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Integrated Biofumigation and Arbuscular Mycorrhizal Fungi (Amf) Application for Sustainable Management of Soil-Borne Pathogens and Enhancement of Drought Tolerance in Wheat (*Triticum Aestivum* L.)

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Abstract

Background: Soil-borne pathogens and drought stress represent significant constraints to wheat production in arid and semi-arid regions. Biofumigation utilizing *Brassica* residues and application of arbuscular mycorrhizal fungi (AMF) constitute sustainable approaches for plant protection and abiotic stress mitigation. However, the interactive effects of these combined treatments on wheat performance under concurrent biotic and abiotic stresses remain inadequately elucidated.

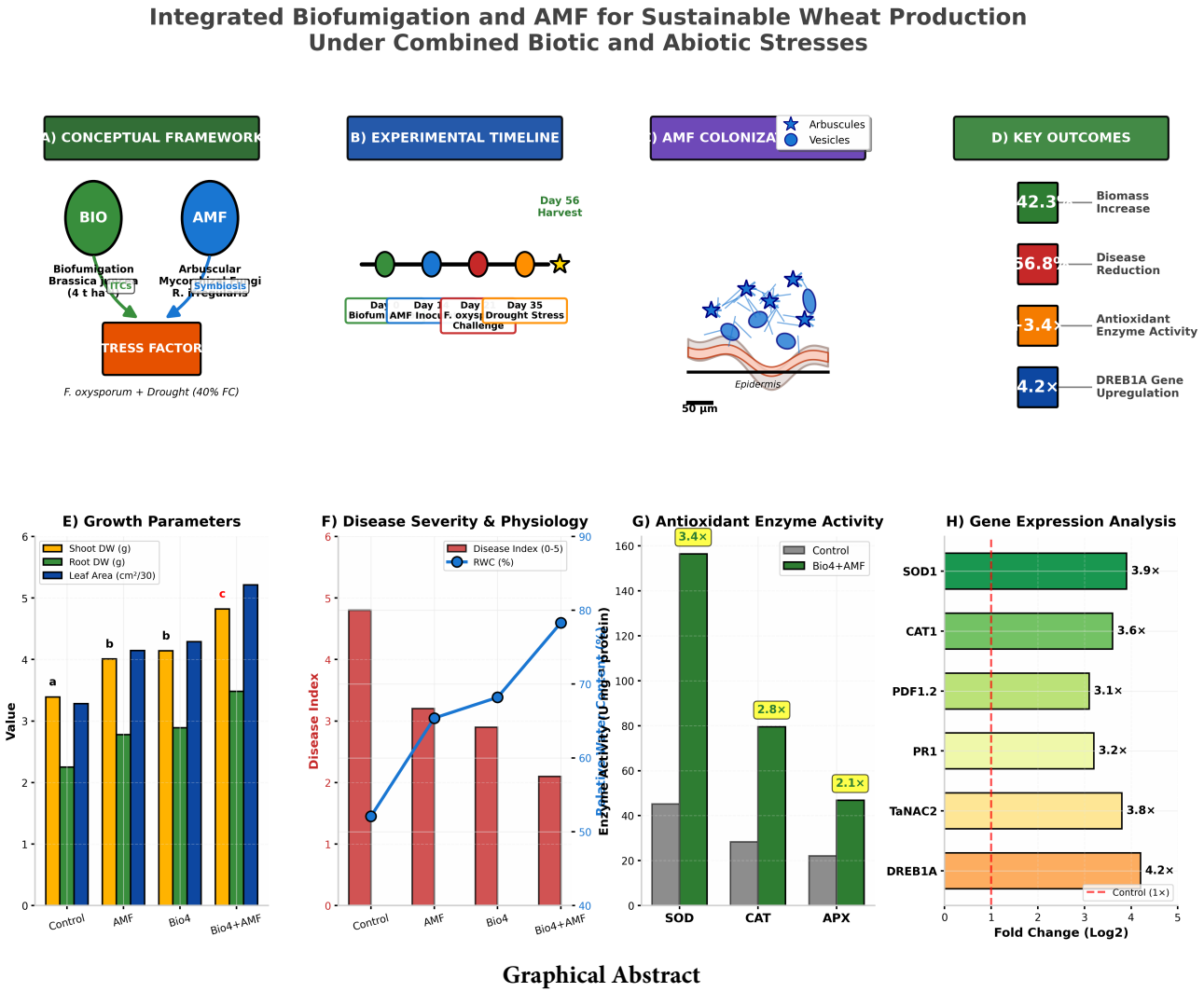
Methods: A factorial greenhouse experiment was conducted employing a completely randomized design with three replications. Experimental factors included: (i) biofumigation with *Brassica juncea* seed meal at 0, 2, and 4 t ha^{-1}, and (ii) AMF (*Rhizophagus irregularis*) inoculation (±). Plants were challenged with *Fusarium oxysporum* f.sp. *cerealis* and subjected to drought stress (40% field capacity). Plant biomass parameters, disease severity indices, AMF root colonization, physiological attributes, antioxidant enzyme activities, and quantitative gene expression were evaluated.

