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IDENTIFICATION OF PATHOGEN ASSOCIATED WITH LEAF SPOT DISEASE AND ITS MANAGEMENT *IN VITRO*

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ABSTRACT

Aloe barbadensis (L.) is a perennial, drought-resisting, xerophytic plant belonging to the family Liliaceae. The leaves are 40-60 cm long, erect, broad, thick and fleshy succulent, green in colour, narrow lanceolate in shape with long acuminate tip with small thorns on both edges. The central bulk of the leaf contains colourless mucilaginous pulp, made up of large, thin walled mesophyll cells. The leaf spot illness caused by *Alternaria alternata* is one of the fore most serious plant diseases touching business cultivation of aloe. This illness is marked by dark brown, death spots, circular to oval with grey canters developing on each surface of leaves. The experiment carried out at ANDUAT, Kumarganj Ayodhya and plant pathology laboratory in 2020-2021. The Aloe vera leaves showing symptoms of disease were collected aseptically from Medicinal & Aromatic Plant (MAP) farm, ANDUA&T, Kumarganj, Ayodhya and brought to the laboratory for isolation and identification of fungi. Pathogen was isolated from infected leaves and identified as *Alternaria alternata* on the basis of cultural and morphological character. The percent growth inhibition was observed in Tebuconazole @ 0.02 (100.00%) at 48 and 72 hrs followed by Bordeaux mixture (81.20%, 75.30%), *Trichoderma harzianum* + *Pseudomonas florescence* (81.09%, 73.96%), Mancozeb (79.46, 74.83%) and Nimbicidine (73.13%, 68.42%) in comparison to control, Minimum growth inhibition was recorded in Tulsi leaf extract (38.52%, 31.71%) followed by garlic bulb extract (46.13, 32.29%) and *Pseudomonas florescence* (68.33, 64.78%) at 48 and 72 hours. Among the systemic fungicides Tebuconazole was found best in an inhibition of spore germination and mycelial growth. It is concluded that Chemical fungicide to be more effective than botanicals and bioagents in controlling *A. alternate.*

Keywords : Aloe vera, Leaf spot disease, *Alternaria alternate,* chemical fungicide, bioagents, plant extract.

Introduction

Aloe plants are used to flavour a number of medicines and drinks that help people stay healthy (Davis and Moro, 1989). Aloe gel is said to be extremely useful for treating sores, wounds, cancer, skin problems, colds and coughs, constipation, piles, and mycosis (Daodu, 2000; Djeraba and Quere, 2000; Olusegun, 2000). Rumoured succulent plants will be used for treatment of respiratory illness, ulceration and polygenic disorder. Aloe is well-known for assisting digestion, blood circulation, and urinary organ, liver, and gall bladder function with at least three medicinal fatty acids, which aids in the smooth functioning of the belly, small intestines, and colonies. The aloe juice concentrates are abundant in vital enzymes that drive digestion and liver activities. It's a natural property to basify biological process liquids, which prevents over-acidity. Saponins, a rare natural substance created naturally to cleanse and drain waste and toxins from the body, are also found in aloe supplements (Kumar *et al*., 2010). In the cosmetics business, succulent is used to produce bath soap, shampoo, hair wash, tooth paste, and body lotions (Daodu, 2000).Aloe is susceptible to fungi-caused foliar diseases such as leaf spot, tip rot, base rot, and leaf rot. Leaf spot is the most serious disease in all of them, despite the fact that various fungi have been linked to it. Leaf spot is caused by *Alternaria alternata* is one of the most common disease found in the field that not only affects the leaf texture but also reduces the quality and quantity of gummy gel used for health and business purposes. Fungicide treatment is the most importantmethod of preventing plant pathogen. But fungicides are harmful and have negative effects on a variety of creatures that dwell in the environment. Some synthetic fungicides are non-biodegradable, they will accumulate in the soil, plants, and water, and so have an organic effect on human health. Natural products appear to be a viable answer to the environmental issues caused by artificial pesticides, and many researchers are working to find an effective natural product to replace artificial pesticides (Kim *et al.,* 2005). Identification of pathogen associated with leaf spot disease and its management is critical. This investigation has been under taken to characterization of pathogen and *In vitro* evaluation of bioagents and oragnics products for management of leaf spot disease of aloe vera.

