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IN VITRO MANAGEMENT OF ANTHRACNOSE OF *PIPER LONGUM* (PIPPALI) THROUGH BIOCONTROL AGENTS

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ABSTRACT

Ten antagonists were screened against *Colletotrichum gloeosporioides* for their antagonistic potential through dual culture and well-in-agar techniques. *Trichoderma harzianum* ISO-1, *T.harzianum* ISO-2, *T.piluliferum*, *Aspergillus niger* and *Penicillium sublateritium* exhibited the antagonism. Isolates of *Trichoderma* showed hyphal interaction and mycoparasitism against *C.gloeosporioides*. *P.frequentans* and *P.herquei* showed minimum antagonistic efficacy and percent inhibition of radial growth. *In vitro* study suggested that biocontrol agents can be applied in field trial (*in vivo*) against anthracnose of *P.longum*.

INTRODUCTION

Piper longum Linn. belonging to the family Piperaceae is a slender aromatic climber with perennial woody roots and found all over India in evergreen forests and is cultivated in Assam, Tamil Nadu and Andhra Pradesh¹. *P.longum* is commonly known as *pippali*. It possesses good medicinal properties such as carminative, stimulant, aromatic, good for constipation, gonorrhoea and paralysis of the tongue. *P.longum* is most commonly used to treat respiratory infections such as bronchitis, diseases of the spleen, cough, tumors, and asthma. When applied topically, it soothes and relieves muscular pains and inflammation. In Ayurvedic medicine, it is said to be a good rejuvenator. It also helps in stimulating the appetite and used as sedative in insomnia and epilepsy. Also as cholagogue in obstruction of bile duct and gall bladder^{2,3}. *P.longum* is attacked by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. causing leaf spot disease⁴. Anthracnose, caused by *C.gloeosporioides* (Penz.) was **reported by⁵ from** Ayurvedic Nursery, Herbertpur, Uttarakhand. ⁶reported that *C.gloeosporioides* causes anthracnose in *P.nigrum* (Black pepper).

Plant disease management through biocontrol agents is in demand nowadays. As the intensive use of fungicides has resulted not only in the accumulation of toxic compounds potentially hazardous to humans and the environment⁷ but also in the built-up of resistance in the pathogens. Increasing awareness of fungicide-related hazards has emphasized the need of adopting biological control methods as an alternative and eco-friendly approach for the disease control in plants⁸.

Biological control of plant disease is the suppression of disease symptoms and disease incidence by the application of a biological agent, usually a microorganism⁹. Antagonistic microorganisms can compete with the pathogen for nutrients, inhibit pathogen multiplication by secreting antibiotics or toxins¹⁰. *Trichoderma harzianum* as a biocontrol agent of plant pathogens was used for the control of soil borne, foliar and post harvest diseases in various crops in the field, in commercial green house and storage deposits^{11,12,13,14}. *Trichoderma* spp. suppresses disease by antagonizing the pathogen¹⁵.

Present study aimed at analyzing the efficiency of culture and culture filtrates of antagonists in the control of *C.gloeosporioides* causing anthracnose in *P.longum*.

MATERIALS AND METHODS

Study of symptoms of anthracnose

The diseased leaves of *P.longum* infected with *C.gloeosporioides* were collected separately in sterilized biodegradable polythene bags from Ayurvedic Nursery, Herbertpur, Uttarakhand and brought into laboratory. Symptoms caused by anthracnose were noted in the field and microscopic characters in laboratory with the help of simple microscope.

Isolation of leaf pathogen

For the isolation of pure culture of fungal pathogen, a portion of leaf containing brown spot was surface sterilized with 0.1% mercuric chloride for 1 min, followed by rinsing with three changes of sterilized distilled water and was placed on potato dextrose agar medium in Petri plates. The plates were incubated in a B.O.D. incubator at $25\pm 1^{\circ}\text{C}$ for mycelial growth. Based on the morphological characters, the organism was identified with the help of standard monographs¹⁶.

