Available online at www.ijpab.com

DOI: http://dx.doi.org/10.18782/2320-7051.6997

ISSN: 2320 – 7051

Int. J. Pure App. Biosci. **6 (5):** 637-648 (2018)

Research Article

International Journal of Pure & Applied Bioscience

OPEN ACCESS

Antagonistic Potential of Bioagents against *Alternaria brassicicola*, the Incitant of Alternaria Leaf Spot of Cabbage

Gunda V. N. S. Madhu Kiran[1*], Thara, S. S.[2] and Brinda, G. B.[3]

[1]Department of Plant Pathology, [2]Assistant Professor, [3]Department of Plant Pathology,
College of Agriculture, Vellayani, Kerala Agricultural University, Kerala, India

*Corresponding Author E-mail: madhuabhi154@gmail.com

Received: 4.07.2018 | Revised: 10.08.2018 | Accepted: 16.08.2018

ABSTRACT

Cabbage (Brassica oleracea var capitata (L.)) is widely grown cool season vegetable crop all over the world belongs to the family cruciferae. Economic part of cabbage is leaves forming the compact head. Now this cool season vegetable crop is cultivated under roof top conditions all over the Kerala. In recent years, losses has been observed at large scale due to Alternaria leaf spot of cabbage caused by Alternaria brassicicola as the severity of the damage was too high right from seedling stage to harvest stage. Application of chemical fungicides against this disease creates residue problems as economic part is leaf portion. Hence the present study was conducted to provide an effective biological management strategy against this disease. For this, fungi have been isolated from the phyllosphere of healthy cabbage plant in Alternaria disease affected field and the efficacy of fungi was evaluated against Alternaria leaf spot of cabbage by dual culture technique. The results revealed that the fungi Myrothecium inundatum isolated from the phyllosphere of cabbage were found to be an effective bioagent against the leaf spot pathogen Alternaria brassicicola. Along with this the antagonistic potential of two fungal bioagents Trichoderma viride, Trichoderma harzianum and two bacterial bioagents Bacillus subtilis, Pseudomonas fluorescens were evaluated against Alternaria leaf spot of cabbage by dual culture technique. In this method, the highest mycelial growth inhibition was recorded with Trichoderma viride (83.3%) followed by Trichoderma harzianum (80%), Bacillus subtilis (63.3%) and Myrothecium inundatum (55.5%).

Key words: *Cabbage, Alternaria leaf spot, Phyllosphere isolation, Molecular identification, Dual culture technique.*

INTRODUCTION

Cabbage (*Brassica oleracea* var. *capitata*) is a profitable vegetable crop in Kerala grown for its compact head formed by the leaves. Cabbage is defenceless to a variety of pathogens which can bring about generous misfortune in yield. Among the fungal diseases of cabbage, Alternaria leaf spot caused by *A. brassicicola* is a noteworthy concern.

Cite this article: Madhu Kiran, G.V.N.S., Thara, S.S. and Brinda, G.B., Antagonistic Potential of Bioagents against *Alternaria brassicicola*, the Incitant of Alternaria Leaf Spot of Cabbage, *Int. J. Pure App. Biosci.* **6(5):** 637-648 (2018). doi: http://dx.doi.org/10.18782/2320-7051.6997

This disease is difficult to manage as the pathogen is primarily seedborne, survive in debris, weeds and wide host range and spread by wind, rain-splash and implements. Application of chemical fungicides against this disease creates residue problems as economic part is leaf portion. Hence the present study was conducted to provide an effective biological management strategy against this disease.

Yadav *et al*.[19] isolated different fungi from phylloplane and tested against *A. brassicae*. The maximum percentage inhibition was recorded with *T. viride* (74%) followed by *Aspergillus flavus* (70%), *Cladosporium cladosporioides* (54%) and *Penicillium citrinum* (40%). Thakur and Harsh[17] tested the *in vitro* fungitoxicity of phylloplane fungi against *A. alternata* and the percentage of inhibition recorded with *T. harzianum* iso-1, *Aspergillus niger* and *Penicillium* sp were 90%, 67.4% and 55.28% respectively. Khanna *et al*.[9] isolated the fungi from the phyllosphere and tested their *in vitro* antagonism against *A. porri* and observed that the percentage inhibition recorded with Trichoderma isolate 3, *Fusarium* sp. and *Penicillium* sp. were 89.44%, 60.55% and 52.94% respectively.

Meena *et al*.[12] observed that the mycelial growth of *A. brassicae* was inhibited by *T. viride* (81%) among different isolates of *Trichoderma* spp. tested under *in vitro* conditions in mustard. Ramegowda *et al.*[16] conducted an experiment and predicted the efficacy of bioagents against *Alternaria macrospora*. The maximum percentage inhibition was observed with *T. viride* (62.3%) followed by *T. harzianum* (60.3%), *Bacillus subtilis* (48.3%) and *Pseudomonas fluorescens* (37.8%).

Amin *et al.*[1] reported that among six isolates of *Trichoderma* spp. tested against *A. brassicicola* the volatile metabolites of *T. viride* (Tv-3) was found to be more effective as it recorded 48.44 per cent inhibition compared to the control under *in vitro* conditions. Rajput *et al.*[15] evaluated the efficacy of six fungal and two bacterial antagonists against *A. alternata* under *in vitro* conditions and reported that *T. viride* (74.77%) and *T. harzianum* (71.25%) were found to be effective among fungal antagonists and percentage growth inhibition recoded with two bacterial antagonists viz., *B. subtilis* and *P. fluorescens* was 69.73% and 56.7% respectively.

