

Research Article

Investigation on the bio-efficacy of fungal and bacterial bio-agents against *Alternaria alternata* inciting little millet leaf blight

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ABSTRACT: Little millet (*Panicum sumatrense* Roth ex Roem. and Schult.) is one of the hardiest minor cereal crop indigenous to Indian sub-continent. The crop is cultivated by tribal and poor farmers for food and feed. Leaf blight disease has been a major production constraint and fungicidal sprays for the management of the disease may not be economically viable and feasible. Hence, the present *in vitro* study was carried out to know the antifungal activity of six fungal and 10 bacterial bio-agents against *Alternaria alternata* inciting little millet leaf blight disease. Among the fungal bio-agents, *Trichoderma harzianum* (ThB5) and among the bacterial bio-agents, *Bacillus velezensis* (P42) showed mycelial growth inhibition of 75.18 and 84.75%, respectively.

KEY WORDS: *Alternaria alternata*, *Bacillus velezensis*, biocontrol, little millet, *Trichoderma harzianum*

(Article chronicle: Received: 04-11-2020; Revised: 21-12-2020; Accepted: 26-12-2020)

INTRODUCTION

Millet is the oldest food crops known to humans and their origin started 4000 years ago (Changmei and Dorothy, 2014). Asia and Africa especially India and Nigeria are responsible for contribution of 97% of world's millet production. During 2014, the contribution of Asian countries towards millet production has increased from 48.72% to 52.25% (Rao *et al.*, 2017). In terms of nutritional composition, millets are superior than major cereals like wheat and rice especially in micronutrients, fiber and phytochemicals. Millets in addition to being good sources of nutrients and phytochemicals, also sustain adverse climatic conditions, thus help to attain food and nutritional security (Bhat *et al.*, 2018).

Among the small millets, little millet (*Panicum sumatrense* Roth ex Roemer and Schultes), locally known as *kutki*, *mejhari*, *medo*, *saama*, *same*, *vari* is one of the hardiest minor cereal crop belonging to the family Poaceae (Gramineae). It is wonderful millet and its grains are suitable for people of all age groups. It helps to prevent constipation and heals all the problems related to stomach. It improves the semen counts of men, besides helps women with irregular menstruation problems. Its high fiber helps to reduce the fat

depositions in the body. The little millet contains 8.7-gram protein, 75.7-gram carbohydrate, 5.3-gram fat and 1.7-gram mineral per 100 grams. Little millet is a rich source of complex carbohydrates, antioxidants and phenolic compounds which help to prevent metabolic disorders like diabetes, cancer, obesity etc. Being eco-friendly, the crop is suitable for fragile and vulnerable agro-ecosystems.

Little millet is affected with many fungal diseases like grain smut (*Macalpinomyces sharmae*), rust (*Uromyces linearis*), banded leaf and sheath blight (*Rhizoctonia solani*) and Udbatta (*Ephelis oryzae*), which are occurring at different stages of plant growth and cause economical yield losses under favourable environmental conditions (Chauhan, 2014). Leaf blight caused by *Alternaria alternata* is one of the emerging maladies in successful cultivation of little millet. No information is available for this disease on little millet in the literature about severity of occurrence, etiology, variability of the pathogen, resistance source and management of the disease.

Plant disease management using chemicals has adverse effect on environment and also there is possibility of resistance development by the pathogen. Biological control offers an environmentally friendly approach to the

management of plant disease and can be incorporated into cultural and physical strategies and limited chemical usage for an effective Integrated Pest Management (IPM) system (Monte, 2001). *Trichoderma* spp. has the ability to produce secondary metabolites such as antibiotics, acids and cell wall degrading enzymes (CDWE) which are potential enough to inhibit phytopathogenic fungi (Vinale *et al.*, 2008). *Trichoderma* spp. exhibit diversified mechanism of action against phytopathogenic fungi and suppress its growth through broad range of antifungal metabolite production, competition, mycoparasitism, occupation of infection court and induced resistance (Elad, 2000). *Bacillus* species has the ability to produce a wide range of secondary metabolic compounds of varied structure and function. The production of secondary antimicrobial compounds determines their capability to control many plant diseases (Silo-suh *et al.*, 1994). Dai *et al.* (2020) reported antagonistic mechanisms of non-volatile lipopeptides (LPs) and volatile organic compounds (VOCs) produced by *B. velezensis* strain C16 against *A. solani* and indicated that VOCs and LPs reduced colony diameter and significantly inhibited germination of conidia. Hence, the present *in vitro* study was taken up to evaluate the efficacy of fungal and bacterial bio-agents for their antifungal activity against *A. alternata* the incitant of little millet leaf blight.

