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Efficacy of *Trichoderma* Strains as Biotic Inducers against Alternaria Leaf Spot of Cauliflower

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ABSTRACT

Alternaria leaf spot is one of the most destructive disease and cause huge economic losses in cauliflower. In the quest of eco-friendly disease management against Alternaria leaf spot of cauliflower, four isolates of *Trichoderma*, namely *T. viride*, *T. harzianum* I-6 strain, *T. harzianum* IMQ-8 and *T. koningii* were evaluated for their antagonistic potential against Alternaria leaf spot disease of cauliflower during the year 2020 at the College of Horticulture and Forestry, Neri, Hamirpur, Himachal Pradesh. In a dual culture assay, *T. harzianum* I-6 strain inhibited the mycelial growth of *Alternaria brassicicola* by 80.64%, followed by *T. viride* (78.49%). Root dip treatment of cauliflower seedlings with *T. harzianum* I-6 strain was found to be most effective in plants challenged with the pathogenic fungus i.e. *A. brassicicola* and resulted in a reduction in disease incidence and disease severity of 32.70% and 40.00%, respectively compared to pathogen-challenged control plants. It was followed by IMQ-8 strain of *T. harzianum* with 29.49 and 34.73% reduction in disease incidence and disease index, respectively. *T. koningii* was least effective against the disease. Among all *Trichoderma* treatments, the maximum plant weight of 6.84 g plant^{-1} was obtained by using *T. harzianum* I-6 strain, followed by *T. harzianum* IMQ-8 strain, *T. viride* and *T. koningii* with 6.45, 5.62 and 4.33 g plant^{-1}, respectively. In comparison, plants inoculated with the pathogen weighed 3.72 g plant^{-1}. Hence, seedling treatment with *T. harzianum* has the potential for eco-friendly management of Alternaria leaf spot of cauliflower.

KEYWORDS: *Alternaria brassicicola*, biocontrol, root dip, *Trichoderma*

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1. INTRODUCTION

Cauliflower (*Brassica oleracea* var. *botrytis* L.) is one of the most important crops grown in both temperate and tropical regions worldwide. It is considered the second most popular crop after cabbage from the cole family (Ma et al, 2022). Cauliflower is a rich source of vitamins A, B, C, fatty acids, omega 3, folate, protein, manganese, phosphorous and dietary fibres (Shaw et al., 2021; Tukassar et al., 2023). It is cultivated on 1.44 m ha of land annually with a production of 26.92 mt across nearly 94 countries. China (39.77%) and India (33.74%) are major producers, while the United States of America (4.63%), Mexico (2.67%), Spain (2.65%), Italy (1.37%) and Turkey (1.17%) also contribute significantly (Anonymous, 2019). In India, the area under cauliflower cultivation is 469 ha having production of 9103 mt with productivity of 19.2 mt ha^{-1}. The crop is mostly grown in Bihar, Uttar Pradesh, Orissa, Madhya Pradesh, Assam, Gujarat and Haryana (Anonymous, 2018). The growth of the crop is affected by both biotic and abiotic factors and the quality as well as quantity of the crop yield is severely affected by many diseases. Alternaria leaf spot is the most damaging disease and is a major constraint in cauliflower production, especially in sub-tropical area. The disease causes yield losses in various parts of the world including India (Pattanamahakul and Strange, 1999; Peddi et al., 2022; Sunitha and Jha, 2023). The disease is caused by two species of Alternaria. The vegetable *Brassicas*, i.e., cauliflower, cabbage and broccoli are mainly affected by *Alternaria brassicicola* (Schwein) Wiltshire and *Alternaria brassicae* (Berkeley) Saccardo whereas, the oleiferous *Brassica* seed crops are mainly affected by *A. brassicae* (Michereff et al., 2012; Kumar et al., 2014; Raj and Sharma, 2022). *A. brassicicola* develops dark-coloured, zonated leaf spots however, *A. brassicae* produces light brown or grey-coloured leaf spots (Selvamani et al., 2014; Siciliano et al., 2017; Javeria et al., 2018; Glory et al., 2022; Kumar et al., 2023). *Alternaria* spp. contributes to 20% of agriculture wastage. In severe cases, the yield losses may go as high as 80%. The loss due to disease in cauliflower vegetable crop in India is about 30–50%, whereas 5–30% loss is caused by *A. brassicae* alone (Sunitha and Jha, 2024). The disease appears annually during the cropping season (from October to February) in various parts of the country and causes a major loss to growers (Chandel et al., 2023). Upto 64% disease incidence and up to 30.4% disease severity were recorded from various parts of the country (Ansar and Ghatak, 2018; Kanna et al., 2023). The disease also causes up to 80% decrease in seed production (Hossain and Mian, 2005). Fungicides are commonly used for the management of Alternaria leaf spot disease (Al-Lami et al., 2023; Chandel et al., 2023) but excessive use of synthetic fungicides have been proven hazardous to ecosystem, human health and cause damage to flora and fauna. The biological control of plant pathogens by antagonistic fungi is an environmentally friendly, dependable, and efficient method that can be successfully integrated into disease management programs. Several *Trichoderma* species have been reported to be effective in the management of various plant diseases including soil borne and aerial disease. *Trichoderma* species not only have direct antifungal activity, but they can also modulate the host defence system for protection against the soil borne pathogens as well as aerial pathogens. Hence the present study was designed to evaluate the efficacy of *Trichoderma* spp. and to manage Alternaria leaf spot disease of cauliflower using various strains of *Trichoderma* spp.

