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In Vitro Evaluation of *Alternaria solani* (Ellis and Mart.) Jones and Grout Causing Fruit Rot of Tomato by Plant Extracts and Bio-Control Agents

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A B S T R A C T

Bio efficacy of six aqueous plant extracts of locally available botonicals at four different concentrations, a check fungicide(Mancozeb) and four biocontrol agents on growth and sporulation of *Alternaria solani* (Ellis and Mart.) Jones and Groutwere evaluated.*Allium sativum* at 1% concentration showed 86.2% inhibition, 94.4% inhibition at 2% concentration, and 100% inhibition was achieved at 3% and 4% concentrations. Mancozeb at 0.05, 0.1 and 0.2% concentrations showed 100% inhibition. Remaining plant extracts showed less than 35% inhibition on growth of the fungus on all four concentrations. All the plant extracts evaluated could inhibit sporulation of fungus completely (100%) in all the four concentrations. The effect of biocontrols agents on the growth and sporulation of the fungus showed that *A. solani* (Ellis and Mart.) Jones and Grout was completely overgrown (100%, class I) by the antagonists *Trichoderma harzianum* and *T.hamatum* while *T. viride* colonized half of the growth of the fungus (50% overgrowth, Class III). Antagonists *Penicillium citrinum* and *P. glabrum* could not overgrow the fungus but formed 0.5 cm and 0.4 cm inhibition zone (Class VI).

Introduction

Tomato (*Solanum lycopersicum* L.) belongs to the family Solanaceae. Tomato is a native to Peruvian and Mexican region. As it is a relatively short duration crop and gives a high yield, it is economically attractive and the area under cultivation is increasing daily. This crop has become widely grown around the world because of its importance and value (Adepoju, 2014). In India, it is cultivated in about 880000 ha in different parts with a production of 18227000 metric tonnes (Anon, 2014). In Manipur, it is cultivated in area of about 2590 ha, with a production of 26159 metric tonnes and productivity of 10.1 metric tonnes per ha (Anon, 2013).

There has been a gradual increase in the area under tomato while the production has been fluctuating due to various diseases. There are several diseases on tomato caused by fungi, bacteria, viruses, nematodes and abiotic factors. Some bacterial/fungal diseases commonly found in tomatoes are Alternaria canker, Early blight, Leafspot, Fruitspot, Blossom end rot and Sunscald. Among the fungal diseases infecting tomato, disease

caused by *Alternaria solani* is one of the most catastrophic disease causing accountable losses (Nikam *et al.,* 2015). The main pathogen causing the economic losses in all regions is *Alternaria solani* (Tomescu and Negru, 2003). The yield loss of tomato fruit was 78% recorded at 72% disease intensity of *A. solani* and each 1% increase reduced tomato yield by 1.36% (Dater and Mayee, 1985). *A. solani* causes diseases on foliage (early blight), basal stems of seedlings (collar rot), stems of adult plants (stem lesions), and fruits (fruit rot) of tomato (Chaerani and Voorrips, 2006). Fruit rot of tomato caused by *A. solani* is most destructive and causes fruit rot in storage, transportation and marketing. The plants are more susceptible to infection by the disease during fruiting period. Tomato fruit loss their nutritional value and becomes unfit for consumption when infected *by A. solani*. Disease symptoms are characteristic dark brown to black lesions with concentric rings, which produce a 'target spot' effect. Symptoms are initially observed on older, senescing leaves (Waal *et al.,* 2001). The maximum incidence of fruit rot disease has been observed in ill drained and low lying fields, where water lodging was common and soil moisture to be high (Chaurasia *et al.,* 2013). Use of fungicides gives good result against *Alternaria solani* but other alternatives such as bio control and plant extracts are also found effective and eco-friendly. Furthermore, they are safe to environment, non-phytotoxic, systemic and easily biodegradable. Recently, many researchers in the world show interest in exploitation of higher plant products as novel chemo therapeutants in plant protection (Singh and Srivastava, 2013). Natural plant products are important sources of new agrochemicals for the control of plant diseases (Kagale *et al.,* 2004). Plant extracts and bio controls agents can also be used as foliar treatment and also as a part of integrated disease management. The present investigation was taken to screen local botanicals to select most effective aqueous plant extracts and bio control agents against *A. solaniin vitro* condition which can be later used in the field and for further research.