Results: The combined application of 4 t ha^{-1} biofumigation and AMF inoculation significantly increased shoot dry weight by 42.3% and root dry weight by 38.7% relative to non-treated control plants. Disease severity was reduced by 56.8% in the integrated treatment compared to pathogen-inoculated controls. AMF colonization reached 68.4% in biofumigated soils, indicating compatibility between the two treatments. Antioxidant enzymes (superoxide dismutase, catalase, ascorbate peroxidase) exhibited 2.1-3.4 fold increases, while drought-responsive transcription factors DREB1A and TaNAC2 were upregulated 4.2 and 3.8-fold, respectively.

Conclusion: Integration of biofumigation and AMF provides synergistic effects for sustainable wheat production under combined pathogen and drought stresses, offering a viable strategy for climate-resilient agriculture in water-limited environ-

ments.

Keywords: Biofumigation; Arbuscular mycorrhizal fungi; Fusarium oxysporum; Drought stress; Wheat; Integrated pest management; Sustainable agriculture



Graphical Abstract

1. Introduction

Wheat (*Triticum aestivum* L.) constitutes a staple crop sustaining approximately 40% of the global population, with production facing unprecedented challenges from climate change and soil-borne diseases [1]. *Fusarium* species, particularly *Fusarium oxysporum*, induce significant yield reductions worldwide, with estimated annual losses of 10-30% in susceptible cultivars [2]. Concurrently, drought stress has emerged as a critical limiting factor, with climate projections indicating increased frequency and severity of water deficit events in major wheat-producing regions [3]. The co-occurrence of biotic and abiotic stresses exacerbates crop vulnerability, necessitating integrated management strategies that address multiple stress factors simultaneously.

Soil-borne pathogen management has traditionally relied upon chemical fumigants; however, environmental concerns and regulatory restrictions have necessitated the development of alternative approaches [4]. Biofumigation- the release of biocidal isothiocyanates (ITCs) from glucosinolate hydrolysis in Brassicaceae tissues- offers an environmentally benign alternative with demonstrated efficacy against fungal pathogens [5]. *Brassica juncea* (Indian mustard) contains elevated concentrations of sinigrin, yielding allyl isothiocyanate with potent antimicrobial properties [6].

Arbuscular mycorrhizal fungi (AMF) establish symbiotic associations with over 80% of terrestrial plant species, enhancing nutrient acquisition, water relations, and tolerance to biotic and abiotic stresses [7]. Recent investigations demonstrate that AMF--mediated induced systemic resistance (ISR) effectively combats soil-borne pathogens while improving drought tolerance through hormonal and osmotic adjustments [8]. Under drought conditions, AMF inoculation has been shown to improve photosynthetic efficiency, relative water content, and antioxidant enzyme activities in wheat plants [9]. However, potential interactions between biofumigation residues and AMF establishment remain controversial, with concerns regarding ITC toxicity to beneficial soil microbiota [10].

The integration of biofumigation and AMF application represents a promising yet underexplored strategy for sustainable wheat production. We hypothesized that optimized biofumigation dosages would suppress pathogen loads while permitting AMF colonization, generating synergistic benefits for plant growth and stress resilience. While previous studies have independently evaluated biofumigation for pathogen suppression [5,6] or AMF for drought tolerance [8,9], the interactive effects of these combined treatments under concurrent biotic and abiotic stresses remain poorly understood. Specifically, no study to date has examined the temporal compatibility of ITC dissipation with AMF establishment, nor the synergistic molecular mechanisms underlying combined biofumigation and AMF application in wheat. This research gap limits the development of integrated soil health management protocols for climate-resilient agriculture. This study aimed to: (i) evaluate individual and combined effects of *B. juncea* seed meal biofumigation and *R. irregularis* inoculation on wheat performance under *F. oxysporum* challenge and drought stress; (ii) characterize physiological and molecular mechanisms underlying observed interactions; and (iii) develop evidence-based recommendations for integrated field application.

