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Biocontrol of *Alternaria tagetica* on marigold using emulsion-based *Trichoderma* bioformulations

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Marigold leaf spot or black spot caused by *Alternaria tagetica* is one of the most prevalent and damaging diseases in India. To manage this disease sustainably, two newly developed emulsion-based bioformulations of *Trichoderma harzianum* AMUTH-1 and *T. viride* AMUTV-3 were explored for the bio-management of *A. tagetica* under field conditions. For comparison, pure culture of *T. harzianum* AMUTH-1, *T. viride* AMUTV-3 and *Pseudomonas fluorescens* AMUPF-1, NIROG™ (commercial *Trichoderma* formulation) and fungicide (azoxystrobin) were used as benchmarking. Inoculation of *A. tagetica* AMUAT-1 under the field caused severe damage to marigold plants with 80% disease severity and 35% reduction in the flower yield. However, foliar application of *T. harzianum* AMUTH-1 and *T. viride* AMUTV-3 bioformulations resulted in a significant enhancement in the plant-growth parameters (35-54%), and flower yield (41-44%), a correspondingly significant decline in leaf spot severity (77-82%) and phylloplane population of *A. tagetica* (80-86%). The effect of both bioformulations was comparable to azoxystrobin and better than Nirog™. The obtained results demonstrated a great potential to use the newly developed bioformulations of *T. harzianum* AMUTH-1 and *T. viride* AMUTV-3 against *A. tagetica*. Foliar application with these bioformulations effectively manage *A. tagetica* on marigold, suggesting that these bioformulations could be used as independent or a component of integrated disease management.

Keywords : Biocontrol agents, disease severity, emulsion formulation, *Tagetes* spp., *Trichoderma* spp.

INTRODUCTION

Marigold (*Tagetes erecta* L.), a member of the family Asteraceae, is a widely cultivated flowering plant grown in India for cut flowers and essential oil (Sidhu and Kumar, 2024). In India, the area of marigold is 74 thousand hectares, and production is 780 thousand tones (Anonymous 2021). Numerous pathogenic microbes, including bacteria, viruses, nematodes, and fungi harm marigold plants and induce diseases that drastically reduce flower production (Anand 2021). The most severe and widespread disease of marigold is leaf spot or black spot, caused by *Alternaria tagetica* (Cheng *et al*. 2019; Anand 2021). It is one of the major destructive and economically important diseases in India, causing 50-60 per cent losses in flower yield (Shinde *et al*. 2018).

Many systemic and non-systemic fungicides are used for managing the leaf spot disease of marigolds (Chandel *et al*. 2010; Sood *et al*. 2024). However, management with fungicides alone is challenging due to the air-borne nature of the pathogen (Shinde *et al*. 2018). Likewise, control through host resistance is unsatisfactory and difficult to adapt by the farmers. Biological control, therefore, is a distinct alternative possibility (Haque *et al*. 2024).

Some *Trichoderma* species/isolates are potential biocontrol agents of plant pathogens, including *Alternaria* species (Ganie *et al*. 2013; Imran *et al*. 2023). They can suppress plant pathogens through antibiosis, hyperparasitism, predation,

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cross-protection, competition for nutrients and colonization site, and induction of systemic resistance (Khan and Mohiddin 2018; Haque and Khan, 2022). However, mass production and delivery systems of these bioagents are considered to be major limitations (Kumar *et al*. 2023; Haque *et al*. 2024).During the last 10-20 years, progress has been made in developing various *Trichoderma* formulations for the management of several plant diseases (Herrera *et al*. 2020; Martinez *et al*. 2023). However, less emphasis has been given to developing *Trichoderma* bioformulation for foliar pathogens.

Hence, this study was mainly focused on exploring the newly developed emulsion-based *Trichoderma* bioformulations and utilising them for alternative disease management strategies through sustainable agriculture methods. The use of emulsion-based *Trichoderma* bioformulations may reduce the cost of applications with an easy delivery system for foliar pathogens and play a dual role by suppressing the disease along with improving plant health.