Chetana *et al.*[4] tested the efficiency of different fungal antagonists against *A. porri* by dual culture technique and stated that highest percentage of inhibition was recorded with *T. harzianum* (79.35%) followed by *Chaetomium* sp. (42.86%). Prakasam and Sharma[14] tested the efficacy of six isolates of *T. harzianum*), seven isolates of *T. viride* and two isolates of *B. subtilis* against *A. porri* under *in vitro* conditions. The highest percentage of inhibition among these isolates were with Th-3 (61.54%), Tv-12 (55.71%) and Bs-1 (48%).

Maheswari and Krishna[11] evaluated the efficacy of different fungal and bacterial antagonists by dual culture technique against *A. alternata* and percentage of inhibition recorded with *T. viride* and *T. harzianum* was 51.7% and 45.3% respectively. The least percentage of inhibition was observed with *P. fluorescens* (19.5%). Gholve *et al*.[6] tested the potency of different species of Trichoderma against *A. macrospora* and maximum percentage inhibition recorded with *T. viride* and *T. harzianum* was 63.64% and 60.88% respectively. Bahekar *et al.*[3] reported that *T. harzianum* can be effectively used to control *A. alternata* as it recorded 65.35% inhibition. Khalse *et al*.[8] found that *T. harzianum* showed 65.21% growth inhibition of *A. brassicae* over control under *in vitro* conditions in cabbage. Jakatimath *et al*.[7] tested the efficacy of different bioagents Trichoderma- 21, Trichoderma- 28, Trichoderma- 29, Trichoderma- 72, Trichoderma- P and *P. fluorescens* and the percentage of inhibition recorded was 83%, 80%, 81.33%, 87%, 61% and 12% respectively.

MATERIAL AND METHODS

1) Isolation and identification of effective fungal antagonists from the phyllosphere of cabbage

Healthy cabbage leaves were collected from Alternaria leaf spot infected field and epiphytic fungi were isolated from the

phyllosphere by leaf washing technique[2]. Under aseptic conditions in laminar airflow chamber, one gram of leaf was taken in 99 ml sterile distilled water in conical flask and shaken on rotary shaker for 20 minutes. Using serial dilution procedure, $10^{-4}$and 10^{-5} dilution was prepared and thereafter one ml of each dilution was poured on rose bengal agar medium using pour plate method in separate petriplates. These plates are incubated for three to four days at room temperature. After incubation, fungal colonies were subcultured to another petriplate and purified. These purified cultures of fungi were stored further for identification and also to test the antagonistic effect on the pathogen under *in vitro* and *in vivo* conditions.

These fungi were identified by studying the morphological and colony characters. Colony and morphological characters like colony colour, hyphae, conidiophores, length and breadth of conidia, shape of conidia were studied to identify the fungi. Morphological characters of the fungi were studied by the slide culture technique which results in identification of the pathogen.

2) *In vitro* evaluation of fungal antagonists isolated from phyllosphere of cabbage against *A. brassicicola*

The efficacy of fungi isolated from serial dilution technique were tested against the virulent pathogen by dual culture technique[13]. In order to study this, PDA medium was prepared and sterilized. Then 15 ml of the medium was poured into sterilized petriplates. Five mm mycelial disc cuts were taken from seven day old culture of the pathogen and placed on PDA plates opposite to the colony of fungal antagonist at a distance of 2.5 cm from the periphery of culture plate. Control plate was maintained by placing the 5 mm mycelial fungal bit of the pathogen at the centre of petriplate. Replicates were maintained for each treatment and plates were incubated at room temperature.

Per cent inhibition of the pathogen over control was determined as described by Vincent[19].

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

Where

I = Per cent growth inhibition

C = Growth of pathogen in control plate

T = Growth of pathogen in treatment plate

3) Molecular identification of effective fungal antagonists

The effective fungal antagonists were identified by slide culture technique and also characterised on molecular basis. Morphological characters of the fungi were studied by the slide culture technique which helps in identification of the pathogen. Molecular identification of the fungus was done by the procedure described below.

Molecular characterisation was done for the virulent isolate by ITS (Internal transcribed spacer) sequencing. Different steps involved during this procedure were as follows:

a) Isolation of DNA
b) Checking the quality of DNA
c) PCR analysis
d) Agarose gel electrophoresis of PCR products
e) ExoSAP-IT treatment
f) Sequencing
g) Post sequencing PCR clean up
h) Sequence analysis

a) Isolation of DNA using GenElute Plant Genomic DNA Miniprep Kit (Sigma):

The mycelium of the fungus was ground in the mixture of lysis solution A (350 µl) and B (50 µl). This was incubated for ten min at 65^0C and mixed with 130 µl of precipitation solution. Then the entire mixture was transferred to an ice and kept for five minutes.

The sample was transferred to an eppendorf tube and initially spinned at 14000 rpm for five min followed by one min to remove the proteins, polysaccharides and the cellular debris. Then the mixture (700 µl) was again spinned at 14000 rpm for one min by placing in Gen Elute nucleic acid binding column.