MATERIALS AND METHODS

Collection of the sample

During October 2018, dark brown, circular to oval necrotic spots surrounded by concentric rings were observed on the upper leaf surface of the little millet variety VS-13 grown in the fields of the University of Agricultural Sciences, GKVK, Bengaluru (13.0784°N, 77.5793°E). As the disease progressed, infected leaves became blighted. Disease incidence up to 53% and severity of 67.33% was recorded. The leaf blight disease incidence and severity was recorded on 1-9 scale (Kiran Babu *et al.*, 2013). Thirty symptomatic leaves were collected from the infected field for isolation of the associated causal organism.

Isolation and identification of the causal organism

The diseased leaves were cut into 5 × 5-mm pieces and initially surface-sterilized in 75% ethanol for 45 seconds followed by 1% sodium hypochlorite for 1 min, rinsed in sterile distilled water three times and placed over filter paper to remove excess moisture. Later, the leaf bits were plated on Petri plates containing PDA medium and incubated at 27±1°C for mycelial growth. Incubated plates were observed for fungal growth. Monoconidial isolation method was employed on water agar for purification of the fungal pathogen (Tutte, 1969). Morphological studies of the causal organism were studied on PDA as described by Simmons (2007).

Molecular characterization of the causal organism

For DNA extraction, the fungal culture was grown on potato dextrose broth (PDB; pH 5.5) for 7 days at 27±1°C in the incubator. Filtered mycelium (200 to 500 mg) was ground to fine powder in liquid nitrogen. Genomic DNA was extracted following the protocols developed by Murray and Thompson (1980) with slight modifications. The genome of the *A. alternata* was amplified in PCR using different set of primers like *Alternaria alternata* species specific primer (AA) mitochondrial Smaller Subunit (SSU) and Internal Transcribed Spacer region (ITS) listed in Table 1. The PCR product was analyzed on 0.8% agarose gel, stained with ethidium bromide and viewed under trans illuminator. The amplified fragments were sequenced and confirmed using NCBI database.

Proving pathogenicity

The conidia from culture plate was harvested by flooding sterile distilled water and diluted to 10⁶ conidia/mL to which 0.02% Tween 20 was added and then sprayed with the help of atomizer on 45 days old little millet plants. Later, inoculated and control plants were covered with transparent polyethylene bags and were maintained in a greenhouse at 28±2°C and 90% RH. The pathogenicity test was repeated three times. Re-isolations were performed from inoculated plants, and the re-isolated pathogen was confirmed as *A. alternata*.

In vitro evaluation of fungal and bacterial bio-agents against *A. alternata*

A total of six fungal bio-agents *Trichoderma viride* (Tv1), *T. viride* (Tv8), *Trichoderma harzianum* (Th55), *T. harzianum* (Th14), *T. harzianum* (ThB5), *T. harzianum* (Th10) and ten bacterial bio-agents *Bacillus velezensis* (A6), *Bacillus* sp. (GPUR-12), *Enterobacter cloacae* (GPUL-19), *B. mojarensis* (UMR-9), *B. cerius* (GPUR-10), *B. velezensis* (P42), *Pennibacillus polymyxa* (GPUS-13), *B. megaterium*, *B. subtilis* and *Pseudomonas fluorescens* were evaluated against *A. alternata* *in vitro* employing dual culture technique. In the dual culture technique, sterilized PDA media was cooled and about 15-20 mL of media was poured into sterile Petri plates. Fungal antagonists were evaluated by inoculating the blight pathogen at one side of the plate and the antagonist on the exact opposite side of the same plate by leaving 3-4 cm gap. Bacterial antagonist was streaked in the corner of the plate after which a fungal disc was placed. Each treatment was replicated three times. After twelve days of incubation the radial growth of the pathogen was measured. Per cent inhibition over control was worked out (Vincent, 1947).

