**Journal of Global Biosciences**
**ISSN 2320-1355**
**Volume 5, Number 2, 2016, pp. 3676-3682**
**Website: www.mutagens.co.in**
**E-mail: submit@mutagens.co.in**
**researchsubmission@hotmail.com**

***Research Paper***

# *IN VITRO* ANTAGONISTIC ACTIVITY OF CULTURE FILTRATES OF PHYLLOPLANE FUNGI AGAINST FOLIAR PATHOGENS

Shikha Thakur and NSK Harsh

Forest Pathology Division,
Forest Research Institute, Dehradun - 248 006
Uttarakhand, India.

**Abstract**

Fungal phytopathogens are considered as causal agents of many plant diseases. Application of biocontrol agents offers a viable alternative to chemical fungicides. Efficacy of culture filtrates of *Trichoderma harzianum* ISO-1, *T. harzianum* ISO-2, *T. piluliferum*, *Aspergillus niger* and *Penicillium sublateritium* were evaluated against foliar pathogens isolated from *Rauwolfia serpentina*, *Spilanthes oleracea*, *Chlorophytum tuberosum* and *Piper longum viz., Alternaria alternata* (*R.s*), *A. alternata* (*S.o*), *Colletotrichum dematium* and *C. gloeosporioides*. The culture filtrates of *T. harzianum* ISO-1, *T. harzianum* ISO-2 and *P. sublateritium* exhibit maximum efficacy in inhibiting the mycelial biomass of *C. gloeosporioides* and *A. alternata* (*R.s*). While *T. piluliferum* and *A. niger* culture filtrates inhibited mycelial biomass of *C. dematium* and *A. alternata* (*R.s*) respectively.

Key words: *In vitro***,** phylloplane, medicinal plant, biocontrol, fungicides.

## INTRODUCTION

The increasing awareness of fungicide-related hazards has emphasized the need of adopting biological control as an alternative disease control method. Biological control of plant disease is the suppression of disease symptoms and disease incidence by the application of a biological agent. Biological control of plant pathogens by microorganisms has been considered a more natural and environmentally acceptable alternative to the existing chemical treatment methods [1]. Aerial plant surfaces provide a suitable habitat for epiphytic microorganisms which are influenced by the nutrients present on the leaf surfaces [2, 3]. The antagonistic potential is the basis for the effective control of phytopathogenic fungi [4, 5]. [6] study the possibility of biological control of fungal pathogens in the phyllosphere and proposed the possibility for the reduction of the incidence and development of the diseases using fungal and bacterial antagonists.

Foliar diseases are of utmost importance as not only photosynthetic area is reduced but also respiration and transpiration processes are adversely affected. Major fungal pathogens for foliar diseases of medicinal plants include species of *Alternaria*, *Botrytis*, *Cercospora*, *Colletotrichum*, *Septoria* etc.

Biocontrol of foliar diseases is a potential alternative means of management of foliar pathogens. Using biocontrol agents to manage the diseases is one of the most important approaches for successful disease management and sustainable production of medicinal plants. [7, 8] had explored the ability of certain antagonistic fungi for the possible control of pathogenic

fungi on aerial plant surfaces. The present study was carried out to examine the efficacy of metabolites produce by culture filtrates of phylloplane fungi of *Rauwolfia serpentina*, *Spilanthes oleracea* against *Alternaria alternata*, *Chlorophytum tuberosum* against *Colletotrichum dematium* and *Piper longum* against *Colletotrichum gloeosporioides* under *in vitro* conditions.

## MATERIALS AND METHODS

### Isolation of leaf pathogen

Leaves of *R. serpentina*, *S. oleracea* infected with *A. alternata*; leaves of *C. tuberosum* infected with *C. dematium* were collected from Non Wood Forest Products Division, Nursery, F.R.I., Dehradun, Uttarakhand. Leaves of *P. longum* infected with *C.gloeosporioides* were collected from Ayurvedic Nursery, Herbertpur, Uttarakhand. For the isolation of pure culture of fungal pathogen, a portion of leaf containing circular brown spot was surface sterilized by submerging in 0.1% mercuric chloride for 1 min, after which it was rinsed with three changes of sterilized distilled water. Then, they were placed on potato dextrose agar medium in Petri plates and incubated in a B.O.D. incubator at 25±1ºC for mycelial growth.