Materials and Methods

Collection of the diseased samples and isolation of causal pathogen involved

Diseased fruits sample were collected from the field around Imphal, Manipur and brought to the laboratory. The diseased portion from fruits were cut with sterilized blade into small pieces of 2-3mm size with half of diseased and half of healthy portion and were surface sterilised with 70% ethanol and 0.1% mercuric chloride. The sterilized pieces were then inoculated on Potato Dextrose Agar slants. The inoculated slants were incubated at 26 ± ^{o}C. The fungal culture was purified by hyphal tip cut method and pure culture was maintained inside the refrigerator at 4^{o}C and periodically sub cultured to fresh medium throughout the experimental period.

Pathogenicity test

Pathogenicity test of the isolated fungus was conducted. Three different types of tomato fruits viz. Green, semi ripe and ripe were collected from the field to study the development of fruit rot disease. Only healthy and uninjured fruits were selected and brought to the laboratory in polythene begs. The fruits were washed thoroughly with sterilized distilled water. Conidial suspension (10^{8} conidia/ml) in 50 ml sterile water was prepared for fungus. Sterilized pins were used to make pin pricks on the fruits and was inoculated by spore spray inoculation method. The inoculated fruits were kept in moistened chamber at 26 ± 2^{o}C for 48 hours for the development of the disease symptoms and fungus from artificially induced diseased

symptoms were reisolated to confirm the pathogenicity of the fungus.

Effect of aqueous plant extracts on the growth and sporulation of *A. solani in vitro*

Eight locally available botanicals namely Aloe vera (*Aloe barbadensis*), Chaste tree (*Vitextri folia*), Garlic (*Allium sativum*), neem (*Azadirachta indica*), Sweet flag (*Acorus calamus*), Wild sage (*Lantana camara*) and a check fungicide Mancozeb were evaluated for their efficacy on the growth and sporulation of the fungus at four levels of concentration *in vitro.*

Collected plant parts were washed in running water for about 2 min then with sterile water. These plants were air dried over a blotting paper. The air dried plants parts were crushed separately at a ratio 1:1(w/v). These extracts were filtered through 2 folds muslin cloth and filtrates were centrifuged at 1500 rpm for 15 minutes and the supernatants were collected. The extracts thus prepared were considered as 100% concentration

The required quantity of each plant extract and check fungicide mancozeb were calculated and were added to the sterilized 50 ml moltened PDA medium to give the desired concentration and shaken well to mix thoroughly. All the plant extracts were tested at four different levels of concentration by using Poisoned Food Technique.

The poisoned PDA medium was poured in Petri plates @ 20ml per plate and allowed to solidify at room temperature. The plates were then inoculated aseptically with 5mm mycelial disc of 7 day old taken from actively growing culture of *A. solani* in inverted position at the Centre of plate. Radial growth of mycelium was measured after six days after inoculation. The medium without any plant extract served as control. Each treatment was replicated thrice. Percentage inhibition on radial growth and sporulation was calculated by following method described by Vincent (1927) as given below:

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

Where,

C= Radial growth of the fungus in control
T= Radial growth of the fungus in treatment

For sporulation 1sq.cm block of the fully-grown mycelium was cut from the periphery. The mycelium was scraped off with the help of a sterilized blade and was then put into a test tube containing 5ml of distilled water and shake properly to make a homogenous spore suspension. The spore counts were done with the help of haemocytometer.

Effect of biocontrol agents on the growth and sporulation of *A. solaniin vitro*

Five biocontrol agents namely, *Trichoderma harzianum*, *Trichoderma hamatum*, *Trichoderma viride*, *Pinicillium glabrum* and *Penicillium citrinum* were taken from Department of Plant Pathology, Central Agricultural University, Imhpalto carry out the experiment under *in vitro* conditions. Dual culture plates technique described by Bell *et al.*, (1982) was followed.