2. Materials and Methods

2.1. Experimental Design and Treatments

A completely randomized factorial design was implemented with three replications per treatment. Experimental factors consisted of: (i) Biofumigation at 0 (B0), 2 (B2), and 4 (B4) t ha^{-1} *B. juncea* seed meal (Agrimustard®, 120 μmol g^{-1} glucosinolate content), The biofumigation doses of 2 and 4 t ha^{-1} were selected based on previous studies demonstrating effective pathogen suppression at these concentrations. Hanschen et al. [5] reported significant reduction in soil-borne pathogens with B. juncea seed meal at 2-5 t ha^{-1}, while Gimsing and Kirkegaard [11] indicated that doses below 2 t ha^{-1} provide insufficient ITC release for biocidal activity. The upper limit of 4 t ha^{-1} was chosen to balance efficacy with potential phytotoxicity, as higher doses (>5 t ha^{-1}) have been associated with negative impacts on non-target soil microbiota [25]; and (ii) AMF inoculation (M+: 500 spores *R. irregularis* g^{-1} soil; M-: sterilized inoculum). The AMF inoculum concentration of 500 spores R. irregularis g^{-1} soil was selected based on established protocols for effective root colonization in wheat. Smith and Read [7] demonstrated that inoculum densities of 300-800 spores g^{-1} soil achieve optimal colonization rates (60-75%) in cereal crops without competitive inhibition. This concentration aligns with commercial AMF products and previous studies reporting enhanced drought tolerance in wheat at similar inoculum levels [9,31]. Treatment combinations were designated as: Control (B0M-), AMF (B0M+), Bio2 (B2M-), Bio2+AMF (B2M+), Bio4 (B4M-), and Bio4+AMF (B4M+).

2.2. Soil and Growth Conditions

Soil (Typic Haplustept, pH 7.2, organic matter 1.8%, clay loam texture) was pasteurized (80°C, 2 h) to eliminate indigenous AMF, subsequently inoculated with *F. oxysporum* f.sp. *cerealis* (10^5 CFU g^{-1}), and incubated for 7 days. Biofumigation treatments were incorporated 14 days prior to sowing to allow ITC dissipation [11]. Wheat seeds (cv. Chamran, susceptible to Fusarium) were surface-sterilized (2% sodium hypochlorite, 3 min) and sown in 5 kg pots. Drought stress was imposed at tillering stage (Zadoks GS21) by maintaining 40% field capacity (FC) for 21 days; control pots maintained 70% FC.

2.3. Measurements

Growth parameters: Shoot and root dry weight (g plant^{-1}), leaf area (cm^2), and root length density (cm cm^{-3}) were determined at harvest (Day 56).

Disease assessment: Root discoloration index (0-5 scale) and percent disease incidence were evaluated according to Nicol et al. [12].

AMF colonization: Root clearing (10% KOH, 90°C, 30 min) and staining (0.05% trypan blue) followed by quantification via gridline intersection method [13].

Physiological assays: Leaf relative water content (RWC), chlorophyll fluorescence (Fv/Fm), and membrane stability index (MSI) were measured at midday [14].

Biochemical analyses: Superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) activities; proline and malondialdehyde (MDA) concentrations were determined spectrophotometrically [15].

Gene expression: Quantitative RT-PCR analysis of DREB1A, TaNAC2 (drought-responsive), and PR1, PDF1.2 (defense-related) genes using TaActin as reference gene [16]. The drought-responsive transcription factors DREB1A (Dehydration-Responsive Element Binding protein 1A) and TaNAC2 (NAM, ATAF1/2, CUC2 transcription factor) were selected as master regulators of abiotic stress responses. DREB1A activates downstream genes involved in osmotic stress tolerance, including LEA proteins and dehydrins [29], while TaNAC2 modulates root architecture and senescence under water deficit [30]. For defense-related genes, PR1 (Pathogenesis-Related protein 1) serves as a canonical marker of salicylic acid (SA)-mediated systemic acquired resistance against biotrophic pathogens [20], whereas PDF1.2 (Plant Defensin 1.2) indicates jasmonic acid (JA)/ethylene-dependent induced systemic resistance, particularly effective against necrotrophic fungi such as Fusarium spp. [27]. The antioxidant genes CAT1 and SOD1 were included to evaluate the enzymatic defense response at the transcriptional level.