MATERIALS AND METHODS

Collection and mass production of Alternaria tagetica

Diseased samples of marigold plants exhibiting typical symptoms of *Alternaria* leaf spot disease were collected from the farmer's fields from the village of Chherat, Aligarh. The samples were brought to the lab for isolation and identification. For isolation, small pieces (2–3 mm) were cut from the juncture of diseased and healthy tissues of infected leaves and allowed for surface sterilization with 0.1 % sodium hypochlorite for 10-15 seconds. Then they were washed 2-3 times in sterile distilled water and transferred to a solidified potato dextrose agar (PDA) in Petri plates under aseptic conditions. The plates were incubated at 25 ± 2°C under a BOD incubator, and after 15 days, microscopic analysis of the conidiophores and conidia was carried out to identify the pathogen following the keys from Shome and Mustafee (1966). Pure cultures were obtained through single- spore isolation (Cheng *et al*. 2019) and examined for colony morphology, colour, mycelial growth, and conidia size. Based on the morphological characters, the pathogen was identified as *Alternaria tagetica* (Fig. 1).

Potato dextrose broth (PDB, Himedia, India) was used to prepare the mass cultures of *A. tagetica*. The PDB media were autoclaved for 15-20 minutes at 121°C under 15 kg/cm^2 pressure. Flasks containing PDB were inoculated with a pure and fresh actively growing culture of *A. tagetica.* After inoculation, the flasks were incubated for 8-10 days in a BOD incubator shaker at 25±2 °C for the production of conidia. After that, the mycelial mat along with conidia were collected from the flasks and filtered through a 0.15 mm mesh sieve. Further, 100 µL of suspension was taken on a haemocytometer and the number of conidia was assessed. The spore (conidial) load was attuned to 3.5 × 10^9 spores/ml using distilled water and was employed as inoculants in field experiments.

Collection of Trichoderma bioformulations and other treatments

Two newly developed emulsion-based bioformulations of *Trichoderma harzianum* AMUTH-1 and *T. viride* (=*T. asperellum*) AMUTV-3 (Haque *et al*. 2025) were selected for this study. The emulsion was prepared using polymer (1% Carboxyl methyl cellulose-10 ml), surfactant (Tween 20 – 0.5 ml), oil (soybean oil – 5 ml), UV-protectant (Ujala™ – 1 drop) and water (distilled water – up to 100 ml). Total nine treatments were evaluated for their relative effectiveness against *A. tagetica* as a foliar application (Table 1).

Field trials

To check the relative effectiveness of both *Trichoderma*-based emulsion formulations, field experiments were conducted in 2024 and 2025 at the Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh, India. The experiment was designed as nine treatments with three replicates each year. Routine agronomical practices were used to grow susceptible marigold var. Hazara in the micro plots (3 × 3.5 m) and the treatments were arranged in a randomized block design (RBD) in an open field. The pathogen *A. tagetica* was inoculated at fifteen days after

transplanting at 2 ml per plant (conidial suspension at 1.0 × 10^9 spores/ml) homogenized with 200 ml water and sprayed on the plants of designated plots with the help of a small hand sprayer. Similarly, on the following day, the *T. harzianum* bioformulations (2 ml/plant at 1.0-2.0 × 10^9 spores/ml), *T. viride* bioformulations (2 ml/plant at 1.0-2.0 × 10^9 spores/ml), *T. harzianum* pure culture (2 ml/plant at 1.0-2.0 × 10^9 spores/ml), *T. viride* pure culture (2 ml/plant at 1.0-2.0 × 10^9 spores/ml), *P. fluorescens* pure culture (2 ml/plant at 1.0-2.0 × 10^9 spores/ml) and fungicide (0.023%) were applied to the plants through a hand sprayer. At the time of harvest (110 days after sowing), plant growth (plant length and fresh weight) and yield/plant, phylloplane population of *A. tagetica* and *Trichoderma* isolates were estimated. After 90 days, the disease severity index was assessed on 0-4 scale given by Cheng *et al*. (2019) and converted into per cent disease index by the formula of Pande *et al*. (2003).

$$\text{Percent Disease Index (PDI)} = \frac{\text{Sum of all numerical ratings}}{\text{Total number of observations x maximum grading scale}} \times 100$$

Phylloplane population of Alternaria tagetica and Trichoderma isolates

At 60 days after inoculation, the phylloplane population of *A. tagetica* and *Trichoderma* isolates were estimated from marigold leaves through leaf washing technique (Inacio *et al*. 2002). After being diluted to 10^9 using DDW, the supernatant was processed using dilution techniques. Following that, the final suspension (0.1 mL) was spread onto potato dextrose agar (PDA, Himedia, India) in Petri plates using a sterile pipette. The BOD incubator was used to keep the Petri plates at a constant temperature of 27 ± 2 °C. The CFU load was measured using a colony counter following a 48-hour incubation period.

