The Review of Contemporary Scientific and Academic Studies
An International Multidisciplinary Online Journal
www.thercsas.com

ISSN: 2583-1380 Vol. 3 | Issue No. 9 | September 2023 Impact Factor: 4.736 (SJIF)

In Vitro Antifungal, Enzymatic and Plant Growth Promotion Activities of Endophytic Fungi Associated with Anaphalis Contorta (D.Don) Hook.f. from Manipur

Nongthombam Kistu Singh (nkistusingh@gmail.com), Corresponding Author
Research Scholar, Department of Life Sciences (Botany), Manipur University, India
Mutum Shyamkesho Singh, Professor, Department of Life Sciences (Botany), Manipur University, India
Radha Raman Pandey, Professor, Department of Life Sciences (Botany), Manipur University, India

 Crossref/DOI: https://doi.org/10.55454/rcsas.3.09.2023.003

Abstract: *Four endophytic fungal isolates, Fusarium sp., Mucor sp., Penicillium sp., and Arthrinium sp., were isolated from different parts of Anaphalis contorta and assessed for antifungal activity, extracellular enzyme production, and plant growth promotion abilities in vitro condition. The antifungal activity was evaluated against ten fungal phytopathogens by using the agar dual culture method. All the endophytes have shown moderate to strong antifungal activity against Curvularia lunata, Fusarium oxysporum, Aspergillus niger, Aspergillus flavus, Sclerotium oryzae, Rhizoctonia solani, Alternaria alternata, Colletotrichum capsici, Ustilaginoidea virens, and Alternaria brassicicola. Maximum inhibition was shown by Penicillium sp. (79.52%) against C. lunata. Potential biocontrol agent plays an important role in environment friendly agricultural practices. Qualitative enzyme activity has shown that protease, lipase, amylase, cellulose, and laccase were all produced by Fusarium sp. These enzymes are very important in industries and pharmaceutical applications. Solubilization of inorganic phosphate, NH4, and HCN production was shown by Penicillium sp., which makes the availability of essential elementsto the host plant and well as protects the host plant from pathogens.*

Keywords: Antifungal, Biocontrol, Endophytic Fungi, Phytopathogens, Protease

Article History: **Received: 31 August 2023;** **Accepted: 16 Sept- 2023;** **Published/Available Online: 30 Sept- 2023;**

1. Introduction

Endophytic fungi colonize the healthy tissues of the host plants for all or a portion of their life cycle without exhibiting any disease symptoms. Tropical medicinal plants offer great promise as hosts for endophytic fungi, which release bioactive secondary metabolites with antibacterial, antifungal, antiviral, anti-inflammatory, anti-tumor, and antioxidant properties. These compounds have potential uses in agriculture, medicine, and the food industry (Abo Nouh, 2019; Banerjee, 2011).*Anaphalis contorta* is a high-altitude perennial herb of Asteraceae with several ethnomedicinal properties (Sharma et al., 2020). Studies reported that *A. contorta* possessed strong antimicrobial properties (Joshi, 2013). Endophytic fungi are useful in modern agricultural applications for their capacity to promote plant growth, suppress pests, withstand environmental stress, and be ecologically friendly (Shah *et al*., 2021; Ripa *et al*., 2019). According to several research, fungal endophytes lessen insect and pathogenic fungal attacks on the host plant (Bamisile *et al*., 2018; Gange *et al*., 2012). Enzymes of microbial origin are important to the food processing, apparel, footwear, and pharmaceutical sectors (Patel *et al*., 2023; Thapa *et al*., 2019). The level of environmental degradation increases as a result of the application of chemicals to control plant diseases. So, the hunt for a novel biological agent is expanding as biological control is viewed as a feasible alternative to chemical treatment. Several mechanisms like competition for food and space, production of volatile and non-volatile metabolites, mycoparasitism, and stimulation of host defence stimulation are employed by antagonistic organisms to encounter pathogens (Köhl *et al*., 2019; Ghorbanpour *et al*., 2018). Endophytic fungi have the ability to absorb macronutrients from the soil and organic matter and increase the supply to the host plant (García-Latorre*et al*., 2021). The need for sustainable agriculture is the main target area for recent scientific research, which will help to protect and reduce the negative impacts on the environment in the future (Umesha *et al*., 2018). In the present study, four isolates of endophytic fungi were evaluated for antifungal activity against ten phytopathogens and further analyzed for extracellular enzymes and growth promotion activities.

