

Green Nanotechnology Approach for Mitigating Trichoderma Threats using Ajwain-derived Silver Nanoparticles

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ABSTRACT

Trichoderma species , particularly those responsible for the green Mold disease , pose a major threat to the commercial cultivation of button mushroom (*Agaricus bisporus*) , leading to a significant yield losses ,reduced quality, and increased production costs, conventional chemical fungicide have shown declining effectiveness due to resistance development and environmental concerns , creating an urgent need for eco- friendly alternatives . in the present study a bio fungicide was formulated using *Trachyspermum ammi* (ajwain) seed extract , known for its antimicrobial activity and antifungal phytochemicals, enhanced through green synthesis of silver nanoparticles(ag NPs) using the same extract as the reducing and stabilizing agent this green synthesis combined the intrinsic antifungal activity if ajwain with the high surface reactivity of a nano silver, producing a synergetic effect against *Trichoderma* species ,and interfere with some spore germination thereby inhibiting the colonization in mushroom substrates. The use of plant- based reducing agents ensures non- toxic , sustainable nanoparticles production, making the formulation suitable for agricultural applications. Overall, the ajwain - mediated Ag Np's bio fungicide demonstrates string potential as an effective and environmentally safe to mange mushroom cultivation.

Keywords: Mushroom cultivation, *Trichoderma* species, silver nanoparticles, Green Mold

I. INTRODUCTION

There are numerous mushroom species that can be used as a food source. Edible mushroom can be considered healthy food with low calorific value and low content of fat but rich in proteins , dietary fibers and minerals . Their nutritional value , antioxidant

activity, and therapeutic properties ,as well as their flavor and unique texture , make them attractive for use as a food ingredient or additive replacement in food. The most widely used and cultivated for use as a food ingredient or additive replace in foods . The most widely cultivated edible mushrooms are *Lentinula edodes*(shiitake) ,*Auricularia auricula* (wood ear) ,

Pleurotus ostreatus (oyster mushroom) and *Agaricus biparous* (champignon).Edible mushroom production is strongly influenced by the presence of fungal pathogens *Trichoderma spp.* that are causative agents of green mold disease , although it can be caused by *Penicillium* and *Aspergillus* , other green mold molds with weak pathogenicity. *Trichoderma* initially produces white and dense mycelia that change color during sporulation and finally become dark green and visible on mushroom – growing substrate. It also causes the appearance of brown spots of necrotic tissue and lesions on mushroom fruiting bodies. More than 30 *Trichoderma* species are pathogens of mushrooms and have been found in the substrate and fruiting bodies of *A. bisporus*, *L. edodes* , *P. ostreatus* etc. Button mushroom or champignon, *Agaricus bisporus* , it the forth most- produced mushroom species in the world and accounts for 15% of world total mushroom production. With China , the USA, the Poland the Netherlands and India as its biggest producers. Since green mold disease in *A. bisporus* production can cause significant yield losses , ranging from 60-100% disease outbreaks outbreak may lead to serious economic costs for the producers . the disease is characterization by a rapid infestation of the compost by the *Trichoderma spp.* and the subsequent inhibition of *A. bisporus* fructification . on the other hand , it also causes mal formation of the fruit bodies . infected white button mushroom are small and abnormally shaped with lesions and dark – browned discoloration. The hat weight and diameter , as well as stipe weight, diameter are also influenced. Severe epidemics of green mold disease on *A . bisporus* mushroom farms in Europe and USA during the 1980 and 1990s were related to aggressive strains or biotypes Th2and Th4 , some authors even suspected it to be due to the release of some *T. harzianum* biocontrol strain. Green mold can be easily spread by contaminated tools , substrate and even clothing of mushroom growers . it can also be spread by contamination air and insect vectors . thus strict hygiene , treatment with disinfections and

applications of fungicides are necessary to minimize contamination in mushroom farm. Among fungicides, prochloraz and metrafenone proved to be effective against strains of various taxa of the genus *Trichoderma* isolated from environmentally friendly ,alternative methods such the use of plant derived biocontrol have become popular against the chemical fungicides. Woo.et.al reported that *Trichoderma spp.* was only found at the initial phase on the substrate preparation and disappeared after pasteurization at 60 °Cfor 10 hr. . . However *Trichoderma spp.* was detected again at the spawn run stage when temperature reached ca. 30 °C. The optimal mycelial growth of the green mold occurred 80% relative humidity . A substrate of around pH8-9 may slow the growth of *Trichoderma spp.* resulting in a disease in infection spread.

