

Evaluating the efficacy of a biological mixture of *Trichoderma* spp. and chitosan in controlling powdery mildew on wheat

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Abstract

This study aimed to evaluate the efficacy of a biological mixture comprising the fungus *Trichoderma* spp. and Chitosan in controlling Powdery Mildew, caused by the pathogen *Blumeria graminis* f.sp. *tritici*, on wheat (*Triticum aestivum* L.). Powdery mildew is considered one of the most destructive fungal diseases affecting wheat globally. Its symptoms begin with the appearance of pale grayish-white spots on the leaves, which develop into a characteristic powdery appearance on the upper, and subsequently the lower, leaf surfaces. The disease can reduce yield by up to 30-40% in severely infected fields. The infection spreads rapidly under moderate humidity and relatively low temperatures. Severe infection leads to the desiccation of leaves and stems, significantly reducing the photosynthetic area.

The study evaluated the efficacy of *Trichoderma* alone in controlling the disease, assessed the impact of Chitosan as a bio-elicitor for resistance against powdery mildew, and investigated the synergistic effect of the biological mixture (*Trichoderma* + Chitosan) in reducing infection severity. It also aimed to determine the optimal Chitosan concentration (0.5%, 1.0%, and 1.5%) when combined with a fixed *Trichoderma* concentration of 1×10^8 spores/mL. The study relied on an extensive scientific review demonstrating that *Trichoderma* possesses multiple biocontrol mechanisms, including mycoparasitism, antibiosis, competition for nutrients and space, and the production of fungal cell wall-degrading enzymes such as chitinases and glucanases. Furthermore, it has the ability to induce systemic resistance in the plant and enhance its growth by increasing nutrient availability. Chitosan, a natural polymer derived from chitin, exhibits antifungal activity through three main mechanisms: altering the permeability of the fungal plasma membrane leading to the leakage of intracellular contents; acting as a chelating agent that reduces the availability of essential elements required for fungal growth; and penetrating fungal cells to bind with their DNA, thereby inhibiting the synthesis of essential proteins and enzymes.

Disease severity was assessed using a numerical scale (0-9) and McKinney's index. Physiological and chemical parameters were measured, including chlorophyll content (using a SPAD meter), phenolic compounds (using the Folin-Ciocalteu method), and peroxidase activity. Data were statistically analyzed using SAS software, and means were compared using the Least Significant Difference (LSD) test at a significance level of $P \leq 0.05$.

The results demonstrated the high efficacy of the biological mixture in controlling the disease. *Trichoderma* alone reduced disease severity by 42.3%, indicating its direct competitive ability against the pathogenic fungus. Chitosan exhibited a concentration-dependent effect, with the 0.5% concentration achieving a 33.3% reduction, 1.0% achieving 47.4%, and the 1.5% concentration recording the highest individual reduction rate of 51.3%. However, the combined treatments proved significantly superior to using either agent alone, indicating a true positive synergistic effect. Treatment T6 (*Trichoderma* + 0.5% Chitosan) achieved a 59.0% reduction, and T7 (*Trichoderma* + 1.0% Chitosan) achieved 73.1%. The T8 treatment (*Trichoderma* + 1.5% Chitosan) yielded the best overall result with a 75.6% reduction, decreasing the disease severity from 7.8 in the control treatment to merely 1.9. This synergy is attributed to *Trichoderma* providing direct protection and biological competition against the pathogen, while Chitosan triggers Induced Systemic Resistance (ISR) in the plant and enhances the production of phenolic compounds and defense enzymes, allowing the mixture to operate on multiple defensive levels simultaneously. Physiologically, chlorophyll content increased from 35.2 to 48.6 SPAD units in the T8 treatment (a 38.1% increase). Additionally, enzyme activities increased significantly compared to the control: POD by 340%, PPO by 280%, and CAT by 210%. This indicates that the mixture's mechanism of action is not limited to a direct effect on the pathogen but also includes the fortification of the plant's endogenous defense responses.