To this nucleic acid column, wash solution was added and centrifuged at 14000 rpm for 1 min. Discarded the flow of liquid and elution solution was added. Then again centrifuged at same rpm and time and kept it as a stock solution and stored in the refrigerator at 5^0C.

b) Checking the quality of DNA

This was done using Agarose gel electrophoresis. For this, agarose gel (0.8%) was prepared with Tris-buffer and EDB (0.5 µg/ml). Then the DNA sample (5 µl) which is mixed with gel loading buffer (1µl) was loaded on the agarose gel and gone for electrophoresis until the bromophenol appear at the lower end of the gel. Ultra Violet transilluminator and Gel doc were used to view the gel and to capture the picture.

c) PCR analysis

For amplifying the DNA the following components were required:

a)	DNA	-	20 ng
b)	dNTPs	-	0.2 mM
c)	Buffer		
d)	Taq polymerase	-	single unit
e)	Bovine serum albumin	-	0.1 mg/ml
f)	Dimethyl sulfoxide	-	4%

Apart from this, two types of primers were used for amplification of DNA viz., Forward and Reverse primer.

ITS Forward 5'- TCCGTAGGTGAACCTGCGG-3'
ITS Reverse 5'-TCCTCCGCTTATTGATATGC-3'

PCR amplification profile:

98^0C - 30 sec
98^0C - 5 sec
72^0C - 10 sec
72^0C - 15 sec
72^0C - 60 sec
} 40 cycles
4^0C - ∞

d) Agarose gel electrophoresis of PCR products:

PCR product (5 µl) was mixed with loading dye (1 µl) and loaded on the agarose gel (1.2%). To execute electrophoresis, the amount of power supply was 75V and allowed until the bromophenol appear at the lower end of the gel. Ultra Violet transilluminator and Gel doc were used to view the gel and to capture the picture. The standard used in this electrophoresis was 2-log DNA ladder.

e) ExoSAP – IT Treatment:

Primarily this treatment contains 2 hydrolytic enzymes – Exonuclease and Shrimp Alkaline Phosphatase used to remove the extra primers and dNTPs from the PCR product. For this, PCR product (5 µl) was added to ExoSAP – IT (2 µl) and kept for incubation for 15 min at 37^0C.

f) Sequencing:

Big dye terminator v3.1 cycle sequencing kit were used for sequencing in thermal cycler. For this, PCR product, primer, sequencing mix, Buffer and sterile distilled water were required and concentrations were 10-20 ng, 3.2 pM, 0.28 µl, 1.86 µl and 10 µl respectively.

Amplification profile:

96^0C - 120 sec

96^0C - 30 sec
50^0C - 40 sec
60^0C - 4 min
} 30cycles

g) Post sequencing PCR clean up:

Master mix I comprised of 10µl milli Q and 2 µl 125 m*M* EDTA per reaction was prepared. Also, master mix II composed of 2 µl 3 *M* sodium acetate (pH 4.6) and 50 µl ethanol was prepared. Master mix I (12 µl) was mixed with master mix II (52 µl) and contents were mixed by inverting, followed by incubation at 26 ± 2 ^{0}C for 30 min. Mixture was further centrifuged at 14,000 rpm for 30 min and supernatant was decanted. Then, 100 µl of 70 per cent ethanol was added and spinned at 14,000 rpm for 20 min. The supernatant was decanted and pellet was air dried. The cleaned up air dried product was sequenced in ABI 3500 DNA Analyzer (Applied Biosystems, USA).

h) Sequence Analysis:

Sequence Scanner Software v1 was used to check the quality of sequence. Alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1[8].

4) *In vitro* evaluation of different biocontrol agents against the pathogen

Five bioagents were selected and evaluated against the pathogen. Of these five bioagents, three were fungal bioagents i.e. *Trichoderma viride* (KAU), *Trichoderma harzianum* (KAU) and one effective fungal antagonist isolated from phyllosphere of cabbage and two were bacterial bioagents i.e., *Bacillus subtilis* (KAU), *Pseudomonas flourescens* (KAU). The efficacy of each bioagents were tested against

the virulent pathogen using dual culture technique.

In case of fungal bioagents, five mm diameter disc cuts were taken from seven day old culture of the virulent pathogen and placed at a distance of 2.5 cm from the periphery on PDA plates. On the opposite side five mm diameter bits of fungal bioagent was placed at a distance of 2.5 cm from the periphery of culture plate. Four replications were maintained for each treatment.

In case of bacterial bioagents, from seven days old culture of pathogen five mm mycelial disc cuts were taken and placed at the centre of PDA plate. Two bacterial streaks of biocontrol agent were done on both sides perpendicular to the disc 2.5 cm apart. Four replications were maintained for each treatment. Control plates were maintained by placing 5 mm diameter disc cuts at the centre of PDA plates.

Per cent inhibition of the pathogen over control was determined as described by Vincent[18].

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

RESULTS AND DISCUSSION

1) Isolation and identification of effective fungal antagonists from phyllosphere of cabbage

Epiphytic fungi were isolated from the healthy leaves collected from the Alternaria infected cabbage field and primarily three fungi were isolated from the phyllosphere viz., *Myrothecium* sp., *Penicillium* sp., and a non sporulating fungus.

The colony of the *Myrothecium* sp. was white in colour and later forms black masses indicates development of spores. Branched conidiophores were developed from the mycelium and produce phialides at the apical portion. Conidia were produced from the tip of these phialides and these conidia were green in colour and cylindrical in shape with round ends (Plate 1).

The colony colour of *Penicillium* sp. was dull green and the mycelium was branched and septate. Conidiophores were produced from the mycelium and produce metulae and phialides at the tip. Globose conidia were produced from these phialides (Plate 1). The colony colour of non-sporulating fungus was white and produce septate mycelium. The morphological characters of fungal antagonists isolated from phyllosphere were mentioned in Table 1.

