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In vitro evaluation of plant extracts, biocontrol agents and fungicides against leaf blight in pigeonpea

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Abstract

Leaf blight is one of the important foliar diseases in pigeon pea. The causal agent associated with pigeonpea leaf blight was identified as *Alternaria alternata* based on the colony morphology and conidial characters. *In vitro* efficacy of five botanicals viz., garlic (bulb extract 5%), zimmu (leaf extract 10%), neem leaf extract (10%) tulasi (leaf extract 10%), and notchi (leaf extract 10%), five *Bacillus subtilis* strains and five fungicides were evaluated against the mycelial growth of *A. alternata*. The botanical viz., garlic bulb extract (5%) recorded 70 per cent inhibition of mycelial growth of *A. alternata* over control. The *B. subtilis* viz., CcB1 strain of, exhibited the least mycelial growth of 2.8cm with the highest growth inhibition of 68.9 percent. Among the five fungicides, Fluopyram SC 500 showed 100 per cent mycelial growth inhibition of *A. alternata* at 1000 ppm concentration.

Keywords: Pigeonpea, leaf blight, botanicals, biocontrol agents, fungicides, evaluation

Introduction

Pigeonpea, commonly known as Redgram or Arhar (*Cajanuscajan* (L) Mill sp.) is one of the primary grain legume crops grown in India for its high quality vegetable protein, animal feed and fodder. India is the world's largest producer and consumer of pigeonpea contributing to almost 80 per cent of the global pigeonpea area and production. It produces 3.17 million tonnes of pigeonpea from 3.88 million hectares with an average productivity of 817 Kg ha⁻¹. In Tamil Nadu it is grown in area of 38,000 ha with the production of 31,000 t and a productivity of 785 kg ha⁻¹ (Anonymous, 2015) [1]. Pigeonpea production is amenable for many biotic and abiotic constraints. Among the biotic constraints leaf blight disease caused by *Alternaria alternata* is one of the yield limiting factors in pigeonpea. The disease was first reported from Varanasi, India during 1971 by Pavgi and Singh (1971) [13]. Later, Kannaiyan and Nene (1977) [9] reported its occurrences from Hyderabad as a disease of minor importance but recently *Alternaria* blight has become one of the serious fungal diseases of pigeonpea. The symptoms on leaves manifested in the form of small, circular, necrotic spots that developed quickly forming typical concentric rings. Later, these spots coalesced and caused blighting of leaves. Spots were initially light brown and later turned dark brown. On stems, spots were sunken with concentric rings. In severe infection, defoliation and drying of infected leaves, branches, and flower buds were observed (Sharma *et al.*, 2012) [16]. This disease has been reported to cause yield losses up to 40-50 percent (Kushwaha *et al.* 2010) [10]. Sharma *et al.* (2012) [16] recorded yield loss up to 20-80 percent due to *Alternaria* blight in pigeonpea. Hence it is highly imperative to develop an effective management strategy for enhancing the yield potential of the pigeonpea.

Several fungicides are reported to be effective against *Alternaria* blight (Prasad and Naik, 2003 and Singh and Singh, 2006) [13, 18]. Repeated use of same fungicides resulted in fungicide resistance in pathogens. Biological control of plant pathogens has been considered as a potential control strategy in recent years and the bacterial biocontrol agent viz., *Bacillus subtilis* proved to be promising against foliar pathogens. *Bacillus subtilis* produces several types of antimicrobial compounds viz., antibiotics, cell wall degrading enzymes and siderophores and they induce growth and defense responses in the host. *Bacillus* sp. able to produce spores that are resistant to UV light and desiccation, and that allow them to resist adverse environmental conditions and permit easy formulation for commercial purposes. Natural plant products are important sources of new agrochemicals for the control of plant diseases (Kagale *et al.*, 2004) [8]. With this in back ground the present work was carried out to evaluate the efficacy of botanicals, biocontrol agents and fungicides against the pigeonpea leaf blight pathogen *Alternaria alternata* *in vitro*.