### Isolation of phylloplane fungi

Phylloplane fungi were isolated from healthy leaves of *R. serpentina*, *S. oleracea*, *C. tuberosum* and *P. longum* through leaf washing technique [9,10] identified with standard monographs and expertise available. To study their antagonistic properties pure cultures were maintained on potato dextrose agar medium at 4°C in a refrigerator for further studies.

### Assay in liquid medium

In this method, 50 ml potato dextrose broth was taken in conical flasks (150 ml) and two sets were maintained. Two discs (5mm) of antagonist were inoculated in each of twelve flasks out of which six flasks (one set) were incubated in shaking incubator (shaking condition) at 150 rpm and six (second set) were maintained in a BOD incubator (stationary condition) at 25±1ºC for 15 days.

After incubation period, all the flasks were harvested using Whatman No.1 filter paper and then filtered with a bacterial syringe filter (0.45µm). Three flasks containing culture filtrate of antagonist from each set were sterilized (autoclaved) and three were kept unsterilized (unautoclaved).

All the flasks containing antagonist culture filtrate were inoculated with two discs (5mm) of pathogens. Then one set was incubated in shaking incubator (shaking condition) at 150 rpm and the other was maintained in a BOD incubator at 25±1ºC for 15 days. One set of control was maintained in each case, in which only pathogen disc was added. After incubation they were harvested as mentioned above. The mycelial biomass in each case was determined by gravimetric measurement after oven drying at 60ºC to constant weight [11,12].

The per cent growth inhibition was calculated as

% Growth Inhibition = C-T/C×100

Where,

C = Dried mycelial weight in control

T = Dried mycelial weight in treatment

## RESULTS

Five phylloplane fungi were identified as viz. *Trichoderma harzianum* Rifai ISO-1 and ISO-2, *T. piluliferum* Webster and Rifai, *Aspergillus niger* van Tieghem and *Penicillium sublateritium* Biourge. While the pathogen were identified as *A. alternata* (*R.s*) from *R. serpentina*, *A. alternata* (*S.o*) from *S. oleracea*, *C. dematium* from *C. tuberosum* and *C. gloeosporioides* from *P. longum*.

Culture filtrates of *T. harzianum* ISO-1, *T. harzianum* ISO-2, *T. piluliferum*, *A. niger* and *P. sublateritium* were assessed to determine their effect on per cent inhibition of mycelial biomass of pathogens viz., *A. alternata* (*R.s*), *A. alternata* (*S.o*), *C. dematium* and *C. gloeosporioides*. Tables 1-5 are the outcomes of two-way Analysis of Variance (ANOVA) carried out to study the effect of culture filtrates of antagonists on per cent inhibition of mycelial biomass of the pathogens under two different conditions viz., condition 1 (sterilized) which was further categorized in

'stationary' and 'agitated' and condition 2 (unsterilized) which was simultaneously categorized in 'stationary' and 'agitated' and their interactions (between pathogens and condition 1, pathogens and condition 2).

During all the experiments it was observed that the per cent inhibition of mycelial biomass of pathogens was found maximum in 'stationary' in comparison to the 'agitated' under both the conditions.

When the effects of the culture filtrate of *T.harzianum* ISO-1 on mycelial biomass of pathogens studied under both conditions. The maximum per cent inhibition of mycelial biomass was observed of *C. gloeosporiodes* (92.09) which was at par with *A. alternata* (*R.s*) (91.70) followed by *A. alternata* (*S.o*) (87.11) and *C. dematium* (80.40) (Table 1). The effect of the interaction between pathogens and condition 1 was non significant. Whereas, the maximum per cent inhibition of mycelial biomass of *C. gloeosprioides* in 'stationary' and minimum of *C. dematium* in 'agitated' was examined during the interaction between pathogens and condition 2.

