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Research Article

Isolation and *in vitro* screening of native *Trichoderma* isolates against economically important pathogens of Japanese Mint (*Mentha arvensis*)

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Abstract

Japanese mint (*Mentha arvensis*) is a common edible aromatic herb of considerable economic importance and the crop is reported to be infected by several fungal pathogens. As Mentha contains essential oil which is used in pharmaceutical industries, the use of biocontrol agents is considered as a potential eco-friendly component in plant disease management. Hence an investigation was conducted in the Department of Plant Pathology, RPCAU, Pusa, Bihar to evaluate native *Trichoderma* isolates against two economically important foliar pathogens of Japanese Mint (*Mentha arvensis*) i.e., *Alternaria alternata* and *Curvularia lunata*. Total 7 *Trichoderma* isolates were isolated from the rhizosphere of various medicinal plants and those were categorized based on their morphological characteristics. Initially, all *Trichoderma* isolates developed whitish mycelium which later differed from light to dark green in color and the growth rate ranged from 11.25 mm to 18.00 mm per day. During the evaluation of antagonistic activity, all the Trichoderma isolates significantly reduced the growth of *Alternaria alternata* and *Curvularia lunata* mycelia *in vitro* condition. Among the seven isolates, Tr6 recorded the highest inhibition of *Alternaria alternata* (65.66%) and *Curvularia lunata* (45.04%) followed by Tr3 which showed 57.00% and 40.32% mycelial growth inhibition of *Alternaria alternata* and *Curvularia lunata* respectively after 168 hours of incubation. Tr6 isolate also exhibited the highest biochemical activities *viz*., indole acetic acid, ammonia, and siderophore production. The results from the current investigation revealed that Tr6 isolate was found most potential in the suppression of pathogens growth as well as in the production of phyto stimulators. In future studies, this isolate can further be evaluated for successful management of diseases of Japanese mint (*Mentha arvensis*).

Keywords biochemical assay, japanese mint, *trichoderma*

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Introduction

Mentha belongs to the family *Lamiaceae* and more than 45 species and subspecies are recognized in the genus *Mentha* [1]. However, the four major species namely *M. Arvensis* (Corn Mint), *M. Spicata* (spearmint), and *M. Piperita* (peppermint) are commonly used in the commercial production of mint oil, and among these, the leaves of the Japanese mint (*Mentha arvensis*) are the common edible aromatic herb of considerable economic importance. The essential oil of the herbages contains 75-80% menthol that is used in food, perfumery and pharmaceutical industries. The crop is infected by several pathogens such as *Alternaria alternata* (Fr.) Keissl [2-3], *Puccinia menthae* Pers [4], *Verticillium dahliae* Kleb [5], *Curvularia lunata* (Wakker) Boedjin [6], *Erysiphe cichoracearum* Jacz [2, 7], *Rhizoctonia solani* Kuhn [8] and

Sclerotium rolfsi Sacc [9] etc. Leaf blight of *Mentha arvensis* [7] which is caused by the fungus *Alternaria alternata* (Fr.) Keissl, has been reported from India and this disease is severe during monsoon season. Leaf spot caused by *Curvularia lunata* (Wakker) Boedjin, is considered as another important disease of *Mentha arvensis* and reported in India by Thakur et al., [6]. To avoid hazardous effects of various toxic chemicals, there is an urgent need to adopt an alternative tool as crop protection measures. Therefore, biological control measures are currently considered as one of the most effective eco-friendly tools in crop protection practices. In the present scenario, *Trichoderma* is considered as a potential fungal antagonist which is massively used in plant disease management [10]. The antagonistic nature of *Trichoderma* is due to various mechanisms such as competition, antibiosis, mycoparasitism, lysis [11]. Many of the *Trichoderma* show antagonistic potential by digesting hydrolytic enzymes such as cellulase, glucanase, and chitinase and also by releasing harmful substances such as ammonia, HCN, siderophore [12]. Therefore, the goal of the study was to assess the antagonistic activity of *Trichoderma* isolates against two foliar pathogens of *Mentha arvensis* i.e., *Alternaria alternata* and *Curvularia lunata*.

Methodology

Isolation of pathogens

Mentha (*Mentha arvensis*) plants showing leaf spot and blight symptoms were observed and two pathogens *viz: Alternaria alternata* and *Curvularia lunata* were isolated from diseased leaf samples. The diseased samples were then subjected to "Surface sterilization" [13]. The surface sterilized samples were then transferred to Potato Dextrose Agar (PDA) plates followed by incubated at 25℃ for 2-3 days. The mycelia were then transferred to Potato Dextrose Agar (PDA) plates by "Hyphal tip technique" [14], and then kept in incubator at 25℃ to obtain a pure culture of the isolated pathogens. Thereafter, based on the cultural and morphological characteristics, the isolated fungal pathogens were identified.