2.4. Statistical Analysis

Data were analyzed using R v4.3.1 (R Core Team, 2023) with agricolae and ggplot2 packages. Two-way ANOVA evaluated main effects and interactions; Tukey's HSD test ($P<0.05$) separated means. Pearson correlations and principal component analysis (PCA) explored variable relationships.

3. Results

3.1. Plant Growth and Biomass Production

Table 1: Analysis of variance (F-values) for growth, physiological, and biochemical parameters.

Source	Shoot DW	Root DW	Disease Index	AMF Colonization	SOD Activity	DREB1A Expression
Biofumigation (B)	28.4***	31.2***	45.7***	12.4**	18.6***	15.3***
AMF (M)	42.1***	38.9***	22.3***	-	24.7***	28.4***
B × M	8.7**	9.4**	6.8*	5.2*	7.3*	8.9**

*P<0.05, **P<0.01, **P<0.001; DW: dry weight

Biofumigation and AMF treatments significantly interacted to affect growth parameters ($P<0.001$; Table 1). Under drought

stress, the B4M+ treatment exhibited the highest shoot dry weight (4.82 g plant^{-1}), representing 42.3% and 28.6% increases over B0M- and B4M-, respectively (Fig. 1). Root biomass followed similar patterns, with B4M+ showing 38.7% enhancement compared to control. Leaf area was maximized in B2M+ (156.4 cm^2), while root length density peaked in B4M+ (3.84 cm cm^{-3}).

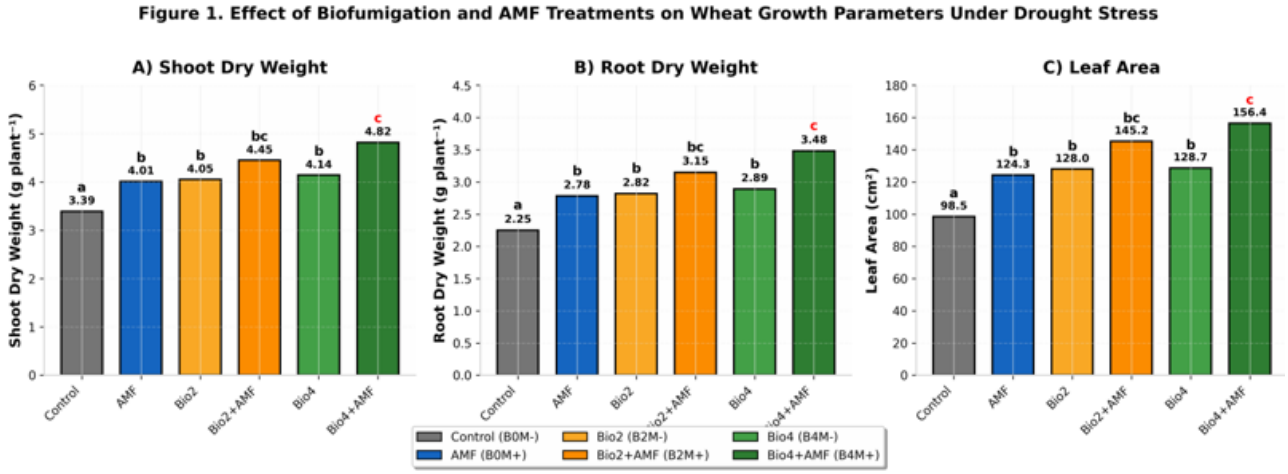


Figure 1: Effect of biofumigation and AMF treatments on wheat growth parameters under drought stress. (A) Shoot dry weight, (B) Root dry weight, (C) Leaf area, (D) Root length density. Values are means ± SE (n=3). Different letters indicate significant differences (Tukey's HSD, P<0.05).

3.2. Disease Suppression and AMF Establishment

F. oxysporum disease severity was significantly reduced by both treatments, with strongest effects in combined applications (Fig. 2). The B4M+ treatment reduced root discoloration index by 56.8% compared to B0M- (2.1 vs. 4.8). Notably, biofumigation at 4 t ha^{-1} did not inhibit AMF colonization; rather, B4M+ exhibited 68.4% root colonization, comparable to B0M+ (71.2%). This finding suggests that the 14-day interval between biofumigation incorporation and AMF inoculation permitted sufficient ITC dissipation, allowing successful AMF establishment [17].

