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Aspergillus terreus Alleviates Salinity-Induced Phytotoxicity and Improves Growth, Photosynthetic Pigment and Osmolyte Status in Barley (*Hordeum vulgare L.*)

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Aspergillus terreus Alleviates Salinity-Induced Phytotoxicity and Improves Growth, Photosynthetic Pigment and Osmolyte Status in Barley (*Hordeum vulgare* L.)

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ABSTRACT

Background. Soil salinization remains one of the most persistent abiotic constraints on cereal productivity, worldwide. Barley (*Hordeum vulgare* L.), although ranked among the more salt-tolerant cereals, still incurs measurable physiological and biochemical penalties. Beneficial rhizospheric fungi have been proposed as a low-cost route to buffer this stress, yet the degree of protection they confer is isolate, host-specific, and needs to be demonstrated experimentally, rather than assumed. The present study investigated the novel use of a rhizospheric *Aspergillus terreus* isolate, originally obtained from the rhizosphere of *Parthenium hysterophorus* to enhance salinity tolerance in barley seedlings.

Methods. The study evaluated the capacity of a rhizospheric fungal isolate, identified as *Aspergillus terreus* and obtained from the Plant-Microbe-Interaction (PMI) Laboratory, Abdul Wali Khan University, Mardan to protect barley seedlings against 150 mM salinity in a pot trial. The trial was arranged as a completely randomized design with four treatments: untreated control, fungal inoculation alone, salinity alone, and salinity combined with fungal inoculation.

Results. Salinity sharply suppressed seedling emergence and vegetative growth, reducing shoot length by 14.3%, root length by 42.5%, fresh biomass by 28.1%, and dry biomass by 64.5% relative to the control, while chlorophyll a, chlorophyll b, carotenoid, and xanthophyll contents fell by 29.0%, 49.6%, 21.0% and 5.1%, respectively. Salt-stressed plants accumulated more total phenolics, flavonoids, and proline, while total lipid content declined and gained soluble sugars by approximately 46%, consistent with an osmotic and ionic stress response. Inoculation with *A. terreus* partially alleviated this damage: seedling emergence and shoot length recovered by approximately 11-25%, root length and dry biomass by 30-109%, and photosynthetic pigments by 2-32%. While, proline and phenolics declined toward the salt-only baseline by 9-17%, though remaining significantly different from the control ($p \leq 0.05$). Further, total lipid content increased by approximately 43% and soluble sugars declined by approximately 14% relative to the salt-only treatments, both

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moving back toward control levels. Light microscopy confirmed the colonization of barley roots by the fungus.

Conclusions. These findings suggest that the *Parthenium hysterophorus* rhizosphere-derived *A. terreus* isolate is associated with reduced proline, phenolic and lipid accumulation, and increased soluble sugar and pigment content in salt-stressed barley. Further, they support *A. terreus* as a candidate inoculant for barley grown under saline conditions, pending validation in non-sterile soil under greenhouse and field conditions.

Keywords: *Aspergillus terreus*, *Hordeum vulgare,* osmolytes, rhizospheric fungi, salt tolerance

Highlights

- *A. terreus* significantly reduces salinity-induced growth inhibition in barley.
- Fungal inoculation is associated with increased photosynthetic pigments and soluble sugar content.
- The isolate is an experimental candidate for salt-affected agricultural soils, pending strain-level biosafety testing, and validation in non-sterile field soil.

1. INTRODUCTION

In recent years, soil salinity has emerged as one of the most significant abiotic limitations to global crop production. It has been growing in both extent and severity due to increasing irrigation intensity and changing groundwater levels resulting from changing precipitation patterns [1]. The higher root zone NaCl levels and, to a lesser extent, Na_2SO_4 affect the ability of the plants to absorb water and cause ionic and osmotic stresses in glycophytic species [2], leading to cell turgor disruption, nutrient imbalance, and increase in ROS levels [3]. Salinization can occur naturally due to mineral weathering of rocks [4], seawater intrusion in coastal aquifers [5], and evaporative concentration of salts in arid and semi-arid soils [6]. It can also occur artificially as a consequence of saline irrigation water [7], as well as over-fertilisation, poor drainage, and disruptions to the natural salt cycle by human activity [8]. The agronomic impact remains similar regardless of the origin and effects of salinity on water relations, nutrient uptake, stomatal conductance, photosynthetic efficiency, and biomass production, significantly limiting crop yields globally [9, 10].

The cellular effects of salinity may occur via three generally overlapping pathways. Osmotic stress decreases the water potential around the roots, causing a drought-like condition that reduces the plasma membrane size, turgidity, and cell wall integrity [11, 9] and affects nutrient status, membrane integrity, and photosynthesis [12]. Ionic toxicity then occurs when Na+ and Cl- build up in the cytosol, affecting K+-dependent enzyme activity [13]. When Cl- builds up, it causes direct damage to the photosynthetic apparatus by chlorophyll degradation and disruption of PSII activity [14]. This electrochemical (ionic) imbalance also removes calcium and potassium, which are important for membrane integrity, enzyme activation, and osmoregulation [15, 16, 17]. Oxidative stress exacerbates both these effects. It disrupts normal metabolism, increasing the likelihood

of the accumulation of reactive oxygen species that damage lipids, proteins, and nucleic acids [18]. Plants respond to this complex set of stresses by via a number of coordinated responses, including the production of compatible solutes, such as proline and glycine betaine [19], preferential sequestration of Na+ into vacuoles by NHX-type transporters, activation of the Salt Overly Sensitive (SOS) signaling pathway (Ahmed et al., 2024), and induction of antioxidant enzymes [20]. Their use, however, is an energy-intensive process and may not be sufficient to stop significant losses in biomass and yield, particularly in sensitive genotypes [21].

Barley (*Hordeum vulgare* L.) is generally regarded as one of the more salt-tolerant cereals, a trait that has made it useful to study salinity adaptation in graminaceous crops [22]. Even so, salinity still measurably depresses its germination, root and shoot elongation, chlorophyll content, and antioxidant balance, with the severity of the response varying across genotypes according to differences in ion transporter expression, such as HvSOS1, HvHKT2, and HvNHX isoforms, and antioxidant enzyme activity [22, 23]. Since conventional approaches to reclaiming saline soils, such as leaching, gypsum amendment, and drainage improvement, tend to be expensive, water-intensive, and often only temporarily effective [24, 25], there has been a growing interest in biological alternatives that work at the level of the plant itself or its immediate root environment [26].