Isolation of phylloplane fungi

Phylloplane fungi were isolated from healthy leaves of *P.longum* through leaf washing technique^{17,18} and identified with the help of standard monographs¹⁹ and expertise available. To study their antagonistic properties pure cultures were maintained on potato dextrose agar medium at 4°C in a refrigerator.

a. Dual-culture technique:

Assay for antagonism was performed on PDA Petri plates by the dual culture technique. Sterilized potato dextrose agar medium was poured aseptically into sterilized Petri dishes of 7 cm dia. Culture discs of 5 mm dia were cut from the periphery of the actively growing colonies using a sterilized cork borer. Disc of test fungus was placed aseptically at the edge of the Petri plate. These plates were incubated at $25\pm 1^{\circ}\text{C}$ for 3 days. Mycelial disc (5 mm) of antagonist growing on PDA was inoculated on opposite side of Petri plate three days after the pathogen to adjust for the slow growth rate of the pathogen. Paired cultures were again incubated at $25\pm 1^{\circ}\text{C}$ for 6-9 days and observed periodically. Each set was made in 3 replicates.

Antagonistic behaviour was measured quantitatively by calculating the area using graph paper to measure the area of the antagonists, test pathogen species and inhibition zone in the Petri plate. Antagonistic efficacy for each antagonist against the pathogen was worked out according to the following formula²⁰:

$$\text{Antagonistic efficacy} = b + c - a$$

Where,

a = % of area of test pathogen sp. with antagonist in the same Petri plate (cm^2)

b = % of area of antagonist, and

c = % area of inhibition zone between antagonist and pathogen or overgrowth of antagonist over test fungus.

The edges of the parasitized pathogen hyphae by microbial antagonists were transferred from the dual culture dish onto clean slides after 7 days of incubation and mounted with a coverslip in a drop of lactophenol cotton blue. Hyphal interaction and morphology were examined under a research microscope.

b) Culture filtrate method (Well in agar assay)

Flasks prepared with PDB medium were inoculated with antagonist fungi and incubated in a BOD incubator at $25\pm 1^\circ\text{C}$ for 15 days. After 15 days of incubation culture filtrate was divided into two parts. One part was filtered with Whatman No.1 filter paper denoted as 'with cell' culture filtrate and the other part was filtered with a bacterial syringe filter (0.45 micron) denoted as 'cell free' culture filtrate.

PDA Petri plates were prepared. Using a flamed cork borer of 5mm diameter, agar plugs were removed at a distance of 1cm from the periphery of the plate to make wells. Five mm mycelial disc of test pathogen was cut from the actively growing colony margin and placed in the centre of the Petri plates. Wells in Petri plates were then filled with culture filtrates (20 μl /well) of the antagonist fungi as prepared above in separate plates. Whereas, in control set the wells were filled with sterilized distilled (20 μl) water. These plates were incubated at $25\pm 1^\circ\text{C}$ for 7 days and the radial growth of the pathogen was measured with the help of metric scale²¹.

Radial growth inhibition was evaluated according to the formula:

$$\% \text{ Growth Inhibition} = R_2 - R_1 / R_2 \times 100$$

Where,

R_2 = Radial growth of test pathogen in control

R_1 = Radial growth of test pathogen in treatment

RESULTS AND DISCUSSION

Pathogen causing leaf anthracnose in *P.longum* was identified as *Colletotrichum gloeosporioides* (Penz.) Sacc. (Fig 1a). Antagonists were identified on the basis of their cultural and microscopic characteristics as *Trichoderma harzianum* Rifai ISO-1 and *T. harzianum* ISO-2, *T. piluliferum* Webster and Rifai, *Aspergillus niger* van Tieghem, *Penicillium sublateritium* Biourge, *P. herquei* Bainier and Sartory, *P.frequentans* Westling, *P.tardum* Thom, *P.citreo-viride* Biourge and *Cladosporium cladosporioides* (Fresen.) de Vries.

Antagonistic activity in vitro**Dual culture technique**

Dual culture assays showed that all antagonistic microorganisms inhibited the mycelial growth of *C.gloeosporioides*, with varying efficiency (Table 1) (Fig 1). Among the ten microbial antagonists, *Trichoderma harzianum* ISO-1 and ISO-2 and *T.piluliferum* significantly exhibited the strongest antagonism against *C.gloeosporioides* with a high percent antagonistic efficacy (90.0%) showing mycoparasitism (lysis of hyphae of pathogen, Fig 2.). Antagonists *A.niger* (62.97%), *P.sublateritium* (61.02%) and *P.herquei* (42.19%) were also found to be effective in

inhibiting the growth of *C.gloeosporioides*, except *P.tardum* (36.91%), *P.citreo-viride* (34.90%) and *C.cladosporioides* (30.97%). Minimum antagonistic efficacy was shown by *P.frequentans* (29.15%).