Isolation of antagonistic fungi

Soil samples were collected from different medicinal plants' rhizosphere in Pusa (Bihar) and nearby districts (Table 1). Serial dilution was carried out and plated on *Trichoderma* selective media (TSM) followed by incubation at 25℃ for isolation of the fungus. The isolates were purified to fresh PDA plates by hyphal tip technique [14] and then morphologically distinguished on the basis of colony characteristics [15].

Table 1. Isolation of Native *Trichoderma* isolates from rhizosphere soil of different medicinal plants

***Trichoderma* isolates**	**Host Plant**	**Place of soil collection**
Tr1	Piper mint	Sihama, Begusarai
Tr2	Ashwagandha	MAP, Pusa, Samastipur
Tr3	Tulsi	Herbal garden, Pusa, Samastipur
Tr4	Sadabahar	Dholi, Muzzafarpur
Tr5	Kalmegh	Khajanpur, Begusarai
Tr6	Bhringraj	MAP, Pusa, Samastipur
Tr7	Sarpagandha	Rosera, Samastipur

Categorization of isolated Trichoderma spp

Trichoderma isolates were categorized macroscopically by observing the fungal colony colour, growth pattern, and growth rate and microscopically categorized on the basis of conidia and phialides size.

Evaluation of antagonistic potential Trichoderma isolates by dual culture technique [16]

The antagonistic behavior of *Trichoderma* spp against test pathogens was evaluated in vitro by dual culture technique [16]. A week-old culture of *Trichoderma* and pathogens were selected to conduct the experiment.

A 5 mm size mycelial disc was cut from colony of test pathogens and transferred on fresh PDA plates at one cm apart from the edge of the Petri plates whereas antagonists were placed opposite to the test fungus one cm from the opposite edge of the plate). Only pathogen inoculated plates were served as control. For each treatment, three replications were used. The inoculated plates were incubated @ 25°C and radial growth of pathogens in *Trichoderma* inoculated plates were measured for 168 hours and compared with control. Inhibition percentage of the growth of pathogens was calculated by using the formula [16].

$$I\ (\%) = \frac{C-T}{C} \times 100$$

Where, C= radial growth of pathogens in control plates
T= radial growth of pathogens in *Trichoderma* inoculated plates

Biochemical assay of Trichoderma spp
Effective *Trichoderma* isolates were subjected to biochemical analysis such as

(A) Indole Acetic acid (IAA) production
Trichoderma isolates were inoculated in conical flasks containing 20 ml of Potato Dextrose Broth (PDB) amended with 0.1% tryptophan whereas the tryptophan free broth was considered as control [17]. The broths were incubated at 28- 30°C for 5 days and then centrifuged at 10000 rpm for ten minutes. After centrifugation, 2 ml Salkwoski reagent (2% 0.5 M Fecl3 in 35% hclo3) were added to 1 ml of filtrate in test tube [18] followed by incubated the mixture for 20 minutes. A changing of broth color to pink indicated the production of IAA.

(B) Ammonia (NH3) production
Fresh *Trichoderma* cultures were inoculated in 10ml peptone broth containing culture tubes and incubated for 4-5 days at 30°C. Subsequently, 1 ml Nessler's reagent was added to each tube and a color change from light yellow to brownish orange indicated the production of NH_3 [19].

(C) Siderophore production
To determine siderophore production, Chrome Azurol- S blue agar (CAS) medium was used [20]. A 5 mm plug of *Trichoderma* isolates were inoculated in agar medium containing Chrome azurol S and incubated at 28°C for 5 days. After incubation, the appearance of a yellow or orange zone around the colony confirmed the production of siderophore [21].

Statistical analysis
The experiment was set in Completely Randomized Design (CRD). The data were statistically analyzed by Analysis of Variance (ANOVA) and means were compared by Duncan's Multiple Range Test (DMRT).