Rhizospheric microorganisms are an obvious candidate. The rhizosphere supports a far denser microbial community than bulk soil, sustained by sugars, amino acids, and organic acids released in root exudates [27]. Several genera within this community, including trichoderma, aspergillus, fusarium, and glomus, are now well documented as plant growth-promoting fungi [28]. These organisms can alleviate salinity stress in various ways, many of which are complementary. They can solubilize phosphate and other nutrients [29]; they produce bioactive metabolites and volatile organic compounds that affect root development and systemic resistance [30]; they can directly improve nutrient uptake and stimulate host resistance to abiotic stress, including salinity and drought [31], in several reported instances. Under salinity stress, salt-tolerant plant genotypes actively recruit beneficial fungi such as talaromyces, cladosporium, and aspergillus species in the root zone [32, 33], Salt-sensitive lines may exhibit greater susceptibility to pathogenic fungi, such as *Fusarium* and *Rhizoctonia*, with pathogen abundance or disease incidence potentially increasing under elevated salinity [34, 35].

A. terreus has been reported from saline, contaminated, and otherwise stressful soil environments. Its ability to persist under adverse conditions has attracted attention as a potential mechanism for improving plant tolerance to different abiotic stresses. Previous studies have demonstrated beneficial effects of *A. terreus* against heavy-metal stress in wheat, as well as drought and waterlogging stress in maize [36, 37, 38]. However, only a few studies have been reported on rhizospheric fungal inoculants for salinity amelioration in barley and most of them involve bacterial PGPR studies [39, 40]. This gap inspired the current study which aimed to determine the response of *Hordeum vulgare* under induced salinity stress and to test if a screened rhizospheric fungal isolate can reduce stress and enhance growth-related traits of *H. vulgare.* The specific objective was to determine whether inoculation with this isolate could counteract the phytotoxic effects of salinity on *H. vulgare* and, if so, to

characterize the physiological and biochemical mechanisms underlying its protection.

2. MATERIALS AND METHODS

2.1. Organisms and Materials used to Isolate Fungi

A. terreus is a rhizospheric fungus that was isolated, screened, and molecularly identified from the rhizospheric soil of *Parthenium hysterophorus* in Plant-Microbe Interaction (PMI) Laboratory, Abdul Wali Khan University, Mardan, Pakistan. The isolate was identified molecularly using ITS sequencing and deposited in GenBank under accession number KT310979.1 (isolate F2) as part of a prior isolate-characterization study. The same deposited isolate was used for inoculation in the present pot trial. For fungal inoculation, a spore suspension of *A. terreus* (isolate F2) was adjusted to a concentration of 1×10^6 spores mL^{-1}. Further, 5 mL of this suspension was applied to the rhizosphere of each seedling at the time of sowing. The seeds of *H. vulgare* (barley) were collected from Cereal Crop Research Institute (CCRI), Nowshehra. Raising and maintenance of fungal culture was done using all glassware, media, and solutions which were autoclaved (Lab Tech LCB-0121V, 121°C, 15 psi, 20 min). Aseptic manipulation was performed in a laminar flow hood. Fungal culture was maintained in a Lab Tech LWB-122D incubator.

2.2. Plant Material and Experimental Design

The plants were cultivated in earthen pots containing autoclaved soil from the Department of Botany, Women University, Mardan. Barley cultivar B-171 was used. The plants were grown in pots measuring 2 inches in diameter and were 12 inches deep. Soil pH and electrical conductivity were not measured in this study. The soil was saturated to field capacity and kept at room temperature for 1 week prior to sowing. After sowing, plants were grown at 25 ± 2°C under a 16 h light / 8 h dark photoperiod and 60–70% relative humidity. Ten seeds were sown per pot. After germination, five uniform seedlings were retained per pot. Each of the four treatments namely (i) untreated control, (ii) fungal inoculation alone, (iii) salinity stress (150 mM), and (iv) salinity + fungal inoculation was replicated in three independent pots arranged as a completely randomized design (CRD) and no blocking factor was applied. Each treatment was replicated in three independent pots (n = 3), with five seedlings retained per pot; pot-level means (averaged across the five seedlings) were used as the unit of statistical analysis to avoid pseudoreplication. The experimental timeline was as follows: seeds were sown in a 1:3 soil mixture on Day 0; fungal inoculation (for treatments ii and iv) was applied at the time of sowing; NaCl treatment (for treatments iii and iv) was applied at the three-leaf stage; root colonization was assessed at Day 14 post-inoculation; and all plants were harvested on Day 30 after sowing.

The four treatments included control (no salinity or fungal inoculation), fungal inoculation (*A. terreus* without salinity), salinity (150 mM NaCl without fungal inoculation), and salinity plus fungus (150 mM NaCl with *A. terreus* inoculation).

NaCl (150 mM) was applied at the three-leaf growth stage (not Day 20) for salinity and salinity + fungus treatments. All plants (including controls) were harvested on Day 30 after sowing, consistent with the timeline given in Section 2.2. This superseded the earlier Day-20/first-toxicity-symptom description used in the original submission. Growth parameters including shoot length, root length, fresh weight, and

dry weight were measured at harvest. Fresh weight was recorded immediately after harvest. For dry weight, tissue was oven-dried at 60–70°C for 48–72 hours until constant weight was achieved. The plant material was then immediately frozen in liquid nitrogen and stored at –80°C for biochemical analysis.

2.3. Determination of Photosynthetic Pigments

Chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid contents were measured after Arnon [41], as modified by Pan et al. [42]. Fresh leaf tissue (100 mg) was homogenized in 10 mL of 80% aqueous acetone and the absorbance of the filtrate was read at 663 and 645 nm (for chlorophyll a and b) and 470 nm (for carotenoids and xanthophyll) on a UV-vis spectrophotometer (T70, UK). The concentrations of pigments were determined as follows:

$$Chlorophyll\ a\ (\mu gmL^{-1}) = (12.25\ A663 - 2.79\ A645)$$

$$Chlorophyll\ b\ (\mu gmL^{-1}) = (21.50\ A645 - 5.14\ A663)$$

$$Carotenoids(\mu gmL^{-1}) = \frac{(1000\ A470 - 1.82\ Chlorophyll\ a - 85.02\ Chlorophyll\ b)}{198}$$

The content of carotenoid was estimated using the equation provided by Pan et al. [42], after correcting for the Chl a and Chl b contribution to A470. Xanthophyll content was calculated as a defined fraction of total carotenoids, following the same source (Xanthophyll = 0.5 × total carotenoids, since carotenoids and xanthophylls are not spectrally resolved by a single A470 reading and so were not measured as fully independent pigment pools. This approximation should be replaced with a chromatographically resolved xanthophyll measurement in any future extension of this work) [42].