Materials and Methods

Collection of diseased sample

The *Aloe vera* leaves showing symptoms of disease were collected aseptically from Main Horticulture Experiment Station, ANDUA&T, Kumarganj Ayodhya, Uttar Pradesh, India. The samples were kept in dry paper envelopes especially meant for the purpose and marked clearly the details of location, variety, crop stage, reaction type and date of collection etc. and brought to the laboratory for isolation of pathogen. The samples were dried for 24 hrs in shade to remove excess surface moisture and repacked in fresh dry paper envelopes and maintained at 6 to 8^0C for further study.

Cleaning and sterilization of glass wares

The standard procedures were adopted for all laboratory studies. The glassware were boiled for half an hour and then washed with vim powder followed by cleaning in tap water. Whenever required, Glassware such as, Petri dishes, culture tubes, funnels, glass rods, beakers and flasks etc., were kept in the cleaning solution containing 60g Potassium Dichromate ($K_2Cr_2O_7$) and 60 ml of concentrated Sulphuric Acid (H_2SO_4) in one litre of water for a day followed by washing in running water. Dry glass wares were sterilized at 160^0C for 2 hrs in hot air oven before use. Metallic objects like tips of inoculation needle, forceps and cork borer were sterilized by dipping in the spirit and heating on blue flame to red hot before inoculation. Laminar flow was sterilized with ultra violet lamp before use. Sprit was used as general disinfectant for hand and surface of laminar flow. All cultural studies were conducted in aseptic condition under laminar flow

Preparation of culture media

Potato Dextrose Agar (PDA) medium was used for isolation and purification of fungal culture and rest of *in vitro* experiments. PDA medium was prepared and sterilized using method described by Johnson and Booth (1983)

Isolation of the pathogen

The diseased leaf samples, showing distinct symptoms were selected for isolation of the pathogen. The selected leaves were washed with fresh sterilized water in order to remove the dust particles and surface contaminants. The washed diseased leaves were cut into small bits having some healthy portions with the help of sterilized scalpel and forceps. The cut leaf pieces were surface sterilized with 0.1 per cent Mercuric Chloride solution under aseptic conditions inside a Laminar flow and washed thoroughly 3 to 4 times with sterilized water to remove the traces of Mercuric Chloride. Excess moisture was removed by placing these in the blotting papers and using sterilized inoculation needle, the pieces were transferred in Petri dishes poured with 20 per cent Potato Dextrose Agar medium. Three to four pieces of diseased leaves were placed per Petri dish at equal distance from each other. Petri dishes were properly marked with glass marking pencil indicating date of isolation and isolate number etc. The Petri dishes were incubated at a temperature of 25± 1ºC and observed for fungal growth.

Purification of the pathogen

The purification of isolated fungi was done by single spore isolation technique. A dilute spore suspension was poured on plain agar in Petridishes and the spores were allowed to settle down on the agar surface. Single spore was selected under the microscope and encircled. They were transferred to Petri plats containing sterilized potato dextrose agar medium after the proper growth of the fungus. The pure culture was again transferred in to potato dextrose agar slants and regularly maintained for further studies by sub culturing at monthly intervals.

Cultural and morphological Studies

Cultural and morphological studies were carried out on the potato dextrose agar medium. Isolated fungi were grown on PDA to study their cultural characteristics like colonial morphology, colony colour, mycelial growth, and morphological characteristics like size shape and colour of conidia and conidiophores.

Micrometry measurement of fungal structures

For calibration of ocular micrometer, a bright-field microscope, an ocular micrometer and a stage micrometer was used. Eye piece of microscope was removed and ocular micrometer glass disc was inserted on the metal diaphragm and eye piece was inserted in the microscope. The stage micrometer glass slide was placed on the microscope stage. Stage micrometer is a special glass slide having one division equals to 10 um (0.01 mm). Fine graduations of stage micrometer were observed under a sharp focus under low-power and high-power objectives. During observations, the ocular micrometer lines were on the top and the stage micrometer image was on the bottom. The lines of the ocular micrometer parallel with those of the stage micrometer were aligned by fine rotation, the lines coincide with stage micrometer at the left end and another line coincides in right (completely parallel at both ends) was observed. Number of divisions of ocular and stage micrometers between the two coinciding lines was counted. 5 readings were taken with the low power (10x) and high-power (40x) objective. These observations are then used to calculate the calibration factor for the objective lens in use.

$$\text{One ocular Division } (\mu m) = \frac{\text{No. of division on stage micrometer}}{\text{No. of division on ocular micrometer}} \times 10$$

After calibration, the ocular micrometer was used to measure the size of conidia and conidiophores, in terms of length and breadth by following formula.

$$\text{Size } (\mu m) = \frac{\text{No. of ocular division occupied by conidia}}{\text{Conidiophores x calibration factor of objective}}$$

Identification of the pathogen

On the basis of morphological and cultural characters, the fungal species were identified by using the standard techniques as mentioned in various manuals and books (Burnett and Hunter, 1972; Ellis, 1971 and Gilman, 1967).