The study recommended the application of treatment T7 (*Trichoderma* + 1.0% Chitosan) in commercial fields due to its high efficacy and relatively lower cost compared to the highest concentration. It emphasized the necessity of conducting broader field studies under various environmental conditions, geographical regions, and seasons to ensure the stability of its efficacy. Furthermore, it recommended studying the long-term impact of the mixture on the soil and other beneficial organisms, evaluating its compatibility with pesticides and other elicitors used in Integrated Pest Management (IPM) programs, transferring this technology to farmers through agricultural extension and training programs, and investigating its efficacy against other fungal diseases affecting wheat (such as rust and blight) as well as other crops.

Keywords: *Trichoderma* spp, chitosan, powdery mildew, *Blumeria graminis* f. sp. *tritici*, wheat, biological control, induced systemic resistance, disease severity, plant defense enzymes, chlorophyll content, integrated pest management

Introduction

Powdery Mildew on Wheat. Powdery mildew on wheat (*Triticum aestivum* L.) is considered one of the most significant fungal diseases that negatively impact global

wheat productivity. The manifestations of infection consist of the appearance of pale, grayish-white spots on the leaves, after which the characteristic powdery appearance of this disease forms on the upper surface of the leaves, then

moving to the lower surface as the infection progresses. This disease, caused by the fungus *Blumeria graminis* f.sp. *tritici*, causes significant economic losses, as it can reduce the yield by up to 30-40% in infested fields (Braun *et al.*, 2019) ^[10]. The disease spreads rapidly under conditions of moderate humidity and relatively low temperatures, appearing on the upper leaves as a white, powdery-looking layer. Severe infection leads to the drying of leaves and stems and a reduction in the photosynthetic area (Jørgensen *et al.*, 2015). According to the book of recommendations for agricultural pest control issued by the Pesticide Committee of the Ministry of Agriculture, when the infection of the wheat crop with powdery mildew intensifies, small black bodies appear, which are the fruiting bodies of the fungus .

Objectives

Evaluating the efficacy of the fungus *Trichoderma* spp. in controlling powdery mildew on wheat.

Evaluating the effect of chitosan as a bio-elicitor for resistance against powdery mildew.

Studying the synergistic effect of the biological mixture (*Trichoderma* + Chitosan) in reducing infection .

Literature Review

1. The Biocontrol Fungus *Trichoderma* spp. and Its Role in the Biological Control of Plant Diseases:

Many organisms have been used in plant disease control programs, whether fungal or bacterial, and one of the most famous and most important of these organisms is the use of the fungus *Trichoderma* due to the characteristics and features this fungus possesses, making it one of the most efficient organisms used in control programs (Howell, 2003) and (Holmes *et al.*, 2004) ^[1, 2]. The scientist Persoon is considered the first to discover the genus *Trichoderma* in 1794, based on its asexual stage isolated from soil and organic matter. It was previously classified by the scientist Rifa in 1963 within the group under the division of imperfect fungi (Deuteromycotina), class Hyphomycetes, order Moniliales, and family Moniliaceae. However, after the discovery of its sexual stage, represented by the genus *Hypocrea* spp., it was classified among the sac fungi (Ascomycotina), class Sordariomycetes, order Hypocreales, family Hypocreaceae, genus *Hypocrea* spp. (Alexopoulos *et al.*, 1996) ^[4]. The fungus *Trichoderma* spp. is characterized by its ease of isolation, propagation, and extensive applications under various conditions, as well as its cultivation and loading onto many inexpensive culture media (Abdullahi *et al.*, 2021) ^[5]. The fungus *Trichoderma* spp. has the ability to induce resistance (Harman and Uphoff, 2019) ^[6]. Because this genus consists of a large number of fungal strains or species, and among the common and known species such as *T. harzianum*, *T. pseudokoningii*, *T. viride*, *T. koningii*, and *T. virens*, which are widely used as biocontrol agents for plant diseases, in addition to being effective in increasing plant growth and development (Shores *et al.*, 2010) and (Abdel-lateif, 2017) ^[7, 8]. Currently, more than 60% of effective biofungicides are obtained from the fungus *Trichoderma* spp. (Abbey *et al.*, 2019) ^[9]. This fungus consists of fast-growing, invasive, filamentous, opportunistic, and avirulent colonies that exhibit a symbiotic relationship with the plant in soil