Results and Discussion

Categorization of isolated Trichoderma spp
Total 7 isolates of *Trichoderma* were isolated from the rhizosphere of various medicinal plants and based on their morphology these were categorized (Table 2). Initially, all the 7 *Trichoderma* isolates developed whitish mycelium which later differed from light to dark green in color. Colony margin varied from irregular to regular pattern and growth rate ranged from 11.25 mm to 18.00 mm per day. Hyphae were hyaline, septate, and branched. Conidiophore verticillately branched and phialides varied from sigmoid to ampulliform, hyaline, solitary or in groups, and size ranged from 7.4×2.6 - 15×3.5 µ (mean of 30). Conidia were globose to sub globose, hyaline to green and size ranged from 1.62 – 3.5 µ (mean of 30). *Trichoderma* isolate Tr6 initially developed light green to whitish center with white margin later center becomes dark green. The verticillately branched conidiophores beard ampulliform (13.72×2.11 µ) phialides (Figure 1). A similar study by Kabir et al., [22] reported that all the tested *Trichoderma* isolates initially formed white colony which later on turned light to dark green.

Table 2. Key morphological descriptions of various *Trichoderma* isolates

Trichoderma isolates	Cultural characteristics			Microscopic characteristics	
	Colony color	Growth pattern	Growth rate (mm/day)	Conidia size (µ)	Phialides Shape/ Size (µ)
Tr1	Initially whitish growth, later patches of light and dark green color	Less sporulation, margin is not dense, initially irregular margin	12.90	2.5	Sigmoid (8×3.5)
Tr2	Initially whitish growth, later turns to light green color with dark green margin	Initially scattered irregular margin, compact growth, profuse sporulation	11.25	3.15	Ampulliform (11.4×3.7)
Tr3	Initially milky white mycelium, later turns to olive green color, reverse side buff white	Dense, vigorous mycelial growth, irregular margin, later on cottony fluffy uniform growth.	18.00	3.5	Ampulliform 13.2 ×1.8
Tr4	Initially dull white mycelium, center light greenish which turns to dark green in color in the later stage	Uniform growth, light mycelial strands in center	15.50	1.62	Sigmoid (7.4×2.6)
Tr5	Initially white mycelium later with light green center and whitish margin	Growth in irregular pattern, loose mycelium	14.25	2.42	Ampulliform 15×3.5
Tr6	Initially light green to whitish center with white margin later center becomes dark green and periphery of the colony turns to creamy to milky white in color	Irregular dense mycelium, profuse sporulation	17.25	1.78	Ampulliform (13.72×2.11)
Tr7	Patches of white and green mycelium	Growth is not uniform, irregular margin, compact growth	16.5	2.9	Ampulliform (11.6×1.9)

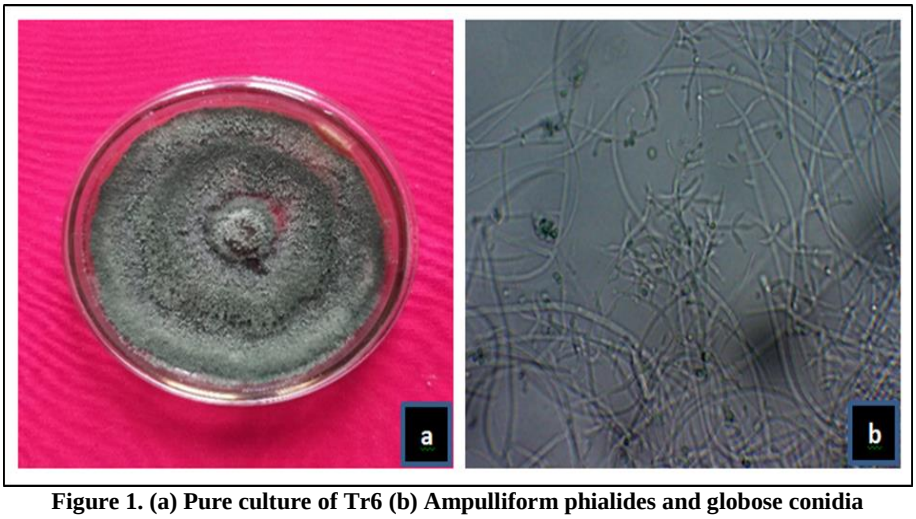

Figure 1. (a) Pure culture of Tr6 (b) Ampulliform phialides and globose conidia

Phialides and spore size was also varied in different isolates. Sigmoid phialides measuring 8-14× 2.4-3µ with globose conidia measuring 4-4.8×3.5-4µ was identified as *Trichoderma* viride whereas ampulliform phialides measuring 3,5-7.5× 2.5-3.8µ with globose to ovoid conidia of 1.7-3.2×1.3-2.5µ was identified as *Trichoderma harzianum*. Another study [23] revealed that *Trichoderma harzianum* formed light to dark green colony with ring like zones. The phialides were globose to nine-pin shaped measuring 8-15×2-3µ whereas conidia were subglobose and measured 3.6-4.5 µ.