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Materials and Methods

Isolation and Identification of Pathogen

The leaf blight pathogen of pigeonpea (*A. alternata*) was isolated from the leaves infected with characteristic symptoms. The infected portions were cut into one cm bit and surface sterilized with 0.1 per cent mercuric chloride (HgCl₂) solution for 30 sec and washed thrice in series of sterile distilled water and transferred to sterilized Petri plate containing Potato Dextrose Agar (PDA) medium amended with a pinch of streptomycin sulphate (Riker & Riker, 1933). The Petri plates were incubated at room temperature (28 ± 2 °C) for 5 days and observed periodically for the growth of the fungus. Isolates were purified by single spore isolation maintained on PDA slants.

Pathogenicity

To confirm pathogenicity, 8 - 10 days old seedlings of pigeonpea cultivar CoRg 7 were grown in pots in three replications (5 plants/pots). Conidial suspension of *A. alternata* was prepared from seven days old culture grown on Potato Dextrose Broth (PDB). Seedlings were spray inoculated with conidial (5 × 10⁵ conidia/ml) suspension and covered with polythene covers and incubated at 28°C ± 1°C and 12 h photo period. Un inoculated pots served as control. Polythene covers were removed after 48 hrs. Plants were regularly watered and monitored for disease development (Sharma *et al.*, 2013)^[13].

Efficacy of plant extracts against *A. alternata*

The botanicals garlic (bulb extract 5%), zimmu (leaf extract 10%), neem leaf extract (10%) tulasi (leaf extract 10%), and notchi (leaf extract 10%) were tested for their antifungal activity against mycelia growth of *A. alternata* and the details are furnished in Table.1

Preparation of plant extracts

The freshly collected plant materials (leaf, bulb) were separately washed with tap water and then with alcohol and finally with repeated changes of sterile distilled water. These were separately ground in sterile distilled water (1ml/gram of the leaf tissue) using a pestle and mortar. The extract was strained through two layers of muslin cloth; subsequently filtered through Whatman no.1 filter paper and finally passed through seitz filter to eliminate the bacterial contamination. This formed the standard plant extract solution (100%). This was further diluted to the required concentration with sterile water. All the leaf extracts were used at 10 per cent concentration and bulb extracts was used at five per cent concentration.

Efficacy of plant extracts against *A. alternata* in vitro

The efficacy plant extracts was tested against the mycelial growth of *A. alternata* by poisoned food technique (Schmitz, 1930)^[19]. The standard plant extracts solution (100%) and PDA medium were mixed at required quantities so as to get the required concentration of the plant extracts. Twenty ml of this mixture was poured into each sterilized Petri dish under aseptic condition and allowed to set. Actively growing 9mm PDA culture disc of *A. alternata* cut out by means of a sterilized cork borer was placed on to the centre of the medium. Three replications were maintained. The plates were incubated at room temperature (28± 2 °C) for 12 days. PDA without plant extract served as control. The radial growth of the mycelium was measured 12 d after inoculation or when the fungal growth occupied the entire plate in the control

which ever was earlier. The results were expressed as percent growth inhibition.

In vitro antagonism of bacterial bio control agents against *A. alternata* – Dual culture technique (Dennis and Webster, 1971)^[6].

Bacterial biocontrol agents were tested for their antagonistic activity against mycelial growth of *A. alternata* by following the dual culture technique (Dennis and Webster, 1971)^[6]. Mycelial disc (5mm diameter) of seven days old culture of *A. alternata* was placed at one side of the Petri plate containing PDA medium 10 mm away from the periphery. Bacterial cultures were streaked on to the medium exactly opposite to the mycelial disc 10 mm away from the periphery. The control plates were inoculated with the pathogen alone. The plates were incubated at room temperature (28 ± 2 °C) for 7 days. The radial growth of the pathogen was measured both in control and treatment plates. Efficacy of the bacterial biocontrol agents against the pathogen was assessed by calculating percent inhibition (PI) over control.