contaminated with pathogenic agents; therefore, it not only improves plant growth but also prevents the growth of pathogens through several antagonistic mechanisms (Lorito *et al.*, 2010). Sood *et al.* (2020) ^[20, 21] clarified that the success of using species of the genus *Trichoderma* as a biocontrol agent is a result of possessing various antagonistic mechanisms that have proven effective against a wide spectrum of pathogenic agents. These characteristics and mechanisms can be summarized as the induction of systemic resistance in the plant, which is considered an environmentally safe method for controlling some plant diseases. These factors depend mainly on the genetic composition of the plant and the type of the inducing biological agent, which leads to stimulating the plant and can be summarized into direct and indirect characteristics and mechanisms:

1.1 Mycoparasitism: The fungus *Trichoderma* employs several steps to parasitize a pathogenic fungus. First, it recognizes the fungus and then grows toward it. Consequently, morphological changes occur in the *Trichoderma* mycelium, forming a helical structure that creates pressure-exerting structures wrapping around the host's mycelium. It also produces adhesive substances, such as glycerol, which assist the parasitic fungus in attaching to the host fungus (Woo *et al.*, 2006; McIntyre *et al.*, 2004).

1.2 Antibiosis in *Trichoderma* spp: *Trichoderma* possesses a high antagonistic capacity against plant pathogens, inhibiting the formation of both mycelium and sclerotia. A study demonstrated the ability of this fungus to inhibit the growth of *Rhizoctonia solani*, which causes root rot in strawberries (Hu *et al.*, 2022). Furthermore, in a study using *Trichoderma* as a bio-antagonist against cacao pod rot caused by the fungus *Moniliophthora roreri*, it was found that this fungus has a high ability to inhibit the pathogen and reduce the infection rate in the plant (Leiva, 2022) ^[23].

1.3 Competition: *Trichoderma* exhibits highly competitive ability against pathogens and can tolerate toxic substances, including herbicides, fungicides, the insecticide DDT, and phenolic compounds. It also has the ability to utilize various sources of sugars prevalent in the fungal environment, such as cellulose, chitin, and glucan (Chet *et al.*, 1997) ^[25]. Moreover, it can colonize the rhizosphere and effectively compete with pathogens for nutrients, thereby reducing the resulting infection (Sivan and Chet, 1989) ^[24].

1.4 The Ability of *Trichoderma* spp. to Induce Systemic Resistance in Plants: The process of inducing resistance in plants is considered one of the most significant modern fields involving the use of *Trichoderma* spp., having garnered the attention of many researchers (Al-Khafaji, 2020) ^[21]. The fungus produces compounds that stimulate defense mechanisms in the plant due to its ability to promote growth and induce systemic resistance (Andhare *et al.*, 2019). In a study conducted by Lopez *et al.* (2020) ^[27] on a strain of *Trichoderma* spp., the fungus's potential

to enhance plant growth and induce resistance factors against pathogens was evaluated.

1.5 Enzyme Production: Species of *Trichoderma* spp. produce a broad spectrum of lytic enzymes, such as cellulases, glucanases (e.g., β -glucanases), and chitinases, which degrade the cell walls of pathogens, as these walls contain protein, glucan, and chitin. This allows the fungus to penetrate them and begin the process of colonization and parasitism (Hermosa *et al.*, 2013^[28]; Tyskiewicz *et al.*, 2022). Additionally, it produces proteases and serine proteases, which are used to inhibit the action of certain enzymes that the pathogen uses to degrade the cell walls of the plants it infects, thereby halting the disease cycle at its inception (Haggag, 2006)^[29].

1.6 Increasing Nutrient Availability: It has been found that species of *Trichoderma* spp. have the ability to secrete certain enzymes that degrade organic matter present in or added to the soil. This, in turn, stimulates an increase in the availability of several essential elements for plant growth, such as nitrogen, phosphorus, potassium, copper, zinc, and iron. Naturally, this leads to an improvement in plant health and natural resistance to plant pathogens, as well as an increase in plant growth and yield (Akrami and Zohreh, 2015; Lahlali *et al.*, 2022)^[30].