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

Where, C = Growth of mycelium in control, T = Growth of mycelium in treatment.

Table 1. Nucleotide sequences of the primers used for PCR amplification of different gene/regions

S. No.	Region	Primer sequence	Reference
1	ITS	ITS-1: 5'-TCCGTAGGTGAACCTGCGG-3' ITS-4: 5'-TCCTCCGCTTATTGATATGC-3'	White <i>et al.</i> (1990)
2	AA	AAF2: 5'-TGCAATCAGCGTCAGTAACAAAT-3' AAR3: 5'-ATGGATGCTAGACCTTTGCTGAT-3'	Konstantinova <i>et al.</i> (2002)
3	SSU	NS1: 5'-GTAGTCATATGCTTGTCTC-3' NS4: 5'-CTTCCGTCGAATTCCTTTAAG-3'	White <i>et al.</i> (1990)

Statistical analysis

The data generated by the experiment were analyzed using the WASP (Web Based Agricultural Statistics *Software* Package) software developed by ICAR-Central Coastal Agricultural Research Institute, Goa.

RESULTS AND DISCUSSION

Pathogen associated with the leaf blight disease was isolated from 30 symptomatic leaf samples of little millet following standard tissue isolation method and cultured on PDA. Initially, fungal colony appeared as olive green which later turned dark grey to greyish black upon maturity. Pure colonies of the fungus initially produced profuse olive green to light grey aerial mycelia that later turned to dark grey (Figure 1). Initially, the hyphae were thin, hyaline and became thick with age. Golden to pale brown conidiophore that arose singly or in small groups were flexuous or straight, geniculate with presence of many scars which represent the point of conidia. Secondary conidiophores were short. The conidia were produced in chains of 4-12, pale brown to light brown, obclavate to pyriform with 0-3 longitudinal and 1-6 transverse septa with constrictions and presence of short beak and corroborate the descriptions of Simmons (1967), Kgatele *et al.* (2018) and Dipak *et al.* (2013).

The pathogen was confirmed as *A. alternata* at molecular level by using *A. alternata* species specific primer (AA), mitochondrial Smaller Subunit (SSU) and Internal Transcribed Spacer region (ITS). The resultant PCR amplicon produced by using *A. alternata* specific primers AAF/AAR yielded fragment size of approximately 345 bp and confirmed the pathogen as *A. alternata*. The results are in accordance with Konstantinova *et al.* (2002) and Huseyin *et al.* (2018) who got similar product size of about 340 bp and 346 bp and confirmed the pathogen as *A. alternata* on potato and carob, respectively (Figure 2). The SSU (MT772257:1020 bp), ITS (MN919390: 585 bp) showed 100 and 99.62% similarity with *A. alternata* with reference strain CBS 916.96 of *A. alternata*. The results are in line with Woudenberg *et al.* (2015) who

described and confirmed the *A. alternata* upto species level by using different gene regions.

The pathogen, which was artificially inoculated on little millet plants produced same symptoms and upon re isolation showed similar characteristic descriptions of mycelium, conidiophores and conidia as of the original. The identity of the re-isolated pathogen was confirmed by comparing with the original descriptions (Simmons, 2007).

Among the six fungal bio-agents tested against *A. alternata*, the mycelial growth inhibition ranged from 68.98 to 75.18% (Table 2, Figure 3A, 3B). Highest mycelial radial growth inhibition was noticed with *T. harzianum* (ThB5) 75.18% followed by *T. harzianum* (Th14) 74.19%. Whereas, *T. viride* (Tv8), *T. viride* (Tv1), and *T. harzianum* (Th10) also showed considerable mycelial growth inhibition of 73.82, 73.20 and 69.52% with minimum inhibition of mycelial growth noticed in *T. harzianum* (Th55).