2.4. Determination of Total Amount of Flavonoids and Phenolics

To extract total flavonoids, 0.5 g of the fresh leaf tissue was homogenized in 5 mL of 80% aqueous ethanol, shaken for 16 h, and then centrifuged at 10,000 ×g for 15 min. The supernatant was kept and stored in the refrigerator at 4°C. Flavonoid content was determined by reacting the supernatant with 80% methanol, 10% $AlCl_3$, and 10% potassium acetate and reading the absorbance at 415 nm on a quercetin calibration curve, as per the method of Li et al. [43].

The total phenolics were determined by flash freezing 2 g leaf tissue in liquid nitrogen and extracting with 16 mL of ethanol at 60°C for 3 h. It was centrifuged at 500×g for 10 min and filtered (Whatman No. 42). The filtrate was concentrated under reduced pressure using a rotary evaporator at 40°C to a final volume of 10 mL with distilled water. The phenolic content was measured using the Folin-Ciocalteu assay based on the principle described by Pérez et al. [44], where the diluted extract was mixed with Folin-Ciocalteu reagent and 7.5% sodium carbonate solution, allowed to stand in the dark for 5 min, and read at 765 nm against the calibration curve of catechol.

2.5. Determination of Proline Contents and Soluble Sugar

Proline was extracted from 0.2 g of leaf material in 1 mL 40:60 (v/v) ethanol, incubated overnight at 4°C, and then centrifuged at 14,000 ×g for 5 min. If a pellet remained, extraction was repeated and the extracts were pooled, with a reported recovery above 93% [45]. Proline was quantified by the acid-ninhydrin method of Khan et al. [45]. The extract was reacted with acid-ninhydrin reagent (1.25 g ninhydrin in 30 mL glacial acetic acid and 20 mL of 6 M phosphoric acid), incubated in a boiling water bath for 1 h, cooled, extracted into toluene,

and read at 520 nm against a proline standard curve (2-10 µg/mL).

Soluble sugars were extracted from 0.1 g of leaf tissue in 1 mL of methanol:chloroform:water (MCW) buffer (MCW ratio 12:5:3 v/v/v), incubated at 50°C for 30 min, and centrifuged at 14,000 ×g for 5 min [46]. Soluble sugars were quantified by the phenol-sulfuric acid method of Xiao et al. [47]. Briefly, 100 µL of supernatant was reacted with 80% phenol concentrated sulfuric acid and absorbance was measured at 490 nm. Sugar content was determined from a calibration curve prepared using glucose standards (0–30 µg/mL) and expressed as mg g^{-1} fresh weight.

2.6. Determination of Lipid Content

Lipids were extracted from 0.2 g of plant material in chloroform:methanol (2:1, v/v), filtered, and partitioned with 0.73% NaCl solution to a final chloroform:methanol:water ratio of 1:1:1 (v/v/v). The lower lipid-containing phase was retained [48]. The extract was evaporated at 50-60°C, treated with the phospho-vanillin reagent in concentrated sulfuric acid, and absorbance was measured at 490 nm using a lipid standard curve of 0-30 µg/mL.

2.7. Root Colonization Assessment

The root colonization by *A. terreus* was assessed after 14 days of inoculation. To verify the presence of fungal structures in the root tissue, fresh root segments were cut into 1 cm pieces, blotted, stained with lactophenol cotton blue for one minute, washed with distilled water, and then mounted on a glass slide for microscopic examination.

2.8. Statistical Analysis

Each of the four treatments was replicated in three independent pots (n = 3 pots per treatment). No additional technical replicates or separate experimental runs were performed. Pot-level means (averaged across the five seedlings retained per pot) were used as the unit of analysis. Since no genuine blocking factor (e.g., spatial block, growth-chamber shelf, or temporal cohort) was applied, the design is more accurately described as completely randomized design (CRD), rather than a randomized complete block design. Data were analyzed by two-way (2 × 2 factorial) ANOVA with salinity and fungal inoculation as fixed factors, testing the main effect of salinity, the main effect of fungal inoculation, and the salinity × fungal-inoculation interaction for every response variable, followed by Duncan's Multiple Range Test (DMRT) for mean separation ($p \leq 0.05$), using IBM SPSS Statistics (version 21). Origin 2024 was used to create figures. Data in figures represent means ± SE of three biological replicates (n = 3). Different letters above bars indicate statistically significant differences among treatments based on ANOVA and Duncan's multiple range test ($p \leq 0.05$).

3. RESULTS

The pattern of physiological injury observed across all four treatments in barley was consistent with a delay in seed establishment which coincided with stunted shoot and root growth, loss of pigments, and a shift in the biochemical profile of leaf tissue to an accumulation of osmolytes and stress metabolites. Co-inoculation with *A. terreus* did not completely resolve this injury, although nearly all of it was resolved under almost all the parameters measured, with the magnitude of this resolution differing slightly by parameter, being closer to 100% for root growth and biomass than for chlorophyll content. The sections below present these effects beginning with germination and ending with the confirmation of root colonization (see Table 1).

Table 1. The Growth, Pigment, and Biochemical Parameters of *Hordeum vulgare* as a Result of the Treatment with 150 mM Salinity with and without inoculation of *Aspergillus terreus*

Parameter	Salinity vs. Control (%)	Salinity + Fungi vs. Salinity (%)
Germination	-42.9	+25.0
Shoot length	-14.3	+11.1
Root length	-42.5	+30.4
Fresh weight	-28.1	+17.4
Dry weight	-64.5	+109.1
Chlorophyll a	-29.0	+31.9
Chlorophyll b	-49.6	+24.5
Carotenoids	-21.0	+15.6
Xanthophyll	-5.1	+2.5
Proline	+16.2	-9.3
Total phenolics	+46.7	-17.0
Flavonoids	+45.3	−17.1
Lipids	-40.2	+43.3
Soluble sugars	+46.3	-13.9

Note. Negative values denote a reduction relative to the comparator treatment; positive values denote an increase. All differences were significant at $p \leq 0.05$ (ANOVA followed by Duncan's Multiple Range Test).