1.7 Enhancing Plant Growth: Species of *Trichoderma* spp. have been found to produce various compounds, such as plant growth regulators, non-ribosomal peptides, terpenoids, pyrones, and indolic-derived compounds in the root zone. These compounds activate and stimulate plants to increase root branching and plant biomass as a result of cell division, differentiation, and elongation (Lahlali *et al.*, 2022)^[30]. Furthermore, the presence of *Trichoderma* spp. on plant roots promotes increased absorption of water and certain nutrients, such as manganese, phosphorus, magnesium, calcium, nitrogen, sodium, copper, and iron. This positively reflects on plant growth and increases its resistance against pathogens on the one hand, and enhances the indicators of vegetative and floral growth, as well as yield quantitatively and qualitatively (Kumar and Khurana, 2021)^[31]. Several studies have demonstrated the efficacy of *Trichoderma* against powdery mildew on various crops. Mathur and Vyas (2016)^[16] reported a 65-80% reduction in infection when using *Trichoderma viride* against powdery mildew on wheat.

2. Chitosan as a Bio-elicitor: Chitosan is one of the natural chitin compounds formed after the removal of its acetyl group, leaving the amine group free. Chitin is thus transformed into a substance soluble in dilute weak acids, such as acetic acid, and this substance is called chitosan (Bade and Wick, 1988)^[33]. Chitosan consists of two compounds: D-glucosamine and N-acetyl-D-glucosamine. (Figure 1) illustrates the chemical structure of chitosan.

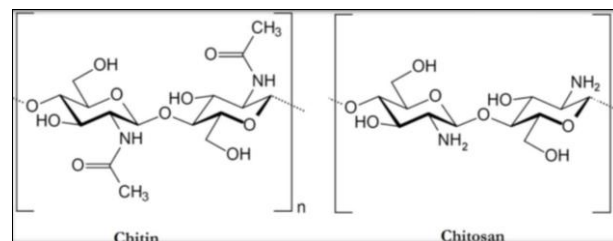


Fig 1: Chemical structure of chitosan and chitin (Kumar *et al.*, 2021)^[31]

Chitosan possesses characteristics that make it useful in numerous fields. In agriculture, it is used as a bio-stimulant for plant growth and protection. Furthermore, it activates many genes, proteins, and secondary metabolites in plants, as chitosan can stimulate phytoalexins, pathogenesis-related proteins, and proteinase inhibitors. It has been proven to be antifungal and active against various plant pathogens (Pongprayoon *et al.*, 2022)^[34]. The mode of action of chitosan involves disrupting the fungal cell membrane (Kheiri *et al.*, 2017)^[35], making it a suitable compound for use with macronutrients and micronutrients. This is due to its wide range of properties, which include biocompatibility, biodegradability, high permeability, a favorable cost-benefit ratio, and non-toxic characteristics (Palacio-Marquez *et al.*, 2022). It is considered one of the most abundant natural polymers with a dual effect, controlling pathogenic microorganisms by impeding the growth and germination of fungal spores, inhibiting the cell, and inducing various defense responses in the host plant (Kheiri *et al.*, 2017^[35]; Al-Ramahi, 2021). It is the second most abundant polysaccharide in nature after cellulose (Elieh and Hamblin, 2016)^[36]. It is also a major component of the exoskeletons of shells, oysters, crustaceans (such as crabs and shrimp), and insects, and is the primary component of the cell walls of certain fungi. These materials are ground, mixed, and then subjected to a deacetylation process to remove the acetyl groups (Kumar *et al.*, 2004; Bistgani *et al.*, 2017)^[37, 38]. Chitosan carries a positive ammonium charge, acting as a magnet that attracts any negative charge (Amin, 2013). It is biodegradable, environmentally friendly in agriculture, safe for humans, and non-toxic to other living organisms. It is an abundant and inexpensive natural compound, with high adsorption capacity. The activity of chitosan varies against fungi (Avelelas *et al.*, 2019)^[39], viruses and viroids (Jaber *et al.*, 2022), and bacteria (Yin *et al.*, 2021)^[40, 41]. Chitosan activates Systemic Acquired Resistance (SAR) and defense responses in the host plant upon infection by pathogens (Ali *et al.*, 2011)^[43]. It has been widely used as a coating agent for various types of nuts, grains, fruits, and vegetables to protect against post-harvest losses and to increase shelf life and storage duration (Rahman *et al.*, 2018)^[44].