In the present study , we characterize the *Trichoderma spp.* associated with *A. bisporus* sigs of green mold disease in order to (1) monitor fungal presence (2) isolation of fungal from soil bed (3) identification and classification of the *Trichoderma spp.*(4) test approved green synthesized bio fungicides to determine the effective doses for controlling *Trichoderma*, without producing undesirable consequences for *A. bisporus* .

II. MATERIALS AND METHODOLOGY

2.1 Fungal Isolation from Button Mushroom Soil Bed

Trichoderma spp. were isolated from the unpasteurized mushroom soil. To isolate the *Trichoderma spp.* from mushroom soil, “Five I ” methods was used which include five essential steps to study microorganisms: inoculation, incubation, isolation, inspection and identification. The sample was isolated from the un attended unpasteurized soil used for mushroom cultivation.1gm of soil was suspended in the 10 ml of distilled water and agitated for 1 hour at room temperature, and aliquots from the logarithmic dilutions series were spread onto potato dextrose agar (PDA). Standard protocol was used to

determine the number of colony – forming units (CFU) over 5 day period, and for isolating and identifying the *Trichoderma* spp.

2.2 Effect of Temperature on Fungal Growth

Young fungal cultures of *Trichoderma* and *Aspergillus Niger* were maintained at PDA at 25 °C. Streaking from the previous plate was done in order to determine the effect of the temperature on mycelium development. The plates were observed after 24-48 hours. In addition, the time of the appearance of green spores, the characteristics of the colony growth, and the pigmentation of the mycelium were recorded. The experiment consists of three replicates were recorded. The experiment consists of three replicates for each fungal isolates and was repeated on three separate occasions.

III. GOLD STANDARD IDENTIFICATION TEST

There are several test that are used for the identification of microorganisms, some of the, were used for the identification of *Trichoderma* these are Indole test, Urease test, Simmon Citrate test, Sugar ferment test, Catalase test, Methyl red test.

3.1 Indole test:

The indole test is used for determining if a bacteria has the ability to convert tryptophan into indole through the enzyme tryptophanase. It is significant because indole production is a reliable biochemical marker for distinguishing indole – positive microorganisms. Since *Trichoderma* does not produce indole, this test differentiates true fungal contamination from bacterial interference, ensuring accurate identification and interpretation of mixed microbial cultures. For indole test SIM media was prepared, components Peptone (0.1g), Sodium chloride (0.05g) and distilled water (10 ml), media was autoclaved at 121°C for 15 min and 15 psi. Autoclaved media was used for inoculation of culture was done with the help of inoculation loop, and was incubated overnight at 37°C. To check the presence, Kovac's reagent was added to the (2-3 drops) test tube.

3.2 Urease test :

The urease test detects an organism's ability to hydrolyse urea into ammonia and carbon dioxide using the enzyme urease. *Trichoderma* species generally show negative or weak urease activity, so the medium rarely turns pink. This helps differentiate *Trichoderma* from strongly urease- positive fungi or bacteria. For urease test media was prepared, components Peptone (0.1g), Sodium chloride (0.05g), Potassium phosphate (0.02g), Glucose (0.01g), Agar (0.2g), Distilled water (10ml), few drops of phenol red and (0.2g) urea after autoclaving the media. Media was autoclaved, fungal culture was inoculated into a test tube and incubated at 37 °C for 24 hours. If color changed to pink or magenta color, indicating strong urease activity. A negative result shows no color change (yellow/ unchanged medium), meaning little to no urease enzyme is produced.

3.3 Simmon's Citrate test

This test checks whether an organism can use citrate as its sole carbon source. When citrate is utilized, alkaline products form, turning the medium Prussian blue – a positive result. If the organism cannot use citrate, the medium remains green, indicating a negative result. Media was prepared using these components Sodium citrate (0.02g), Ammonium dihydrogen phosphate (0.01g), Dipotassium phosphate (0.01g), sodium chloride (0.05g) little bit of Magnesium sulfate, agar (0.2g) and distilled water (10ml), 2-3 drops of bromothymol blue. Media was autoclaved, after cooling down the media the fungal culture was inoculated with the help of inoculating loop. Further inoculation test tube was kept at 37°C for overnight.