Evaluation of antagonistic potential Trichoderma isolates by dual culture technique

All *Trichoderma* isolates significantly reduced the mycelial growth of *Alternaria alternata* and *Curvularia lunata in vitro* condition (Table 3 and 4).

Table 3. *In vitro* evaluation of *Trichoderma isolates* on radial growth and percent of *Alternaria alternata* at different time interval

Treatments	72 hrs		120 hrs		168 hrs	
	Radial Growth (mm)	Inhibition Percent (%)	Radial Growth (mm)	Inhibition Percent (%)	Radial Growth (mm)	Inhibition Percent (%)
Tr1	27.33	32.92^{e}	33.17	33.77^{e}	35.08	42.17^{e}
Tr2	31.42	22.90^{f}	36.25	27.62^{f}	38.25	36.95^{g}
Tr3	20.08	50.72^{b}	24.25	51.58^{b}	26.08	57.00^{b}
Tr4	25.42	37.63^{d}	27.92	44.26^{d}	30.17	50.28^{d}
Tr5	27.83	31.70^{e}	34.08	31.94^{e}	36.58	39.70^{f}
Tr6	17.42	57.26^{a}	20.17	59.73^{a}	20.83	65.66^{a}
Tr7	21.67	46.83^{c}	25.58	48.92^{c}	28	53.85^{c}
Control	40.75	0g	50.08	0g	60.67	0h
SE(m)	**0.58**		**0.69**		**0.73**	
C.D at 5%	**1.76**		**2.09**		**2.21**	
C.V.	**3.80**		**3.81**		**3.68**	

Table 4. *In vitro* evaluation of *Trichoderma isolates* on radial growth and percent growth inhibition of *Curvularia lunata* at different time interval

Treatments	72 hrs		120 hrs		168 hrs	
	Radial Growth (mm)	Inhibition Percent (%)	Radial Growth (mm)	Inhibition Percent (%)	Radial Growth (mm)	Inhibition Percent (%)
Tr1	19	13.32^{d}	22.67	24.57^{e}	26.17	25.94^{e}
Tr2	21	4.19^{f}	25.17	16.25^{g}	28.92	18.15^{g}
Tr3	16.5	24.73^{b}	18.17	39.54^{b}	21.08	40.32^{b}
Tr4	19.83	9.52^{e}	24.42	18.74^{f}	27.17	23.11^{f}
Tr5	18	17.88^{c}	19.83	33.99^{c}	22.17	37.26^{c}
Tr6	15.33	30.05^{a}	16.42	45.37^{a}	19.42	45.04^{a}
Tr7	17.75	19.02^{c}	21.5	28.45^{d}	22.92	35.14^{d}
Control	21.92		30.05	0h	35.33	0h
SE(m)	**0.43**		**0.45**		**0.57**	
C.D at 5%	**1.30**		**1.37**		**1.74**	
C.V.	**3.97**		**3.52**		**3.92**	

Among the seven isolates, Tr6 recorded the highest inhibition of *Alternaria alternata* (65.66%) and *Curvularia lunata* (45.04%) (Figure 2) followed by Tr3 which showed 57.00% and 40.32% mycelial growth inhibition of *Alternaria alternata* and *Curvularia lunata* respectively after 168 hours of incubation. The isolate Tr2 was found least effective by recording 36.95% mycelial growth inhibition of *Alternaria alternata* and 18.15% in growth inhibition of *Curvularia lunata* after 168 hours of incubation. In an experimental finding [24] isolated *Alternaria alternata* from *Mentha arvensis* and observed that after seven days of incubation, inhibition percentage of *Alternaria alternata* by *Trichoderma asperellum* was 86.25 ± 7.34%. Comparative findings were reported by Tagaram et al., [25] isolated *Alternaria alternata* from