**Table 1 Per cent inhibition of mycelial biomass of pathogens in *T. harzianum* ISO-1 culture filtrate**

| Pathogens | Condition 1 Sterilized | | Condition 2 Unsterilized | | Mean |
|---|---|---|---|---|---|
| | Stationary | Agitated | Stationary | Agitated | |
| *A.alternata* (*R.s*) | 92.96±1.87 | 90.45 ±4.50 | 92.29± 2.93 | 91.12±4.26 | 91.70 |
| *C.dematium* | 82.17±7.75 | 78.64 ±6.68 | 84.95± 5.25 | 75.86±5.92 | 80.40 |
| *C.gloeosporioides* | 93.07±4.54 | 91.11±5.29 | 95.11± 3.49 | 89.04±4.05 | 92.09 |
| *A.alternata* (*S.o*) | 90.60±3.17 | 83.62 ±4.13 | 86.44± 6.98 | 87.78±2.56 | 87.11 |
| **Mean** | 89.70 | 85.95 | 89.70 | 85.95 | |
| | P | C1 | C2 | P x C1* | P x C2 |
| SEM± | 1.21 | 0.85 | 0.85 | - | 1.71 |
| CD at 5% | 3.49 | 2.47 | 2.47 | 4.94 | 4.94 |

P-Pathogen; C1-Sterilized; C2-Unsterilized; NS*- Non-significant

Effects of the culture filtrate of *T. harzianum* ISO-2 studied under both the conditions illustrates that maximum per cent inhibition of mycelial biomass was observed in *C. gloeosporoides* (92.68) followed by *A. alternata* (*R.s*) (89.62). Per cent inhibition of mycelial biomass of *C. dematium* (81.59) was found to be at par with *A. alternata* (*S.o*) (79.59) (Table 2).

Maximum per cent inhibition of mycelial biomass was found in *C. gloeosporioides* in 'stationary' and 'agitated' conditions and minimum was in *C. dematium* and *A. alternata* (*S.o*) in 'agitated' condition during the interaction between (pathogens and condition 1). Similarly, maximum per cent inhibition of mycelial biomass of *C. gloeosporioides* was observed in 'stationary' condition and minimum was in *C. dematium* and *A. alternata* (*S.o*) in 'agitated' condition during the interaction between (pathogens and condition 2).

In the culture filtrate of *T. piluliferum*, maximum per cent inhibition of mycelial biomass was observed of *C. dematium* (89.62) which was at par with *A. alternata* (*R.s*) (87.09) and minimum was of *C. gloeosporioides* (83.87) which was at par with *A. alternata* (*S.o*) (83.86) under both the conditions (Table 3).However, the effect of condition 2 (unsterilized), interaction between pathogens and condition 1 and pathogens and condition 2 were found to be non significant.

**Table 2 Per cent inhibition of mycelial biomass of pathogens in *T. harzianum* ISO-2 culture filtrate**

| Pathogens | Condition 1 | | Condition 2 | | Mean |
|---|---|---|---|---|---|
| | Sterilized | | Unsterilized | | |
| | Stationary | Agitated | Stationary | Agitated | |
| *A.alternata* (*R.s*) | 91.80±3.39 | 87.44±4.45 | 90.22±4.22 | 89.02±4.91 | 89.62 |
| *C.dematium* | 86.31±4.42 | 76.87±6.54 | 85.85±4.45 | 77.33±7.24 | 81.59 |
| *C.gloeosporioides* | 92.45±4.19 | 92.91±4.68 | 95.06±2.23 | 90.30±4.59 | 92.68 |
| *A.alternata* (*S.o*) | 82.59±2.91 | 76.59±1.50 | 79.76±5.06 | 79.42±2.57 | 79.59 |
| **Mean** | 88.29 | 83.46 | 87.73 | 84.02 | |
| | P | C1 | C2 | P x C1 | P x C2 |
| SEM± | 0.97 | 0.69 | 0.69 | 1.37 | 1.37 |
| CD at 5% | 2.81 | 1.98 | 1.98 | 3.97 | 3.97 |