3.1. Seedling Emergence

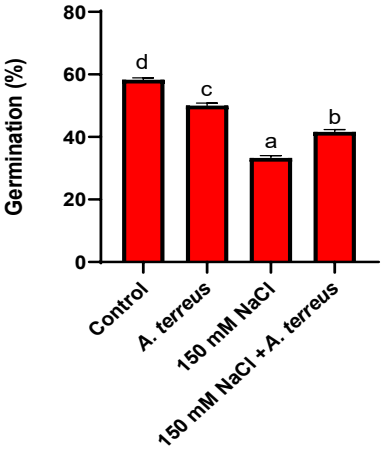


Figure 1. Effect of 150 mM NaCl and Inoculation with *A. terreus* on barley (Hordeum vulgare L.) Seedling Emergence Percentage, Recorded after the Three-leaf Growth Stage

Barley seedlings were evaluated for changes in seedling emergence percentage, recorded after the three-leaf stage (i.e., after NaCl application; see Section 2.2), under salinity stress, and treated with rhizospheric fungi. Salinity at 150 mM sharply suppressed seedling emergence relative to untreated control, with the emergence rate reduced by 42.9% ($p \leq 0.05$). Inoculation with *A. terreus* under the same saline conditions substantially reversed this inhibition, raising seedling emergence by 25.0% relative to the non-inoculated, salt-stressed treatment, indicating a protective effect of the fungus on seedling establishment under saline conditions (Figure 1).

3.2. Shoot and Root Elongation

The effects of salinity on shoot and root length were examined in barley seedlings. Shoot length was reduced by 14.3% under salinity stress, as compared with the control ($p \leq 0.05$). Fungal inoculation counteracted this effect, increasing shoot length

by 11.1% relative to the salt-stressed, non-inoculated plants. The effect of salinity on root elongation was more severe, with root length reduced by 42.5% relative to the control. Fungal inoculation improved root length, increasing it by 30.4% relative to the salt-stressed, non-inoculated plants (Figure 2).

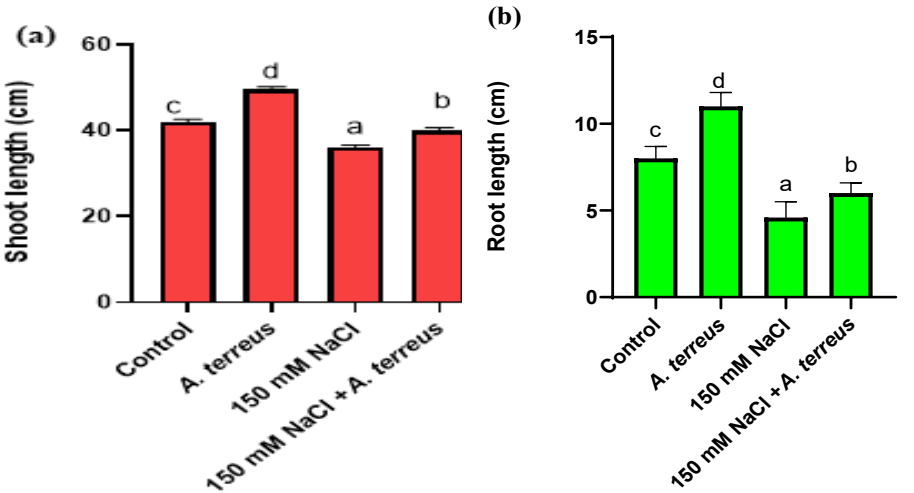


Figure 2. Effect of 150 mM NaCl and *A. terreus* Inoculation on (a) Shoot Length and (b) Root Length of Barley (*Hordeum vulgare* L.) Seedlings

3.3. Biomass Accumulation

The influence of salinity stress on biomass accumulation was examined in barley seedlings inoculated with rhizospheric fungi. Salinity reduced both fresh and dry biomass substantially: fresh weight was reduced by 28.1% and dry weight by 64.5% relative to the control ($p \leq 0.05$ for both). This is consistent with a marked, though not near-total, arrest of growth at the salinity level in the absence of any ameliorative treatment. Inoculation with *A. terreus* partly reversed this loss, increasing fresh weight by 17.4% and dry weight by 109.1% relative to the salt-stressed, uninoculated plants (Figure 3). The dry-weight values reported here have been verified against the original balance records.

3.4. Photosynthetic Pigments

Salinity-induced changes in chlorophyll content were evaluated in barley seedlings, treated with rhizospheric fungi. It was found that the salinity of 150 mM caused a decrease in chlorophyll a content by 29.0% as compared to the control ($p \leq 0.05$), while chlorophyll b decreased by 49.6%. Fungal inoculation partially helped the plants regain pigment content as compared with salt-stressed, uninoculated plants, increasing chlorophyll a by 31.9% and chlorophyll b by 24.5%. Carotenoid content decreased by 21.0% under salinity and increased by 15.6% after inoculation with *A. terreus*. Xanthophyll (estimated as a fixed fraction of total carotenoids; see Section 2.3) decreased by 5.1% under salinity and increased by 2.5% following inoculation, the smallest change of the four pigments measured, rather than the biggest (Figure 4).

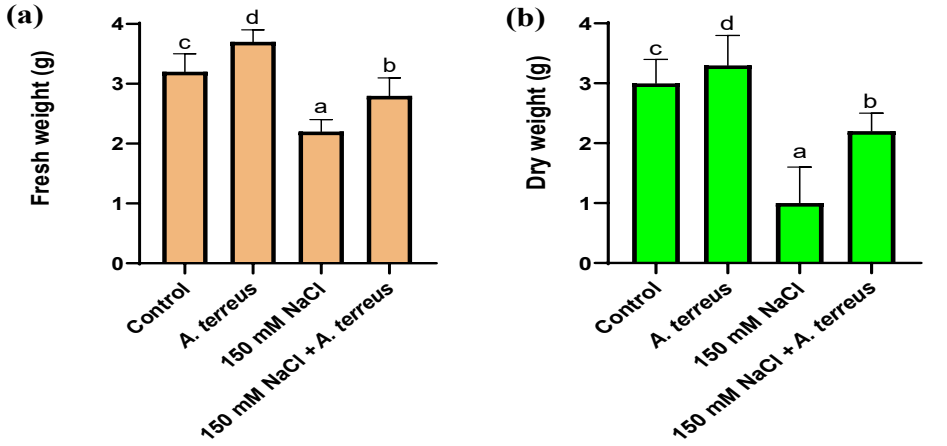


Figure 3. Effect of 150 mM NaCl and *A. terreus* Inoculation on (a) Fresh Weight and (b) Dry Weight of Barley (*Hordeum vulgare* L.) Seedlings