3.4 Sugar ferment test

The sugar fermentation test is used to check whether a microorganisms can ferment a specific carbohydrate (like glucose, lactose, sucrose). During fermentation, bacteria break down the sugar to produce acid, gas, or both. Media was prepared using these components Peptone (0.1g), sodium chloride (0.05g), few drops of phenol red indicator and 10 ml

of distilled water. Several media's were prepared to confirm the presence of *Trichoderma* {Dextrose, Lactose, Sucrose, and Mannitol}. After the autoclaving the media's the fungal culture was inoculated and incubated for overnight at 37°C. Color change will determine the presence of the *Trichoderma*.

3.5 Methyl red test

The MR test detects mixed – acid fermentation of glucose, producing stable acids that keep the pH below (4.4). *Trichoderma* is a fungus, not a bacterium, and not perform mixed-acid fermentation, so it cannot lower the pH enough to give positive MR reaction. *Methyl red Voges Proskauer broth* was used to determine the presence of fungus. Media's components Peptone (0.05g), glucose(0.07g), potassium hydrogen (0.05g), 10 ml of distilled water. Broth was autoclaved and culture was inoculated further it was incubated at 37°C for 24 hours. Color change determine the presence or absence, in case of *Trichoderma* it was negative as the solution remained yellow even after adding methyl red, no red color was formed.

3.6 Catalase test

The catalase test detects the presence of the enzyme catalase, which breaks down hydrogen peroxide(H₂O₂) into water and oxygen. The rapid release of oxygen forms visible bubbles. For this test fungal culture was used, on a clean glass slide add 1-2 drops of 3%hydrogen peroxide and with the help of inoculating loop culture was taken, made a smear that result in the bubble formation.

IV. GREEN SYNTHESIZED NANOPARTICLES

For bio synthesis of nanoparticles from ajwain there were two main components used 1. Ajwain extract 2. Silver nitrate nanoparticles.

4.1 Ajwain extract : fresh leaves of ajwain was used to make the extract. 5g of leaves were crushed in order to get the extract out of them, around 10 ml of distilled water is used to make the extract (0.1mM) after getting crushed with mortar and

pestle they are transferred to a beaker following along with 40 ml of distilled water and was further autoclaved for 15 min to get rid of any microorganism. After autoclaving extract was given some time to settle down (30min) and the supernatant was taken out leaving us with just extract.

4.2 Silver nitrate nanoparticles: Around 0.17 g of silver nitrate was weighed and was kept aside.

4.3 Ajwain Nanoparticles: 90 ml of distilled water was measured adding 10 ml of extract of ajwain, measured 0.17g of silver nitrate all was added to a amber bottle in order to make the biosynthesized nanoparticles. Color change was noticed every 10 minutes, color went from light to dark brown in around 30 minutes that indicates the starting synthesis of nanoparticles. Its OD was checked after one day and after 1 week.

V. SPORE GERMINATION INHIBITION TEST

In this study, the bio – extract was evaluated for its antimicrobial potential using the agar well – diffusion assay. Antibacterial activity was tested against *Klebsiella*, while antifungal activity was assessed against *Trichoderma* spp, *Penicillium*, and *Aspergillus* spp. the extract was introduced into the wells cut in pathogen agar plates, and zones of inhibition was measured. Additionally antioxidants activity was examined to determine the extract's free- radical scavenging ability, supporting its overall biological potential.

5.1 Antibacterial Well Diffusion

The antimicrobial activity of the biosynthesized nanoparticles was evaluated against the *Klebsiella pneumonia* using the agar well diffusion method. Nutrient agar medium (NAM) plates were prepared and uniformly spread with the bacterial suspension. Well was punched into the agar with the help of pipette tip, and extract was introduced into the well. The plates were incubated at 37 °C for overnight. Formation of clear zone of inhibition around the wells

indicate effective antibacterial activity of the extract against *Klebsiella pneumoniae*.