The present findings are in accordance with Babu *et al.* (2000), who evaluated the efficacy of six *Trichoderma* species on early blight of tomato, of which *T. harzianum* exerted the highest inhibition of mycelial growth (50.22%) of the pathogen over control followed by *T. viride*. Thaware *et al.* (2011) studied the antagonistic effect of the fungal bio-agents against *A. alternata* and found *T. harzianum* as most effective that caused 85.88 per cent mycelial growth



Fig. 1. Pure culture of *A. alternata* and conidia (100x)

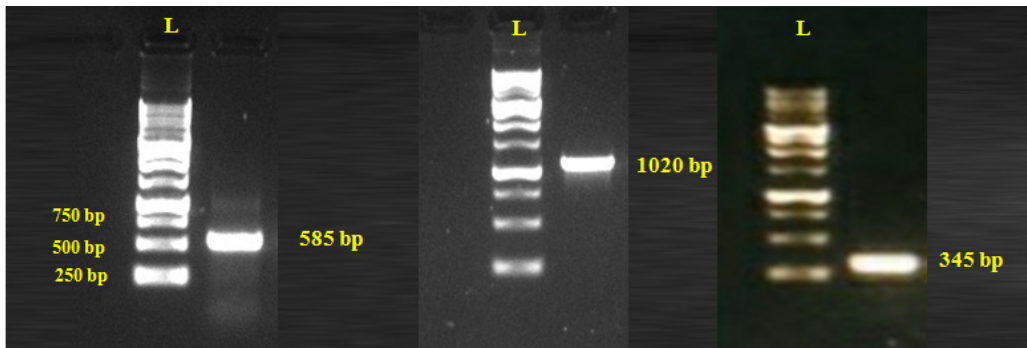


Fig. 2. PCR amplification of ITS (585 bp), SSU (1020 bp) and AA (345 bp) (Lane L: 1kb ladder)

inhibition followed by *T. viride* (81.88%). Zade *et al.* (2018) revealed that *T. harzianum* and *T. asperellum* gave the best result against *A. alternata* recording maximum mycelial inhibition of 79.65 and 76.55% respectively. Chethana *et al.* (2012) recorded the maximum mycelial inhibition of 79.50% with *T. harzianum* against *A. porri*. Hariprasad *et al.* (2018) recorded the maximum inhibition (100%) with *Trichoderma harzianum* (NBAIR), followed by *T. viride* (81.38%) against *A. tenuissima*. Inhibition of the test pathogen can be possibly because of the competitive ability of *Trichoderma* spp. which includes mycoparasitism, siderophore formation and antibiosis. According to Ghaffar (1969) the test pathogen encountered interactions such as inhibition, stimulation, over growth of antagonistic organism over target pathogen.

Among the bacterial bio-agents tested against *A. alternata*, the mycelial growth inhibition ranged from 20.64 to 84.75% (Table 3, Figure 5A, 5B). *Bacillus velezensis* strain P₄₂ showed highest mycelial growth inhibition (84.75%) whereas, least inhibition of mycelial growth was recorded with *B. subtilis*.

Dai *et al.* (2020) studied antagonistic mechanisms of non-volatile Lipopeptides (LPs) and Volatile Organic Compounds (VOCs) produced by *B. velezensis* strain C16 against *A. solani* and indicated that VOCs and LPs reduced

Table 2. *In vitro* efficacy of fungal bio-agents against *A. alternata*

S. No.	Fungal bio-agent	Per cent inhibition over control*
1	<i>Trichoderma viride</i> Tv1	73.20 (58.82)
2	<i>Trichoderma harzianum</i> Th55	68.98 (56.15)
3	<i>Trichoderma harzianum</i> Th14	74.19 (59.46)
4	<i>Trichoderma harzianum</i> ThB5	75.18 (60.12)
5	<i>Trichoderma harzianum</i> Th10	69.52 (56.49)
6	<i>Trichoderma viride</i> Tv8	73.82 (59.22)
	S. Em (±)	0.31
	CD (0.01%)	1.06

*Mean of three replications; Figures in parenthesis are arc sine transformed values