Table 1: Microscopic and colony characters of effective fungal antagonists isolated from phyllosphere

Sl. No.	Fungal antagonist	Colony colour	Conidia		
			Length	Breadth	Shape
1	*Myrothecium* sp.	White	15.42 µm	5.34 µm	Cylindrical with round ends
2	*Penicillium* sp.	Dull green	2.3 µm	2.2 µm	Globose
3	Unidentified fungi	White	No sporulation		

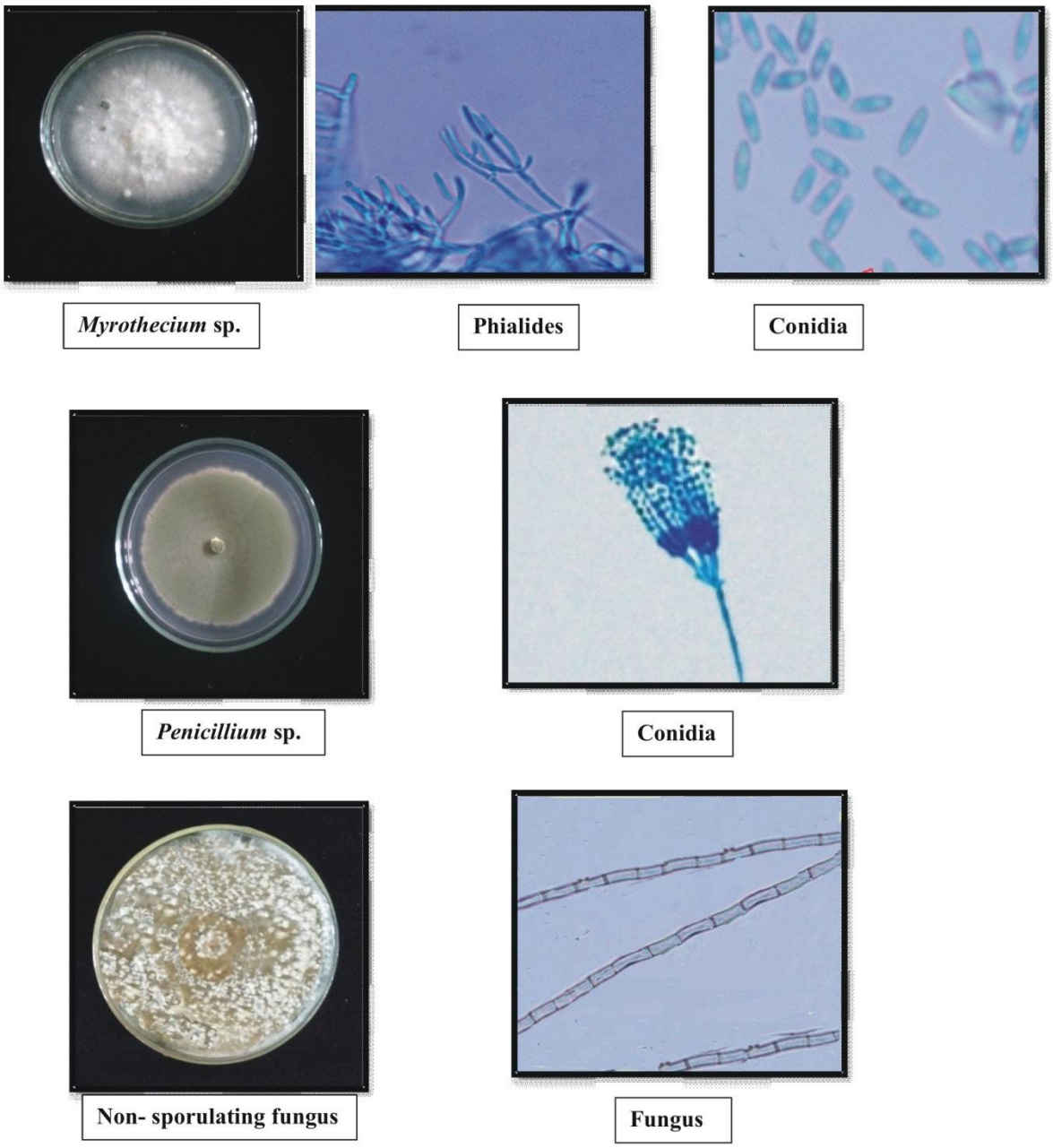

Plate 1: Fungal antagonists isolated from phyllosphere and their microscopic characters

2) *In vitro* evaluation of fungal antagonists isolated from phyllosphere of cabbage against *A. brassicicola*

The fungi isolated from the phyllosphere of cabbage were tested against *A. brassicicola* by dual culture technique and the results revealed that the maximum percentage of inhibition was recorded with *Myrothecium* sp. (54.4%) which differed significantly from *Penicillium* sp. and showed 50% inhibition. The least percentage inhibition was observed with non sporulating fungus which was 46% (Table 2, Plate 2).

Epiphytic fungi were isolated from the phyllosphere of cabbage and tested their *in vitro* antagonism against *A. brassicicola*, the incitant of Alternaria leaf spot of cabbage. Primarily three fungi were isolated from the phyllosphere. Based on the colony and spore morphology, these fungi were identified as *Myrothecium* sp., *Penicillium* sp. and one non-sporulating fungus. The results from *in vitro* antagonism showed that highest percentage inhibition of pathogen was recorded with *Myrothecium* sp (54%) which differed significantly with *Penicillium* sp. (50%). Yadav *et al.*[19] observed that *Penicillium citrinum* reduced the growth of *A. brassicicola* by 40 per cent. The percentage inhibition of *A.*

alternata by *Penicillium* sp. was 55.28%[17]. The most effective fungal antagonist *Myrothecium* sp. was subjected to molecular identification at RGCB, Trivandrum and confirmed as *Myrothecium inundatum* with accession number MH915568.