2. Materials and Methods

Plant Collection: Mature *Anaphalis contorta* plants were collected from the Ukhrul district (latitude 25° 8' 41.464" N; longitude 94° 27' 38.289" E; altitude 2062.14 m a.s.l.) of Manipur during the month of

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www.thercsas.com
ISSN: 2583-1380 Vol. 3 | Issue No. 9 | September 2023 Impact Factor: 4.736 (SJIF)

December. The collected plants were processed within 18 hrs of collection. The plant was authenticated and deposited at the Manipur University Museum of Plants (MUMP), Manipur University.

Isolation: The isolation was conducted by following the protocol of Hallmann *et al*. (2006) with minor modifications. A three-step surface sterilization process was performed, first with 70% ethanol for 3 minutes, followed by 4% sodium hypochlorite (NaOCl) solution for 2 minutes, and 70% ethanol for 1 min. Leaf, stem, root, and inflorescence of *A. contorta* were used for the isolation of endophytic fungi. The sterilized plant parts were cut into the size of 0.5 cm using a sterile blade and transferred into Petri plates containing PDA medium. The Petri plates were incubated for a period of 5 to 7 days at 28±1°C.The fungal growth that emerged around the plant segments were pure cultured for 7 days and proceeded for identification.

Identification: Morphological identification of the fungal endophytes were carried out based on the colony morphology, colony margin, texture, pigmentation, spore and spore-bearing structures after referring to books on fungal identification (Barnett & Hunter, 1998; Watanabe, 2002). The isolates were deposited at the National Fungal Culture Collection of India (NFCCI), Agharkar Institute, Pune.

Antifungal Activity: Four isolates of endophytic fungi, namely, *Fusarium* sp., *Penicillium* sp., *Arthrinium* sp., and *Mucor* sp. were assessed for antifungal activity against economically important ten fungal plant pathogens:*Curvularia lunata* (ITCC 7170), *Fusarium oxysporum* (ITCC 4998), *Aspergillus niger* (ITCC 5406), *Aspergillus flavus* (ITCC 6972), *Sclerotium oryzae*(ITCC 4107), *Rhizoctonia solani* (ITCC 4576), *Alternaria alternate* (ITCC 6778), *Colletotrichum capsici* (ITCC 6078), *Ustilaginoidea virens* (ITCC 7046), and *Alternaria brassicicola* (ITCC 6193) by Petri plate dual culture method. The PDA plates were incubated at 28±1°C for a period of 7 days and the antifungal inhibition (%) were calculated by using the formula, I% = [(r1–r2)/r1] × 100; r1 = growth of the pathogen on the control plate, r2 = growth of the pathogen on dual culture plate (Hajieghrari *et al*., 2008).

Enzyme Activity: The endophytic fungal isolates were evaluated for qualitative production of protease, lipase, amylase, cellulase and laccase. The enzyme production was observed by the digestion of a particular substrate in agar medium (Rajput *et al*., 2016; Yadav *et al*., 2015).

Protease: For protease activity, the endophytic isolates were grown on glucose yeast peptone agar (GYPA) media containing 1% skim milk and observed for clear region after 5 days of incubation at 28±1°C.

Lipase: The fungal endophytes were enoculated on the peptone agar (PA) medium with added Tween 20 and observed for clear zone after 5 days of incubation at 28±1°C.

Amylase: The endophytes were inoculated on GYPA medium containing 1% soluble starch. After 5 days of incubation at 28±1°C, iodine solution (1% iodine and 2% potassium iodide) was poured over the plates containing the fungal colony and observed for the appearance of a halo region around the colony.

Cellulase: Endophytic fungi were inoculated on GYPA medium with added carboxy methyl cellulose (CMC) and incubated for a period of 5 days at 28±1°C. After incubation, the Petri plates were flooded with Congo red dye solution (1%) for 20 minutes and destained with NaCl solution for 15 minutes and observe for yellowish colour around the fungal colony.

Laccase: The isolates were inoculated on GYPA medium supplemented with 1-naphthol (0.005%)and incubated for 8 days at 28±1°C.The culture medium was observed for colour change from transparent to purple or violet.