Table 1. Antagonistic efficacy against *C.gloeosporioides*

S.No.	Antagonists	Percent Antagonistic efficacy (Mean±S.D.)
1	<i>A.niger</i>	62.97(79.33)±0.83
2	<i>C.cladosporioides</i>	30.97(33.65)±5.56
3	<i>P.citreo-viride</i>	34.90(32.77)±2.12
4	<i>P.frequentans</i>	29.15(23.73)±0.56
5	<i>P.herquei</i>	42.19(45.10)±0.42
6	<i>P.sublateritium</i>	61.02(76.43)±2.96
7	<i>P.tardum</i>	36.91(36.07)±0.88
8	<i>T.harzianum</i> ISO-1	90.00(100)±0.00
9	<i>T.harzianum</i> ISO-2	90.00(100)±0.00
10	<i>T.piluliferum</i>	90.00(100)±0.00
	Mean	57.08
	SEM±	1.24
	CD at 5%	3.65

*Original values are given in parentheses

Microscopic observations of mycelial interactions

Microscopic observations were carried out on the interaction of *C.gloeosporioides* with *Trichoderma harzianum* ISO-1, *T.harzianum* ISO-2 and *T.piluliferum* in which lysis of hyphae occurred and the pathogen could not be re-isolated from the point of contact (Fig.2). Antagonists *Aspergillus niger*, *Penicillium sublateritium*, *P.citreo-viride*, *P.frequentans*, *P.herquei*, *P.tardum* and *Cladosporium cladosporioides* did not show any clear pattern of hyphal interaction.

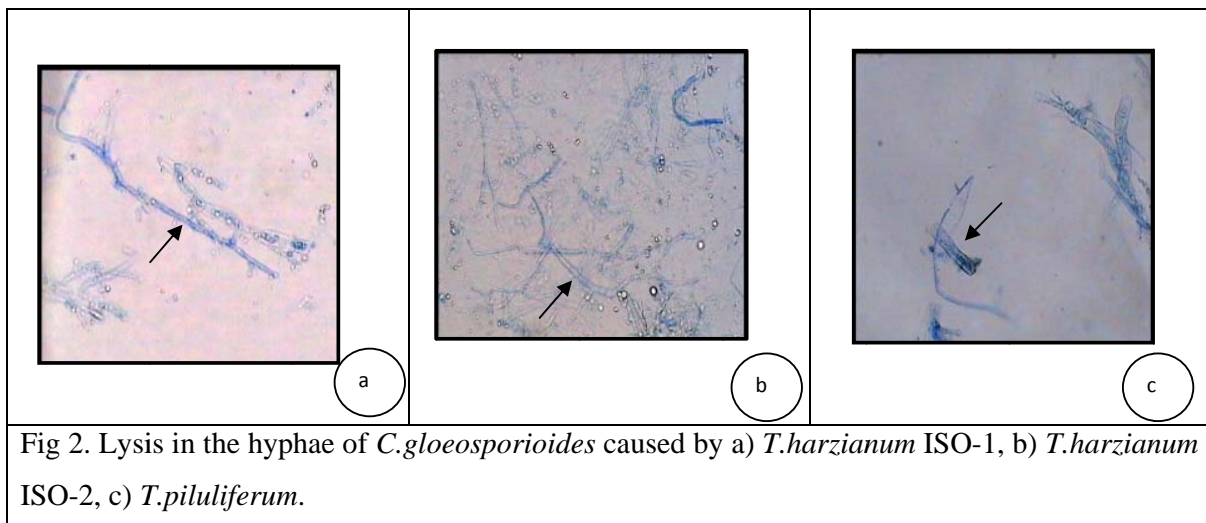


Fig 2. Lysis in the hyphae of *C.gloeosporioides* caused by a) *T.harzianum* ISO-1, b) *T.harzianum* ISO-2, c) *T.piluliferum*.

Culture filtrate method (Well-in-agar assay)

Antagonist *T.piluliferum* exhibited highest percent radial growth inhibition (89.35) of *C.gloeosporioides* which was at par with *T.harzianum* ISO-1 (88.0) followed by *T.harzianum* ISO-2 (61.22). Per cent radial growth inhibition of *C.gloeosporioides* exhibited by *P.sublateritium* (57.0) was found to be at par with *A.niger* (53.65). Whereas, minimum suppression was shown by *P.herquei* (15.87) (Table 2) (Fig 3). During interaction between (antagonists and conditions) it was observed that *A.niger*, *P.sublateritium*, *P.citreo-viride*, *P.frequentans*, *P.herquei*, *P.tardum*, *C.cladosporioides* and *T.harzianum* ISO-2 exhibited more inhibition under 'with cell' as compared to the 'cell free' condition except *T.harzianum* ISO-1 and *T.piluliferum* which showed similar effect under both the conditions.

