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In-vitro antagonistic potential of fungal endophytes against chilli anthracnose pathogen, *Colletotrichum acutatum*

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Abstract
Chilli anthracnose is one of the destructive diseases of chilli affecting its yield and quality. There are various *Colletotrichum* species associated with that disease in India. A lot of chemical fungicides are being used for the management of chilli anthracnose which is drastically affecting the environment. So, there is an urgent need to augment alternate management strategies for the management of this disease. Bio-control agents plays a major role as they are cost effective and does not harm the environment. Endophytes comes into play as they not only protect the plants from invading pathogens but also activates plants defenses. A total of 122 fungal endophytic isolates were isolated from healthy chilli plants (54 from leaves, 42 from fruits and 26 from roots) and their antagonistic activity was tested against anthracnose pathogen *Colletotrichum acutatum.* Among all the isolates tested ENRF 7 was found to be superior in inhibiting the growth of pathogen (61.45%). Similar results were obtained by ENRF 7 while testing the effect of culture filtrate/ metabolites/ non-volatile compounds at all the concentrations i.e., 5%, 10%, and 15%. Based on the cultural, morphological and molecular characters the isolate ENRF 7 was identified as *Trichoderma asperellum.*

Keywords: chilli anthracnose, *Trichoderma*, *Colletotrichum acutatum*, fungal endophytes

Introduction

Chilli is one of the most important and widely cultivated spice crops across India and it alone contributes to the 25% of the total chilli production in the world. The area under chilli cultivation in the world is 17,64,284 ha for producing fresh chillies and 18,37,419 ha for producing dry chillies with production of 2,99,08,102 tons (FAO 2007). Chillies are low in sodium and cholesterol, rich in vitamins A and C and are a good source of potassium, folic acid and vitamin E (Osuna-Garcia *et al* 1998; Marin *et al*. 2004) [19]. The production of chilli in India is 17. 64 lakh tons with productivity of 2400 kg per ha (Chilli outlook). In India, chilli is cultivated mainly in the states of Andhra Pradesh, Telangana, Madhya Pradesh, Karnataka, and Tamil Nadu (www.horticulturalstatistics.com, 2019-2020). There are various factors which limit the realization of the yield potential of the chilli crop. Among them, diseases are of main importance as they cause extensive damage to the crop and its produce. Among different diseases which affect Chilli production, anthracnose disease caused by *Colletotrichum* spp. is of major importance as it also affects the harvested produce. It is a complex disease which is caused by various *Colletotrichum* spp. *Colletotrichum* spp. has been reported among ten most destructive plant pathogens in the world. Different *Colletotrichum* species associated with chilli anthracnose disease in India are *C. capsici*, *C. gloeosporioides*, *C. acutatum* and *C. truncatum* (Rohan D Lokhande *et al*., 2019) [23].

The disease appears as multiple sunken circular or angular lesions which often coalesce to form severe fruit rot. These lesions are characterized by the presence of orange or pink spots in concentric rings which turn black when matured. These are called acervuli containing setae entrapping conidia. Similar symptoms also appear on stems and leaves which results in defoliation. The infection of the growing tip results in necrosis of branches which proceeds backward and killing it (Die backstage). This will lead to death of whole plant

Presence of a small lesion on the fruits will drastically reduce the marketability of the produce (Ahila Devi and Prakasam, 2016). *Colletotrichum* spp. causes some of the most economically important plant diseases which results in the reduction of yield from 10 – 80% of total production in some developing countries (Poonpolgul and Kumphai 2007) [22]. The most important aspect of this disease is that the pathogen also causes latent infection and affects ripe fruits which results in pre- and post-harvest fruit decay (Jeffries *et al*., 1990) [10]. Yield loses up to 50% have been reported (Liu *et al*., 2016) [13]. Pre-harvest fruit loses up to 25% and post-harvest loses up to 25 - 40% have been reported (Sharma and Shenoy, 2004). Severe yield loses up to 60% have been reported (Bansal and Grover, 1969). Yield loses up to 30% and 60% were reported from Punjab and Assam (Sahitya *et al*., 2014) [24].