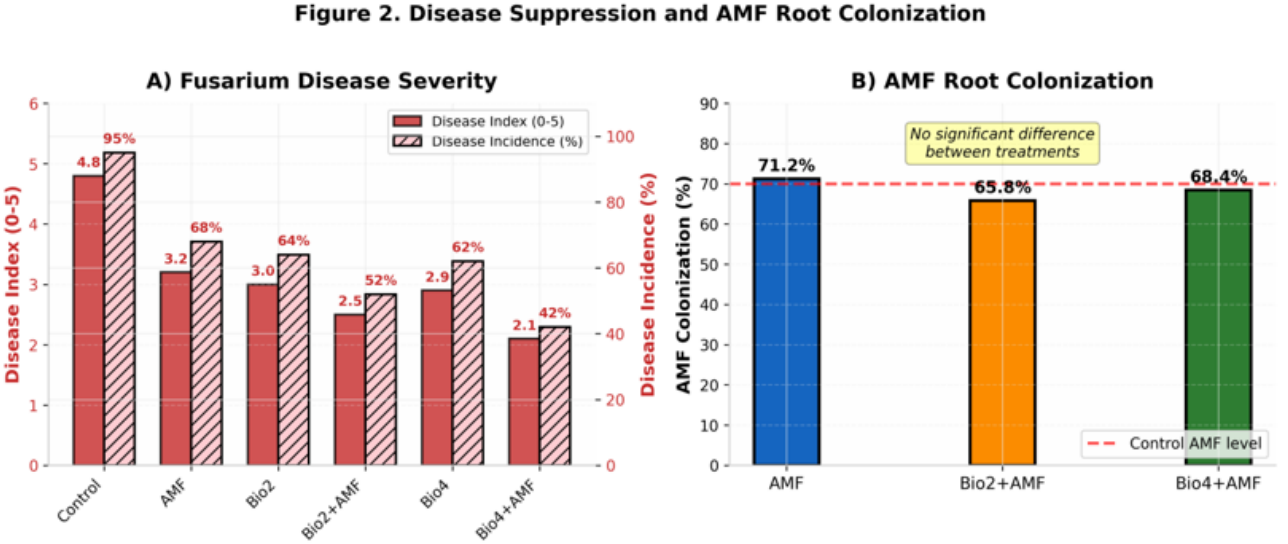


Figure 2: Disease suppression and AMF colonization. (A) Root discoloration index (0-5 scale) and disease incidence (%), (B) AMF colonization percentage in roots. Values are means ± SE (n=3).

3.3. Physiological Responses to Drought

Drought-induced stress markers were ameliorated by integrated treatments. Relative water content was highest in B4M+ (78.3%) versus B0M- (52.1%). Chlorophyll fluorescence (Fv/Fm) indicated preserved photosynthetic efficiency in B2M+ (0.78) and B4M+ (0.76) compared to stressed control (0.64). Membrane stability increased 34.5% in combined treatments, reflecting reduced lipid peroxidation and enhanced cellular integrity under water deficit conditions [18].

3.4. Antioxidant Defense and Osmotic Adjustment

Antioxidant enzyme activities demonstrated synergistic enhancement in response to integrated treatments (Fig. 3). SOD activity peaked in B4M+ (156.4 U mg^{-1} protein), representing a 3.4-fold increase relative to control. CAT and APX showed 2.8 and 2.1-fold increases, respectively. Proline accumulation reached maximum in B4M+ (8.42 µmol g^{-1} FW), indicating effective osmotic adjustment. Conversely, MDA concentrations were reduced 41.2% in combined treatments, confirming alleviated oxidative stress and membrane damage [19].