Growth Promotion Activity

Phosphate (PO_4) Solubilization: Phosphate solubilization was assessed by inoculating the endophytic fungi on Pikovskaya's agar medium containing insoluble calcium phosphate. After 7 days incubation at 28±1°C, the appearance of a clear zone around the colony was observed (Ripa *et al*., 2019).

Ammonia (NH_4) Production: The endophytic fungal isolates were placed in test tubes with 10 ml peptone water and incubated for 72 hrs at 28±1°C. The Nessler's regent (0.5ml) was added to each tube and observed for change in colour in the peptone water extract (Mahfooz *et al*., 2017).

Hydrogen Cyanide (HCN) Production: The fungal isolates were grown in test tubes containing Bennett agar amended with glycine (4.4 g/L). Whatman No. 1 flooded with a solution of 0.5% picric acid and 2%

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sodium carbonate was inserted inside the test tubes. The tubes were parafilm-sealed and incubated at 28±1°C for 10 days. The development of a brown to red colour in the filter paper was observed (Potshangbam *et al*., 2017).

3. Results

Isolation and Identification of Endophytic Fungi: Four isolates of endophytic fungi were isolated from different plant parts and identified viz. *Fusarium* sp. from inflorescence, *Mucor* sp. from root, *Penicillium* sp. from leaf, and *Arthrinium* sp., from stem (Table 1, Fig. 1).

Antagonistic Inhibition (I%): After calculation of the inhibition percentage (I%) of the 4 isolates, it was found that highest inhibition of *C. lunata*, *A. flavus*, *R. solani*, *S. oryzae*, and *C. capsici* was shown by *Penicillium* sp.; *F. oxysporum*, *A. tenuissima*, and *A. alternata* by *Mucor* sp.; *A. niger* by *Fusarium* sp.; and *U. virens* by *Arthrinium* sp. (Table 2, Fig. 2).

Enzyme Production Activity: The endophytic fungi in this study are good producers of extracellular enzymes. *Fusarium* sp. produces allfive enzymes; *Mucor* sp. and *Arthrinium* sp. do not produce laccase enzyme; and *Penicillium* sp. shows negative results in lipase and laccase enzyme production (Table 3).

Plant Growth Promotion Activities: *Penicillium* sp. produces both NH_4 and HCN; *Mucor* sp. produces NH_4; *Fusarium* sp. produces HCN; and *Arthrinium* sp. does not show any positive result for plant growth promotion (Table 3).

Discussion

The purpose of this study was to assess the antifungal properties as well as screen for the production of extracellular enzymes and plant growth-promoting potentials of endophytic fungi isolated from the medicinal plant *Anaphalis contorta*. All the endophytic fungi in the study show good antifungal properties. This highlights their potential as novel biocontrol agents, which are highly needed in organic agricultural practices and integrated farming methods. Several isolates of endophytic fungi have been known to show strong antagonistic activities against plant pathogens. The endophytic fungi *Trichoderma viride*, *Epicoccum nigrum*, *Fusarium tricinctum*, *Alternaria alternata*, *Sclerotinia sclerotiorum*, and Cytospora sp. were reported to control the "oak decline" disease of oak trees caused by *Diplodia corticola* (Campanile et al., 2007). In another study, the endophytic fungus Fusarium solani isolated from cotton plants could control Verticillium wilt in cotton caused by *Verticillium dahliae* (Wei*et al*., 2019).

Almost all the endophytic fungi produce extracellular enzymes, which indicate their protective nature for host plants against invading pathogens. Enzymes of microbial origin, especially those of endophytic fungi, are of great interest due to low time consumption, little inoculum requirement, no negative impacts on the environment, and cost efficiency. The proteases are used in several industries, such as the pharmaceutical industry, the leather industry, the detergent industry, and the food industry. In medical applications, protease is used to treat a variety of diseases such as cancer, inflammatory diseases, glaucoma, etc. An endophytic fungus, Alternaria alternata isolated from *Eremophilia longifolia*, produces a high amount of proteases in a wide range of pH (3-9) (Zaferanloo*et al*., 2014).