P-Pathogen; C1-Sterilized; C2-Unsterilized

**Table 3 Per cent inhibition of mycelial biomass of pathogens in *T. piluliferum* culture filtrate**

| Pathogens | Condition 1 | | Condition 2 | | Mean |
|---|---|---|---|---|---|
| | Sterilized | | Unsterilized | | |
| | Stationary | Agitated | Stationary | Agitated | |
| *A.alternata* (*R.s*) | 87.54±3.43 | 86.63±4.35 | 86.43±3.77 | 87.75±3.99 | 87.09 |
| *C.dematium* | 91.16±3.53 | 88.08±3.62 | 88.89±4.79 | 90.35±2.66 | 89.62 |
| *C.gloeosporioides* | 86.97±2.76 | 80.77±6.38 | 84.85±4.09 | 82.88±7.24 | 83.87 |
| *A.alternata* (*S.o*) | 87.57±5.44 | 80.16±4.56 | 86.58±5.88 | 81.15±5.57 | 83.86 |
| **Mean** | 88.31 | 83.91 | 86.69 | 85.53 | |
| | P | C1 | C2* | P x C1* | P x C2* |
| SEM± | 1.28 | 0.90 | - | - | - |
| CD at 5% | 3.69 | 2.60 | 2.60 | 5.21 | 5.21 |

P-Pathogen; C1-Sterilized; C2-Unsterilized; NS*- Non-significant

In table 4 showed that maximum per cent inhibition of mycelial biomass was found of *A. alternata* (*R.s*) (80.21) followed by *C. dematium* (66.21) which was at par with *C. gloeosporioides* (68.28) and *A. alternata* (*S.o*) (69.04) in the culture filtrate of *A.niger* under both the conditions.

Maximum per cent inhibition of mycelial biomass was observed in *A. alternata* in 'stationary' and minimum in *C. dematium* in 'agitated' condition during the interaction between pathogens and condition 1. However, the interaction between pathogens and condition 2 were found to be non significant.

**Table 4 Per cent inhibition of mycelial biomass of pathogens in *A. niger* culture filtrate**

| Pathogens | Condition 1 | | Condition 2 | | Mean |
|---|---|---|---|---|---|
| | Sterilized | | Unsterilized | | |
| | Stationary | Agitated | Stationary | Agitated | |
| *A.alternata* (*R.s*) | 87.19±10.48 | 73.24±4.75 | 85.22±12.61 | 73.24±5.48 | 80.21 |
| *C.dematium* | 68.46±2.84 | 63.97±4.81 | 67.76±5.49 | 63.97±2.72 | 66.21 |
| *C.gloeosporioides* | 69.33±5.54 | 67.23±4.68 | 70.91±3.95 | 67.23±4.80 | 68.28 |
| *A.alternata* (*S.o*) | 72.69±3.50 | 65.38±2.99 | 71.37±4.85 | 65.38±4.06 | 69.04 |
| **Mean** | 74.41 | 67.46 | 73.82 | 68.05 | |
| | P | C1 | C2 | P x C1 | P x C2* |
| SEM± | 1.12 | 0.79 | 0.79 | 1.58 | - |
| CD at 5% | 3.22 | 2.28 | 2.28 | 4.56 | 4.56 |

P-Pathogen; C1-Sterilized; C2-Unsterilized, NS*- Non-significant

In both conditions, the effects of culture filtrate of *P. sublateritium* was studied. The maximum per cent inhibition of mycelial biomass was observed of *C. gloeosporioides* (83.57) which was at par with *A. alternata* (*S.o*) (83.20) and *A. alternata* (*R.s*) (82.84) and minimum of *C. dematium* (65.04) (Table 5).