5.2 Well-Diffusion Assay for *Trichoderma* spp.

The antifungal activity of the extract against *Trichoderma* spp. was assessed using the agar well-diffusion technique. Potato dextrose Agar (PDA) plates were prepared and allowed to solidify, followed by swabbing of fungal culture *Trichoderma* to create a lawn of fungal growth. With the help of pipette tip wells of equal diameter were punched into the agar, and measured volumes of the extract were carefully transferred into the wells. The plates were incubated at 28-30°C for 48-72 hours to allow fungal growth and its interaction with the extract. After incubation the plates were examined for the clear zones or areas of the suppressed mycelial growth indicates the antifungal effectiveness of the extract against *Trichoderma*. The size of the inhibition zone was recorded.

5.3 Antifungal well – diffusion Assay for *Aspergillus* spp.

The antifungal activity of the extract against *Aspergillus* spp. was evaluated using the agar well-diffusion method. Potato Dextrose agar (PDA) plates were prepared, allowing it to solidify, and then inoculated with the uniform layer of *Aspergillus* with the autoclaved swab all over the plate to ensure consistent fungal growth. With pipette tip wells were made in the plate. Volume of extract was added into the each well. Plates were incubated at 28°C for 48-72 hours to permit fungal development and extract diffusion. After incubation, the plates were observed after the incubation, for the clear inhibition zones or reduction of fungal growth around the wells. These inhibition zones indicate the antifungal activity of extract against *Aspergillus* spp.

5.4 Antifungal Well -Diffusion Assay for *Trichoderma* spp. using different extract concentration

The antifungal activity of the biosynthesized extract against *Trichoderma* spp. was evaluated using agar well – diffusion method with four wells containing

progressively lower extract concentration. Potato dextrose agar (PDA) plates were prepared and uniformly inoculated with fresh *Trichoderma* spore to ensure fungal growth. Wells were made in the agar plates, and filled with different concentrations (100%, 75%, 50%, 25%). The plates were incubated at 28°C for 48 -72 hours to allow fungal growth and proper diffusion of the extract. After incubation, the zone of inhibition around each well was observed and measured.

5.5 Antioxidant Activity (DPPH Radical Scavenging Assay)

The antioxidant activity of the plant extract was evaluated using the DPPH free radical scavenging methods. A methanolic solution of DPPH, which exhibits a deep purple color, was mixed with different concentrations of the extract. The mixture was incubated in the dark for 30 minutes to allow the reaction between the DPPH radicals and antioxidant compounds present in the extract. A decrease in absorbance and color change from purple to yellow indicated the extract ability to donate hydrogen or electrons and neutralize free radicals. The percentage of radical scavenging activity was calculated by comparing the absorbance of the control and ajwain sample. Higher reduction in the absorbance reflected stronger antioxidants potential of the extract.

VI.RESULTS



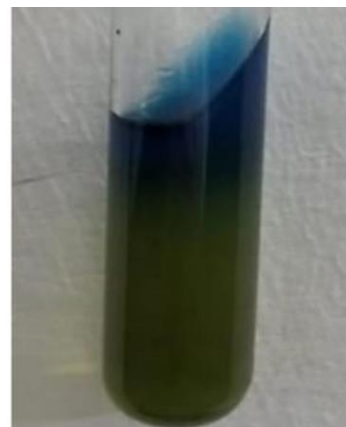
- a.) Isolation of fungus "*Trichoderma*" from the mushroom soil bed, with using four I method it was isolated and made a plate .



- b.) Indole test : confirmatory test for the *Trichoderma* , it involve the reaction with **p-dimethylaminobenzaldehyde** in acidic environment to form **rosindole dye**.

For positive test : cherry – red ring on the surface

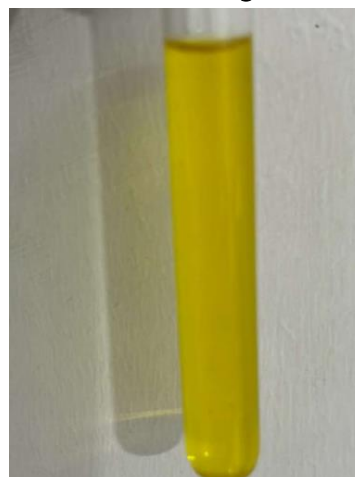
For negative test : reagent layer remains yellow or pale brown.