Data analysis

All data were analyzed using analysis of variance (ANOVA) with R programme and graphs were made with Microsoft Excel 2023. Since differences between repeated data from the two-year studies were not statistically significant at *Pd"*0.05, the data were pooled, with six replicates per treatment. Data on disease severity, plant growth, yield, and phylloplane population were subjected to single-factor ANOVA at a significance level of *Pd"*0.05 to determine the least significant differences (LSD) and used to indicate Tukey HSD test among the treatments.

RESULTS AND DISCUSSION

Effect of bioformulations on disease severity of Alternaria tagetica

Marigold plants infected with *A. tagetica* showed typical symptoms of target-shaped spots, most frequently on the leaves, flowers and infrequently on the stem. The inoculated plants exhibited 80% disease severity index over control (Fig.2). However, the bioformulations and fungicide application significantly reduced the disease severity but varied with the treatments (Fig.2). Foliar application of *T. harzianum* AMUTH-1 bioformulation caused maximum suppression in the disease severity (82%) followed by *T. viride* AMUTV-3 bioformulation (77%). The effects of the rest treatment are in order of azoxystrobin>*T. harzianum* AMUTH-1 pure > *T. viride* AMUTV-3 pure culture > *P. fluorescens* > Nirog™ (Fig.2). Statistical analysis using one-way ANOVA showed significant differences among treatments. The calculated p-value was less than 0.001, indicated that the treatments significantly affected the disease severity index at $P \leq 0.05$,and the *F*-value was also significant at $P \leq 0.05$.

The present study has revealed that marigold cultivar Hazara was more prone to *A. tagetica* infection and severe disease developed at the flowering stage. Cheng *et al.* (2019) also reported 27-30% disease intensity of *A. tagetica* on marigold. Among the seven treatments evaluated,the bioformulations of *T. harzianum* AMUTH-1 and *T. viride* AMUTV-3 exhibited the maximum inhibitory effect and reduced the disease severity by 82% and 77%, respectively in compared to the inoculated control, whereas the pure culture of the same isolates showed significantly lower effectiveness. There are numerous reported antagonists of *A. tagetica,* including *Trichoderma* isolates and their application resulted in a significant decrease in *Alternaria* blight severity (Ganie *et al*. 2013; Imran

Table 1: List of treatments used in the study with details

Treatments	Details
T-1	Control plant without any treatment
T-2	*Alternaria tagetica* infested plant
T-3	*A. tagetica* infested plant + *T. harzianum* AMUTH-1 emulsion formulation
T-4	*A. tagetica* infested plant + *T. viride* AMUTV-3 emulsion formulation
T-5	*A. tagetica* infested plant + *Pseudomonas fluorescens* AMUPF-1 (pure culture, NCBI accession no.KY072889)
T-6	*A. tagetica* infested plant + *T. harzianum* AMUTH-1 (pure culture, NCBI accession no. KM435269)
T-7	*A. tagetica* infested plant + *T. viride* AMUTV-3 (pure culture, NCBI accession no. KY062571)
T-8	*A. tagetica* infested plant + Nirog™ (commercial *T. viride* formulation, Nav Durga Agroscience Pvt. Limited)
T-9	*A. tagetica* infested plant + Score™ 23 SC (Azoxystrobin, Syngenta, India)

et al. 2023). According to our earlier study, *T. harzianum* AMUTH-1 was capable of producing indole acetic acid and siderophores and also exhibited significant enzymatic activity, including the ability to synthesize cellulase, ligninase, protease and chitinase (Haque *et al*. 2024). It is well-documented that these hydrolytic enzymes are released as secondary metabolites that break down the fungal cell wall and play a significant role against pathogen infections (Imran *et al*. 2023). The successful suppression of *A. tagetica* by *T. harzianum* bioformulation may have been facilitated by the activity of these secondary metabolites.

Effect of bioformulations on plant growth and yield

A. tagetica caused a considerable reduction in plant growth and biomass parameters such as plant length (37%), plantweight (31%), number of flower (35%) and flower yield per plant (35%) of marigold when compared to the uninoculated control (Fig. 3). However, bioformulations and fungicide gives the best result and significantly increase the plant growth and biomass of inoculated plants over respective controls (Fig. 3). *T. harzianum* AMUTH-1 and *T. viride* AMUTV-3 bioformulations proved most effective and significantly enhanced the plant growth (35-54%) and yield (35-44%) over respective control (Fig. 3). Next in effectiveness was *T. harzianum* AMUTH-1 pure culture followed by *T. viride* AMUTV-3 (Fig. 3). A similar effect was also observed with *P. fluorescens* but the effects were lower than with former treatments. The commercial formulation NIROG exhibited the lowest effectiveness over inoculated control (Fig. 3).The calculated p-value was less than 0.001 for plant length, 0.011 for number of flowers and 0.05 for yield per plant, indicated that the treatments significantly affected these parameters at $P \leq 0.05$.*F*-value was also significant at $P \leq 0.05$.