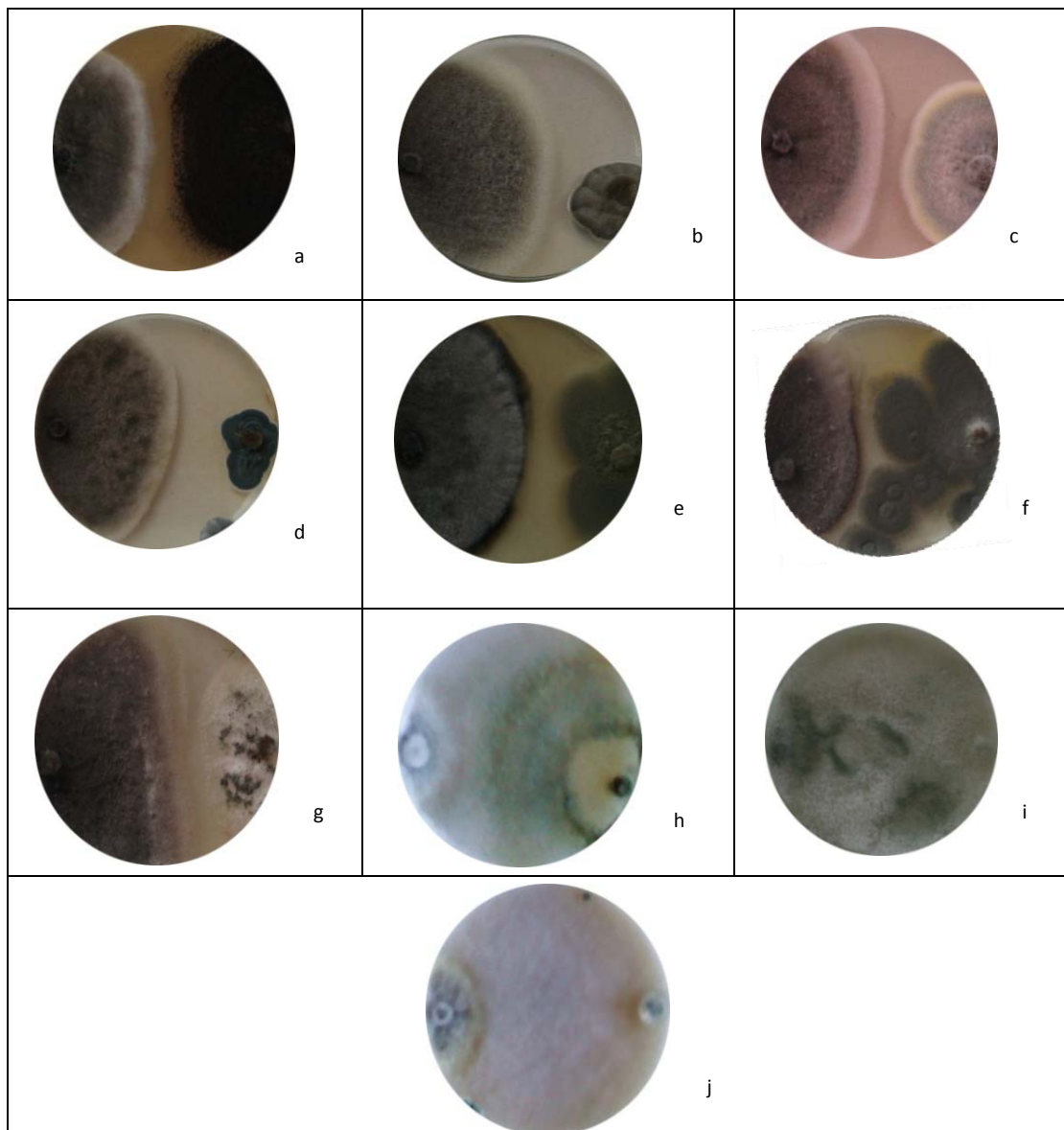


Table 2. Per cent inhibition of radial growth of *C.gloeosporioides*

S.No.	Antagonists	Conditions		Mean
		With cell (Mean±S.D.)	Cell free (Mean±S.D.)	
1.	<i>A. niger</i>	68.47±3.96	38.83±0.56	53.65
2.	<i>C.cladosporioides</i>	34.66±2.19	1.53±2.65	18.10
3.	<i>P.citreo-viride</i>	37.10±1.44	4.23±0.98	20.67
4.	<i>P.frequentans</i>	28.80±2.42	16.43±0.25	22.62
5.	<i>P.herquei</i>	27.46±2.33	4.26±0.92	15.87