- c.) Citrate utilization test , where an organism can use citrate as its sole carbon source and inorganic ammonium salts as the sole nitrogen source

For positive test : color change of **the medium from green to blue**.

For negative test: **no color change** .



- d.) the urease test detects the enzyme urease, which hydrolyzes urea into Ammonia+ carbon dioxide

For positive test: rapid or gradual color change of the medium from yellow to orange to bright pink

For negative test: no color change



- e.) **(Dextrose)** the sugar fermentation test determines whether a microorganism can ferment a specific carbohydrate (e.g, glucose, lactose, sucrose) to produce acid / or gas.

For positive test : color change from red to yellow



- f.) Sugar fermenter test : **(Lactose)**
for positive test : medium turns yellow
for negative test : medium remains red

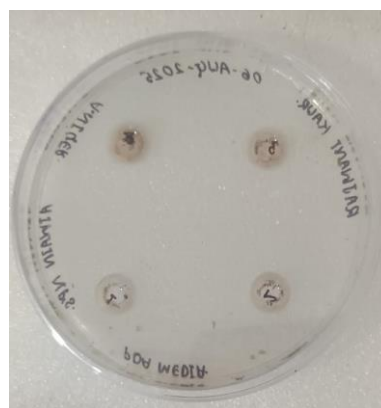


- g.) Sugar ferment test:
For positive test : medium turns yellow
For negative test : medium turn red or pink.



- h.) Catalase test :

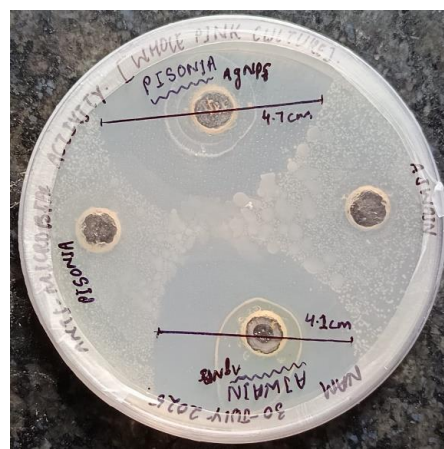
This shows release of oxygen gas is observed as visible bubbles.



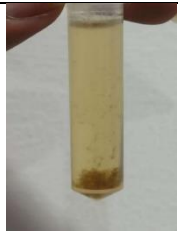
- i.) **Before** : It shows the amount of bio fungicide in the wells about .02ml,.04ml,.06ml,.08ml



- j.) **After** : incubation at 28 °C for 16-18 hours



- k.) It shows the antibacterial property of the bio fungicide

Name of test	Control	Ajwain extract	Ajwain nanoparticles	Vitamin C
Antioxidant Activity				

VII.DISCUSSION

The present work explores the effect of ajwain extract and its synthesized nanoparticles on the growth of *Trichoderma* fungus, a major cause of green mold disease in button mushroom. Several confirmatory test were performed to evaluate their antifungal potential. The ajwain extract produced a strong inhibition zone, conforming to their nanoparticles showed their nanoparticles showed antifungal efficiency and natural fungicide properties. Of Ajwain. The antioxidant potential of the nanoparticles was observed to nearly that of the crude extract , suggesting that the smaller particle size enhancer the available of the active components that can disrupt the fungal cell structures. In additional to suppressing fungal growth , the nanoparticles also inhibited the test bacterium , indicating a wide range of antimicrobial activity within mushroom compost.

VIII. CONCLUSION

There is a growing need for safer and more natural ways to control disease , especially those caused by *Trichoderma aggressive*. Chemical fungicides can be harmful, so better alternatives are needed Ajwain extract appears to be a good option because it has antifungal properties and is eco- friendly. Using such natural methods can help reduce the loses caused by *Trichoderma* in mushroom farms. It is also important to know that not all *Trichoderma* species are harmful some actually help plants grow and protect them from other fungi . this is why correctly identifying the harmful strain is necessary before applying any treatment. Overall, natural methods like Ajwain

extract can support safer and more environmentally friendly disease control.

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