Lipases are used in various industries such as dairy, food, detergents, textiles, pharmaceuticals, cosmetics, biodiesel industries, and agriculture. The lipase enzyme produced by the endophytic fungi *Emericella nidullans* and *Dichotomophtora boerhaaviae* has shown good antileishmanial activity against *Leishmania amazonensis* (Alves*et al*., 2018). Amylases are important in the food, textile, detergent, paper, and biofuel production industries. A huge amount of amylase was reported to be produced by the endophytic fungi *Cylindrocephalum* sp. from *Alpinia calcarata* (Sunitha *et al*., 2012); *Alternaria alternata* from *Cupressus torulosa* (Rajput *et al*., 2016); and *Discosia* sp. from *Calophyllum inophyllum* (Hegde *et al*., 2011). Cellulases are widely used in different industries like animal feed, agriculture, laundry and detergent, textile, paper and pulp, brewing, food processing, olive oil extraction, and biofuel production. Cellulase is recorded to be produced by the endophytic isolates *Penicillium oxalicum* from *Taxus cuspidate* (Li *et al*., 2021) and *Pycnoporus sanguineus* from *Baccharis dracunculifolia* (Onofre *et al*., 2015).Laccase is an important enzyme as it can oxidize both toxic and nontoxic substrates and is utilized in the textile industry, food processing industry, wood processing industry, pharmaceutical industry, and chemical industry. Previous studies reported that laccase production by endophytic fungi, *Monotospora* sp. from *Cynodon dactylon*

(Wang *et al*., 2006) and *Colletotrichum gloeosporioides* from *Piper betle* (Sidhu *et al*., 2014). Laccase produced by the endophyte *Penicillium megasporum* has been shown to decolorize synthetic dyes (Agarwal *et al*., 2021).

The plant growth promotion abilities enhance the growth of the host plant at times of environmental stress. Phosphorus is one of the major growth-limiting macronutrients required for proper plant growth. Most soils possess considerable amounts of phosphorus, but a large proportion is bound to soil constituents. In a study, *Penicillium* spp. and *Aspergillus* spp. isolated from the roots of *Taxus wallichiana*solubilized insoluble phosphate at different temperatures (Adhikari & Pandey, 2019). Another study shows that an endophytic fungus, *Curvularia geniculate*, from the roots of *Parthenium hysterophorus* improves plant growth through phosphate solubilization of three different sources, $AlPO_4$, $FePO_4$, and $Ca_3(PO_4)_2$ (Priyadharsini & Muthukumar, 2017).Nitrogen is the most essential element required by the plant for the formation of biomolecules like proteins, enzymes, chlorophylls, and nucleic acids. Endophytic fungi provide nitrogen in the form of ammonia to the host plants. The endophytic fungi *Penicillium chrysogenum* and *Penicillium crustosum* isolated from *Teucrium polium* were reported to produce a high amount of ammonia (Hassan, 2017). Hydrogen cyanide is a bioactive compound produced by endophytic fungi that protect the host plant from invading phytopathogens and herbivores. It has been reported that HCN produced by the endophytic fungi *Aspergillus alabamensis*, *Aspergillus oryzae*, and *Aspergillus tubingensis* could suppress the wilt disease of pepper plants (Attia *et al*., 2022).

Conclusion

The endophytic fungal isolates *Fusarium* sp., *Penicillium* sp., *Arthrinium* sp., and *Mucor* sp. isolated from healthy parts of *Anaphalis contorta* can be used as potential biocontrol agents against a wide range of phytopathogens. Till now there are few commercially available biocontrol agents on the market and endophyte-origin biocontrol agents could provide the safest alternative to chemical control of plant diseases. *In vivo* experimentation and field trials are still required to evaluate the complete antifungal potential. Endophytic enzymes and plant growth-promoting compounds are industrially, medicinally, and agriculturally important and further studies on quantification are needed.

Conflicts of Interest: The authors declare that they have no conflict of interest and agreed for submission.

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ISSN: 2583-1380 Vol. 3 | Issue No. 9 | September 2023 Impact Factor: 4.736 (SJIF)

Tables:

Table 1: Identification and accession of endophytic fungi from *Anaphalis contorta*

Endophytic fungi	Plant parts used	Accession no.	Family
Fusarium sp.	Inflorescence	NFCCI 4767	Nectriaceae
Mucor sp.	Root	NFCCI 4771	Mucoraceae
Penicillium sp.	Leaf	NFCCI 4735	Aspergillaceae
Arthrinium sp.	Stem	NFCCI 4726	Apiosporaceae

Table 2: Antagonistic inhibition percentage (I %) of endophytic fungi against phytopathogens