Table 2: *In vitro* antagonism of Phyllosphere fungi against *A. brassicicola*

Sl. No.	Fungal antagonist	Percentage mycelial inhibition of *A. brassicicola**
1	*Myrothecium* sp.	54.4
2	*Penicillium* sp.	50
3	Unknown	46
	CD (0.05 level)	2.2
	S. Em	1.02

*Mean of five replications

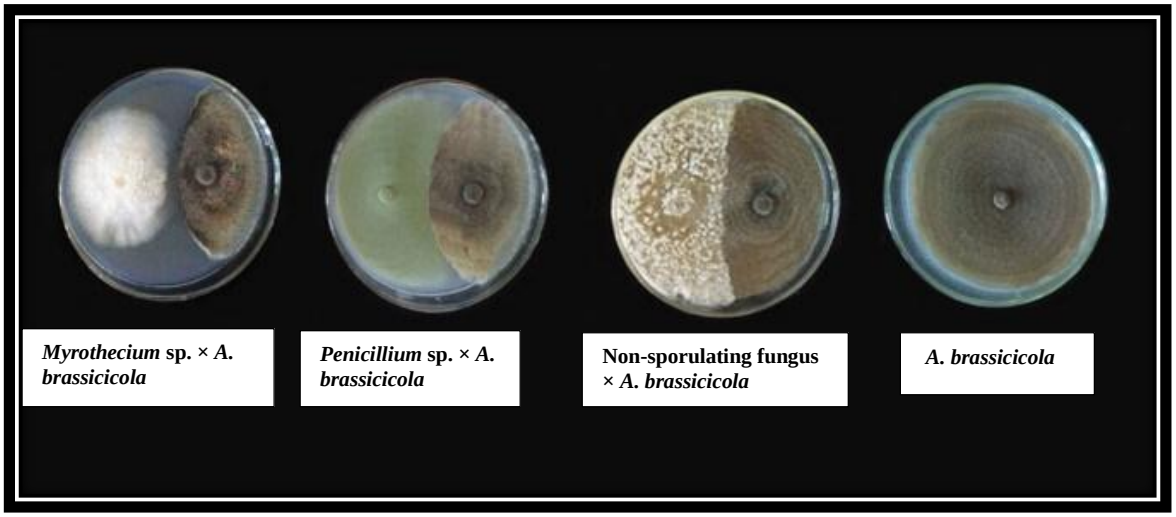

Plate 2: *In vitro* antagonism of fungal antagonists against *A. brassicicola*

3) Molecular identification of the effective fungal antagonist

The most effective fungal antagonist against *A. brassicicola* isolated from phyllosphere of cabbage was *Myrothecium* sp. The culture was subjected to molecular characterisation to identify the species of this fungus through ITS (Internal Transcribed Space) sequencing. Sequencing of this fungus was done with a universal primers (ITS – 1F and ITS -4R). The sequence obtained was amplified using PCR and quality of PCR product was tested by Sequence Scanner Software. The sequence of this isolate is as follows:

- MH915568

GTTCGGGAAGTTGAAACTCCCAAACCC
TTTGTGACCTTACCTATCGTTGCTTCGG
CGGGATCGCCGCCGGGCCGGCCCCTTC
GCGGGGGCCCTCCGGAGCCAGGCGCCC
GCCGGAGAACCCAAACTCTTTGTTTTTT
ATGGTTTTCTCCTCTGAGTGGATTATAA
ACAAATAAATCAAAACTTTCAACAACG
GATCTCTTGGTTCTGGCATCAATGAATA
ACGCAGCGAAATGCGATAAGTAATGTG
AATTGCAAAATTCAGTGAATCATCGAA
TCTTTGAACGCACATTGCGCCCGCCAGT
ATTCTGGCGGGCATGCCTGTTCGAGCG
TCATTTCAACCCTCAAGCCCCCATTGCC
TGGCGTTGGAGATCGGCCGTACGGCGC
GCACCCCCCCCCGGGGGTTTGCGCGGC
GGGCCGGCTCCGAAATCTAGTGGCGGT
CTCGCTGTAGTCCTCCTCTGCATAGTAG
CACAACCTCGCAGTTGGAACGCGGCGG
TGGCCATGCCGTTAAACACCCCACTTCT
GAAAGTTGACCTCGGATCAGGTAGGAA
TACCCGCTGAACTTAAGCATATCAATA
ACCGGAGGAA

The sequence length of this phyllosphere isolated fungus was 618 bp. The phylogenetic relationship of this isolate with the known isolates from different countries was constructed using Clustar X2, Treeview software and the dendrogram was mentioned in Fig 1. The chromatogram with 100 blast hits based on the query sequence of *M. inundatum* was given in Fig 2.

Nucleotide blast were done in NCBI site with the above sequence in FASTA format and the results obtained from this blast analysis showed 98% nucleotide identity with *M. inundatum* isolates of Malaysia, China, Vietnam and USA and 96% identity with Denmark and Indian isolates of *M. inundatum* as mentioned in Table 3.