2. MATERIALS AND METHODS

2.1. Isolation and purification of the pathogen

The cauliflower leaves showing the typical symptoms of Alternaria leaf spot of cauliflower were collected from the nearby field of Hamirpur, Himachal Pradesh, India during October–November, 2020. The samples were brought to the laboratory of the Department of Plant Pathology, College of Horticulture and Forestry, Dr Y. S. Parmar University of Horticulture and Forestry, Neri, Hamirpur, Himachal Pradesh for the isolation of the pathogen. First, leaf samples were washed with running tap water to remove soil debris. Isolation was done by cutting small pieces of infected portion of leaves along with the healthy portion and immersed in 1% sodium hypochlorite (NaOCl) for 30–60 seconds. The samples were kept thrice in Petri plates having sterile distilled water for 2–3 min followed by drying on sterile filter paper and then placed aseptically on the sterilized Petri plates containing sterilized potato dextrose agar (PDA) medium. These plates were incubated at 28°C temperature for growth of the pathogen. Fungal growth on each plate was sub-cultured to a new plate. The growth of the pathogen was observed daily.

2.2. Pathogenicity test

The pathogenicity was proved by following Koch's postulates. The leaves of cauliflower were artificially inoculated with the pathogen *A. brassicicola* using pin prick method (Sharma et al., 2021). The spore suspension ($4x10^5$ spores ml^{-1}) was used as inoculum for spraying on 35 days old cauliflower seedlings grown in pots having dimensions 7×8.5 cm^2. Before inoculation the leaves were surface sterilized with 70% ethanol with the help of a cotton swab. Gentle pricking was done with the help of sterilized needle. After inoculum spraying, seedlings were covered with perforated and moistened transparent plastic cover at a temperature ranging from 20–25°C. Inoculated plants were labelled and kept under humid conditions to maintain proper moisture for disease development. High relative humidity

was maintained with water spray inside the polythene bags after every 12 hours. Leaves were observed for symptom development at regular intervals. In case of control plants, sterile distilled water was sprayed.

2.3. Morphological characterization of A. brassicicola

Morphological characters of *A. brassicicola* were studied by observing the temporary slides prepared from the seven day old pure culture of the fungus. Various attributes like hyphal colour, hyphal width, length, breadth, colour, shape, size and septation of conidia and conidiophore characteristics were observed under the microscope.