6.	<i>P.sublateritium</i>	71.46±6.36	42.53±1.56	57.00
7.	<i>P.tardum</i>	45.06±2.35	1.20±1.03	23.13
8.	<i>T.harzianum</i> ISO-1	88.36±4.39	87.80±3.11	88.08
9.	<i>T.harzianum</i> ISO-2	75.53±6.21	46.90±0.78	61.22
10.	<i>T.pliluliferum</i>	90.90±0.62	87.80±3.48	89.35
Mean		56.78	33.15	
		A	C	A x C
SEM±		1.20	0.53	1.70
CD at 5%		3.43	1.53	4.86

A-Antagonist; C-Condition (with cell, cell free)

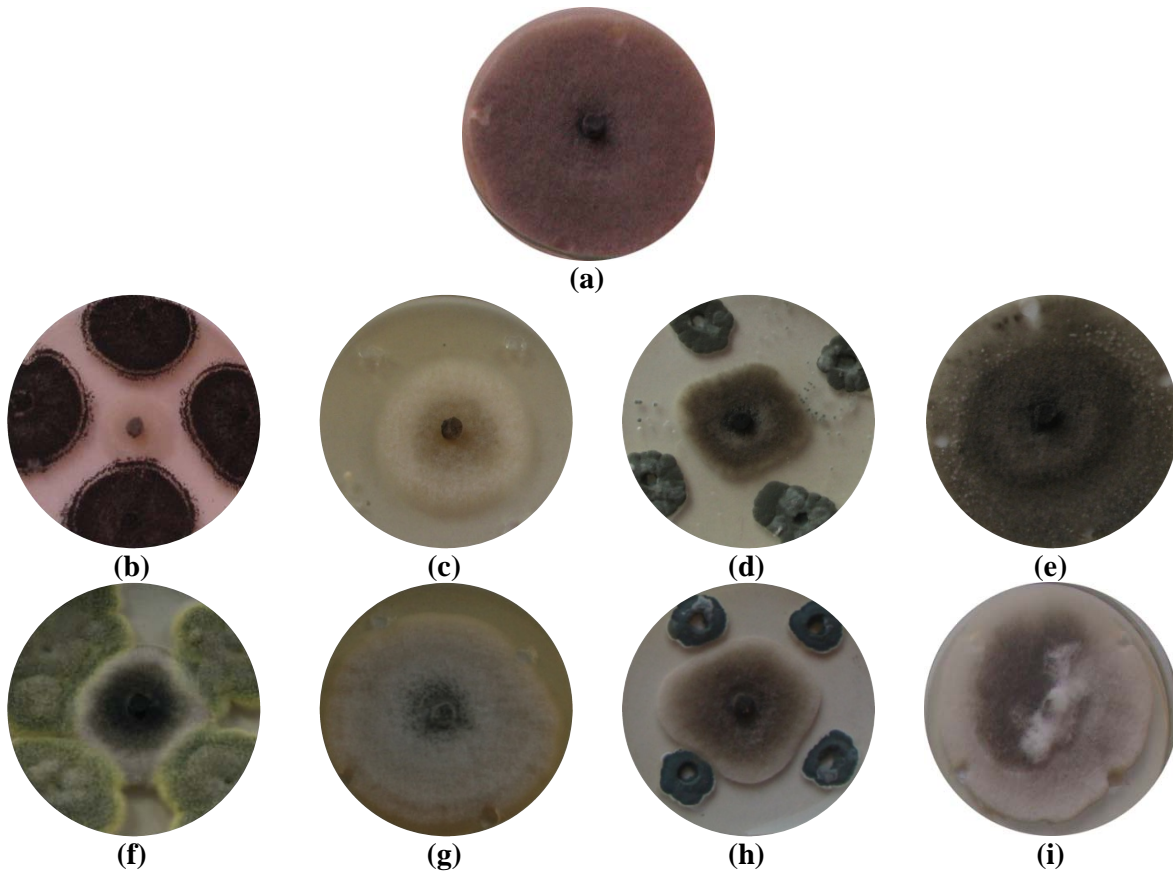
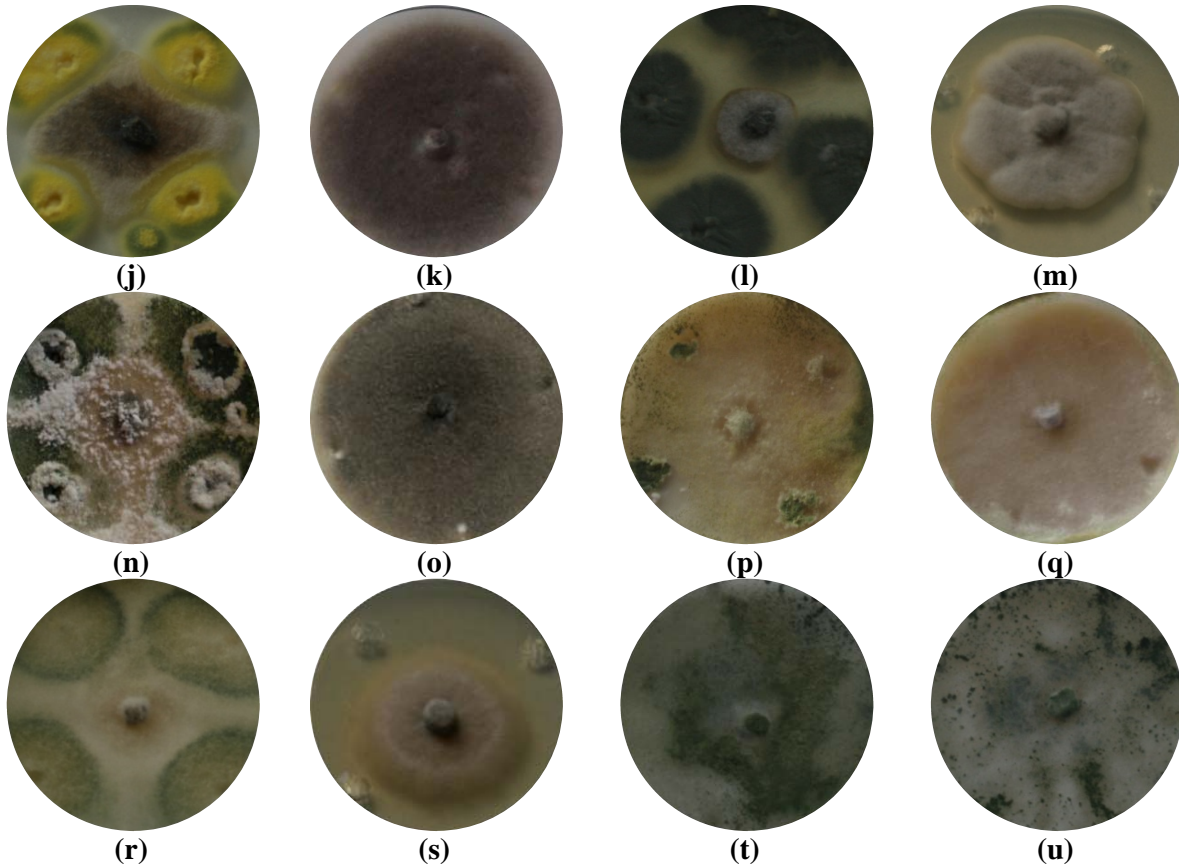