**Table 5. Per cent inhibition of mycelial biomass of pathogens in *P. sublateritium* culture filtrate**

| Pathogens | Condition 1 | | Condition 2 | | Mean |
|---|---|---|---|---|---|
| | Sterilized | | Unsterilized | | |
| | Stationary | Agitated | Stationary | Agitated | |
| *A.alternata* | 85.70±5.20 | 79.98±3.14 | 82.92±5.13 | 82.77±5.50 | 82.84 |
| *C.dematium* | 69.90±5.89 | 60.18±7.52 | 65.70±10.27 | 64.38±6.44 | 65.04 |
| *C.gloeosporioides* | 85.23±3.73 | 81.92±5.57 | 83.42±5.73 | 83.73±4.32 | 83.57 |
| *A.alternata*(*S.o*) | 87.67±2.83 | 78.73±2.89 | 82.75±5.36 | 83.65±5.91 | 83.20 |
| **Mean** | 82.13 | 75.20 | 78.70 | 78.63 | |
| | P | C1 | C2* | P x C1* | P x C2* |
| SEM± | 1.48 | 1.05 | - | - | - |
| CD at 5% | 4.28 | 3.03 | 3.03 | 6.06 | 6.06 |

P-Pathogen; C1-Sterilized; C2-Unsterilized; NS*- Non-significant

## DISCUSSION

Biological control methods could be considered as an alternative of chemical control. Successful biological control of foliar diseases has been achieved by a number of researchers under greenhouses and field trials using fungal and bacterial antagonists [13,14,15,16,17,18,19]. [20] found that leaf spot diseases of wheat can be controlled *in vitro* and *in vivo* by the use of potential microorganisms such as fungi, bacteria and yeast isolated from phylloplane of wheat.

Culture filtrates of *T. harzianum* ISO-1 and *T. harzianum* ISO-2 effectively inhibited mycelial growth of *C. gloeosporioides*. Similar observations were made by [21] where *T. harzianum* was found to be more effective antagonist in inhibiting the mycelial growth of *Colletotrichum gloeosporioides* causing leaf spot of Kokum. The culture filtrate of *A.niger* caused inhibition (68.28%) of *C. gloeosporioides* mycelial biomass. This finding was in accordance with the study conducted by [22] where the culture filtrates of *A.niger* also caused more than 50% inhibition of *C. gloeosporioides*. [21,23, 24] reported the efficacy of culture filtrates of antagonists in inhibiting the mycelial biomass of the pathogenic fungi.

## CONCLUSION

*In vitro* findings demonstrated that culture filtrates of *T. harzianum* ISO-1, *T. harzianum* ISO-2 and *P. sublateritium* caused maximum per cent inhibition of mycelial biomass of *C. gloeosporioides* and *A. alternata* (*R.s*). *T. piluliferum* and *A. niger* culture filtrates caused maximum per cent inhibition of mycelial biomass of *C. dematium* and *A. alternata* (*R.s*). Therefore, phylloplane fungi can be applied as biocontrol agents for the management of foliar diseases in medicinal plants in the field conditions.

## ACKNOWLEDGEMENT

The authors express their gratitude to the Director, Forest Research Institute, Dehradun, for providing the facilities to complete this study successfully.

## REFERENCES

[1] Baker, R., Paulitz, TC., 1996. Theoretical basis for microbial interactions leading to biological control of soilborne plant pathogens In: Principles and Practice of Managing Soil borne Plant Pathogens. Hall, R. (ed.). The American Phytopathol. Soc. St. Paul, MN. pp. 50-79.