2.4. Evaluation of biotic resistance inducers of microbial origin

2.4.1. In vitro evaluation of biotic resistance inducers of microbial origin

The relative efficacy of various species of *Trichoderma* i.e *T. viride*, *T. harzianum* I-6 strain, *T. harzianum* IMQ-8 and *T. koningii* were evaluated under *in vitro* conditions against the pathogen through dual culture technique (Sharma et al., 2021). Mycelial discs of 5 mm diameter were taken from 7 days old culture of *A. brassicicola* and from antagonist *Trichoderma* spp. The discs were placed at periphery but at the opposing end of the respective Petri plate. In control plate, *A. brassicicola* was inoculated in the centre of the Petri plate. The plates inoculated with both organisms as well as control were placed in incubator at 25°C. Seven days after incubation, radial growth of *A. brassicicola* and *Trichoderma* isolates were measured. Colony diameter of *A. brassicicola* in dual culture plate was observed and compared with control.

Mycelial growth inhibition (%) was calculated by following formula:

Mycelial growth inhibition (%)={(C-T)/C}×100 Where, C: Growth of test pathogen in absence of *Trichoderma* spp. (mm), T: Growth of test pathogen in presence of *Trichoderma* spp. (mm)

2.4.2. Evaluation of biotic resistance inducers in pot conditions

The bio agents were given as root dip treatment. The spore suspension (32×10^6 spores ml^{-1}) of each *Trichoderma* spp. i.e *T. viride*, *T. harzianum* I-6 strain, *T. harzianum* IMQ-8 and *T. koningii* was prepared by harvesting spores of the fungus (Ahmad and Ashraf, 2016). Sterile distilled water was added to 7 days old culture growing on PDA Petri plates. The fungal spores were gently scrapped with the help of sterilized slide and spore suspension was filtered through sterilized muslin cloth. Roots of 35 days old seedlings of cauliflower were dipped in spore suspension of each *Trichoderma* spp. for 1 hour. Then, seedlings were transplanted in pots. After 5 days, *A. brassicicola* was inoculated as foliar spray of conidial suspension (4×10^5 conidia ml^{-1}) as described above. After pathogen inoculum spraying, plants were covered with perforated and moistened transparent plastic cover at a temperature ranging from 20–25°C. All treatments were labelled and high relative humidity was maintained with water spray inside the polythene bags after every 12 hours. Simultaneously, pathogen inoculated and control plants were also maintained in which no root dip treatment of *Trichoderma* spp. was given. The data on disease incidence (%), per cent disease index and plant weight (g) was recorded after 10 days of transplanting. Further, reduction in disease incidence and reduction in disease index was also calculated. The data on disease incidence was calculated according to following formula:

Disease incidence (%)=Number of diseased plants/Total no. of plants observed×100

The data on severity of Alternaria leaf spot in cauliflower was recorded by following 0–5 scale (Chandel et al., 2023). Further. Per cent disease index was calculated according to following formula:

Per cent disease index (PDI) =Sum of observed all numerical ratings/Total no. of ratings×Maximum disease grade×100

Per cent reduction in disease incidence and disease index was calculated according to following formula:

Percent reduction in disease incidence/index = {(C-T)/C}×100

where, C: Disease incidence/disease index (%) in untreated control

T: Disease incidence/disease index (%) in treatment

2.5. Statistical analysis

The data obtained from laboratory as well as field experiments were subjected to appropriate statistical analysis wherever necessary. The differences exhibited by the treatments in experiments were tested for their significance using standard procedures (Gomez and Gomez, 1984). Statistical analysis was also performed by one way ANOVA using OPSTAT software (Sheoran et al., 1998).

3. RESULTS AND DISCUSSION

3.1. Isolation and purification of the pathogen

The fungus causing Alternaria leaf spot disease was isolated from cauliflower leaves on potato dextrose agar (PDA) medium following the standard procedure (Chandel et al., 2023). The pathogen was purified by sub-culturing the hyphal tips on the PDA (Potato Dextrose Agar) Petri plates. The mycelium of the pathogen was initially white in colour then it produced sporulation which was initially light green in colour and later became dark olivaceous brown to black.