Fig 3. Radial growth inhibition of *C.gloeosporioides* in (with cell, cell free) culture filtrates of antagonists a) Control, b) In *A.niger* (with cell) culture filtrate, c) In *A.niger* (cell free) culture filtrate, d) In *C.cladosporioides* (with cell) culture filtrate, e) In *C.cladosporioides* (cell free) culture filtrate, f) In *P.citreo-viride* (with cell) culture filtrate, g) In *P.citreo-viride* (cell free) culture filtrate, h) In *P.frequentans* (with cell) culture filtrate, i) In *P.frequentans* (cell free) culture filtrate.



j) In *P.herquei* (with cell) culture filtrate, k) In *P.herquei* (cell free) culture filtrate, l) In *P.sublateritium* (with cell) culture filtrate, m) In *P.sublateritium* (cell free) culture filtrate, n) In *P.tardum* (with cell) culture filtrate, o) In *P.tardum* (cell free) culture filtrate, p) In *T.harzianum* ISO-1 (with cell) culture filtrate, q) In *T.harzianum* ISO-1 (cell free) culture filtrate, r) In *T.harzianum* ISO-2 (with cell) culture filtrate, s) In *T.harzianum* ISO-2 (cell free) culture filtrate, t) In *T.piluliferum* (with cell) culture filtrate, u) In *T.piluliferum* (cell free) culture filtrate.