Traditionally, chemical fungicides were used for controlling chilli anthracnose disease under field conditions. There are numerous reports of using chemicals on farmers income and health, and toxic contamination to the environment, particularly in developing countries (Voorrips *et al*., 2004) [30]. Thus, emphasis should be given for using bioagents for the management of the plant disease which is not only cost effective but also environment friendly. Bioagents are

known to induce systemic resistance against several plant diseases and also improve the yield of the plants.

Endophytes are microorganisms which colonize the plant cells without causing any harmful effects and eventually establish mutualistic relationship with its plant host (Padhi *et al*. 2013) [20]. The host will supply nutrients and protection to the endophytic microorganisms while they produce bioactive compounds which improves the host's ability to withstand biotic and abiotic stress and aid in promoting plant growth by supplying essential nutrients (Tan and Zou 2001; Gouda S *et al*. 2016) [9]. Studies done earlier demonstrated that endophytic microorganisms have a very good potential in controlling plant pathogens (Phongpaichit *et al*. 2006; Bivi *et al*. 2010; Landum *et al*. 2016; Marcellano *et al*. 2017) [21, 7, 12, 15]. This study is aimed at screening different fungal endophytes against chilli anthracnose pathogen *Colletotrichum acutatum* under in-vitro conditions. Thus, identified bio-agents can be subjected to further studies under in-vivo conditions to assess their performance alone and in combinations with other inputs.

Materials and Methods

Isolation of chilli anthracnose pathogen

The plants with chili anthracnose disease were identified based on the symptoms and diseased plant samples were collected in separate polythene bags from different chilli growing areas of Andhra Pradesh and Tamil Nadu. The plant samples were brought to laboratory and washed in tap water to remove the adhering debris. A small portion of infected tissue (2×2 mm) along with a healthy portion was exercised by using a sterile scalpel. These bits are now surface sterilized in 70% alcohol for 20 seconds followed by 1% sodium hypochlorite for 1 min. Then they are washed thrice with sterile distilled water to get rid of the surface sterilizing agents and dried on a sterilized filter paper in the air of the laminar airflow chamber. After drying, these bits were aseptically transferred to Petri-plates containing potato dextrose agar medium (PDA) in such a way that each plate containing four bits towards the periphery of the plates. These plates are now incubated in biological oxygen demand (BOD) incubator at 28 ± 1^0C for 3 days. Purification is done by single spore isolation method and the pure cultures thus obtained are inoculated in PDA slants and stored in the refrigerator at 4^0C for future studies.

Isolation of endophytic fungi

For the isolation of the endophytic fungi; leaf, fruit and root samples were collected from healthy chilli plants (*Capsicum annuum*) separately in different polythene bags.

The isolation of the endophytic fungi was carried out according to the procedure described by Kunihiko *et al*., 2002. A small portion (2×2 mm) of each sample was exercised using a sterile scalpel and was dipped in 70% ethanol for 1 min followed by dipping in 15% H2O2 for 15 min. The sample bit was again dipped in 70% ethanol and rinsed thrice in sterile distilled water.

These bits were then aseptically placed on 2% malt extract agar (MEA) medium. Then the plates were incubated at 27ºC in an incubator for 6 days. Fungi which grow out of those leaf/ fruit/root bits were purified on PDA (potato dextrose agar) slants and are recorded as endophytic fungi.

In-vitro screening of endophytic fungi against *Colletotrichum acutatum*

Antagonistic effect of endophytic fungal cultures was tested against *Colletotrichum acutatum* by employing dual culture technique as described by Webber and Hedger (1986). Six mm discs of pathogen and the endophytic fungi were placed on opposite sides in the petri plates containing PDA and the plates are incubated in BOD incubator at 27 ± 1°C for 7 days. Control plates were maintained only with *Colletotrichum acutatum* culture. Radial growth of *Colletotrichum acutatum* was measured after 7 days and the percent inhibition of was calculated using the formula. The same procedure is adopted for all the endophytic fungal isolates.

$$\text{Percent inhibition} = \frac{C-T}{C} \times 100$$

C: Radial growth of *C. acutatum* in control plates

T: Radial growth of *C. acutatum* in plates with test organism

Effect of cell-free culture filtrate/non-volatile compounds/metabolites of endophytic fungal antagonists against mycelial growth of *Colletotrichum acutatum*