3.2. Pathogenicity test

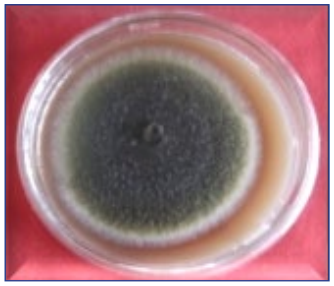
To prove the pathogenicity of *A. brassicicola* on cauliflower leaves, Thirty-five days old seedlings of cauliflower were artificially inoculated with conidial suspension of *A.*

brassicicola at a concentration of $4x10^5$ conidia ml^{-1} by spraying with a hand automizer. The inoculated plants showed initial symptoms after 72 hours of pathogen inoculation. Initially, minute dark green spots appeared on lower leaves. Spots gradually enlarged forming concentric rings and became dark brown to black surrounded by yellow halo. Eventually the leaves turned chlorotic and fell down. The fungus was re-isolated from the infected leaves and was identified as *A. brassicicola*. Hence, pathogenicity of *A. brassicicola* was proved by following Koch's postulates. These findings are in accordance with Doullah et al. (2006) who demonstrated the pathogenicity of *A. brassicicola* on one-month-old mustard seedlings by spray inoculation with spore concentration of 4×10^5 conidia ml^{-1}. The disease severity was observed after 72 hours of incubation period. Similarly, Meena et al. (2016) tested three inoculation methods viz., seed inoculation, cotyledon inoculation and seed and cotyledon inoculation method for proving pathogenicity of *A. brassicae* on mustard crop. Conidial suspension was adjusted to 5×10^5 conidia ml^{-1} with the aid of a hemocytometer. Out of three methods, seed and cotyledon inoculation method was found highly effective where mean Alternaria blight severity was 84.60% in comparison to 49.30% in the inoculation of seed and 62.5% in inoculation of cotyledon method. On cotyledonary leaves, disease appeared after four days of pathogen inoculation, indicating the incubation time of 96 hours.

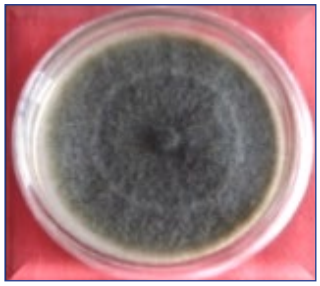
3.3. Morphological characterization of A. brassicicola

It was observed that mycelium of *A. brassicicola* completely filled PDA medium Petri plate of 70 mm diameter in seven days at 28°C temperature. The colony colour of *A. brassicicola* was initially white, turned light green and finally turned to brownish-black. The old culture of *A. brassicicola* was black in colour. Colony surface appearance of the pathogen was velvety (Plate 1 A, B, C).

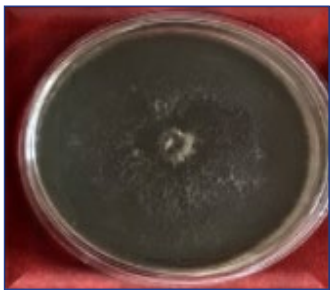
In Microscopic examination, the pathogen hyphal cells were septate, hyaline which later on turned to olivaceous brown in colour measuring 47.5 µm to 50.1 µm in length and 14.1 µm to 16.7 µm in breadth. The average length of hyphal cell was 48.82 µm and average breadth of hyphal cell was 15.34 µm. The conidiophores of *A. brassicicola* were septate, branched and olivaceous. Conidia were septate, cylindrical, dark olivaceous brown without beak measuring 25.1 to 41.8 µm in length and 16.7 to 33.4 µm in breadth. The average length of conidial cell was 33.4 µm and average breadth of conidial cell was 21.2 µm. Most of mature conidia were of 33.4×16.7 µm dimension. The conidia were with 0–2 longitudinal septa and 4 to 7 transverse septa (Plate 1 E). Thus, the pathogen causing leaf spot of cauliflower was identified as *A. brassicicola*. Similar morphological characters of *A. brassicicola* were also described by Chandel et al. (2023) who described mycelium of *A. brassicicola* as septate, olive grey to greyish black in colour. Further, the conidiophores were branched, olivaceous, septate and measuring 35–45 µm in length and 5–8 µm in width. Conidia were dark, cylindrical to oblong, muriform without beak measuring 44–55 µm in length and 11–16 µm in width with 5–8 transverse and 0–4 longitudinal septa.