Fungal pathogens	Inhibition percentage shown by endophytic fungi			
	Fusarium sp.	*Mucor* sp.	*Penicillium* sp.	*Arthrinium* sp.
C. lunata	50.18±0.04	77.52±0.07	79.52±0.13	71.21±0.22
F. oxysporum	33.33±0.03	71.48±0.09	30.00±0.03	41.48±0.02
A. niger	76.47±0.04	58.58±0.02	66.91±0.03	63.48±0.02
A. flavus	40.32±0.25	44.09±0.14	66.67±0.11	53.22±0.22
S. oryzae	44.00±0.08	46.66±0.05	68.00±0.04	50.66±0.03
R. solani	48.89±0.11	43.33±0.10	70.00±0.08	44.67±0.10
A. alternata	40.00±0.02	44.44±0.02	42.22±0.02	39.22±0.01
C. capsici	55.22±0.09	59.70±0.09	62.68±0.09	58.20±0.14
U. virens	57.14±0.03	63.26±0.09	46.93±0.02	65.26±0.11
A. tenuissima	45.23±0.04	52.38±0.09	35.71±0.02	38.09±0.17

Data are means of inhibition percentage (triplicate) ± standard deviation (SD)

Table 3: Qualitative analysis of enzyme production and plant growth promotion abilities

Endophytic fungi	Enzyme activities					Plant growth promotion activities		
	Protease	Lipase	Amylase	Cellulase	Laccase	PO_4	NH_4	HCN
Fusarium sp.	+	+	+	+	+	-	+	+
Mucor sp.	+	+	+	-	-	+	-	-
Penicillium sp.	+	-	+	+	-	+	+	+
Arthrinium sp.	+	+	+	+	-	-	-	-

+ indicates positive result; - indicates negative result

Figures:

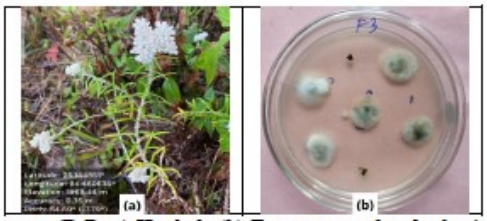

Figure 1: (a) Anaphalis contorta (D.Don) Hook.f.; (b) Emergence of endophytic fungi from plant segments

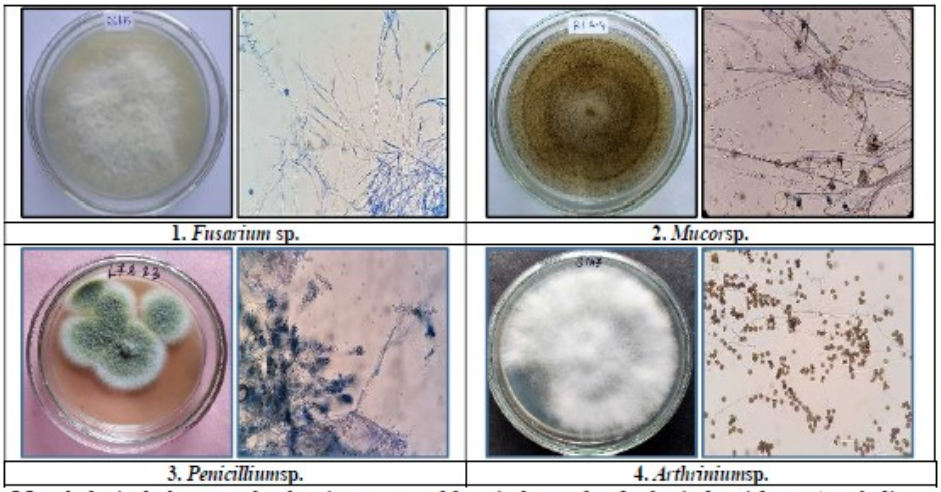

Figure 2: Morphological photographs showing spores of four isolates of endophytic fungi from *Anaphalis contorta*

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ISSN: 2583-1380 Vol. 3 | Issue No. 9 | September 2023 Impact Factor: 4.736 (SJIF)