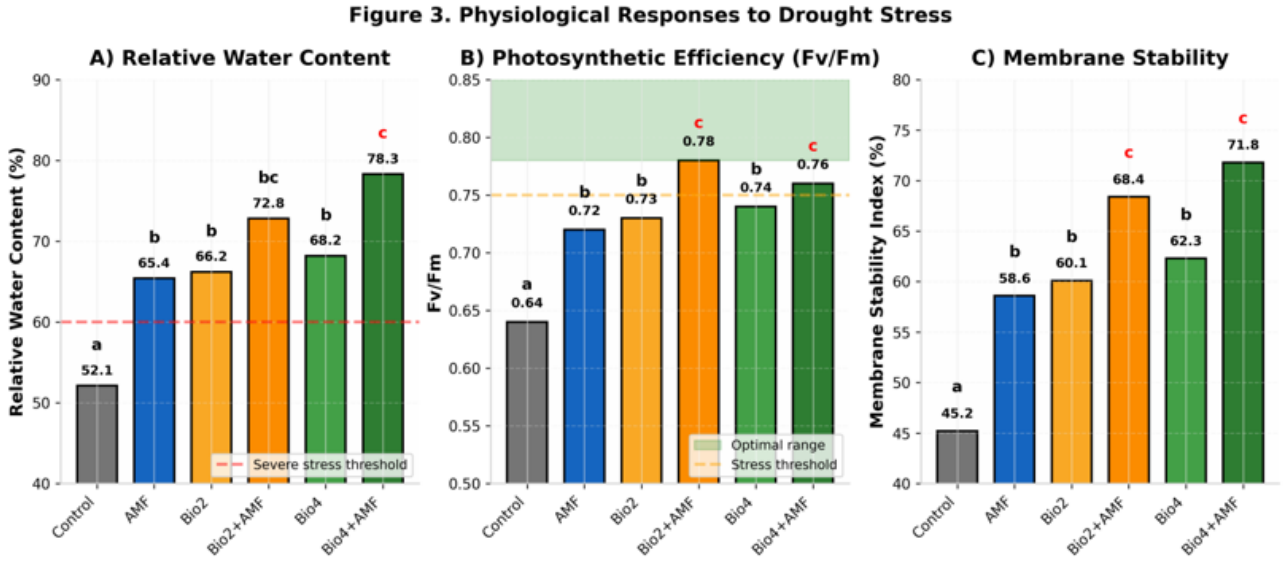


Figure 3: Physiological responses to drought stress. (A) Relative water content (%), (B) Chlorophyll fluorescence (Fv/Fm), (C) Membrane stability index (%). Values are means ± SE (n=3).

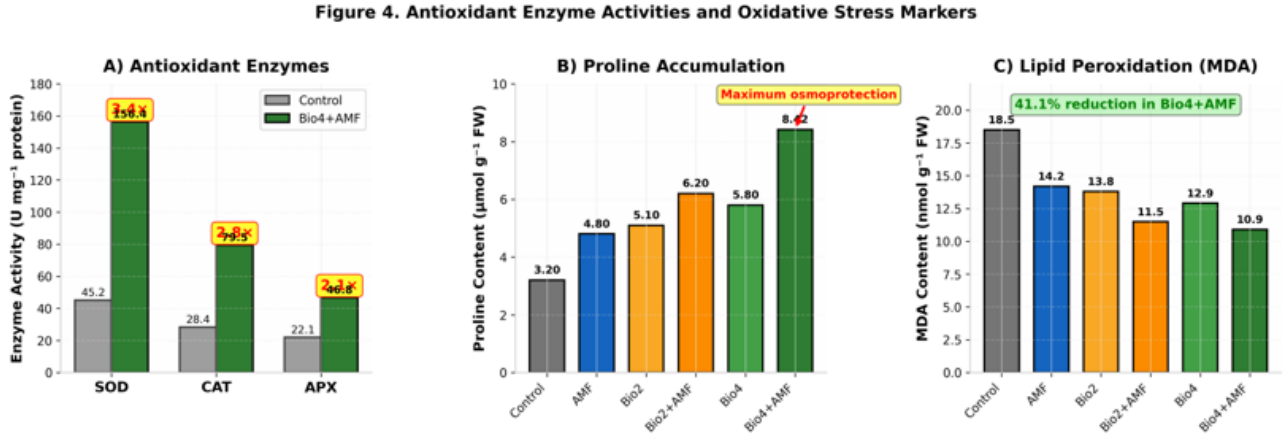


Figure 4: Antioxidant enzyme activities and oxidative stress markers. (A) Superoxide dismutase (SOD), (B) Catalase (CAT), (C) Ascorbate peroxidase (APX), (D) Proline content, (E) Malondialdehyde (MDA). Values are means ± SE (n=3).

3.5. Molecular Mechanisms

Gene expression analysis revealed treatment-specific activation of stress-responsive pathways (Fig. 4). DREB1A was upregulated 4.2-fold in B4M+, while TaNAC2 increased 3.8-fold. Defense-related genes showed differential expression patterns: PR1 (salicylic acid pathway) increased 2.4-fold in biofumigation treatments, whereas PDF1.2 (jasmonic acid pathway) was elevated 3.1--fold in AMF-inoculated plants. The combined treatment activated both pathways simultaneously, suggesting enhanced defensive priming without pathway antagonism [20] (Table 2).