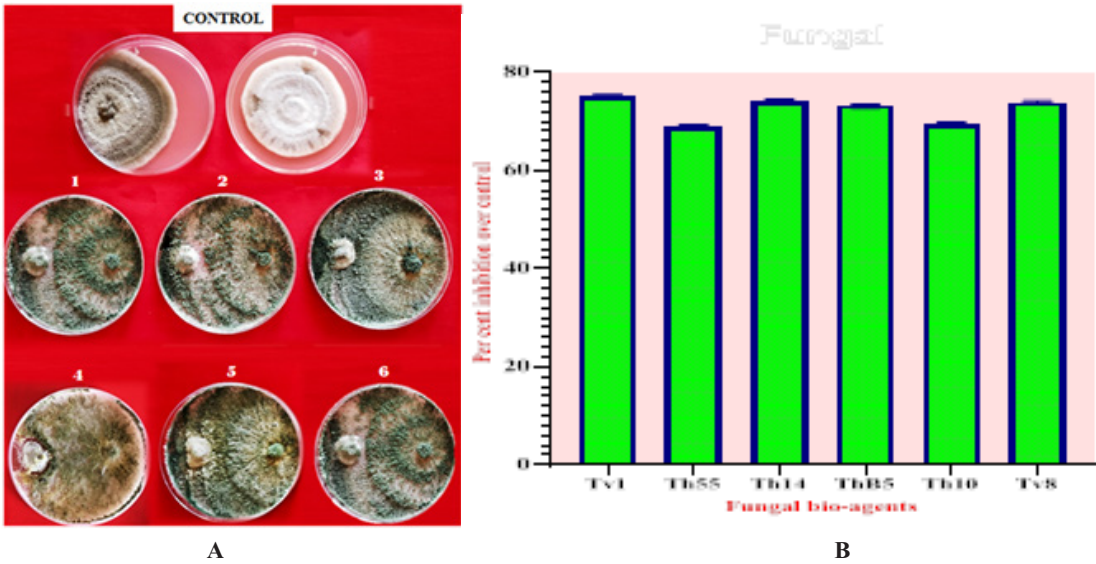


Fig. 3A, B. *In vitro* evaluation of fungal bio-agents against *A. alternata*
1. *T. harzianum* (Th14), 2. *T. harzianum* (ThB5), 3. *T. harzianum* (Th55), 4. *T. harzianum* (Th10), 5. *T. viride* (Tv1), 6. *T. viride* (Tv8).

Table 3. *In vitro* evaluation of bacterial bio-agents against *A. alternata*

S. No	Bacterial bio-agent	Per cent inhibition over control*
1	<i>Bacillus velezensis</i> (A ₆)	81.10 (64.24)
2	<i>Bacillu</i> sp (GPUR-12)	62.16 (52.04)
3	<i>Enterobacter cloacae</i> (GPUL-19)	56.01 (48.45)
4	<i>B. mojavensis</i> (UMR-9)	58.64 (49.98)
5	<i>Bacillus cerius</i> (GPUR-10)	78.46 (62.35)
6	<i>Bacillus velezensis</i> (P ₄₂)	84.75 (67.01)
7	<i>Pennibacillus polymyxa</i> (GPUS-13)	53.64 (47.09)
8	<i>Bacillus megaterium</i>	34.70 (36.09)
9	<i>Bacillus subtilis</i>	20.64 (27.02)
10	<i>Pseudomonas fluorescens</i>	26.18 (30.76)
	S. Em (±)	0.43
	CD (0.01%)	1.71

*Mean of three replications; Figures in parenthesis are arc sine transformed values

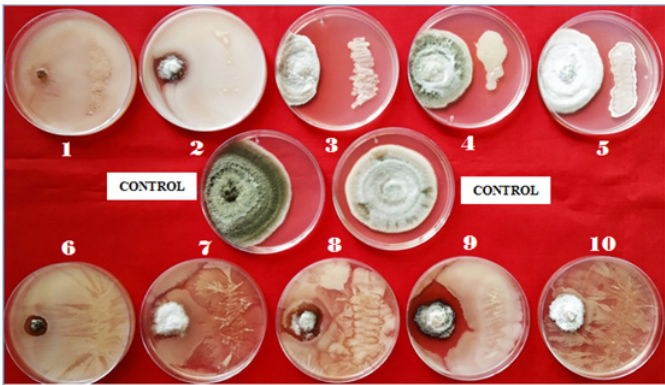


Fig. 5A, 5B. *In vitro* evaluation of bacterial bio-agents against *A. alternata*

1. *B. velezensis* (P42), 2. *B. cerius* (GPUR-10), 3. *B. megaterium*, 4. *P. fluorescens*, 5. *B. subtilis*, 6. *B. velezensis* (A6), 7. *E. cloacae* (GPUL-19), 8. *Bacillus* sp (GPUR-12), 9. *P. polymyxa* (GPUS 13), 10. *B. mojavensis* (UMR-9).