Five mm mycelial disc of both *A. solani* and the antagonist from 5 day- old cultures were taken and aseptically transferred to Petri dish containing PDA by placing 3 cm apart from each other. The seeded plates were then incubated at 25 ± 1^{o}C. Each treatment was replicated three times. All the ratings were done after contact between the pathogen and the antagonist using a modified Bell's scale (Bell *et al.,* 1982) developed as:

Statistical analysis

All data obtained were subjected to analysis of variance (ANOVA) and significant differences between the means were determined using Randomized Block Design (RBD) at *P*=0.05.

Results and Discussion

Pathogenicity test of the isolated pathogen

When the isolated fungus from the diseased samples was artificially inoculated on healthy pin pricked green, semi ripe and ripe fruits of tomato, all the taken fruits of various ages were found to be susceptible for the development of fruit rot. Semiripetomato fruit was found to be most susceptible for pathogen as it could induce characteristic symptoms of the disease after 10 days. The ripe fruit has been found to be moderately susceptible as symptoms of the disease developed after 12 days. The green fruit was found to be very less susceptible with disease symptoms developing after 15 days. On reisolation from the three fruits, the fungus was found and thus proves the pathogenicity of *Alternaria solani* (Ellis and Mart) Jones and Grout.

The present findings agree with those of Shrivastava and Tondon (1966) who reported *Alternaria solani* as a pathogen for fruit rot disease of tomato in India.

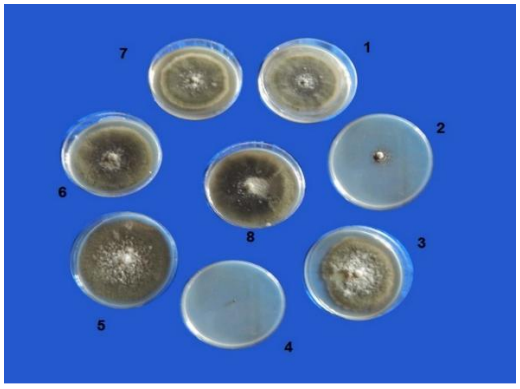

Fig.1 Effect of different aqueous plant extract on growth and sporulation of *A. Solani* at first level concentration. 1. sweet flag (3%), 2. garlic (1%), 3. aloe (3%), 4. mancozeb (0.05%), 5. neem (3%), 6. wild sage (3%), 7. chaste tree (3%), 8. control

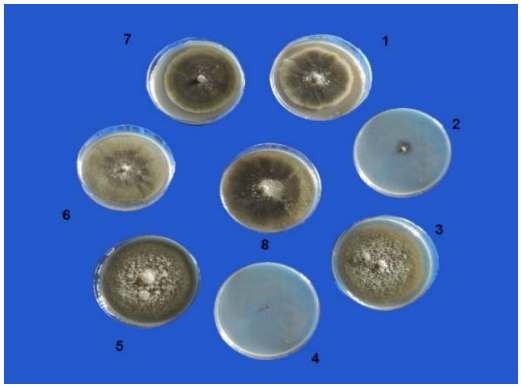

Fig.2 Effect of different aqueous plant extract on growth and sporulation of *A. solani* at second level concentration. 1. sweet flag (5%), 2. garlic (2%), 3. aloe (5%), 4. mancozeb (0.1%), 5. neem (5%), 6. wild sage (5%), 7. chaste tree (5%), 8. control