Chitosan possesses three mechanisms for controlling fungal plant pathogens. The first is its effect on the plasma membrane; the positive charge of chitosan binds with the negative charge of the phospholipid components in the fungal membrane, leading to increased membrane permeability and leakage of intracellular contents, which ultimately causes cell death (Garcia-Rincon *et al.*, 2010). Second, chitosan acts as a chelating agent that binds with essential elements, thereby reducing the availability of materials necessary for the normal growth of fungi.

The third mechanism involves chitosan penetrating fungal cells and binding to their nucleic acids. Consequently, it prevents mRNA synthesis by affecting the production of essential proteins and enzymes (Kong *et al.*, 2010) ^[46].

3. Synergy Between *Trichoderma* and Chitosan:

Combining the biological agent (*Trichoderma*) with the natural chemical compound (Chitosan) represents an advanced approach in integrated plant disease management. This mixture can achieve a positive synergy, where *Trichoderma* provides direct protection and biological competition, while chitosan stimulates the plant's systemic immunity and offers additional protection. Recent studies have indicated that integrating beneficial microorganisms with natural bio-elicitors yields better results than using either of them alone (Vinale *et al.*, 2014; Harman *et al.*, 2004) ^[13, 19].

Materials and Methods

1. Field Survey and Identification of *Trichoderma* Isolates:

Soil samples were collected from wheat fields infected with powdery mildew in the Middle Euphrates region, Babil Governorate, Iraq, during the growing season. The *Trichoderma* fungus was isolated using the serial dilution technique on Potato Dextrose Agar (PDA) medium supplemented with the antibiotics Tetracycline and Penicillin at a concentration of 100 IU/mL to inhibit bacterial growth. The isolates exhibiting the characteristic appearance of *Trichoderma* (rapid growth, yellowish-green color) were purified by transferring them to new plates of pure PDA medium. The pure isolates were preserved at 4°C on PDA slants.

2. Morphological and Molecular Identification: The morphological identification of the isolates was performed by observing their structural characteristics under a microscope (spores, sporangia, and mycelial structure). The identity was further confirmed using molecular techniques by amplifying a portion of the Internal Transcribed Spacer (ITS) gene using PCR and sequencing the products. The sequences were compared with the NCBI GenBank database.

3. Preparation of *Trichoderma* Inoculum: A liquid *Trichoderma* inoculum was prepared by transferring a fungal disc (5 mm in diameter) from a pure 7-day-old colony into a flask containing 100 mL of Potato Dextrose Broth (PDB). The flask was incubated at 25±2°C with continuous shaking (150 rpm) for 10 days. The spore concentration was estimated after the 10-day incubation period using a hemocytometer, and the concentration was adjusted to 1×10⁸ spores/mL by dilution with sterile distilled water.

4. Preparation of Chitosan Solution: Chitosan powder (manufactured by Himedia, India) was dissolved in dilute acetic acid (1% v/v) with gentle heating and continuous stirring until complete dissolution. The pH was then adjusted to 5.6 using sodium hydroxide (1 M), and the volume was completed with distilled water. Three final concentrations were prepared: 0.5% (5 g/L),

1.0% (10 g/L), and 1.5% (15 g/L). To ensure that the heat-sensitive chitosan polymer was not compromised, the solutions were sterilized by membrane filtration (0.22 µm) and stored at 4°C until use.