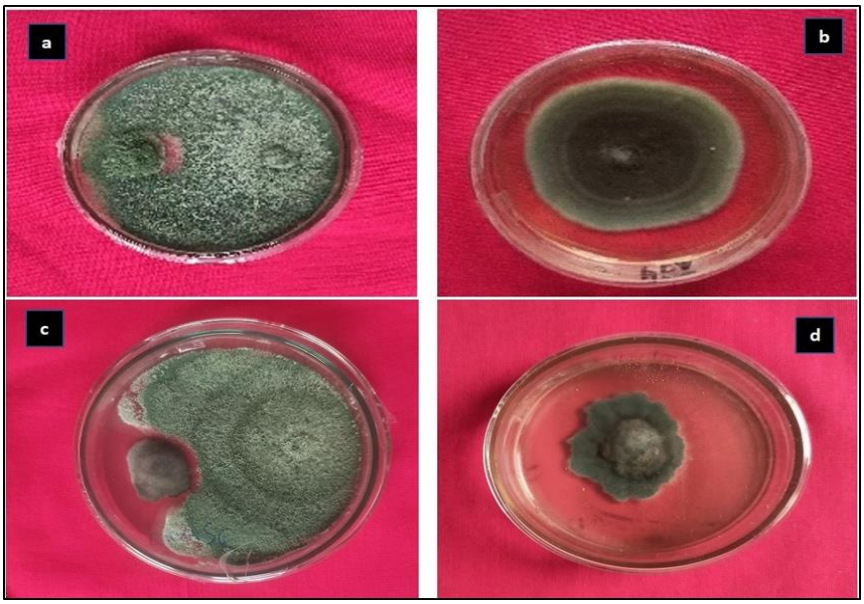

Figure 1. (a) Dual culture of Tr6 and *Alternaria alternata* after 168 hours of inoculation (b) Control after 168 hours of inoculation (*Alternaria alternata*) (c) Dual culture of Tr6 and *Curvularia lunata after* 168 hours of inoculation (d) Control after 168 hours of inoculation (*Curvularia lunata*)

infected Senna leaves and reported that inhibition of mycelial growth of the pathogen by *Trichoderma viride* and *Trichoderma harzianum* were 80% and 72% respectively. Similarly, Iftikhar et al., [26] tested 12 *Trichoderma* isolates against *Curvularia lunata* causing leaf spot of tomato. All *Trichoderma* isolates inhibited the growth of the pathogen by more than 80% and among those, *Trichoderma viride* showed highest mycelial inhibition (93.8%) followed by *Trichoderma hamatum* (92.2%) [10] reported that *Trichoderma harzianum* and *Trichoderma viride* recorded 76.3% and 72.0% mycelial inhibition of *Curvularia lunata* respectively.

Biochemical assay of Trichoderma isolates

Biochemical responses of *Trichoderma* isolates are given in Table 5. All the isolates except Tr2 and control were recorded to be positive in Indole Acetic Acid (IAA) production by developing a light to reddish pink color. The highest IAA production was observed in Isolates Tr6 and Tr7 followed by isolate Tr3. During the IAA production test [27] reported that out of twenty *Trichoderma* isolates,

Table 5. Biochemical assay of *Trichoderma isolates*

SN.	Isolate	IAA production	NH3 production	Siderophore production
1	**Tr1**	+	+	+
2	**Tr2**	-	+	-
3	**Tr3**	+	++	++
4	**Tr4**	+	+	+
5	**Tr5**	+	+	-
6	**Tr6**	++	++	++
7	**Tr7**	++	+	+
8	**Control**	-	-	-

Positive reaction (+ + > +), Negative reaction (-)

only eight isolates showed positive result IAA production and among those eight isolates, maximum IAA production was exhibited by TG 4 (90 µg/ml). All *Trichoderma* isolates were observed to be positive in Ammonia (NH_3) production test by developing dark yellow to brownish orange color whereas

no change in color was observed in uninoculated broth. Among the isolates, Tr3 and Tr6 showed highest production of Ammonia (NH_3) by production of dark brownish orange color whereas other isolates were observed to produce dark yellow color. Similarly, Ayyandurai et al., [28] reported that all six *Trichoderma* spp developed yellowish to brown color and hence found positive reactions in the ammonia production test. *Trichoderma* isolates namely Tr1, Tr3, Tr4, Tr6, and Tr7 siderophore production where developed yellow zone around the mycelial margin, and among these isolates, Tr6 showed the highest production of siderophore. In a similar study, Dixit et al., [29] reported that among 18 *Trichoderma* isolates, only 4 isolates exhibited positive results of siderophore production by developing pink halo in the medium.

Conclusion

The present study revealed that out of seven *Trichoderma* isolates, T6 was most effective in reducing radial growth of the foliar pathogens of Japanese mint i.e., *Alternaria alternata* (65.66%) and *Curvularia lunata* (45.04%) which was followed by T3 isolate. The present study also reported that the majority of the *Trichoderma* isolates exhibited positive results in ammonia, IAA, and siderophore production. Hence, the preparation of microbial formulation and consortium development using the potential isolates is required to confirm their antagonistic ability. In subsequent investigations, the potential *Trichoderma* isolates must undergo molecular characterization in order to be proper identification and these isolates should be included in IDM program for the management of diseases of Japanese mint.

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