The antagonistic activity of endophytic fungal isolates was tested according to the poisoned food technique described by Nene and Thapliyal (1993) [18]. The metabolites were obtained from the endophytic fungal antagonists by inoculating a 6 mm disc of 7 days old culture in 50 ml PD (potato dextrose) broth and incubating them at 28 ± 1°C for 15 days in the dark. Then the broth was subjected to centrifugation at 3000 rpm for 20 min. The resultant supernatant was used for the bioassay at three different concentrations i.e., 5%, 10% and 15%. PDA media seeded with the culture filtrate was poured into petri plates and was inoculated with 5 mm disc of 7 days old *Colletotrichum acutatum* at the center and incubated at 28 ± 1°C for 15 days. Control plates were inoculated without the antagonist culture filtrate and three replications were maintained. The mycelial growth was recorded and percent inhibition over control is calculated as per the formula given by Vincent (1927).

$$\text{Percent Inhibition (I)} = \frac{C-T}{C} \times 100$$

Where,

I: Percent inhibition of growth of test pathogen

C: Radial growth of pathogen in the control plate

T: Radial growth of pathogen in the treatment plate

Cultural and morphological characterization of antagonistic fungal endophytes

The cultural and morphological characters of best 10 endophytes were observed. Five mm discs of the 7 days old cultures were placed at the center of Petri plates containing 20 ml of PDA aseptically and incubated at 27 ± 1^0C for 15 days in BOD incubator. The cultural and morphological characters like colony color, growth pattern, elevation, color, shape of conidia of the isolates were observed.

Results and Discussion

A total of 20 isolates of *Colletotrichum* were isolated from plant samples collected from different chilli growing areas of Andhra Pradesh and Tamil Nadu and among them C 7 isolate exhibited highest disease incidence and percent fruit rot. Based on the morphological and molecular studies, the isolate C 7 was identified as *Colletotrichum acutatum* (NCBI GenBank accession: OL348327).

Isolation of endophytic fungi

A total of 122 endophytic fungal cultures were isolated from healthy chilli plants (54 leaf, 42 fruit and 26 root). The endophytic fungal isolates include plant pathogens such as *Alternaria* spp., *Cercospora* sp., *Colletotrichum* spp., *Curvularia* spp., *Chaetomella* sp., *Cunninghhamella* sp., *Fusarium spp*., *Aspergillus* spp., *Mucor* sp., *Myrothecium* sp., *Periconia* sp., *Pythium* spp., *Phoma* sp., *Phomopsis* sp., *Glomerella* sp., *Helicostylum* sp., *Leptosphaera* sp., *Mycosphaerella* sp., *Nigrospora* spp., *Stemphylium* spp., etc. and antagonists like *Beauveria bassiana*, *Trichoderma* spp., *Pencillium* spp., *Chaetomium globosum* and *Gliocladium virens* etc.

They were tested against Chilli anthracnose pathogen for antagonistic activity by employing dual culture technique. Among them, 4 leaf endophytic fungi, 3 fruit endophytic fungi and 4 root endophytic fungi exhibited strong antagonistic activity against *Colletotrichum acutatum.* The remaining endophytic fungi showed light to moderate activity against the pathogen. Endophytes which showed strong antagonistic activity were subjected to further studies.

***In vitro* efficacy of antagonistic fungal endophytes against *Colletotrichum acutatum* by dual culture technique**

Antagonistic activity of the isolated fungal endophytes was tested by employing dual culture technique. The tested endophytic fungal isolates resulted into the percent inhibition of pathogen varying from 19.77 to 61.45%. Among all the endophytic isolates tested, ENRF 7 recorded maximum inhibition percent of 61.45% followed by ENFF 19 at 52.91%. The least percent inhibition was recorded from ENFF 7 at 19.77%. The inhibition percent of the isolates ENLF 8 and ENLF 31 are significantly on par with each other. Similarly, ENFF 7, ENFF 36 and ENRF 18 resulted into lower and significantly similar inhibition percent over control (Table 1). Talapatra *et al*., 2017 [26] had previously reported that endophytic *Trichoderma* spp. isolated from roots resulted into highest inhibition zone against *Colletotrichum capsici* at both 7 (18.6mm) and 14 (7.3mm) days after inoculation. *Trichoderma* spp. are known to produce lytic enzymes which enhances its antagonistic ability (Benitez T *et al*., 2009). It was also previously reported that *Trichoderma* isolate CA-60 inhibited the growth of *Colletotrichum capsici* with inhibition percent of 52.1 (Abhishek Mishra *et al*., 2017). Amrita Saxena *et al*., 2015 had isolated BHUF4 isolate of *Trichoderma* spp. from phyllosphere which resulted into an inhibition percent of 77.67% against the chilli anthracnose pathogen. Mukesh Yadav *et al*., 2021 [17] assessed *Trichoderma* harzianum, *T. asperellum* and *Paenibacillus dendritiformis* against *Colletotrichum truncatum* the causal agent of Chilli anthracnose and reported that *T. harzianum* showed highest percent inhibition of pathogen growth (75.46%) followed by *T. asperellum* and *P. dendritiformis*