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

Where C is the growth of test pathogen (cm) in the absence of the antagonist strain; T is the Growth of test pathogen (cm) in the presence of the antagonist strain

In vitro evaluation of fungicides

The efficacy of various fungicides *viz.*, Tebuconazole 50% + Trifloxystrobin 25 WG, Propiconazole 25% EC, Mancozeb 75 WP, Carbendazim 12% + Mancozeb 63% WP, Fluopyram SC 500 against the pathogen was evaluated by poisoned food technique (Schmitz, 1930)^[19]. PDA medium was amended with 100, 500 and 1000 ppm of respective fungicide and poured separately in a Petri plate and allowed for solidification. The seven day old actively growing cultures of *A. alternata* was used for the study. Fungal disc (5mm dia) was placed in the middle of the Petri plate and appropriate control was maintained without adding fungicides. The treatments were replicated thrice. The plates were incubated at room temperature (28+2 °C) and the diameter of colonies were recorded on seventh day expressed in centimetre (cm). The per cent inhibition of growth was calculated.

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

Where

PI –Percent inhibition

C-Rate of growth of pathogen in control

T-Rate of growth of pathogen in treatment

Results and Discussion

Isolation and identification of the pathogen

The pathogen associated with the leaf blight of pigeonpea was isolated from the infected samples collected from Pulse Breeding Station, Tamil Nadu Agricultural University, Coimbatore. The fungal mycelium was initially whitish grey later turned into black colour. The reverse side of the culture was brown to black due to pigment production. Microscopic observation was done on the fungal hyphae and the hyphae were highly branched, septate and brown in colour. Septate brown coloured conidiophore bearing chain of conidia was

observed in the culture. Conidia was observed to be muriform in shape with transverse and longitudinal septations and arranged singly in acropetal chains. Based on the morphology of mycelia and conidia the pathogen was identified as *Alternaria alternata*. The observation were in accordance with the description of *A.alternata* by (Sharma *et al.* 2013) [13].

Pathogenicity

The pathogenicity of *A. alternata* on pigeonpea was proved by spraying conidial suspensions of *A. alternata* (5×10^5 conidia/ml) on 8 – 10 days seedlings. The seedlings showed symptoms 12 days after inoculation in the form of light brown spot and later turns into dark brown colour with concentric rings.

Efficacy of botanicals against mycelial growth of *A. alternata* in vitro

Botanicals are good reservoirs of effective therapeutant and would constitute an inexhaustible source of harmless pesticides. Five botanicals viz; garlic (bulb extract 5%), zimmu (leaf extract 10%), tulasi (leaf extract 10%), and notchi (leaf extract 10%) were evaluated for their antifungal activity against mycelial growth of *A. alternata* by poisoned food technique. Among these, garlic bulb extract (5%) was significantly superior in inhibiting the mycelial growth by recording the least mycelial growth of 2.7 cm as against 9.0 cm in the control. This accounted for the growth inhibition of 70 percent. Similar results obtained by Chaudary *et al.* (2003) [5] who found that bulb extract of garlic was highly inhibitory to the *A.alternata*. Garlic bulb extract showed the highest reduction mycelial growth of *A.solani* (Raja, 2010) [14]. Garlic bulb extract inhibited the mycelial growth of *A. carthemi* by 79.6 per cent (Chattopadhyay, 2001) [4]. Zimmu leaf extract (10%) ranked next in inhibiting the mycelial growth with 3.6 cm growth and 60 percent growth inhibition. Nochi leaf extract (10%) reduced the mycelial growth by 48.9 percent. Leaf extracts of *Vitex negundo* possess anti-oxidant potential and antifungal activities (Tiwari and Tripathi, 2007) [27]. The leaf extract of neem inhibited the mycelial growth of *A. alternata* only by 33.3 percent (Table 2). Mesta *et al.*, (2009) [11] found that neem leaf extract was effective in controlling *A.helianthi* with 38.49 per cent inhibition of spore germination and 43.90 per cent inhibition of mycelial growth.