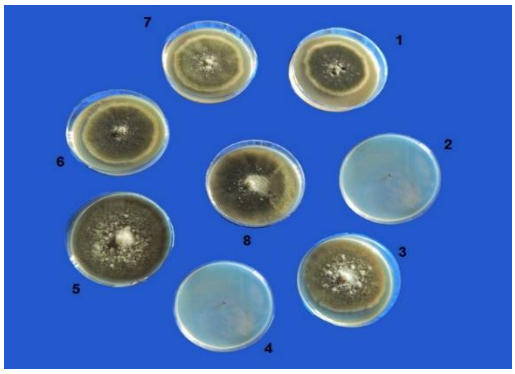

Fig.3 Effect of different aqueous plant extract on growth and sporulation of *A. solani* at third levelconcentration. 1. sweet flag (7%), 2. garlic (3%), 3. aloe (7%), 4. mancozeb (0.2%), 5. neem (7%), 6. wild sage (7%), 7. chaste tree (7%), 8. control

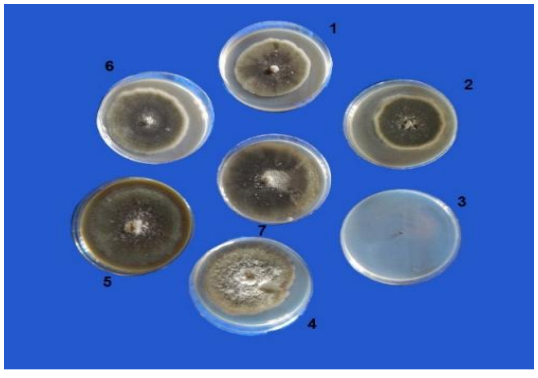

Fig.4 Effect of different aqueous plant extract on growth and sporulation of *A. solani* at fourth level concentration. 1.chaste tree (10%) 2. sweet flag (10%), 3. garlic (4%), 4. aloe (10%), 5. neem (10%), 6. wild sage (10%), 7. control

Table.1 List of plants and parts used with a check fungicide along with their concentrations

Common name	Botanical name	Plant parts used	Concentration (%)			
Aloe	*Aloe barbadensis* Mill.	leaf	3	5	7	10
Chaste tree	*Vitextri folia* L.	leaf	3	5	7	10
Garlic	*Allium sativum* L.	clove	1	2	3	4
Neem	*Azadirachta indica* L.	leaf	3	5	7	10
Sweet flag	*Acorus calamus* L.	rhizome	3	5	7	10
Wild sage	*Lantana camara* L.	leaf	3	5	7	10
Mancozeb	-	-	0.05	0.1	0.2	-
Control(PDA)	-	-	-	-	-	-

Table.2 Effect of Aqueous plant extracts and a check fungicide on growth and sporulation of *A. solani* at first level of concentrations

Treatments	Details	Concentrations (%)	Growth(cm) *	Inhibition(%) over control	Sporulation (cfu/ml)
T_1	Sweet flag	3%	6.77 (2.69)	20.35	-
T_2	Garlic	1%	1.17 (1.29)	86.23	-
T_3	Aloe	3%	7.27 (2.79)	14.47	-
T_4	Mancozeb	0.05%	0 (0.71)	100	-
T_5	Neem	3%	7.73 (2.87)	9.05	-
T_6	Wild sage	3%	7.23 (2.78)	14.94	-
T_7	Chaste tree	3%	6.82 (2.70)	19.76	-
T_8	Control	_	8.5 (3)	0	$40X10^3$
	SE (d)±		0.06		
	$CD_{(0.05)}$		0.13		

*All insertion is an average of three replications

Figures in parenthesis are ($\sqrt{x+0.5}$) transformed values.

-No sporulation

Table.3 Effect of Aqueous plant extracts and a check fungicide on growth and sporulation of *A. solani* at second level of concentrations

Treatments	Details	Concentrations (%)	Growth (cm)*	Inhibition (%) over control	Sporulation (cfu/ml)
T_1	Sweet flag	5%	6.30 (2.61)	25.80	-
T_2	Garlic	2%	0.47 (0.97)	94.40	-
T_3	Aloe	5%	7.17 (2.77)	15.64	-
T_4	Mancozeb	0.1%	0 (0.71)	100	-
T_5	Neem	5%	7.67 (2.86)	9.76	-
T_6	Wild sage	5%	6.87 (2.71)	19.17	-
T_7	Chaste tree	5%	6.47 (2.64)	23.88	-
T_8	Control	_	8.5 (3)	0	$40X10^3$
	SE (d)±		0.08		
	$CD_{(0.05)}$		0.18		

*All insertion is an average of three replications

Figures in parenthesis are ($\sqrt{x+0.5}$) transformed values.