Table 1: *In vitro* efficacy of antagonistic fungal endophytes against *Colletotrichum acutatum*

S. No	Isolate Code	Mycelial Growth (mm)		Percent inhibition over control
		Endophytes	*Colletotrichum acutatum*	
1	ENLF 8	46.7	43.3	38.40
2	ENLF 11	40.3	49.7	29.30
3	ENLF 31	44.9	45.1	35.84
4	ENLF 44	49	41	41.67
5	ENFF 7	33.6	56.4	19.77
6	ENFF 19	56.9	33.1	52.91
7	ENFF 36	36	54	23.18
8	ENRF 2	41.7	48.3	31.29
9	ENRF 7	62.9	27.1	61.45
10	ENRF 18	35.7	54.3	22.75
	Control	-	70.3	-
C.D.			3.718	
SE(m)			1.26	
SE(d)			1.781	
C.V.			4.6	

*Mean of three replications

Effect of cell-free culture filtrate of endophytic fungal antagonists against mycelial growth of *Colletotrichum acutatum*

The effect of cell-free culture filtrate of fungal antagonists was tested against *Colletotrichum acutatum* at three different concentrations 5%, 10% and 15% using poisoned food technique. The fungal antagonists significantly inhibited the growth of the pathogen at all the tested concentrations. Observations revealed that the increase in the concentration of culture filtrate resulted in to increase in the inhibition of the pathogen. Among all the isolates tested, ENRF 7 recorded highest percent inhibition of the pathogen at all the concentrations tested 5% (68%), 10% (74.82%) and 15 % (87.11%). This was followed by ENFF 19 at 5% (59.64), 10% (67.03) and 15% (81.09). This was followed by ENLF 44 and ENLF 8 which are significantly on par with each other at 5% and 10%. Similarly, ENLF 11, ENLF 31 and ENRF 2 recorded significantly similar results at all the tested concentrations (Table 2).

The lowest percent inhibition of pathogen was recorded from ENRF 18 at all the tested concentrations. Based on the morphological and molecular studies, the isolate ENRF 7 was identified as *Trichoderma asperellum* (NCBI GenBank accession: OL868970).

Similar experiment was earlier performed by Abhishek Mishra *et al*., 2017 and found that *Trichoderma harzianum* isolates CA-06 and CA-07 isolates showed highest inhibition of mycelial growth at all concentrations against the tested pathogens. Ahsanur Rahman *et al*., 2013 [13] has studied the effect of non-volatile compounds of different *Trichoderma* species on the mycelial growth of

Colletotrichum capsici and reported that highest inhibition was recorded from *Trichoderma harzianum* (81.96%) followed by *T. pseudokoningi* (59.67%).

The inhibition by the culture filtrate of antagonists may be due to the presence of cell wall degrading enzymes and antifungal antibiotics (Tronsmo and Hjeljord, 1997).

Table 2: Effect of cell-free culture filtrate/ non-volatile compounds/ metabolites of endophytic fungal antagonists against mycelial growth of *Colletotrichum acutatum*