^{22,23} emphasized the biological significance of phyllosphere fungi. Fungi present on phylloplane such as (*Aspergillus* spp., *Trichophyton* spp. and *Gliocladium* spp.) have been reported for their antagonistic activity against *Colletotrichum* leaf disease of *Hevea brasiliensis* (rubber plant) ²⁴. In present study *Trichoderma harzianum* ISO-1, ISO-2 and *T.piluliferum* effectively antagonized mycelial growth of *C.gloeosporioides* causing anthracnose in *P.longum* through dual culture technique. Similar observations were made by²⁵ where *Trichoderma harzianum* and *T.viride* overgrew the colonies of *C.gloeosporioides*. Antagonistic effect of *T.harzianum*, *A.niger* against *C.gloeosporioides* was reported by²⁶. Potential of *T.harzianum* as an effective antagonist against *Colletotrichum* species has been reported earlier by²⁷.

‘With cell’ and ‘cell free’ culture filtrates of antagonists were analyzed *in vitro*. It was examined that *Trichoderma harzianum* ISO-1, *T.harzianum* ISO-2, *T.piluliferum* caused maximum per cent radial growth inhibition of the pathogens through well-in-agar technique followed by *A.niger* and *P.sublateritium*. During study it was observed that *A.niger* (with cell and cell free) culture filtrates effectively inhibited radial mycelial growth of *C.gloeosporioides* which was found to be in agreement with the findings made by²⁸. ²⁹examined that culture filtrate of *Trichoderma harzianum* effectively controlled the growth of *Colletotrichum capsici*. Similar findings were made by³⁰ where non-volatile compounds produced by *T.harzianum* inhibited the growth of *C.lindemuthianum*.

Thus *T.harzianum* ISO-1, *T.harzianum* ISO-2, *T.piluliferum*, *A.niger* and *P.sublateritium* were found to be strong and potent antagonists against *C.gloeosporioides*.

CONCLUSION

It can be concluded that biological control agents *T.harzianum* ISO-1, *T.harzianum* ISO-2, *T.piluliferum*, *A.niger* and *P.sublateritium* showed potential in *in vitro* techniques. Therefore, they can be recommended as eco-friendly method for the management of foliar diseases of *Piper longum* for enhancing the productivity and can be applied for field trials.

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REFERENCES

1. Anon., “The Wealth of India, A dictionary of Indian raw materials & industrial products-Raw Material Series”, 1969; Vol.VIII:96-99. Publications and Information directorate, Council of Scientific and Industrial Research, New Delhi.

2. Dahanakur S.A., Karandikar S.M. and Desai M., "Efficacy of *Piper longum* in childhood asthma", Indian drugs., 1984; Vol. 21:384-388.
3. Dhiman A.K., "Wild medicinal plants of India" (With Ethnomedicinal Uses) Bishen Singh, Mahendra Pal Singh Publishers, 2005; pp.330-332.
4. Puni L. and Harsh N.S.K. Studies on nursery diseases of important medicinal plants of Uttarakhand Non wood Forest Products Division Forest Research Institute (Indian Council of Forestry Research and Education). Dehradun. 2009; 78p.
5. Thakur, S. Biological control of some important foliar diseases of medicinal plants. Thesis submitted for the degree of Doctor of Philosophy in Forest Pathology. Forest Research Institute, Dehradun, India. 2014; 289 p.
6. Kurien P.S., Josephraj Kumar A., Backiyarani S and Murugan, M. "Case study of "Pollu" disease epidemic of black pepper in high ranges of Idukki District", Proceedings, 12th Kerala Science Congress 2000, Kumily, Kerala, 2000, pp. 497-498. State Committee on Science, Technology and Environment, Thiruvananthapuram.
7. Reshu and Khan M.M., "Role of different microbial-origin bioactive antifungal compounds against *Alternaria* spp. causing leaf blight of mustard", Plant Pathology Journal, 2012; Vol.11(1):1-9.
8. Kumar S., Upadhyay J.P. and Kumar S. Biocontrol of *Alternaria* leaf spot of *Vicia faba* using antagonistic fungi. Journal of Biological Control, 2005; Vol. 20:247-251.
9. Baker K.F. and Cook R.J., "Biological control of plant pathogens", W.H. Freeman and Company. San Fransisco. 1974; pp. 433.
10. Blakeman J.P., "Potential for biological control of plant diseases on the phylloplane", Annual Review of Phytopathology, 1982; Vol.20:167-192.
11. Papavizas G.C., "*Trichoderma* and *Gliocladium*: Biology, ecology and potential for biocontrol", Annual Review of Phytopathology, 1985; Vol. 23:23-54.
12. Elad Y., "Biological control of grape gray mold of *Trichoderma harzianum*", Crop Protection, 1994; Vol. 13:35-38.
13. Jegathambigai V.M., Wijeratnam R.S.W. and Wijesundera R.L.C., "Effect of *Trichoderma* sp. on *Sclerotium rolfsii*, the causative agent of collar rot on *Zamioculcas zamiifolia* and an on farm method to mass produce *Trichoderma* species". Plant Pathology Journal, 2010; Vol.9:47-55.
14. Joshi Kirti and Hash N.S.K. "Population assessment of *Trichoderma piluliferum* using a modified selective medium". *Indian Forester.*, 2009; Vol. 135:960-964.