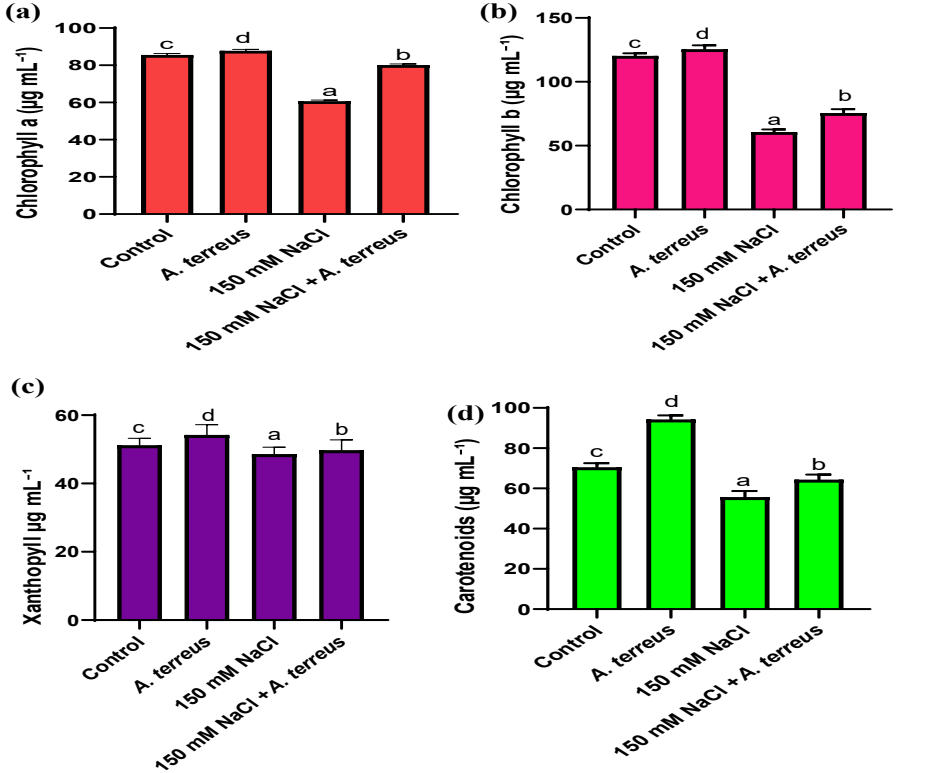


Figure 4. Effect of 150 mM NaCl and *A. terreus* Inoculation on (a) Chlorophyll a (b) Chlorophyll b (c) Xanthophyll and (d) Carotenoids Contents of Barley (*Hordeum vulgare* L.) Seedlings

3.5. Proline Accumulation

Proline content in barley seedlings were examined for their response to salinity stress, inoculated with rhizospheric fungi. Proline content increased by 16.2% under 150 mM salinity, relative to the control ($p \leq 0.05$). This is consistent with the activation of an osmoregulatory stress response. Inoculation with A. terreus reduced proline accumulation by 9.3% relative to the salt-stressed, uninoculated plants, moving levels back toward those of unstressed plants. This is consistent with, but does not on its own demonstrate, a reduction in the underlying osmotic and oxidative pressure resulting in proline accumulation (Figure 5).

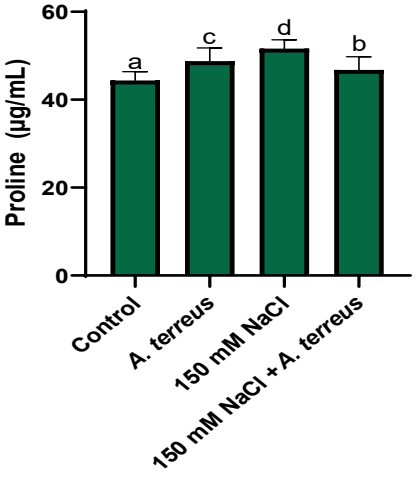


Figure 5. Effect of 150 mM NaCl and *A. terreus* Inoculation on Proline Content of Barley (*Hordeum vulgare* L.) Seedlings

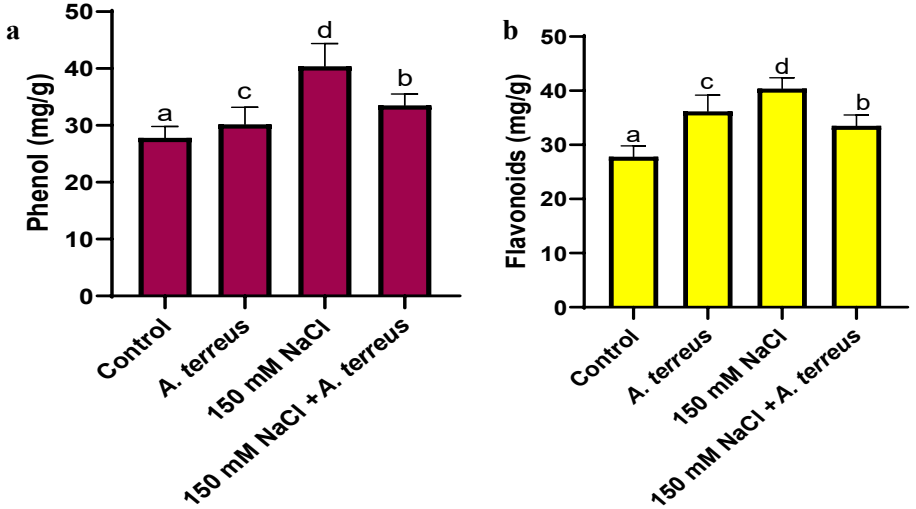


Figure 6. Effect of 150 mM NaCl and *A. terreus* Inoculation on (a) Phenol and (b) Flavonoids Content of Barley (*Hordeum vulgare* L.) Seedlings

3.6. Total Phenolic and Flavonoid Content

The impact of salinity on phenolic content was assessed in barley seedlings, treated with selected rhizospheric fungi. Total phenolic content rose by 46.7% under salinity stress relative to the control ($p \leq 0.05$), indicating an enhanced phenolic biosynthetic response to salt-induced oxidative

pressure. Flavonoid content followed a broadly similar pattern, increasing by 45.3% under salinity. Fungal inoculation lowered both metabolite pools, reducing phenolic content by 17.0% and flavonoid content by 17.1% relative to the salt-stressed, uninoculated plants. This is consistent with a reduction in oxidative burden that triggered their accumulation, pending confirmation of flavonoid values (Figure 6).