S. No	Isolate Code	Radial growth of pathogen (mm)			Percent inhibition over control		
		5%	10%	15%	5%	10%	15%
1	ENLF 8	37.6	29.4	22.8	48.20	59.78	68.06
2	ENLF 11	41.6	33.6	25.6	42.69	54.03	64.14
3	ENLF 31	42.1	35.1	21.6	42.01	51.98	69.74
4	ENLF 44	37.2	29.1	18.9	48.76	60.19	73.52
5	ENFF 7	51.2	39.2	27.8	29.47	46.37	61.06
6	ENFF 19	29.3	24.1	13.5	59.64	67.03	81.09
7	ENFF 36	48.2	36.2	28.4	33.60	50.47	60.22
8	ENRF 2	42	35	26	42.14	52.12	63.58
9	ENRF 7	23.2	18.4	9.2	68.04	74.82	87.11
10	ENRF 18	60	53	46	17.35	27.49	35.57
11	Control	72.6	73.1	71.4	-	-	-
C.D.		4.147	3.711	2.559	-	-	-
SE(m)		1.405	1.257	0.867	-	-	-
SE(d)		1.987	1.778	1.226	-	-	-
C.V.		5.462	5.897	5.308	-	-	-

*Mean of three replications

Cultural and morphological characterization of antagonistic endophytic fungi

Best 10 endophytic fungal isolates which showed antagonistic activity against *Colletotrichum acutatum* were selected and grown of PDA plates for studying their colony and morphological characters. Among the different endophytic isolates, ENFR 7 and ENFF 19 recorded highest colony diameter of 90mm followed by ENLF 31 and ENLF 44. The colony colour and shape also varied from one isolate to another. The texture of most of the isolates was cottony whereas it was smooth in ENLF 8 and rough in ENLF 11.

Six isolates also produced pigments, ENLF 31 produced yellowish pigment, ENLF 44 producing light yellow, ENFF 7 producing dark grey pigment, ENFF 36 producing Beige pigment, ENRF 2 producing yellow pigment and light violet pigment is produced by ENRF 18. Based on the conidial colour, morphology and cultural characters, the endophytic isolates were identified and presented in Table 3.

Table 3: Cultural and Morphological characterization of antagonistic endophytic fungi

S. No	Isolate Code	Colony Colour	Growth Pattern	Colony Shape	Texture	Pigmentation	Colony Diameter (mm)	Elevation	Conidial Morphology	Conidia Colour	Isolate Name
1	ENLF 8	White	Disperse	Round	Smooth	No	46	Flat	Round to oval	Hyaline	*Beauveria* spp.
2	ENLF 11	Dark brown	Floccose	Oval	Rough	No	81	Raised	Globose	Dark brown	*Aspergillus* spp.
3	ENLF 31	Green	Fluffy	Round	Cottony	Yellowish	89	Flat	Globose	Blue green	*Penicillium* spp.
4	ENLF 44	Greenish white	Fluffy	Oval	Cottony	Light yellow	87	Raised	Round	Dark brown	*Chaetomium globosum*
5	ENFF 7	Black	Floccose	Oval	Cottony	Dark grey	78	Raised	Globose	Black	*Nigrospora* spp.
6	ENFF 19	White and green at edges	Floccose	Round	Cottony	No	90	Raised	Cylindrical	Hyaline	*Gliocladium virens*
7	ENFF 36	White	Velvety	Round	Cottony	Beige	38	Raised	Cylindrical	Hyaline	Unknown
8	ENRF 2	Green	Fluffy	Round	Cottony	Yellow	47	Flat	Ellipsoid	Green	*Penicillium* spp.
9	ENRF 7	Green	Floccose	Round	Cottony	No	90	Raised	Slightly Ovoidal	Hyaline	*Trichoderma* spp.
10	ENRF 18	White	Floccose	Round	Cottony	Light violet	81	Raised	Falcate, tapered edge	Hyaline	*Fusarium* spp.

Conclusion

A total of 122 fungal endophytic isolates were isolated from leaves, fruits and roots of healthy chilli plants which include most of pathogens and a few bio-control fungi. It clearly states that there was a wide diversity of microorganisms present in healthy chilli plants. All the isolates were screened for their antagonistic ability against *Colletotrichum acutatum* in-vitro and found that 10 isolates exhibited antagonistic activity. Among the 10 antagonistic isolates, ENRF 7 (*Trichoderma asperellum*) isolate resulted into maximum percent inhibition of the pathogen under dual culture assay and poisoned food technique at all the concentrations.

It was followed by ENFF 19 and ENLF 44. Based on the cultural and morphological characters they were identified as *Gliocladium virens* and *Chaetomium globossum* respectively.

The antagonistic ability of ENFR 7 has to be further tested under field conditions alone and in combinations with other bio agents and organic amendments for developing a new approach for managing Chilli anthracnose disease.

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