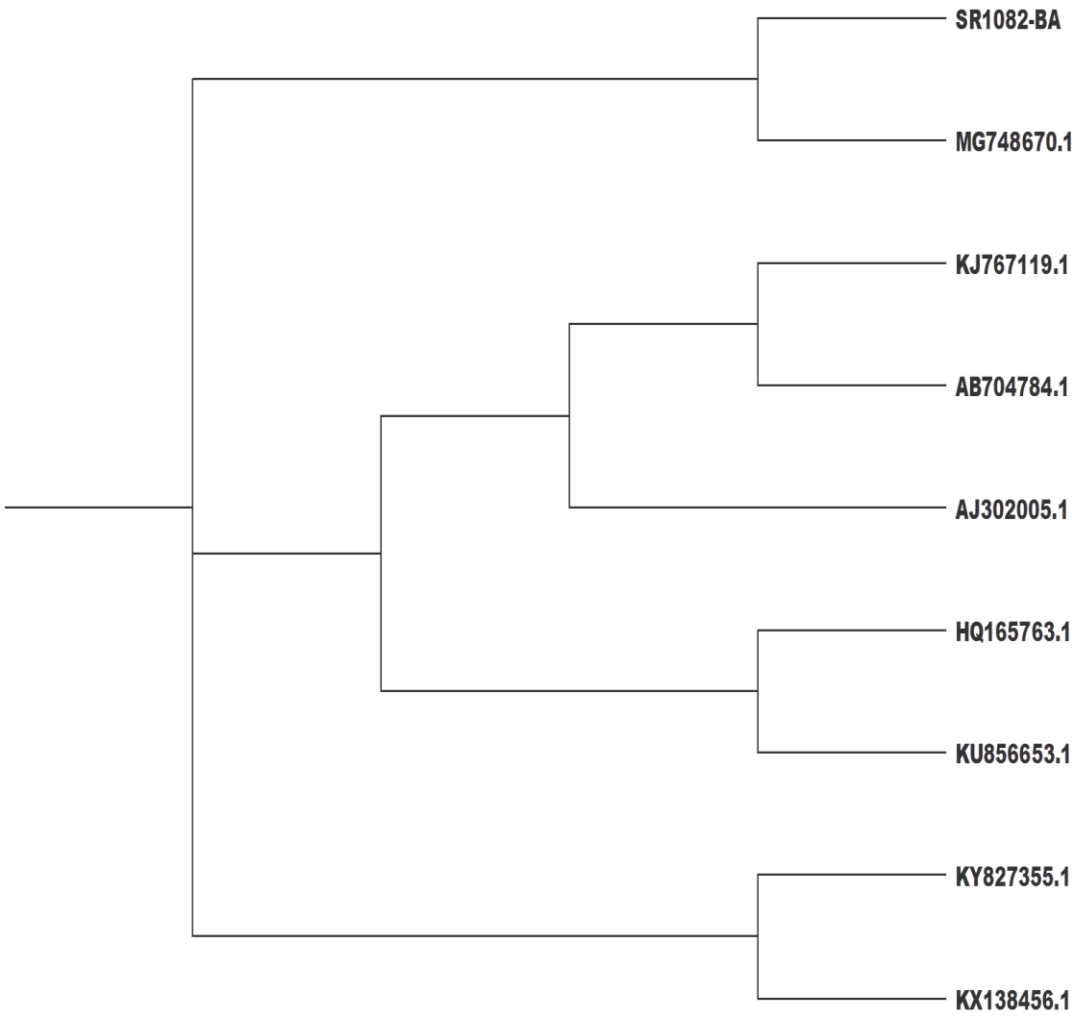

Fig. 1: Dendrogram showing the relationship of effective fungal antagonist with reported isolates of *M. inundatum*