Efficacy of *Bacillus subtilis* strains against the mycelial growth of *A.alternata*

Five strains of *B. subtilis* viz; CcB1, CcB2, CcB3, CcB4 and CcB5 were evaluated for their efficacy against growth of *A. alternata* by dual culture technique. All the strains were effective in inhibiting the growth of *A. alternata*. The strain CcB1 exhibited the least mycelial growth of 2.8 cm and the highest growth inhibition of 68.9 percent. It was on par with CcB4 in arresting the mycelia growth of *A. alternata* which showed growth inhibition of 64.4 percent. The strain CcB2 showed the highest radial growth of 5.2 cm and the lowest

inhibition of 42.2 percent (Fig.1). Chaurasia *et al.* (2005) [3] demonstrated that *B. subtilis* was antagonistic to *A alternata* in vitro. Sharma and Sharma (2008) [16, 17] studied the biocontrol efficacy of *B.subtilis* strain UK-9, against *Alternaria* leaf spot of mustard and proved that antifungal metabolites produced by the bacteria caused morphological alterations of vegetative cells and spores, disruption and lysis of the cell wall. Basim and Katircioglu (1990) [2] evaluated antagonistic activity of 12 isolates of *B. subtilis* against *A. alternata* and *A.solani* by dual culture technique. Among these, *B. subtilis* AB-2 and AB-27 isolates were the most antagonistic against *A. alternata* and *A.solani*.

In vitro assay of fungicides against mycelia growth of *A. alternata*

Five fungicides viz; Tebuconazole 50% + Trifloxystrobin 25 WG, Propiconazole 25% EC, Mancozeb 75 WP, Carbendazim 12% + Mancozeb 63% WP, Fluopyram SC 500 screened for their fungicidal activity on radial growth of *A. alternata* at three different concentrations viz; 100, 500 and 1000 ppm. All the tested fungicides were found to inhibit the pathogen at varying degrees compared to the control. Among the five fungicides, Fluopyram SC 500 was found to be significantly superior showing cent per cent inhibition (100%) of growth at 1000 ppm concentrations. The mycelial growth was only 0.8 cm was recorded at 100 ppm concentration of Fluopyram SC 500. The protective action of Fluopyrom against *Botrytis cinerea* causing fruit rot in strawberry was earlier reported by Veloukas and Karaogladinin (2012). Spraying of fluopyram 200 + tebuconazole 200 SC at a concentration of 0.0625 per cent concentration reduced the *Alternaria* leaf spot severity by 94.55 per cent in apple (Shalini Verma and Kishore Khosla, 2018) [20].

Propiconazole and pre mixture fungicide viz., Tebuconazole + Trifloxystrobin were on par with each other in arresting the growth of *A.alternata* by recording growth inhibition of 92.2 and 91.1 per cent respectively at 1000 ppm concentration. The premixture fungicide Carbendazim 12% + Mancozeb 63% WP at 1000 ppm exerted growth inhibition of 88.9 per cent. Mancozeb at 1000 ppm concentration inhibited the mycelial growth of *A.alternata* to the extent of 74.4 percent (Table. 3) The fungicidal action of Tebuconazole + Trifloxystrobin against *Alternaria* leaf spot of cabbage was demonstrated by Sujoy Saha *et al.*, (2018) [21]. Ganesha Naik and Jayalakshmi (2017) [7] found that Propiconazole 25 EC (0.1%) and Carbendazim + mancozeb were highly effective against leaf spot of bhendi.