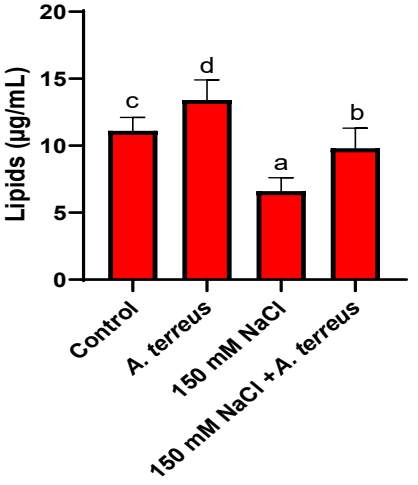


Figure 7. Effect of 150 mM NaCl and *A. terreus* Inoculation on Lipids Content of Barley (*Hordeum vulgare* L.) Seedlings

3.7. Total Lipid Content

Total lipid content, measured by the phospho-vanillin assay (a measure of total lipid content, not of lipid peroxidation, membrane leakage, or oxidative membrane damage), was examined in barley seedlings under salinity stress with and without rhizospheric fungal inoculation. Total lipid content decreased by 40.2% under salinity stress relative to the control ($p \leq 0.05$). Under saline conditions, inoculation with *A. terreus* partly reversed this effect, increasing total lipid content by 43.3%, as compared with the salt-stressed, uninoculated plants. This describes the observed change in total lipid content only; it does not, on its own, indicate the restoration of normal membrane lipid metabolism or membrane stability, neither of which was directly assessed in this study (Figure 7).

3.8. Soluble Sugar Content

Soluble sugar content responses to salinity stress were determined in barley seedlings inoculated with rhizospheric fungi. Salt stress increased soluble sugar content by 46.3% as compared to the control ($p \leq 0.05$), indicating that carbohydrate metabolism was affected by salt stress. Inoculation with *A. terreus* partly reversed this increase, lowering the level of sugar in inoculated plants by 13.9% as compared with the uninoculated, salt-stressed plants, moving the soluble-sugar pool back toward the control level (Figure 8).

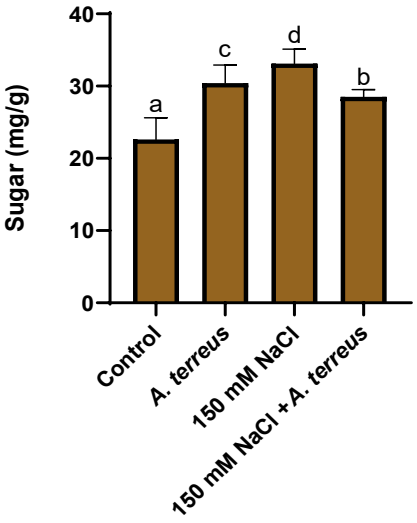


Figure 8. Effect of 150 mM NaCl and *A. terreus* Inoculation on Sugar Content of Barley (*Hordeum vulgare* L.) Seedlings

3.9. Root Colonization

Light microscopy of stained root sections indicated apparent colonization of barley roots by *A. terreus*, with hypha-like structures visible within the root cortex of inoculated plants over the 14-day period, in both non-saline and salt-stressed treatments (Fig 9). Lactophenol cotton blue staining alone does not confirm that these structures are specifically *A. terreus* rather than another root-associated fungus, which is noted as a limitation in Section 4.

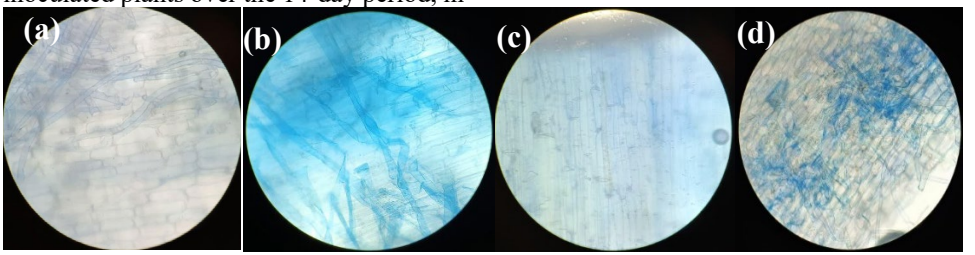


Figure 9. Light Microscopy (40x Magnification) of Lactophenol Cotton Blue-stained Barley Root Sections after 14 days, Showing *A. terreus* Colonization. This Panel Shows A Single Representative Field of View; the Corresponding Images for the Other Three Treatments (a) Control, (b) *A. terreus* (c) 150 mM NaCl and (d) 150 mM NaCl + *A. terreus.*

3.10. Differential Effect of Fungal Inoculation under Non-saline versus Saline Conditions

To evaluate whether *A. terreus* affected barley differently depending on the presence of salinity (Comment 13), the salinity × fungal inoculation interaction term from the two-way factorial ANOVA (Table 2) was examined for every response variable. For seedling emergence, as well as fresh weight and root length, the interaction term was not significant (p = 0.122, 0.412 and 0.149, respectively), indicating that fungal inoculation alone (in the absence of salinity) produced a broadly similar proportional effect to fungal inoculation under salinity for these traits. Notably, the main effect of fungal inoculation on seedling emergence was itself not significant ($F(1,8)$ = 0.00, p = 1.000), so the modest numerical change seen for the fungi-only treatment in this trait should not be over-interpreted.