[2] Fokkema, NJ., 1976. Antagonism between fungal saprophytes and pathogens on aerial plant surfaces. In: Dickinson C.H.; Preece T.F., eds. Microbiological of aerial plant surfaces. London: Academic Press: 486–506.
[3] Pandey, RR., Arora, DK., Dubey, RC.,1993. Antagonistic interaction between fungal pathogens and phylloplane fungi of guava. Mycopathol.124, 31–39.
[4] Papavizas, GC.,1985. *Trichoderma* and *Gliocladium*: biology, ecology and potential for biocontrol. Annu Rev Phytopathol. 23, 24–54.
[5] Samuels, G.J.,1996. *Trichoderma*: A review of biology and systematic of the genus. Mycol Res.100, 923–935.
[6] Anderson, LM., Stockwell, VO., Loper, JE., 2004. An extracellular protease of *Pseudomonas flourescens* inactivates antibiotics of *Pantoea agglomerans*. Phytopathol. 94, 1228–1234.
[7] Euveh, AG., Okhuoya, JA., Osemwegie, OO., Hamad, AI., Ogbebor, ON., 2011. Evaluation of phylloplane fungi as biocontrol agent of Corynespora diseases of rubber (*Hevea brasiliensis* Muell.Arg.).World Journal of Fungal and Plant Biology. 2, 1–5.
[8] Dickinson, CH., 1967. Fungal colonization of Pisum leaves. Canadian Journal of Botany 45, 915–927.
[9] Aneja, KR., 2003. Experiments in microbiology, plant pathology and biotechnology, 4th Edn., New age international publications limited.176p.
[10] Dennis, C., Webster, J., 1971.Antagonistic properties of species-groups of *Trichoderma* hyphal interaction. Transactions of British Mycological Society 57, 363–369.
[11] Dhingra, DO., Sinclair, BJ., 1985. *Basic Plant Pathology Methods*.CRC Press, Inc., of Boca Raton, 355 p.
[12] Singh, UP., Prithviraj, B., Singh, KP., Sharma, BK., 2000. Control of powdery mildew (*Erysiphe pisi*) of pea (*Pisum sativum*) by combined application of plant growth promoting. Zeitschrit-planzenkrankheiten und planzenschutz. 107, 59–66.
[13] Abd, El-Moneim., Maisa, L., 2001. Evaluation of some non-chemical methods to control soil borne fungi and foliage diseases of cucumber. Ph.D. Thesis, Fac. Agric., Zagazig Univ., Egypt. 143pp.
[14] Mosa, AA., 2002. Induced resistance in rice against blast disease using biotic agents. *Ann. Agric. Sci*., Ain Shams Univ. Cairo, 47(3), 993–1008.
[15] El-Gamal, Naida, G., 2003. Usage of some biotic and abiotic agents for induction of resistance to cucumber powdery mildew under plastic house conditions. Egypt.J.Phytopathol. 31(1-2), 129–140.
[16] Kamel, SMH., 2003. Antagonistic effects of some microbial inhabitants on phylloplane of squash plants towards *Sphaerotheca fuliginea*. M.Sc. Thesis Fac. of Agric. Kafr El-sheikh, Tanta Univ. 94pp.
[17] Saber, MM., Abdou, YA., ElGantiry, SM., Ahmed, SS., 2003.Biocontrol of anthracnose disease of soybean caused by *Colletotrichum dematium*. Egypt. J. Phyopathol. 31(1-2), 17–29.
[18] Hussein, MAM., Hassan, MHA., Allam, ADA., Abo-El-Yous, KAM., 2007. Management of stemphylium blight of onion by using biological agents and resistance inducers. Egypt. J. Phytopathol. 35(1), 49–60.
[19] Perello, AE., Monaco, C., 2007. Status and progress of biological control of Wheat (*Triticum aestivum* L.) foliar diseases in Argentina. Fitosanidad. 11(2), 85–105.
[20] Jadav, SK., Diwakar, MP., Sawant, UK., Kadam, JJ., 2008. Management of leaf spot disease of Kokum incited by *Colletotrichum gloeosporioides*. J. Pl. Dis. Sci. 3(2), 193–196.
[21] Pandey, RR., Arora, DK., Dubey, RC., 1993. Antagonistic interaction between fungal pathogens and phylloplane fungi of guava.Mycopathol.124, 31–39.
[22] Adebola, MO., Amadi, JE., 2012. Studies on *Penicillium digitatum, Botryodiploidia theobromae*, *Alternaria tenuis* and *Trichoderma harzianum* for bicontrol of *Phytophthora palmivora* Cocoa black pod disease pathogen. American-Eurasian J. Agron 5(2), 30–34.
[23] Bokhari, NA., Perveen, K., 2012. Antagonistic action of *Trichoderma harzianum* and *Trichoderma viride* against *Fusarium solani* causing root rot of tomato. Afr. J.Microbiol. Res. 6(44), 7193–7197.

[24] Reena, A., Anitha, M., Aysha, OS., Valli, S., Nirmala, P., Vinothkumar, P., 2013. Antagonistic activity of *Trichoderma viride* isolate on soil borne plant pathogenic fungi. Int. J. Bioassays., 2(1), 294–297.