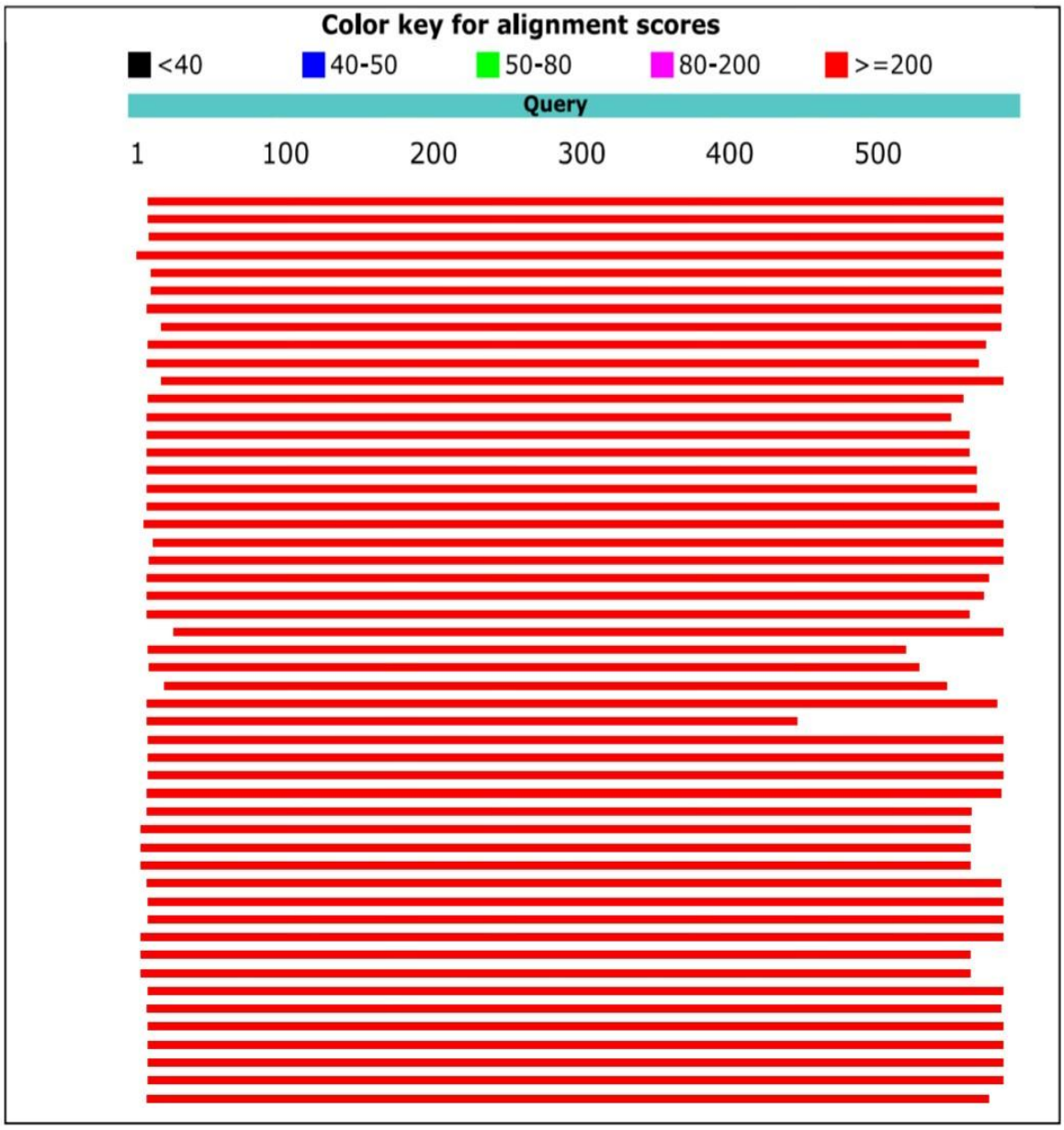

Fig. 2: Chromatogram showing distribution of 100 blast hits on the Query sequence of *M. inundatum*

Table 3: Nucleotide identity of *Myrothecium* spp. isolate with reported isolates of *M.inundatum*

Accession no	Description	Nucleotide identity (%)
KJ767119.1	*Myrothecium inundatum* isolate A2S4-D45,Malaysia	98
KY827355.1	*Myrothecium inundatum* strain SCAU025, china	98
AB704784.1	*Myrothecium inundatum*, Vietnam	98
HQ165763.1	*Myrothecium inundatum* strain IN-5, USA	98
MG748670.1	*Myrothecium inundatum* isolate M51, Brazil	95
KU856653.1	*Myrothecium inundatum* isolate 159, Denmark	96
KX138456.1	*Myrothecium inundatum* strain S9, Jammu and Kashmir , India	96
AJ302005.1	*Myrothecium inundatum*, Germany	91

4) *In vitro* studies of different biocontrol agents against the pathogen

The five bioagents viz., *T. viride*, *T. harzianum*, *B. subtilis*, *P. fluorescens* and *M. inundatum* were evaluated against *A. brassicicola* by dual culture technique under *in vitro* conditions as explained under 3.5. The results revealed that the highest percentage of inhibition was recorded with *T. viride* (82.2%) which is on par with *T. harzianum* (80%). The percentage of inhibition recorded with *B. subtilis* was 64.4%. The percentage inhibition by phyllosphere isolated fungus *M. inundatum* was only 54.4% which was less compared to fungal bioagents. The least percentage of inhibition was recorded with *P. fluorescens* (14.4%) (Table 4, Plate 3).

In vitro antagonism of bioagents by dual culture technique revealed that percentage of mycelial inhibition of the pathogen was 82.2% with *T. viride* and 80% with *T. harzianum* (Fig 8). Meena *et al.*[12] stated that among 10 isolates of *Trichoderma* spp. tested against *A. brassicae* the highest percentage of inhibition was recorded with *T. viride*- GR isolate (81%). Amin *et al.*[1] tested the *in vitro* antagonism of six Trichoderma isolates against *A. brassicicola* and the mycelial inhibition percentage varied between 34.62 to 48.44%. Khalse *et al.*[8] reported the maximum percentage of inhibition of *A. brassicae* with *T. harzianum* (65.21%) and stated that the antagonistic properties was due to competition for nutrients, mycoparasitism and antibiosis.

The percentage of inhibition recorded with *B. subtilis* against *A. brassicicola* was 64.4% and *P. fluorescens* failed to check the growth of pathogen under *in vitro* conditions and the percentage of inhibition recorded was 14.4%. Maheswari and Krishna[10] tested the *in vitro* antagonism of *P. fluorescens* against *A. alternata* and recorded growth inhibition of 19.5%. Jakatimath *et al.*[7] also reported that *P. fluorescens* was found to be effective to control the pathogen as the percentage of inhibition was only 12%.

Table 4: *In vitro* antagonism of bioagents against *A. brassicicola*

Sl. No.	Bioagents	Percentage mycelial inhibition of *A. brassicicola* *
1	*Pseudomonas fluorescens* (KAU)	14.4 (22.29)[d]
2	*Trichoderma viride* (KAU)	82.2 (65.05)[a]
3	*Trichoderma harzianum* (KAU)	80.0 (63.43)[a]
4	*Bacillus subtilis* (KAU)	64.4 (53.37)[b]
5	*Myrothecium inundatum*	54.4 (47.73)[c]
CD (0.05 level)		2.2
SE		0.98

*Mean of three replications

Values in parenthesis are arcsine transformed.