In contrast, shoot length, dry weight, and every biochemical/biochemical-pigment variable measured (chlorophyll a, chlorophyll b, carotenoids, xanthophyll, proline, total phenolics, and soluble sugars) showed a significant salinity × fungus interaction ($p < 0.05$ to $p < 0.001$; Table 2), indicating that the fungus's effect was condition-dependent rather than a fixed, additive effect. In practical terms, *A. terreus* increased shoot length, dry weight, and pigment content in both non-saline and saline plants. However, the magnitude of this benefit was larger under salinity for some traits (e.g., dry weight, where fungal inoculation raised salt-stressed plants by 109.1% versus a smaller proportional gain in non-saline plants) — consistent with a condition-dependent, partially stress-alleviating role for the fungus, rather than a generic growth-promotion effect independent of the plant's stress status. For the total lipid content, the interaction did not reach significance (p = 0.058). So, this pattern should be treated as suggestive rather than confirmed for this variable.

The exact F-values, degrees of freedom, and p-values for all main effects and interactions are reported in Table 2.

Table 2. Two-way (2 × 2) Factorial ANOVA Summary (salinity × fungal inoculation) for All Measured Response Variables, Based on $n = 3$ Independent Pots Per Treatment ($df = 1, 8$ for Each Term)

Response Variable	Salinity $F(1,8)$	Salinity p	Fungi $F(1,8)$	Fungi p	Salinity×Fungi $F(1,8)$	Interaction p
Seedling emergence (%)	12.00	0.009	0.00	1.000	3.00	0.122
Shoot length	180.19	<0.001	99.19	<0.001	9.19	0.016
Root length	70.33	<0.001	19.30	0.002	2.55	0.149
Fresh weight	270.75	<0.001	60.75	<0.001	0.75	0.412
Dry weight[1]	720.75	<0.001	168.75	<0.001	60.75	<0.001
Chlorophyll a	14138.7	<0.001	6244.9	<0.001	4128.5	<0.001
Chlorophyll b	904203.0	<0.001	30603.0	<0.001	6912.0	<0.001
Carotenoids	112560.3	<0.001	59536.0	<0.001	12882.3	<0.001
Xanthophyll[2]	3675.0	<0.001	1323.0	<0.001	243.0	<0.001
Proline	354.68	<0.001	11.05	0.010	1179.41	<0.001
Total phenolics	1418.73	<0.001	44.18	<0.001	3318.55	<0.001
Total lipids[3]	962.18	<0.001	368.73	<0.001	4.91	0.058
Soluble sugars	5547.0	<0.001	768.0	<0.001	11532.0	<0.001

Note. [2] Xanthophyll values are derived as a fixed fraction of total carotenoids (Section 2.3), rather than an independently resolved pigment pool. [3] Total lipid content (phospho-vanillin assay); not a direct measure of lipid peroxidation or membrane damage.

4. DISCUSSION

The study observed the stress in barley exposed to 150 mM salinity, that is, suppressed germination, root and shoot elongation, depleted biomass, degraded photosynthetic pigments, and an elevated burden of osmolytes and oxidative stress markers, fits the established physiological signature of salt stress in cereals. The especially severe reduction in root length (95%) relative to shoot length (64%) is consistent with reports that salinity disproportionately disturbs root mitotic activity and architecture, given that roots are the first tissue to encounter the saline solution and bear the immediate brunt of ionic and osmotic disruption before any signal reaches the shoot [49, 50]. The near-complete collapse in fresh and dry biomass (97-98%) at this single salinity level indicates that 150 mM represents a severe rather than marginal stress for this barley line under our pot conditions, a useful benchmark for future dose-response work even though only one concentration was tested in this study.

Salinity-induced decrease of chlorophyll a, chlorophyll b, carotenoids, and xanthophylls is broadly in agreement with previously reported effects on the activity of the chlorophyllase, the stability of the PS II reaction center, and the functioning of the Calvin cycle, which would result in a loss of pigments similar to that reported for PSII structure under salinity in barley and other cereals [21]. It is noteworthy that the reduction in xanthophyll content as compared to chlorophyll a and b is relatively large, suggesting the sensitivity of the xanthophyll cycle to oxidative stress, considering that xanthophylls are directly involved in photoprotection and are used for photoprotection during stress [51]. Simultaneously, the increase in proline, phenolics, flavonoids, and lipid content, along with the decrease in soluble sugars, suggests a synchronised stress response, whereby osmotic adjustment, secondary metabolite mediated antioxidant defense, and the change in membrane lipid turnover were simultaneously activated. Whereas, the carbohydrate me-

tabolism was disrupted, possibly due to reduced photosynthetic carbon fixation coupled with the diversion of carbon skeletons toward the biosynthesis of stress metabolites [52, 53]. These findings represent a phenotypic and biochemical association between *A. terreus* inoculation and salinity tolerance, rather than a mechanistically demonstrated pathway; targeted assays (e.g., MDA/TBARS, antioxidant enzyme activity) would be needed to directly confirm the physiological basis of this protection.

In view of the above, inoculation with *A. terreus* resulted in a general yet not complete recovery. Germination and shoot growth were also significantly improved (11-25%), with the greatest improvement seen in root length and dry biomass (30-109%), indicating that the fungus predominantly affected nutrient and water uptake at or in close association with the root-soil interface, where direct physical contact with fungal hyphae would be expected to have the greatest effect. The beneficial effect of *A. terreus* observed in the present study is also consistent with previous findings in other crop species. Saifullah et al. reported that *A. terreus* improved physiological and biochemical responses of *Allium sativum* under lead stress, supporting the broader potential of this fungus to enhance plant performance under adverse environmental conditions [54]. The pattern is similar to previous studies which established that *A. terreus* isolates from stressed or contaminated soils can grow inside the root systems of host plants and provide measurable abiotic stress tolerance, such as the ability to alleviate salinity and alkalinity stress in wheat through enhanced growth and various physiological parameters [38] and through the modulation of primary and secondary metabolism of wheat under salt stress [37]. This is consistent with more recent evidence that *aspergillus* species mitigate salinity stress across diverse hosts and salt concentrations. *A. niger* VN2, for example, alleviated oxidative stress and enhanced growth in *Vigna radiata* exposed to 150–250 mM NaCl, partly through improved antioxidant enzyme activity and reduced DNA damage [55]. The ability of *A. terreus* isolates to withstand extreme environmental conditions, such as effluent contaminated and metal stressed soils, has been observed in the fields of biocontrol and stress amelioration [36], supporting the fact that isolates obtained from stressed environments including the salt-affected rhizosphere of *P. hysterophorus* sampled here are suitable candidates to perform stress amelioration in other host species.