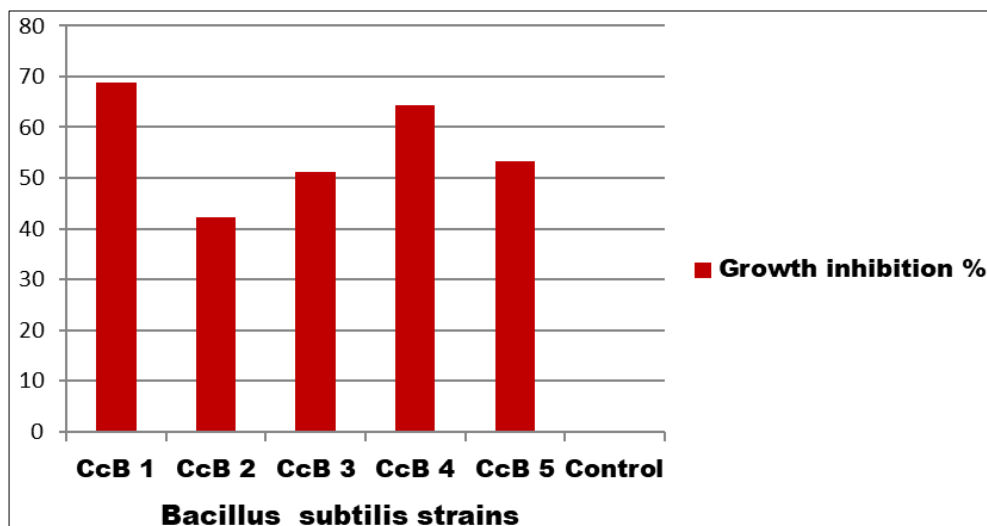
From this study it was concluded that garlic bulb extract, biocontrol agent *B. subtilis* strain CcB1 and the fungicides viz., Fluopyrom, Propiconazole and pre mixture fungicide Tebuconazole + Trifloxystrobin was promising in reducing the mycelial growth of *A. alternata*. From the garlic bulb extract antifungal component can be identified and further used for developing formulation to manage the leaf spot disease of crop plants.

Table 1: Botanicals screened for antifungal activity against *A. alternata*

S. No	Common name	Botanical name	Family	Plant parts used	Concentration (%)
1	Garlic	<i>Allium sativum</i> . L	Alliaceae	Bulb	5
2	Zimmu	<i>Allium cepa</i> L X <i>Allium sativum</i> L	Alliaceae	Leaf	10
3	Neem	<i>Azadirachta indica</i> A.Juss	Meliaceae	Leaf	10
4	Tulsi	<i>Ocimum sanctum</i> L	Labiatae	Leaf	10
5	Notchi	<i>Vitex negundo</i> (L)	Verbenaceae	Leaf	10

Table 2: *In vitro* assay of plant extracts against mycelial growth of *A. alternata*

S. No	Botanicals	Mycelial growth (cm)*	Growth inhibition (%)
1	Garlic (bulb extract 5%)	2.7 ^a	70.0
2	Zimmu (Leaf extract 10%)	3.6 ^b	60.0
3	Neem (Leaf extract 10%)	6.0 ^d	33.3
4	Thulasi Leaf extract 10%)	5.6 ^d	37.8
5	Nochi (Leaf extract 10%)	4.6 ^c	48.9
6	Control	9.0 ^e	-

**Fig 1:** Efficacy of *Bacillus subtilis* strains against mycelial growth of *A. alternata* (Dualculture technique)**Table 3:** Efficacy of fungicides against mycelial growth of *A. alternata*

S. No	Fungicides	100 ppm concentration		500 ppm concentration		1000 ppm concentration	
		Growth in cm*	Growth inhibition %	Growth in cm*	Growth inhibition %	Growth in cm*	Growth inhibition %
1.	Tebuconazole 50% + Trifloxystrobin % 25 WG	1.8 ^b	80.0	1.4 ^b	84.4	0.8 ^{ab}	91.1
2.	Propiconazole 25%EC	2.5 ^b	72.2	1.2 ^b	86.7	0.7 ^{ab}	92.2
3.	Mancozeb 75 WP	4.3 ^c	52.2	3.5 ^c	61.1	2.3 ^c	74.4
4.	Carbendazim 12% + Mancozeb 63% WP	4.1 ^c	54.4	2.9 ^c	67.8	1.0 ^b	88.9
5.	Fluopyram SC 500	0.8 ^a	91.1	0.45 ^a	95.0	0.0 ^a	100.0
6.	Control	9.0 ^d	-	9.0	-	9.0 ^d	-

*Mean of three replications

In a column means followed by same letter are significantly different by DMRT

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