-No sporulation

Table.4 Effect of Aqueous plant extracts and a check fungicide on growth and sporulation of the fungus at third level of concentration

Treatments	Details	Concentrations (%)	Growth (cm)*	Inhibition (%) over control	Sporulation (cfu/ml)
T_1	Sweet flag	7	6.23 (2.59)	26.70	-
T_2	Garlic	3	0 (0.71)	100	-
T_3	Aloe	7	7.13 (2.76)	16.11	-
T_4	Mancozeb	0.2	0 (0.71)	100	-
T_5	Neem	7	7.56 (2.84)	11.05	-
T_6	Wild sage	7	6.53 (2.65)	23.17	-
T_7	Chaste tree	7	6.27 (2.60)	26.23	-
T_8	Control	_	8.5 (3)	0	$40X10^3$
	SE (d)±		0.05		
	$CD_{(0.05)}$		0.11		

*All insertion is an average of three replications

Figures in parenthesis are ($\sqrt{x+0.5}$) transformed values.

-No sporulation

Table.5 Effect of Aqueous plant extracts on growth and sporulation of *A. solani* at fourth level of concentration

Treatments	Details	Concentrations (%)	Growth(cm)*	Inhibition(%) over control	Sporulation (cfu/ml)
T_1	Sweet flag	10	5.90 (2.53)	30.58	-
T_2	Garlic	4	0 (0.71)	100	-
T_3	Aloe	10	7.1 (2.76)	16.47	-
T_4	Neem	10	7.47 (2.82)	12.11	-
T_5	Wild sage	10	6.37 (2.62)	25.05	-
T_6	Chaste tree	10	6.13 (2.58)	27.88	-
T_7	Control	_	8.5 (3)	0	$40X10^3$
	SE (d)±		0.02		
	$CD_{(0.05)}$		0.06		

*All insertion is an average of three replications

Figures in parenthesis are ($\sqrt{x+0.5}$) transformed values.

-No sporulation

Table.6 Effect of biocontrols agents on the growth and sporulation of the fungus *in vitro*

Biocontrol agents	Duration of point of contact	Bells's scale	Sporulation (cfu/ml)	Per cent inhibition of spore
Trichoderma viride	2	Class - III	_	100
Trichoderma hamatum	3	Class-I	_	100
Trichoderma harzianum	3	Class-I	_	100
Penicilliumcitrinum	_	Class-VI	_	100
Penicilliumglabrum	_	Class-VI	_	100
Control	_	_	$40X10^3$	0

Effect of biocontrol agents on the growth and sporulation of *A. solaniin vitro*

Class I	The antagonist completely overgrew the pathogen (100% overgrowth),
Class II	The antagonist overgrew at least 3/2rd of the pathogen's surface (75% overgrowth),
Class III	The antagonist colonizes on half of the growth of the pathogen (50% overgrowth),
Class IV	The pathogen and the antagonist locked at the point of contact,
Class V	The pathogen overgrew the mycoparasite and
Class VI	Formation of inhibition zone between pathogen and antagonist.