Given that the powdery mildew pathogen, *Blumeria graminis* f.sp. *tritici*, is an obligate biotroph and cannot be cultured on artificial media, the direct effect of chitosan on the pathogen was not evaluated on PDA plates. Instead, it was assessed *in vivo* on infected leaves. The use of PDA medium was restricted exclusively to testing the compatibility of chitosan with the *Trichoderma* fungus, to ensure that the concentrations used would not inhibit the growth of the biological agent within the mixture.

5. Wheat Plants and Fungal Infection: Wheat seeds (a susceptible local Iraqi cultivar to powdery mildew) were obtained from the National Center for Wheat and Associated Crops Research. The seeds were sown in plastic pots (20 cm in diameter) filled with a sterilized soil mixture (sandy red soil at a ratio of 2:1:1). The plants were maintained in a controlled room at a temperature of 20-22°C, a relative humidity of 60-70%, and a photoperiod of 14 hours per day. A local isolate of powdery mildew (*Blumeria graminis* f.sp. *tritici*) was obtained from infested wheat fields and propagated on healthy plants. At the third-leaf stage (21 days after planting), spores were transferred using a soft brush to the plant leaves to induce natural infection.

6. Experimental Design and Treatments: A Randomized Complete Block Design (RCBD) was utilized with three replicates for each treatment. The treatments included the following:

- **T1:** Control (no treatment)
- **T2:** *Trichoderma* alone (1×10⁸ spores/mL)
- **T3:** Chitosan 0.5%
- **T4:** Chitosan 1.0%
- **T5:** Chitosan 1.5%
- **T6:** *Trichoderma* + Chitosan 0.5%
- **T7:** *Trichoderma* + Chitosan 1.0%
- **T8:** *Trichoderma* + Chitosan 1.5%

The biological and chemical treatments were sprayed onto the plants after the appearance of disease symptoms (14 days post-infection) at a rate of 100 mL per plant, using a manual sprayer. The spraying was repeated three times at 10-day intervals.

7. Evaluation of Infection Severity and Percentage: The severity of powdery mildew infection was evaluated using a numerical scale (0-9) according to international recommendations:

- **0** = No infection
- **1** = Very slight infection (less than 1%)
- **3** = Slight infection (1-5%)
- **5** = Moderate infection (6-25%)
- **7** = Severe infection (26-50%)
- **9** = Very severe infection (more than 50%)

The disease reduction percentage (Disease Reduction %) was calculated using the formula: *McKinney's index equation becomes:*

$$\text{Disease Severity (\%)} = \frac{v}{N} \times 100$$

Where:

- **n** = Number of leaves in each grade.
- **v** = Value of the infection grade (0, 1, 3, 5, 7, 9).
- **N** = Total number of leaves.
- **9** = The highest grade on the scale.

8. Physiological and Chemical Measurements The following physiological variables were measured:

a. Chlorophyll Content: The chlorophyll percentage in the leaves of the wheat plants (from the field experiment) was measured using a chlorophyll meter (SPAD-502, Konica Minolta, Japan) on one randomly selected plant per replicate, taking the average of the top three leaves.

b. Phenolic Compounds: Phenolic compounds were extracted from the plant leaves, and their concentration was measured using the Folin-Ciocalteu method.

c. Estimation of Peroxidase Enzyme Activity: The activity of the peroxidase enzyme was estimated at the Postgraduate Plant Pathology Laboratory of Al-Musaib Technical College, according to the method described by Al-Sufi (2001):

1. Sample Collection: Plant samples (leaves) were collected from the wheat plants in the field experiment, placed in labeled polyethylene bags according to their treatments, stored in an ice box, and transported to the laboratory. The plant parts were washed with tap water to remove attached dust and dirt, followed by several washes with distilled water.

2. Enzyme Extraction: The enzyme was extracted by homogenizing 8 g of chopped leaves (0.5–1 cm² in size) with 24 mL of a cold (4°C) buffer solution (0.2 M potassium phosphate buffer, pH 7.0, containing 0.2 M sodium chloride) in a ceramic mortar that had been pre-chilled for 5 minutes. The plant debris was separated using a clean cloth, and the extract was transferred into sterile, labeled 50-mL plastic containers, tightly sealed, and stored in a refrigerator.