Other studies also showed that *A. tagetica* is a devastating fungus that causes up to 60% yield loss (Shinde *et al.* 2018). Further, to mitigate this problem and develop a sustainable management module, novel bioformulations of multifaceted *Trichoderma* isolates were developed by solid-state fermentation techniques in our previous study (Haque *et al*., 2025) and applied as a foliar treatment. The foliar application with the *Trichoderma* bioformulations lowered the leaf spot

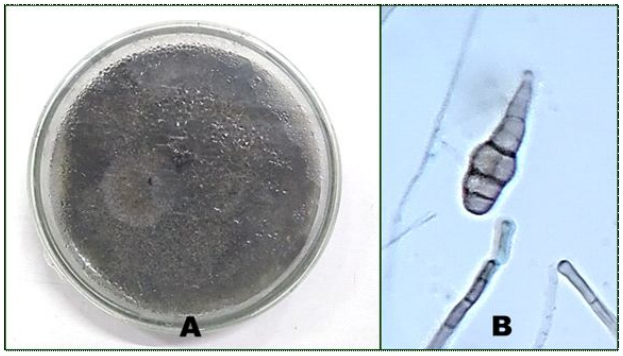

Fig.1: Pure culture of *Alternaria tagetica* on PDA (A) and morphological character of conidia and condiophores (B)

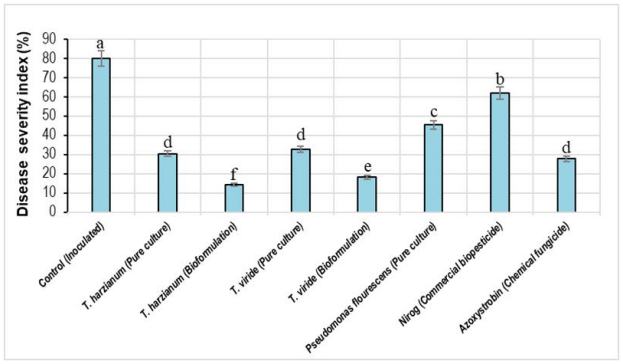

Fig. 2: Effects of different treatments on the per cent disease severityindex of marigold plants inoculated with *Alternaria tagetica* under field conditions.Each value is a mean of six replicates (three per year). Bars indicate the standard error of mean (SEM)and marked by different alphabets are statistically different at Pd" 0.05 according to the Tukey test

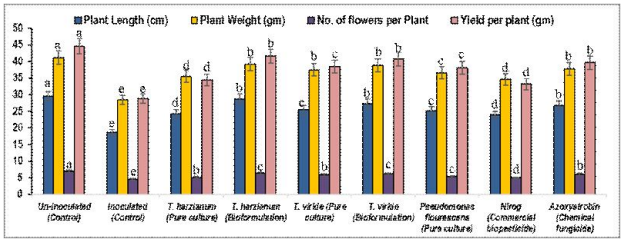

Fig. 3: Effects of different treatments on plant growth, number of flowers and yield per plantof marigold plants inoculated with *Alternaria tagetica* under field conditions.Each value is a mean of six replicates (three per year). Bars indicate the standard error of mean (SEM)and marked by different alphabets are statistically different at Pd" 0.05 according to the Tukey test

disease and significantly increased the marigold yield.

In the present study, the foliar application of *Trichoderma* bioformulations significantly enhances the plant growth and flower yield of marigold, and the highest response was observed with *T. harzianum* AMUTH-1 bioformulations. It was demonstrated that the aforementioned