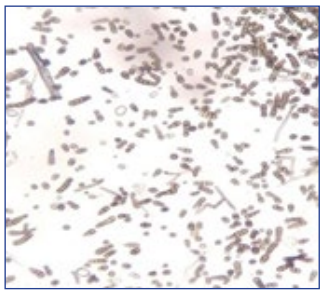
A. Light green sporulation in four- days old culture of *A. brassicicola*



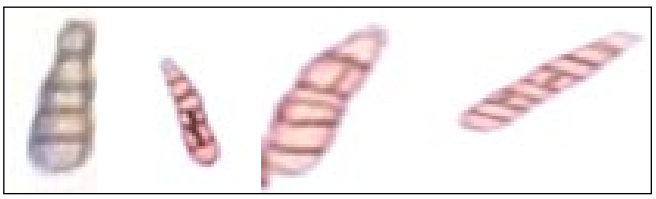
B. Brownish black sporulation in seven-days old culture of *A. brassicicola*



C. Black sporulation in ten-days old culture of *A. brassicicola*



D. Conidia of *A. brassicicola*



E. Magnified view of conidia of *A. brassicicola*

Plate 1: Cultural and morphological characters of *Alternaria brassicicola*

3.4. Evaluation of biotic resistance inducers of microbial origin

3.4.1. In vitro evaluation of biotic resistance inducers against A. brassicicola

In order to assess the antagonistic potential of different *Trichoderma* spp. against *A. brassicicola*, four strains of *Trichoderma* i.e. *T. harzianum* I-6 strain, *T. harzianum* IMQ-8 strain, *T. viride* and *T. koningii* were screened against the pathogen by dual culture technique. Seven days after incubation, the radial growth of both *A. brassicicola* and *Trichoderma* spp. was measured and the inhibition in mycelial growth of *A. brassicicola* by different *Trichoderma* spp. was calculated, as presented in Table 1. All *Trichoderma* spp.

Table 1: Effect of biotic resistance inducers on mycelia growth of *Alternaria brassicicola*

Treatments	Biocontrol agents	Radial growth of mycelium (mm)	Inhibition in mycelia growth (%)
T_1	*Trichoderma viride*	15.06[b]	78.49 (62.34)[b]
T_2	*Trichoderma harzianum* (I-6 strain)	13.55[a]	80.64 (63.87)[a]
T_3	*Trichoderma harzianum* (IMQ-8 strain)	22.22[c]	68.26 (55.69)[c]
T_4	*Trichoderma koningii*	30.00[d]	57.14 (49.09)[d]
T_5	Control	70.00[e]	-
CD (p=0.05)		0.49	0.82 (0.54)

*Figures in parentheses are angular transformed values; *Figures denoted by same letter do not differ significantly