Effect of plant extracts on the growth and sporulation of *A. solani in vitro*

The aqueous extracts of six plant species and a check fungicide were tested against *A. solani* to exploit their antifungal properties (Table 1). Data presented in Table 2, 3, 4 and 5 showed that there was a considerable range of efficacies of aqueous plant extracts at four different concentrations against growth and sporulation of the fungus. Garlic at 1% concentration showed 86.2% inhibition, 94.4% inhibition at 2%, and 100% inhibition at 3% and 4% concentrations. Mancozeb at 0.05, 0.1 and 0.2% concentrations showed 100% inhibition. Remaining plant extracts showed less than 35% inhibition on growth of *A. solani* on all four concentrations. Data in Table 2 showed that Sweet flag at 3% concentration showed 20.35 % inhibition followed by Chaste tree (3%) with 19.76% inhibition, Wild sage (3%) 14.94%, Aloe (3%) 14.47 % and least inhibition by neem(3%) with 9.05% (Figure 1). It was further observed from Table 3 that Sweet flag (5%) could inhibit 25.8% followed 23.8% by chaste tree (5%). However, Wild sage, Aloe and Neem each at 5% concentration showed 19.17%, 15.64% and 9.76% inhibition over control (Table 3, Figure 2).

Higher fungistatic effect was shown by plant extracts at higher concentrations. Sweet flag at 7% could inhibit 26.70% followed by Chaste tree (26.23%), wild sage (23.17%), Aloe (16.11%) and Neem (11.05%) each at 7% concentration (Table 4, Figure 3). At 10% concentration, Sweet flag could inhibit 30.58% followed Chaste tree (27.88%), wild sage (25.05%), Aloe (16.47%) and each at 10% concentration. Neem showed least fungistatic effect (12.11%) at 10% concentration. (Table 5, Figure 4). All the plant extracts evaluated could inhibit sporulation of fungus completely (100%) in all the four concentrations. The result were in accordance with the findings of Nguyen *et al.*,(2013) who reported that among the plant extract tested *in vitro*, garlic bulb extract each at 5,10,15% were best in inhibiting the mycelial growth of *A. solani.* Singh *et al.*, (2010) also reported that *Acorus calamus* (Sweet flag) crude extract at 5000 µg/ml is found to be highly effective against *Alternaria solani*. Some of the aqueous plant extracts under *in vitro* revealed that higher doses were relatively more efficient than the lower doses. This statement is in agreement with the findings of Shivpuri *et al.*, (1997) also observed that effectiveness of plant extracts at higher doses than lower dose of the 10 plant species evaluated (*Allium cepa, Allium sativum, Azhardirachta indica, Calotropis procera, Datura stramonium, Ocimum sanctum, Polialthia longifolia, Tagetus erecta, Vinca rosea, Withamia somnifera*) against five pathogenic fungi viz. *Alternaria brassicicola, Colletotrichum capsici, Fusarium oxysporium, Rhizoctonia solani* and *Sclerotinia sclerotiorum* under laboratory condition.

Effect of biocontrol agents on the growth and sporulation of *Alternaria solaniin vitro*

The data presented in Table 6 revealed that all the species of *Trichoderma* and *Penicillium* showed differential antagonistic potential against *A. solani* associated with fruit rot of tomato. It was observed that *T. viride* could come in contact with the fungus after 2 days of incubation while *T. hamatum, T. harzianum* could come in contact with *A. solani* after 3 days of incubation. The growth of *A. solani* was completely overgrown (100%, class I) by the antagonists *T. harzianum* and *T. hamatum* while *T. viride* colonized half of the growth of the fungus (50% overgrowth, Class III). Antagonists *P. citrinum* and *P. glabrum* could not overgrow *A. solani* but formed 0.5 cm and 0.4 cm inhibition zones (Class VI). All the antagonists could inhibit the sporulation of the fungus. Sobia *et al.*, (2015) reported that

Trichoderma harzianum could inhibit 67.78% on the growth of *A. solani* whereas *Trichoderma viridae* showed less inhibition (59.63%). Chethana *et al.*, (2012) also reported that maximum inhibition of *Alternaria porri* (Ellis) Cif. was recorded in *T. harzianum* with 79.5% inhibition. The antagonistic effects of species of *Trichoderma* against pathogen might be due to (a) production of non-volatile toxic substances diffuse in the substrate and (b) parasitism by the antagonists. The formation of inhibition zone indicates the production of antibiotics by the antagonists which diffused in the medium.

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