Plate 3: (T1-T6): Evaluation of bioagents against *A. brassicicola*

T1: *T. viride* × *A. brassicicola*
T2: *T. harzianum* × *A. brassicicola*
T3: *B. subtilis* × *A. brassicicola*
T4: *P. fluorescens* × *A. brassicicola*
T5: *M. inundatum* × *A. brassicicola*
T6: *A. brassicicola*

REFERENCES

1. Amin, F., Razdan, V. K., Mohiddin, F. A., Bhat, K. A., Sheikh, P. A., Effect of volatile metabolites of *Trichoderma* species against seven fungal plant pathogens under *in vitro*. *J. Phytol*. **2(10):** 34-37 (2010).
2. Aneja, K. R., Experiments in Microbiology, Plant Pathology and Biotechnology, in isolation of microorganisms from phyllosphere (phylloplane). (4th Ed.), New Age International Publications Limited, 176p (2003).
3. Bahekar, A. M., Ingle, R. W., Kendre, V. P., Efficacy of fungicides and bioagents against fungal pathogens of *Aloe vera*. *Int. J. Chem. Stud.* **5(4):** 1540-1543 (2017).
4. Chetana, B. S., Ganeshan, G., Rao, A. S., Bellishree, K., *In vitro* evaluation of plant extracts, bioagents and fungicides against *Alternaria porri* (Ellis) Cif, causing purple blotch disease of onion. Pest Manag. *Hort. Ecosyst*. **18(2):** 194-198 (2012).
5. Drummond, A. J., Ashton, B., Buxton, S., Cheung, M., Cooper, A., Heled, J., Kearse, M., Moi,r R., Stones-Havas, S., Sturrock, S., Thierer, T., Wilson, A., Geneious **5(1):** (2010).
6. Gholve, V. M., Jogdand, S. M., Suryawanshi, A. P., Evaluation of fungicides, botanicals and bioagents against Alternaria leaf blight caused by *Alternaria macrospora* in cotton. *J. cotton Res*. **28(2):** 327-331 (2014).
7. Jakatimath, S. P., Mesta, R. K., Biradar, I. B., Mushrif, S. K., Ajjappalavar, P. S., *In vitro* evaluation of fungicides, botanicals and bioagents against *Alternaria alternata*: Causal agent of fruit rot of brinjal. *Int. J. Curr. Microbiol. App. Sci*. **6(5):** 495-504 (2017).
8. Khalse, K. D., Lal, A. A., Simon, S., Efficacy of bioagents and plant extracts against the Alternaria leaf spot of cabbage. *J. Pharmacogn. Phytochem*. **6(4):** 1980-1982 (2017).

9. Khanna, M. G., Devi, G. U., Sagar, B. V., Shankar, A. S., Screening of phylloplane microflora against *Alternaria porri* under in vitro condition. *Prog. Res. Int. J*. **10(3):** 1477-1479 (2015).
10. Maheswari, S. K., Krishna, H., Field efficacy of fungicides and bio-agents against Alternaria leaf spot of Mungbean. *Ann. Plant Prot. Sci*. **21(2):** 364-367 (2013).
11. Maheswari, S. K., Krishna, H., Field efficacy of fungicides and bio-agents against Alternaria leaf spot of Mungbean. Ann. *Plant Prot. Sci*. **21(2):** 364-367 (2013).
12. Meena, P. D., Meena, R. L., Chattopadhyay, C., Kumar, A., Identification of critical stage for disease development and biocontrol of Alternaria blight of indian mustard. *J. Phytopathol*. **152(4):** 204-209 (2004).
13. Mishra, B. K., Mishra, R. K., Mishra, R. C., Tiwari, A. K., Yadav, R. S., Biocontrol efficacy of *Trichoderma viride* isolates against fungal plant pathogens causing disease in *Vigna radiata L. Archives App. Sci. Res*. **3(2):** 361-369 (2011).
14. Prakasam, V., Sharma, P., *Trichoderma harzianum* (Th-3) a potential strain to manage the purple blotch of onion (*Allium cepa* L.) caused by *Alternaria porri* under north indian plains. *J. Agric. Sci*. **4(10):** 266-272 (2012).
15. Rajput, R. B., Solanky, K. U., Prajapati, V. P., Pawar, D. M., Suradkar, S. R., Effect of fungal and bacterial bioagents against *Alternaria alternata* (FR.) Keissler *in vitro* condition. *Bioscan*. **8(2):** 627-629 (2010).
16. Ramegowda, G., Naik, M. K., Sunkad, G., Patil, M. B., Sataraddi, A., Evaluation of fungicides, Botanicals and Bioagents against leaf spot caused by *Alternaria macrospora* in cotton. *Indian J. Plant Prot.* **35(2):** 316-319 (2007).
17. Thakur, S., Harsh, N. S. K., Phylloplane fungi as biocontrol agent against Alternaria leaf spot disease of (Akarkara) *spilanthes oleracea*. *Biosci*. **5(2):** 139-144 (2014).
18. Vincent, J. M., Distortion of fungal hyphae in the presence of certain inhibitors. *Nature*. **159:** 800 (1927).
19. Yadav, S. L., Mishra, A. K., Dongre, P. N., Rashmi, S., Assessment of fungitoxicity of phylloplane fungi against *Alternaria brassicae* causing leaf spot of mustard. *J. agric. Tech*, **7(6):** 1823-1831 (2011).