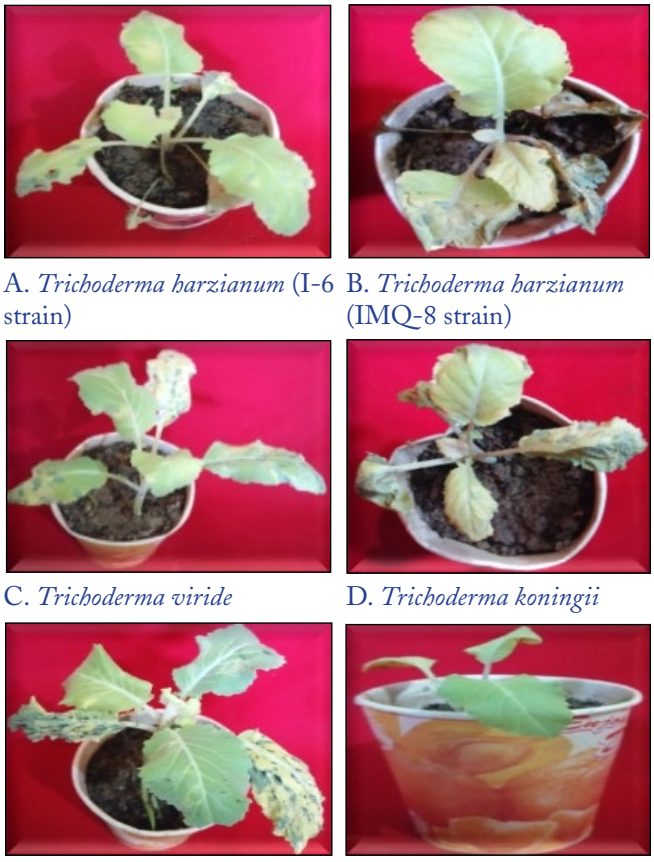
exhibited an antagonistic effect against the mycelial growth of *A. brassicicola*. The minimum average radial growth of *A. brassicicola* was observed when using the antagonistic *T. harzianum* I-6 strain, with a colony diameter of 13.55 mm and maximum mycelial inhibition of 80.64% followed by significantly different *T. viride* and *T. harzianum* IMQ-8 strain, with colony growth of *A. brassicicola* measuring 15.06 and 22.22 mm and inhibition rates of 78.49% and 68.26%, respectively. Meanwhile, the maximum average radial growth of 30.00 mm was observed by using *T. koningii* as the antagonist, with a minimum mycelial inhibition of 57.14%. All treatments were significantly different from each other. In contrast, the control Petri plate showed a growth of 70.00 mm for *A. brassicicola*.

Several other reports have documented the efficacy of *Trichoderma* spp. as antagonists against *A. brassicicola* and other species of the genus *Alternaria*. Yadav et al. (2011) reported in a dual assay that *T. viride* caused the maximum inhibition (74%) of *A. brassicae* causing leaf spot in mustard. Ganie et al. (2013) showed in their reports that higher mycelial growth inhibition of *A. solani* was recorded with *T. harzianum* (71.85%) followed by *T. viride* (65.93%) while, *T. virens* (58.65%) was found to be least effective. Sadana and Didwania (2015) evaluated five *T. harzianum* strains and found that the T_5 strain of *T. harzianum* was the most effective with 81% inhibition in the mycelial growth of *A. solani* in dual culture test. Glory et al. (2022) evaluated *Trichoderma* isolates against *A. brassicicola* causing black leaf spot on cabbage. Among the bioagents, *T. viride* recorded highest level of mycelial growth inhibition (76.84%) followed by *T. harzianum* (64.90%) and caused lysis and mycoparasitism on *A. brassicicola* after 20 days of incubation.

3.4.2. Evaluation of biotic resistance inducers in pot conditions

It is clear from Table 2 that root dip treatment with biotic resistance inducers resulted in a reduction in disease incidence and disease severity of Alternaria leaf spot disease of cauliflower (Plate 2).

The disease incidences for *Trichoderma harzianum* strains I-6 and IMQ-8 treatments were only 58.33% and 61.11% with control efficiencies of 32.70% and 29.49%, respectively. These values were statistically at par with each other. Next



A. *Trichoderma harzianum* (I-6 strain) B. *Trichoderma harzianum* (IMQ-8 strain)

C. *Trichoderma viride* D. *Trichoderma koningii*

E. Pathogen-challenged control F. Non-challenged control

Plate 2: Effect of biotic resistance inducers against Alternaria leaf spot of cauliflower

in order of effectiveness were *T. viride* and *T. koningii* with disease incidences of 68.05% and 72.22% with control efficiency of 21.48% and 16.67%, respectively. The values were statistically similar to each other. In pathogen-inoculated plants, a disease incidence of 86.67% was observed.