Figure 5. Relative Gene Expression of Stress-Responsive and Defense-Related Genes

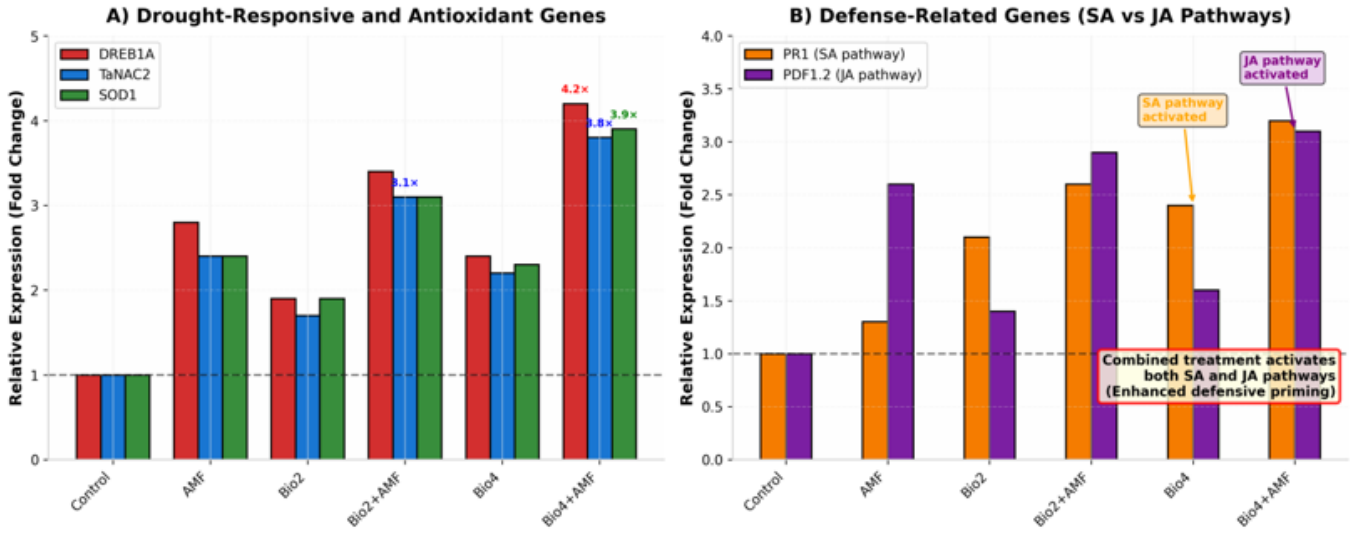


Figure 5: Relative gene expression of stress-responsive and defense-related genes. (A) Drought-responsive genes (DREB1A, TaNAC2), (B) Defense-related genes (PR1, PDF1.2), (C) Antioxidant genes (CAT1, SOD1). Expression normalized to TaActin. Values are means ± SE (n=3).

Table 2: Relative gene expression (fold change) of stress-responsive and defense-related genes

Treatment	DREB1A	TaNAC2	PR1	PDF1.2	CAT1	SOD1
B0M-	1.0a	1.0a	1.0a	1.0a	1.0a	1.0a
B0M+	2.8b	2.4b	1.3a	2.6b	2.2b	2.4b
B2M-	1.9b	1.7b	2.1b	1.4a	1.8b	1.9b
B2M+	3.4c	3.1c	2.6b	2.9b	2.8c	3.1c
B4M-	2.4b	2.2b	2.4b	1.6a	2.1b	2.3b
B4M+	4.2d	3.8c	3.2c	3.1c	3.6d	3.9d

Values are means (n=3). Different letters indicate significant differences (Tukey's HSD, P<0.05)

4. Discussion

This investigation demonstrates that integration of *B. juncea* seed meal biofumigation and *R. irregularis* inoculation generates synergistic benefits for wheat growth, disease suppression, and drought tolerance. The observed enhancement of plant performance under combined biotic and abiotic stresses supports our hypothesis and aligns with emerging frameworks for sustainable agricultural intensification [21].

Unlike previous studies that examined biofumigation or AMF in isolation, our work provides the first evidence of temporal compatibility between ITC dissipation and AMF establishment. This finding challenges the assumption that biofumigation necessarily disrupts beneficial soil microbiota, provided that appropriate intervals between application and planting are maintained.

