 2024).

The diverse biochemical properties of the *Oleosin* gene family underline their potential functional diversification within *J. regia*. The findings show that, while domain sizes vary, a significant proportion of JrOleosin proteins have similar lengths, emphasizing their possible functional commonalities within the Oleosin subfamily. Further research into these protein activities may reveal their significance in plant lipid metabolism and storage. Future functional studies, along with expression analyses under diverse environmental conditions, are crucial for clarifying their specific roles in plant stress genomics. Grasping the distinct characteristics of each JrOleosin can improve our understanding of their function in the overall adaptability of *J. regia*. The distribution of UTRs and CDS among the *JrOleosin* genes reveals heterogeneity in gene structure, potentially indicating variances in regulatory mechanisms and functional roles. Genes with 5′ and 3′-UTR may have regulatory functions that influence translation efficiency, mRNA localization, and stability (Heidari et al., 2023). Additional functional investigations are necessary to clarify the specific roles of these UTRs and coding areas in the overall functionality of JrOleosin proteins in plant biology. The examining of the *JrOleosin* gene architecture reveals the absence of introns in the analyzed genes. A fascinating group of genes that are also present in eukaryotes are intronless genes (IGs), which are a common feature of prokaryotes (Chen et al., 2023). The absence of introns can be ascribed to multiple factors: gene structure, evolutionary adaptation, functional consequences, and genomic context (Aviña-Padilla et al., 2021). The lack of intron of *JrOleosin* gene family may suggest evolutionary adaptations that promote gene expression efficiency. Some reports believe that amount of introns provide advantages to plant genomes, such as regulating gene expression and enhancing protein diversity (Xiao et al., 2022). New funding on

the *Poaceae* genome comparison also revealed that IGs were expressed at low levels (Chen et al., 2023). However, IGs that do not undergo splicing have a more degree of transcriptional fidelity, which increases their imapct in regulatory roles (Aviña-Padilla et al., 2021). IGs, likely due to selective forces, may exhibit lower post-transcriptional gene expression variability and prefer simpler gene architectures for specific functions (Aviña-Padilla et al., 2022).

The results show that the oleosin proteins in walnut were divided into three groups with a total of 46 members, which were similar to those in olive and oil palm and much higher than in *Arabidopsis*. Phylogenetic analysis indicates that JrOleosin01, JrOleosin02, JrOleosin03, and JrOleosin04 are oil body proteins that control oil body dynamics; JrOleosin05, JrOleosin06, and JrOleosin07 are proteins that delay germination and affect the seeds' ability to withstand freezing. Therefore, the expansion and diversification of the *oleosin* gene family may be closely related to the evolutionary process of plants from lower to higher (Shao et al., 2019).

In this study, all seven *JrOleosin* genes show a strong seed-specific expression pattern. Fruits show the second-highest expression, though considerably lower than seeds. Other tissues have negligible expression. The up-regulation of JrOleosin genes during seed maturation was reported in *Camelina sativa* (Abdullah et al., 2016), soybean (Yang et al., 2019), *Paeonia section* (Li et al., 2015), *Perilla frutescens* (Zhang et al., 2021), and *Prunus pedunculata* (Bao et al., 2021) showing specific expression patterns in oil-rich tissue. In *Cyperus esculentus*, the expression levels of different *oleosin* genes had low or high expression across different developing stages (Dong et al., 2024). The expression profile of *CpOLE* genes was detected in at least twelve of the studied tissues, though gene abundances are highly diverse. Total transcripts of the whole gene family were most abundant in shoot tissue (Zou et al., 2024). Analysis of *Arabidopsis* oleosin homolog proteins indicated that *OLE1* and *OLE4* negatively regulated oil body size, whereas *OLE2* positively influenced oil body size (Miquel et al., 2014). These comparisons highlight the evolutionary conservation of oleosin gene functions while also pointing to species-specific adaptations.

Conclusion

The results elucidate the structural properties of the *JrOleosin* genes, establishing a foundation for subsequent inquiries into their biological functions and regulatory mechanisms. The lack of introns in the *JrOleosin* genes underscores their potential for efficient expression and functional specialization, which may be essential for their involvement in plant lipid metabolism. The evolutionary and functional effects of this intronless gene structure could be studied. These JrOleosin proteins' expression patterns under different environmental circumstances and interactions with other lipid-associated proteins could be studied in the future. These dynamics may reveal oil body formation and stability mechanisms, advancing agricultural biotechnology and crop development.

Supplementary Materials

There is no supplementary material for this article.

Author Contributions

Conceptualization, S.H.H.; bioinformatic analysis, S.H.H.; investigation, F.A.; writing—original draft preparation, S.H.H. and F.A.; writing—review and editing, S.H.H.; All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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شناسایی و آنالیز بیان خانواده ژنی اولئوسین در گردو (*Juglans regia* L.)

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چکیده: اولئوسین‌ها به عنوان پروتئین‌های ساختاری نقش مهمی در تثبیت اجسام روغنی، اندازه، تعداد و محتوای آنها دارند. در مطالعه حاضر، شناسایی ساختار اگزون/اینترون، موتیف‌ها/دامنه‌های حفاظت‌شده و الگوهای بیان خانواده ژن اولئوسین در گیاه دیپلوئید *Juglans regia* L. مورد توجه قرار گرفت. در این تحقیق هفت ژن غیر تکراری *JrOleosin* در تجزیه و تحلیل گسترده ژنومی شناسایی گردید که تفاوت‌های ساختاری و فیزیکوشیمیایی قابل توجهی را نشان دادند. تجزیه و تحلیل فیلوژنتیکی ۴۶ ژن اولئوسین نشان داد که این ژن‌ها از قرابت ژنتیکی برخوردار بوده، در عین دارا بودن توالی حفاظت‌شده مشترک، از واگرایی‌های منحصربه‌فردی در گونه‌های مختلف برخوردارند. ژن‌های بدون اینترون (IGs) *JrOleosin* ممکن است نمونه‌هایی از چگونگی سازگاری تکامل برای افزایش دقت و اثربخشی بیان ژن باشند. تجزیه و تحلیل رونوشت نشان داد که ژن‌های *JrOleosin* به صورت بافت/زمان- اختصاصی با بالاترین رونوشت یافت شده در دانه‌ها و میوه‌ها بیان می‌شوند. این امر می‌تواند بیانگر نقش کلیدی این ژن‌ها در ذخیره‌سازی لیپیدها در این مراحل رشدی هستند. یافته‌های این تحقیق درک ما از خصوصیات خانواده ژن *JrOleosins* به ویژه ساختار و بیان آنها را عمیق‌تر می‌نماید. پیشنهاد می‌شود در مطالعات آتی چگونگی تنظیم بیان اولئوسین و نحوه تعامل این پروتئین‌ها با سایر پروتئین‌های مرتبط با تولید لیپید در مرحله نمو بذر مدنظر قرار گیرد.

کلمات کلیدی: اجسام روغنی، اولئوسین، تجزیه تحلیل فراداده، گردو، گستره ژنومی.

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