The root dip treatment with *T. harzianum* I-6 and IMQ-8 strains was found to be the most effective, resulting in a disease index of 47.50% and 51.67% with control efficiencies of 40.00% and 34.73%, respectively. The next best root dip treatment was with *T. viride* and *T. koningii* with disease

Table 2: Effect of biotic resistance inducers against Alternaria leaf spot of cauliflower in pot conditions

Treatments	Biocontrol agents	Disease incidence (%)	Reduction in disease incidence (%)	Disease index (%)	Reduction in disease index (%)	Plant weight (g plant^{-1})
T_1	*Trichoderma viride*	68.05 (55.63)^{bc}	21.48	57.50 (49.30)^{b}	27.37	5.62^{c}
T_2	*Trichoderma harzianum* (I-6 strain)	58.33 (49.81)^{b}	32.70	47.50 (43.54)^{b}	40.00	6.84^{b}
T_3	*Trichoderma harzianum* (IMQ-8 strain)	61.11 (51.47)^{b}	29.49	51.67 (45.94)^{b}	34.73	6.45^{b}
T_4	*Trichoderma koningii*	72.22 (58.22)^{c}	16.67	68.33 (55.81)^{c}	13.69	4.33^{d}
T_5	Control	86.67 (68.57)^{d}	--	79.17 (62.85)^{d}	--	3.72^{d}
T_6		0.00 (0.00)a	--	0.000 (0.00)^{a}	--	7.52^{a}
CD (p=0.05)		10.65 (6.37)		9.71 (5.76)		0.61

*Figures in parentheses are angular transformed values; *Figures denoted by same letter do not differ significantly

index of 57.50% and 68.33% having control efficiencies of 27.37% and 13.69%, respectively. In contrast, in pathogen-challenged plants, the disease index was 79.17%.

The data presented in Table 2 indicates that all treatments of biotic resistance inducers resulted in an increase in plant weight compared to pathogen-challenged plants. The maximum plant weight of 7.52 g plant^{-1} was obtained in un-challenged healthy plants. Among all *Trichoderma* treatments, the maximum plant weight of 6.84 g plant^{-1} was obtained using the *T. harzianum* I-6 strain, followed by *T. harzianum* IMQ-8 strain, *T. viride* and *T. koningii* with 6.45, 5.62 and 4.33 g plant^{-1}, respectively. In comparison, pathogen-inoculated plants yielded a plant weight of 3.72 g plant^{-1}.

Environment-friendly plant disease management largely depends on antagonistic ability of different *Trichoderma* species (Debanath et al., 2012). Several other reports of field evaluations of *Trichoderma* spp. as biotic resistance inducer have been documented by other researchers. Ahmad and Ashraf (2016) evaluated six biological agents, namely *T. viride, T. harzianum, T. hamatum, T. koningii, T. reesei* and *T. aureoviride,* both under *in vitro* and *in vivo* conditions for their effectiveness in managing Alternaria leaf spot of mustard caused by *A. brassicae*. Among them, maximum pathogen growth inhibition was recorded with *T. harzianum* (61.44%) followed by *T. viride* (55.42%) and *T. konngii* (40.96%). Under field conditions, the per cent disease index of Alternaria leaf spot of mustard with treatment of *T. harzianum* was 60.86% and with *T. viride,* it was 58.57%, compared to the maximum disease index of 88.02% in the control plot. Glory et al. (2022) studied that under field conditions, *T. harzianum* and *T. viride* were found to be less effective in the management of black leaf spot disease of cabbage and resulted in a 16.25% and 13.89% reduction of disease severity, and a 6.43% and 5.21% increase in yield over the control, respectively. Two foliar sprays of *Trichoderma viride* provided 28.31% reduction in Alternaria leaf spot of soybean (Wasule et al., 2023).

4. CONCLUSION

Different *Trichoderma* isolates were found to be effective in *in vitro* dual culture assays. *T. harzianum* I6 and IMO-8 strains resulted in the maximum reduction in disease incidence and disease index in the pot experiment, as well as an increase in plant weight. Thus, the results of this study indicate that the *T. harzianum* reduced the Alternaria leaf spot disease of cauliflower.

6. ACKNOWLEDGEMENT

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