In vitro pathogenicity test of isolated fungi

Based on Koch postulate pathogenicity test was carried out on similar size healthy leaves of *Aloe vera* plant. The leaves were cleaned and surface sterilized with 0.1% mercuric chloride for one minute and rinsed three times with distilled water. Then leaves were placed on the surface of wet-cotton in petri dishes. 2 mm mycelial disc of isolated fungi were placed on the leaves. Disc of PDA only were placed on another leaves for control. Three replications of each treatment were used. The plants were maintained at

room temperature until the development of symptoms. When the symptoms appeared after inoculation, the test pathogens were re-isolated from the infected leaves.

***In vitro* efficacy of fungicides, bioagents and plant extracts**

Food poison technique was followed to test the efficacy of four fungicides namely Tebuconazole (0.02%), Bordeaux mixture (5000 ppm), Mancozeb (0.3%), Nimbicidine (2.0%), bioagents namely *Trichoderma, Pseudomonas*, and *Trichoderma*+ *Pseudomonas* at 2.0% concentrations on mycelial growthof *Alternaria alternate* isolate collected from Kumarganj, Ayodhya. Petri dishes having PDA with no fungicide were served as control. After solidification, 5 mm mycelia disc of *Alternaria alternata* from 10 days old culture was taken and placed at the center of petriplate containing poisoned PDA medium and incubated at 25±1ºC. Three replications were maintained for each treatment. After 48 and 72 hrs inoculation, radial growth of test fungus in the entire poisoned medium was observed. The colony diameter of the pathogen in control plates was also recorded and per cent inhibition over control was worked out according to the equation given by Vincent (1947).

***In vitro* evaluation of plant extracts**

Fresh parts of the test plants (*Allium sativum* and *Ocimum sanctum*) were collected and washed thoroughly in clean water. Hundred grams of each washed samples were grinded in mortar and pestle by adding equal amount (100 ml) of sterilized distilled water (1:1 W/V) and boiled at 80^0C for 10 minutes in a hot water bath. The grinded material was filtered through muslin cloth followed by filtering through sterilized Whatman No.1 filter paper and treated as standard 100 per cent plant extract To study the antifungal mechanism of plant extracts, the poisoned food technique was used (Nene and Thapliyal, 1982). For preparing required concentration, 5, 10, 15 ml of plant extract were mixed with 95, 90 and 85 ml of sterilized molten PDA media, respectively so as to get 5, 10 and 15 percent concentration. The mediumwas thoroughly shaken for uniform mixing of extract. Petri dishes having PDA with no Plant extract were served as control. After solidification, 5mm mycelia disc of *Alternaria alternata* from 10 days old culture was taken and placed at the center of petriplate containing poisoned PDA medium and incubated at 25±1ºC. Three replications were maintained for each treatment. The radial growth of mycelium was recorded after 48 and 72 hrs of inoculation. The percent growth inhibition was calculated over the control with the formula described by Vincent (1947).

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

Where,

I = Per cent inhibition of mycelium

C = Colony diameter (mm) in control

T = Colony diameter (mm) in treatment

Result and Discussion

The results obtained during the present investigation are described under following heads-

Symptoms of Alternaria leaf spot of Aloe vera:

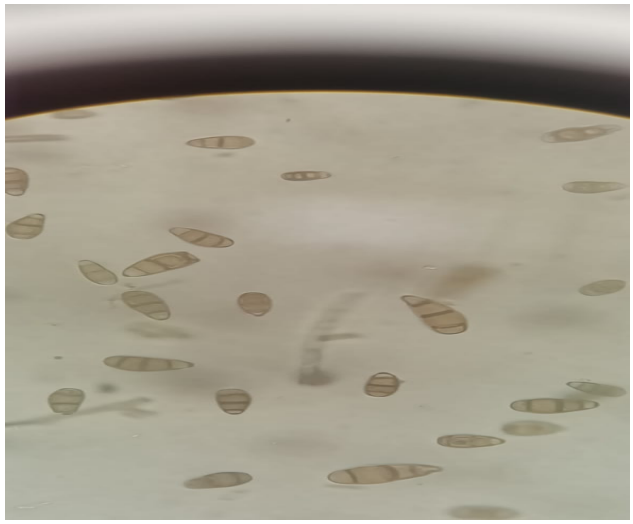
Symptoms are generally circular to oval dark brown necrotic sunken spots appeared on the both side of leaves. These spots were located commonly on the leaf tip. The spots were initially smaller in size about 1-2 mm later enlarged reached up to 4-5mm. As infection increases the affected leaves dried from the tip downwards and mucilaginous jel.