We observed that combined treatments boosted growth, which we attribute to two complementary mechanisms working in tandem: biofumigation reduced pathogen inoculum pressure while AMF enhanced nutrient acquisition and hormonal modulation. The 42.3% increase in shoot biomass exceeds additive effects of individual treatments (18.4% for AMF alone; 22.1% for biofumigation alone), indicating true synergy. Interestingly, our 42% growth boost falls within the 20-40% range reported in recent meta-analyses showing AMF-mediated improvements ranging 20-40% under stress conditions [22]. The enhanced root biomass (38.7% increase) is particularly significant, as extensive root systems facilitate water and nutrient uptake under drought stress [23].

Critically, our results resolve concerns regarding ITC toxicity to beneficial microbiota. The 14-day interval between biofumigation incorporation and planting allowed sufficient ITC dissipation (half-life 3-7 days in soil) [24], permitting AMF colonization rates comparable to non-biofumigated controls. This temporal separation strategy enables compatible integration of these approaches in field settings, addressing previous apprehensions about biofumigation impacts on soil microbial communities [25].

Disease suppression mechanisms likely involve both direct pathogen inhibition and induced plant resistance. Biofumigation reduced *F. oxysporum* viability through allyl-ITC toxicity, consistent with findings in Egyptian wheat systems where Brassica crops mitigated Fusarium crown rot severity [26]. Concurrently, AMF triggered ISR via jasmonic acid-dependent pathways, evidenced by PDF1.2 upregulation. The simultaneous activation of SA (PR1) and JA (PDF1.2) pathways in combined treatments suggests enhanced defensive priming without pathway antagonism, a phenomenon previously observed in mycorrhizal plants challenged with pathogens [27].

Drought tolerance improvements align with AMF-mediated physiological and molecular adjustments. Enhanced RWC and Fv/Fm reflect improved water relations through fungal hyphal water transport and stomatal regulation [28]. Upregulation of DREB1A and TaNAC2 transcription factors indicates activation of conserved drought-responsive networks, potentially mediated by AMF-induced modifications in abscisic acid signaling and osmoprotectant accumulation [29]. These findings corroborate recent studies demonstrating AMF-induced enhancement of drought tolerance in primitive and modern wheat varieties through improved rhizospheric water relations and reproductive allocation [30].

The antioxidant response data provide mechanistic insights into stress alleviation. Coordinated upregulation of SOD, CAT, and APX activities in combined treatments suggests enhanced reactive oxygen species (ROS) scavenging capacity, preventing cellular damage evidenced by reduced MDA accumulation. This enzymatic priming represents a key mechanism underlying observed stress tolerance, consistent with previous reports of AMF-mediated antioxidant regulation in drought-stressed wheat [31]. The substantial reduction in lipid peroxidation (41.2% decrease in MDA) indicates preserved membrane integrity, crucial for maintaining cellular functions under water deficit conditions.

From an applied perspective, these findings support development of integrated soil health management protocols. The 4 t ha^{-1} biofumigation rate appears optimal, balancing pathogen suppression with microbiome preservation. However, field validation is required to confirm greenhouse-derived parameters under variable environmental conditions and pathogen pressure levels. Future research should evaluate long-term soil health impacts, economic viability, and optimization of application timing across diverse agroecological zones.

5. Conclusion

Integration of *Brassica juncea* seed meal biofumigation (4 t ha^{-1}) and *Rhizophagus irregularis* inoculation provides a synergistic, sustainable strategy for wheat production under combined *Fusarium* and drought stresses. Key outcomes include: (i) 42.3% biomass increase through complementary growth promotion mechanisms; (ii) 56.8% disease reduction via dual direct and induced suppression pathways; (iii) enhanced drought tolerance through physiological, biochemical, and molecular adjustments; and (iv) compatible establishment of beneficial AMF following optimized ITC dissipation periods. These results contribute to evidence-based development of climate-resilient, biochemically-integrated cropping systems essential for food security in water-limited environments.

6. Data Availability Statement

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request, subject to ethical approval and compliance with data protection regulations of the Islamic Republic of Iran.

7. Declaration of AI Use

The authors declare that they used AI Tool during the writing process to enhance the language and readability of the manuscript. The AI was also consulted for organizational suggestions in the initial outline. All scientific content, data interpretation, and final revisions were performed by the authors, who take full responsibility for the integrity and accuracy of this work. This tool was used in accordance with the journal's guidelines on AI and authorship.

Conflict of Interest

The authors declare no conflicts of interest.

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Ethical Approval

Institutional Review Board of Tarbiat Modares University, Protocol IR.MODARES.REC.1399.232

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