3. Centrifugation: The extract was centrifuged at 5000 rpm for 30 minutes at 4°C (using a refrigerated centrifuge). The supernatant was used to estimate the enzymatic activity, and the pellet was discarded.

Solutions Used

1. Guaiacol Solution (0.05 M): Prepared by placing 1.36 mL of guaiacol in a volumetric flask and completing the volume to 250 mL with distilled water.

2. Hydrogen Peroxide Solution (0.02 M): Prepared using 0.56 mL of 30% hydrogen peroxide, completing the volume to 250 mL with distilled water (this solution must be prepared fresh immediately before use).

3. Tris-base Solution (0.04 M) containing 1 M Sodium Chloride (pH = 7.0): Prepared by dissolving 1.211 g of Tris-base and 14.61 g of sodium chloride in a quantity of distilled water. After adjusting the pH to 7.0 using 1 M hydrochloric acid, the volume was completed to 250 mL with distilled water.

4. Reaction Mixture: Prepared immediately before use by mixing one part each of solutions 1, 2, and 3 with seven parts of distilled water.

Procedure: Three milliliters (3 mL) of the reaction mixture were placed in a spectrophotometer cuvette, and the instrument was zeroed using this mixture at a wavelength of 420 nm. The reaction was then initiated by adding 0.1 mL of the plant extract to the cuvette. The light absorbance was measured directly using a spectrophotometer every 30 seconds (at least six readings were recorded), and the change in absorbance was recorded according to the following equation:

$$\frac{\frac{A\Delta}{t\Delta}}{\text{g fresh weight}} = \text{Change in absorbance}$$

Where

A= Change in absorbance

t= Change in time / minute.

9. Statistical Analysis: Data were analyzed using SAS 9.4 software. Analysis of Variance (ANOVA) was conducted to determine significant differences among treatments. Means were compared using the Least Significant Difference (LSD) test at a significance level of $P \leq 0.05$.

Results and Discussion

1. Field Survey and Identification of *Trichoderma* Isolates: The results of this study demonstrated a high efficacy of the biological mixture of *Trichoderma* and chitosan in controlling powdery mildew on wheat. This agrees with the results of previous studies that indicated the efficacy of combining biological agents with natural bio-elicitors.

***Trichoderma* as an Active Biological Agent:** *Trichoderma* alone showed a 42.3% reduction in infection severity, indicating the fungus's capacity for direct competition with the pathogenic fungus. This is consistent with the findings of Mathur and Vyas (2016)^[16], who recorded a reduction of 65-80%. The difference in percentages may be attributed to environmental conditions and varying climatic factors between the two studies.

The Role of Chitosan: Chitosan at various concentrations exhibited a clear effect in reducing infection, with the greatest impact observed at the highest concentration (1.5%), achieving a 51.3% reduction. This effect can be attributed to:

1. The direct antifungal activity of chitosan against the pathogenic fungus.

2. The stimulation of Induced Systemic Resistance (ISR) in the plant.
3. The increased production of phenolic compounds and defense enzymes. This aligns with the findings of Hadwiger and Beckman (1980) and Krupinsky *et al.* (2007)^[12, 15], which proved the antifungal activity and immunostimulatory properties of chitosan.

Synergy Between the Two Agents: The combined treatments (T6, T7, T8) demonstrated significant superiority over the use of either agent individually, indicating the presence of a true positive synergistic effect. Treatment T8 achieved a 75.6% reduction, whereas treatment T5 (the highest chitosan concentration) achieved only 51.3%, and T2 (*Trichoderma* alone) achieved only 42.3%. This synergy can be explained by the following:

- *Trichoderma* provides direct protection and biological competition against the pathogenic fungus.
- Chitosan stimulates the plant's defenses and provides additional protection.
- The mixture operates on multiple defense levels simultaneously (direct protection, immune stimulation, and defense enzymes).