colony diameter and significantly inhibited germination of conidia. Hyphae treated with antagonistic compounds from C16 exhibited serious structural destruction, with thin or gapped structures and swollen sacs. Regassa (2020) reported that *B. velezensis* AR1 inhibited the growth of *A. sesami* by $54.6 \pm 3.7\%$ and the volatile organic compound activity limited the pathogen's growth by about $81.3 \pm 0.9\%$. Dragana *et al.* (2012) noticed 61.75% mycelial growth inhibition of *A. alternata* with *Bacillus* strain Q3 isolate. Priyanka *et al.* (2018) reported that bacterial strain *B. subtilis* subsp. *spizizenii* (MM19) inhibited mycelial growth of *A. alternata* to an extent of 83.99% followed by

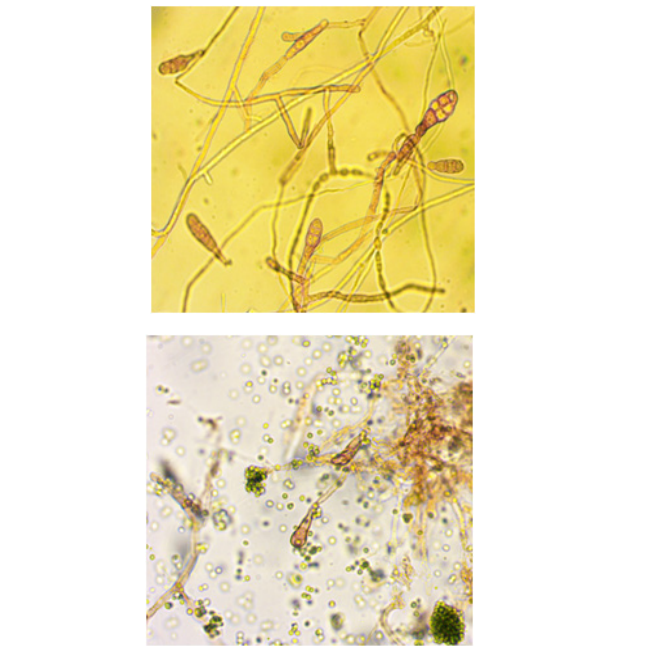
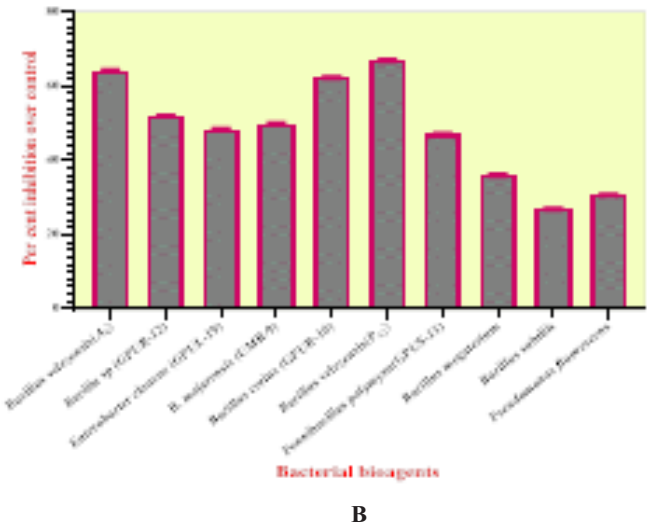


Fig. 4. A. Normal growth. B. Effect of *T. harzianum* (ThB5) on *A. alternata*



B. amyloliquefaciens strain MM12 (80.05%) and *B. subtilis* VB3 (73.23%).

CONCLUSION

The present *in vitro* investigation suggests that the pathogen *Alternaria alternata* inciting little millet leaf blight could be efficiently suppressed by fungal bio-agent, *Trichoderma harzianum* (ThB5) and the bacterial bio-agent, *Bacillus velezensis* (P42) which showed mycelial growth inhibition of 75.18 and 84.75%, respectively. The present study paves the better way for further testing of these

two bio-agents to find out the major metabolic compounds involved in hindering the pathogen, which can be further used as an efficient and better biocontrol agents against leaf blight pathogen.

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