15. Shah, S and Nasreen, S., "Evaluation of bioagents against the infection of green mold (*Trichoderma* spp.) in *Pleurotus sajor-caju* cultivation", International Journal of Plant Pathology., 2011; Vol. 2:81-88.
16. Sutton B.C., The Coelomycetes: fungi imperfecti with pycnidia, acervuli and stromata. 1st ed. U.K.: Commonwealth Mycological Institute Kew, Surrey, England, 1980; 696 p.
17. Dickinson C.H., "Fungal colonization of Pisum leaves", Canadian Journal of Botany., 1967; Vol. 45:915-927.
18. Aneja K.R., "Experiments in microbiology, plant pathology and biotechnology", 4th Edn., New Age International Publications Limited. 2003; pp.176.
19. Ellis M.B., "Dematiaceous Hyphomycetes", Commonwealth Mycological Institute, Kew, Surrey, England, 1971; 608 p.
20. Ojha B.M., "Studies on biological control of root diseases caused by *Fusarium* species in some multipurpose tree species in forest nurseries". Ph.D. thesis G.D.D. University, M.P., 2000; 117p.
21. Dhingra D.O. and Sinclair B.J., "Basic Plant Pathology Methods". CRC Press, Inc., of Boca Raton, 1985, 355 p.
22. Kapooria R.G. and Sinha S., "Phylloplane mycoflora of pearl millet and its influence on the development of *Puccinia penniseti*", Transactions of British Mycological Society, 1969; Vol.53:153-154.
23. Skidmore A.M. and Dickinson C.H., "Colony interaction and hyphal interference between *Septoria nodorum* and phylloplane fungi", Transaction of British Mycological Society, 1976; Vol. 66:57-64.
24. Eueh G.A. and Ogbebor N.O., "Use of phylloplane fungi as biocontrol agent against *Collectotrichum* leaf disease of rubber (*Hevea brasiliensis*)". African Journal of Biotechnology., 2008; Vol.7(15):2569-2572.
25. Deshmukh P.P. and Raut J.G., "Antagonism by *Trichoderma* spp. on five plant pathogenic fungi", New Agriculturist, 1992; Vol. 3:127-130.
26. Deshmukh A.J., Mehta B.P and Patil V.A., "In vitro evaluation of some known bioagents to control *Colletotrichum gloeosporioides* Penz. And Sacc., causing anthracnose of Indian bean", International Journal of Pharma and Biosciences, 2010; Vol.1(2):1-6.
27. Barros S.T., Oliveira N.T. and Bastos S.T.G., "*Trichoderma* spp. in the biological control of *Colletotrichum lindemuthianum*, causal agent of bean (*Phaseolus vulgaris* L.) anthracnose", Bulletein Mycologia., 1995; Vol.10(1/2):5-11.

28. Pandey R.R., Arora D.K. and Dubey R.C., “Antagonistic interactions between fungal pathogens and phylloplane fungi of Guava”, *Mycopathologia*, 1993; 124:31-39.
29. Ajith P.S. and Lakshmidēvi N., “Effect of Volatile and Non-volatile compounds from *Trichoderma* spp. against *Colletotrichum capsici* incitant of anthracnose on Bell peppers”, *Nature and Science*, 2010; Vol. 8(9):265-269.
30. Shaikh F.T. and Nasreen, S., “*In vitro* assessment of antagonistic activity of *Trichoderma viride* and *Trichoderma harzianum* against pathogenic fungi”, *Indian Journal of Applied Research*, 2013; Vol. 3(5):57-59.