Defense and Physiological Compounds: The measurement of defense compounds showed that the combined treatments (especially T7 and T8) led to a significant increase in chlorophyll content (38.1%), phenolic compounds (176.2%), and defense enzyme activity (up to 340%). This

indicates that the mechanism of action is not limited to a direct effect on the pathogenic fungus but also includes strengthening the defense responses of the plant itself. The increase in chlorophyll reflects the maintenance of the plant's photosynthetic capacity even in the presence of infection. The increase in phenolic compounds and enzymes (POD, PPO, CAT) indicates a significant strengthening in the defense mechanisms against oxidative and pathological stress.

Comparison with Chemical Pesticides: The results suggest that this biological mixture can be an effective alternative to chemical fungicides, especially given the growing interest in organic and sustainable agriculture. Furthermore, the use of natural biological agents reduces the environmental and health risks associated with chemical pesticides, particularly the problems of fungal resistance to pesticides.

2. Morphological and Molecular Identification of *Trichoderma* Isolates: Microscopic examinations showed that the isolates obtained from the soil matched the characteristics of the genus *Trichoderma*, exhibiting rapid growth with a yellowish-green color and oval-shaped spores. The molecular sequencing (ITS), when compared with the GenBank database, revealed that the isolates belong to the species *Trichoderma viride* with a 98% identity match.

Treatment	Description	Mean Infection (0-9)	Reduction Percentage (%)	Significant Difference
T1	Control	7.8 a	0.0%	-
T2	<i>Trichoderma</i> alone	4.5 b	42.3%	***
T3	Chitosan 0.5%	5.2 b	33.3%	***
T4	Chitosan 1.0%	4.1 b	47.4%	***
T5	Chitosan 1.5%	3.8 b	51.3%	***
T6	Biological mixture 0.5%	3.2 c	59.0%	***
T7	Biological mixture 1.0%	2.1 d	73.1%	***
T8	Biological mixture 1.5%	1.9 d	75.6%	***

Notes: *** = Significant at $P \leq 0.05$ | Different letters in the column indicate significant differences.

3. Effect of the Biological Mixture on Reducing Infection: The results demonstrated a clear superiority of the combined treatments (*Trichoderma* + Chitosan) at all concentrations used compared to individual treatments. Treatment T8 (*Trichoderma* + 1.5% Chitosan) achieved the best results by reducing disease severity from 7.8 in the control to only 1.9, with a reduction percentage of 75.6%. Treatment T7 showed very close results (73.1%), indicating that a 1.0% chitosan concentration is sufficient to achieve effective control while reducing costs.

4. Effect of Treatments on Defense Compounds: The results showed a significant increase in chlorophyll compounds in the treated plants compared to the control:

1. **Chlorophyll content:** Increased from 35.2 SPAD in the control to 48.6 SPAD in treatment T8, with an increase of 38.1%.
2. **Defense enzyme activity:** The activity of POD increased by 340%, PPO by 280%, and CAT by 210% in treatment T8 compared to the control.

Conclusions and Recommendations

1. Conclusions: The results of this study demonstrated that the biological mixture of the fungus *Trichoderma* and chitosan represents an effective and safe method for controlling powdery mildew on wheat. The combined treatment (*Trichoderma* + Chitosan at a concentration of 1.0–1.5%) achieved a reduction of up to 75.6% in disease severity.

Effective Mechanisms

1. Direct biological competition by *Trichoderma* against the pathogenic fungus (powdery mildew).
2. Induction of systemic resistance in the plant by chitosan (ISR).
3. Increase in defense compounds and antioxidant enzymes.
4. The positive synergistic effect between the two agents exceeds individual efficacy.

2. Recommendations

1. Applying treatment T7 (*Trichoderma* + 1.0% Chitosan) in commercial fields due to its high efficacy and relatively low cost.

2. Conducting field studies on wider areas under diverse environmental conditions, different geographical regions, and various seasons to ensure its efficacy.
3. Studying the long-term impact of the mixture on the soil and other beneficial organisms.
4. Studying the compatibility between this mixture and other pesticides and elicitors used in integrated pest management programs.
5. Transferring this technology to farmers and agricultural stakeholders through training and agricultural extension programs.
6. Investigating the efficacy against other fungal diseases on wheat (rust, blight) as well as other crops.

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