The results of chlorophyll a, b, carotenoid, and xanthophyll recovery after inoculation are consistent with the recovery of chloroplast integrity after inoculation under salinity, which has been reported in the literature due to the reduction of oxidative load on the photosynthetic apparatus by beneficial fungi [56]. The relative efficiency of the recovery of the photoprotective pigment pool (16% and 3% of carotenoids and xanthophylls were recovered, respectively) was smaller than that of the chlorophyll a and b photoprotective pools (32% and 25%, respectively), which may simply reflect differences in the turnover rate or biosynthetic priority of these pigment classes. However, it would require more time-course data than collected in this study for confirmation.

The decrease in the levels of proline, phenolics, flavonoids, and lipids in inoculated salt stressed plants and the partial recovery of soluble sugars might be the most telling, when inferring mechanism. What is seen is not a series of individual stress markers ‘recovering. Rather, the pattern

seems to be that all the markers react to one negative stressor: the decrease in oxidative and osmotic forces that initially caused them to accumulate. The osmoregulatory and/or ROS-scavenging response to salinity-induced stress [52] is also demonstrated by the reduction in proline contents towards the baseline after inoculation, indicating that the fungus was able to alleviate the osmotic stress sufficiently, so that there was no longer any need for ROS-scavenging and/or osmoregulatory defense at the same level. The same can be said for the phenolic pool and the flavonoid pool, which build up under stress, and their decrease following inoculation is likely to indicate the decrease of the oxidative burden, rather than the loss of antioxidant capacity [38]. This is further supported by the partial recovery of soluble sugars, as improved photosynthetic function, indicated by the pigment data, would also lead to enhanced carbohydrate availability which is also needed for osmotic adjustment and general metabolism, as previously reported for sugars in salt-stressed cereals.

Some other factors that might contribute, but were not directly tested in this work, are worth calling out as they are well supported in the general literature of aspergillus-mediated rhizospheric fungal stress amelioration. These include the ability to produce antioxidant enzymes to withstand and remove high levels of salinity reported for salt-tolerant fungal isolates growing in saline soil and to produce organic acids and solubilze phosphates, which has been reported for aspergillus species colonizing stressed plant roots [33, 31]. The widespread physiological recovery and the isolate's source from a salt-affected rhizosphere suggests that multiple signaling pathways are more likely to have been responsible for the physiological recovery observed, with improved nutrient acquisition and reduction of the ionic load reaching the root likely being the most important. This is an inference, rather than a demonstrated mechanism; in this study, tissue Na+ and K+ content, organic acid production, and antioxidant enzyme activity of fungi were not quantified directly.

The novelty of this work is the discovery of a specific isolate of *A. terreus*, recovered from the rhizosphere of a non-crop weed, and the demonstration of its ability to provide broad-spectrum protection to barley against salinity under a range of germination, growth, pigment, and biochemical stress endpoints. The literature on using fungal salinity amelioration in cereals focuses on bacterial PGPR consortia or on arbuscular mycorrhizal fungi. Indeed, there have been only a few studies where individual strains of aspergillus have been isolated and characterised for their properties of salinity amelioration in barley. Hence, this study contributes a data point to the latter group and does so through a relatively extensive set of physiological and biochemical endpoints that are not measured exclusively by growth. The practical advantage of using a fungal isolate from a weed that colonizes the marginal salt-affected land is also appealing for biofertilizer prospecting, as it does not involve the maintenance and import of specialized microbial collections.

However, some of the results are subject to some limitations in generalizability. Only one barley genotype and one level of salinity (150 mM) were investigated. Further, the dose-response relationship and possible genotype-dependent variation in the fungal response have yet to be fully characterized. The trial was conducted on pots under controlled lab conditions and over a relatively short growth period. However, the same protections cannot be as-

sumed in field conditions where soil heterogeneity, native microbial competition, and the variable field environmental conditions would all be factors creating differences. We did not quantify directly the content of Na+, K+, and Ca2+ in the tissue, the activity of antioxidant enzymes (including superoxide dismutase, catalase, or peroxidase), or the amount of production of phytohormones by the fungi, which could distinguish among the candidate mechanisms discussed above, leaving them as plausible inferences based on the existing biochemical data. Finally, only one fungal isolate was evaluated and thus it is not possible to conclude whether the degree of protection observed here is typical of *A. terreus* or is specific to this isolate as compared to other isolates from the same or different sites.

4.1. Conclusion

High salinity of 150 mM caused severe phytotoxic effects on barley, such as the reduction of germination and vegetative growth, decrease in photosynthetic pigments, disturbance of carbohydrate metabolism, and increase in proline, phenolics, flavonoids, and lipids. Rhizospheric isolate of *A. terreus* from *P. hysterophorus* rhizosphere significantly ameliorated this damage across all parameters, moving seedling emergence, growth, and pigment content substantially toward (but in most cases not fully back to) control levels and shifting the biochemical stress parameters and soluble sugar pool toward control levels. The treated, salt-stressed plants nonetheless remained statistically distinct from the unstressed control for most variables (Duncan's test letters, Table 1). The results indicate that *A. terreus* is a potential candidate to be considered in the development of biofertilizers to enhance the productivity of barley on salt-affected soils and highlight the need for further research across different salinity levels and barley cultivars. Based on a single isolate tested at one salinity level in autoclaved soil, *A. terreus* shows promise as a candidate inoculant under controlled conditions. Moreover, strain-level biosafety and metabolite screening, together with validation in non-sterile soil and under greenhouse/field conditions, are needed before any practical agricultural application can be considered.

Author Contribution

Naveen Dilawar: conceptualization, writing - review & editing, supervision. **Hafiz Zeeshan Wadood:** investigation. **Hira Bashir:** formal analysis, writing - review & editing. **Maria Muhammad:** conceptualization, writing - original draft. **Karishma Sahar:** methodology. **Muhammad Usman:** formal analysis. **Shakir Ullah:** writing - review and editing. **Rahid Khan:** writing - review and editing. **Habib Allah:** writing - original draft.

Conflict of Interest

The authors of the manuscript have no financial or non-financial conflict of interest in the subject matter or materials discussed in this manuscript.

Data Availability Statement

Data supporting the findings of this study will be made available by the corresponding author upon request.

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