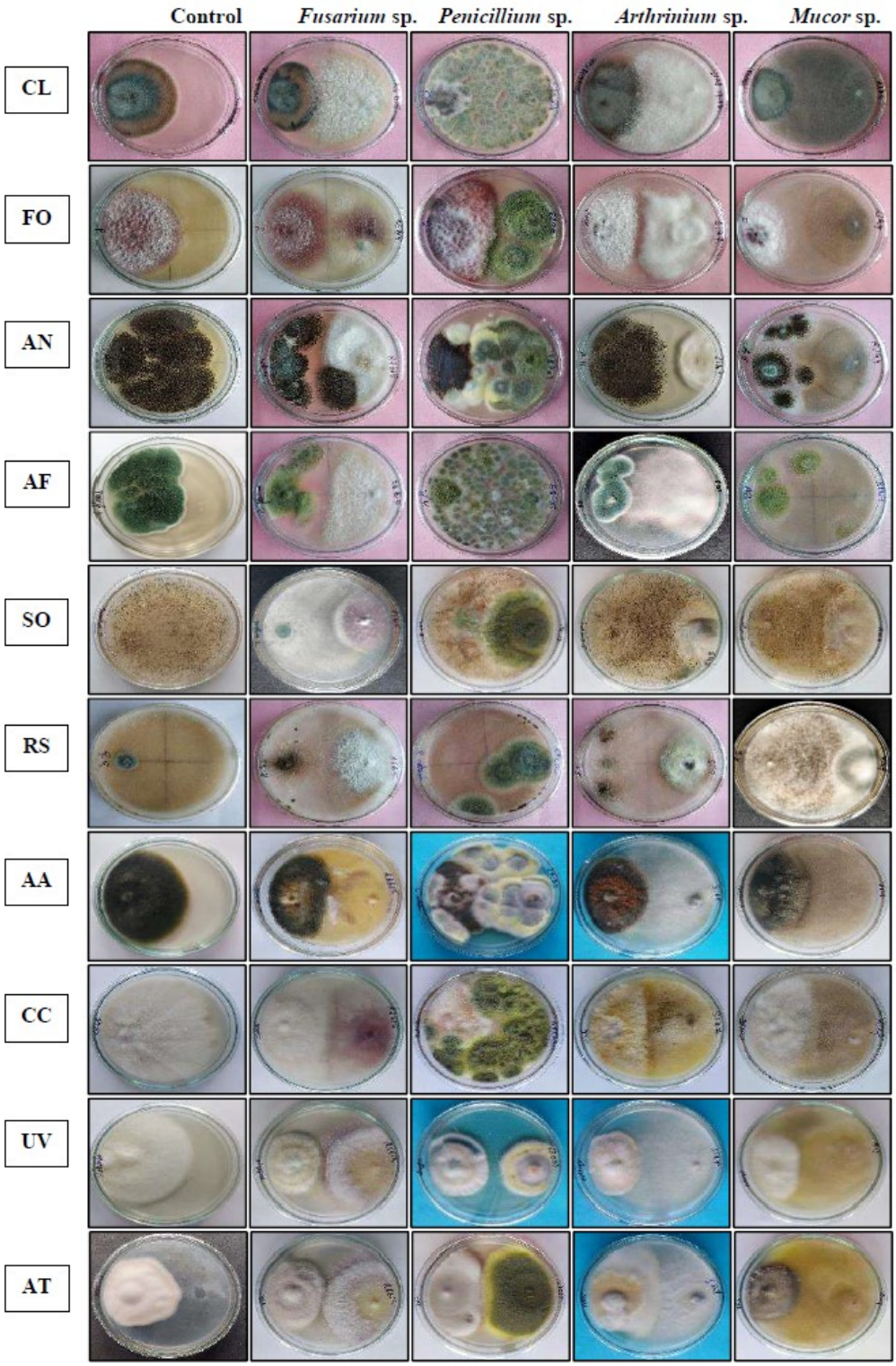

Figure 3: Antagonistic activity of endophytic *Fusarium* sp., *Penicillium* sp., *Arthrinium* sp., and *Mucor* sp. against pathogenic*C. lunata* (CL), *F. oxysporum* (FO), *A. niger* (AN), *A. flavus* (AF), *S. oryzae* (SO), *R. solani* (RS), *A. alternata* (AA), *C. capsici* (CC), *U. virens* (UV) and *A. tenuissima* (AT)using petriplate dual culture method.

Pathogen inoculated on left side of plate; endophytic fungi inoculated on right side of plate.