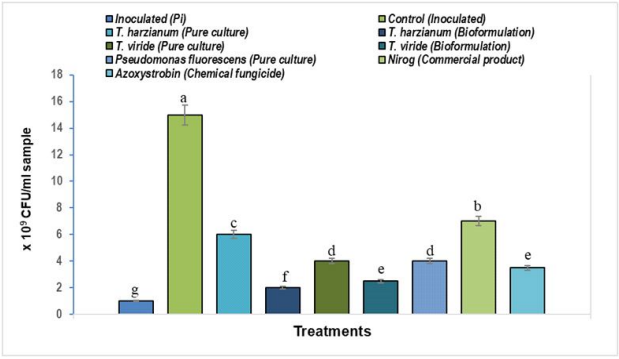

Fig. 4: Effects of different treatments on the phylloplane population of*Alternaria tagetica*under field conditions.Each value is a mean of six replicates (three per year). Bars indicate the standard error of mean (SEM) and marked by different alphabets are statistically different at Pd" 0.05 according to the Tukey test

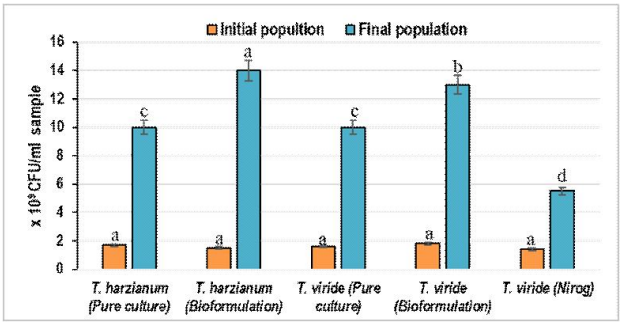

Fig. 5: Phylloplane population of *Trichoderma* treatments under field conditions. Each value is a mean of six replicates (three per year). Bars indicate the standard error of mean (SEM) and marked by different alphabets are statistically different at Pd" 0.05 according to the Tukey test

treatments were more effective than the fungicide, azoxystrobin. *Trichoderma* species are one of the best-known and potential biocontrol agents for wide range of plant pathogens (Khan and Mohiddin, 2018; Haque and Khan, 2023). Many species of this genus have been recognized as opportunistic avirulent plant symbionts and proven antagonists to control the inoculum of *Alternaria* spp.(Mohammedi *et al*. 2022).

Phylloplane population of Alternaria tageticaand Trichoderma isolates

The phylloplane population of *A. tagetica* was reduced maximum in *T. harzianum* AMUTH-1 bioformulation (86%), followed by *T. viride* AMUTV-3 bioformulation (80%), compared to their initial population (Fig. 4). However, the third lowest phylloplane population of *A. tagetica* (82%) was in azoxystrobin treatment (Fig. 4).

The phylloplane populations of *Trichoderma* isolates were varied with the treatments. In general, it was increased in the foliar-treated plants at the time of observation in comparison to their initial application population (Fig. 5).The highest increase was observed in *T. harzianum* AMUTH-1 bioformulation, followed by *T. asperellum* AMUTV-3 bioformulation. The lowest soil population increase was of NIROG (*T. viride*).

The mass production and delivery system of *Trichoderma* are considered to be major limitations in its field applications (Hewavitharana *et al*. 2018; Kumar *et al*. 2023). Several workers evaluated various substrates for the mass multiplication of *Trichoderma* (Prathibha *et al*. 2015; Mohiddin *et al*. 2017; Haque *et al*. 2023). The newly developed liquid bioformulation simplifies the application processes for the large-scale application of *Trichoderma* in fields either soil or foliar application (Haque *et al*. 2025).Based on the preliminary data, it was revealed that these bioformulations can be stored for up to 6 months under room conditions without any significant reduction in spore viability. The newly developed bioformulationshave a higher spore load, longer shelf life, and lower cost of production due to the use of cheaper substrates (Haque *et al*. 2025), which makes them more novel than existing *Trichoderma*-based products. However, cost-benefit ratio, application methods, effect of field environmental conditions such as ultraviolet radiation, relative humidity, temperature and storage conditions may have a negative influence on the viability and shelf-life of the bioformulation and need to be explored in future studies.

The study has demonstrated that *A. tagetica* is a devastating pathogen of marigold and can be effectively managed by the foliar application of *T. harzianum* AMUTH-1 and/or *T. viride* AMUTV-3 bioformulation. The effects of both bioformulations were comparable to fungicides, azoxystrobin, hence, the application of these bioformulations may offer an effective alternative for managingleaf spot of marigold, and other foliar pathogens like *A. solani, A. alternata, A. tenuissima*,etc. These findings can contribute to the development of integrated disease management strategies to protect marigold plants in the context of multi-pathogenic diseases. However, shelf-life studiesand multi-locational field experiments are necessary to validate the efficacy of the above *Trichoderma* bioformulations before recommending them for use by farmers and growers.

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