Plate: Symptoms of Alternaria leaf spot caused by *Alternaria alternata*on Aloevera leaf

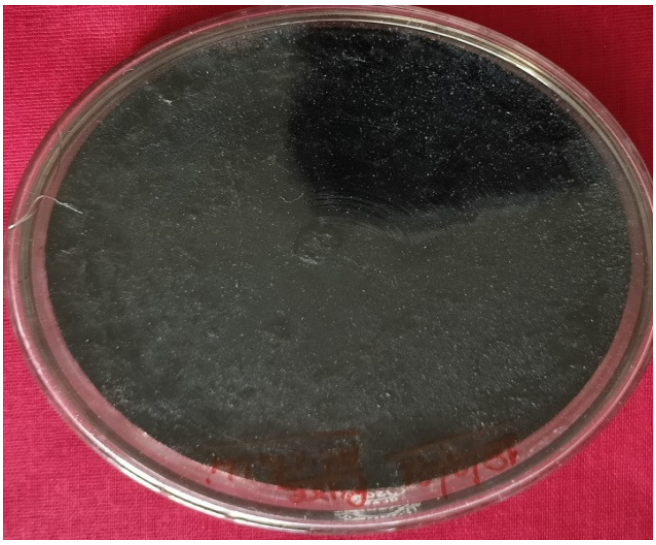
The small dark brown to black spots on the both sides of leaves was also reported by Ghosh and Banerjee (2014). The symptomatology of disease studies on artificially inoculated plants as well as a natural plantation at several locations in Rajasthan revealed that leaf spot is damaging more foliage.

Characterization of pathogen

Colonies of *Alternaria alternate* was found black to grey with branched in small group. Growth of mycelium was slightly raised with fluffy colour, some time it was flat and velvety. Conidiophore pale to mid olive or golden brown up to 28.43-64.04×2.61-3.3µm in size. Conidia of *Alternaria alternata* were pale smooth found light brown to dark gray, circular to sub circular thick 1-3 transverse septa and 2-7 longitudinal septa with 27.55-37.98 x 17.66-22.30 µm in diameter. The measurements fall within the range as measurements described by Maiti *et al.* (2006).



Conidia of *Alternaria alternate*



Pure culture of *Alternaria alternata*

Characterization of *Alternaria alternata* in the treatments

Perusal of the table-1revealed that the mycelium growths were slightly raised and fluffy in Tebuconazole, raised and fluffy in Bordeaux mixture, Nimbicidine and Garlic bulb extract. Medium fluffy to fluffy was in Tulsi leaf extract, *Trichoderma* and *Pseudomonas*. Flat and velvety was in Mancozeb. Sporulation was found less in Bordeaux mixture, garlic bulb extract and *Trichoderma*. Shape of conidia was circular to sub circular in all the treatments with variable transverse and longitudinal septa. Colour of conidia were light brown to dark brown in Tebuconazole and Bordeaux mixture, light black to black in mancozeb and Tulsi leaf extract, whitish gray to whitish black in Nimbicidine, garlic bulb extract, *Trichoderma harzianum Pseudomonas and Trichoderma + Pseudomonas florescence's. Trichoderma harzianum* and *Pseudomonas florescence* was found effective for inhibition of mycelia growth due to volatile and non-volatile substances or cell wall degradation enzymes like glucanases and β-1-3 glucanases produced by the antagonics (Baby *et al.,* 2000; Ghush *et al.*, 2018).

Efficacy of bioagents, Plant extract and fungicides

From the results of table-2 showed that all the treatments were found effective for inhibition of mycelium growth. The percent growth inhibition was observed cent per cent in Tebuconazole at 48 and 72hrs followed by Bordeaux mixture (81.20%, 75.30%), *Trichoderma harzianum + Pseudomonas florescence* (81.09%, 73.96%), Mancozeb (79.46, 74.83%) and Nimbicidine (73.13%, 68.42%) in comparison to control. Whereas minimum growth inhibition was recorded in Tulsi leaf extract (38.52%, 31.71%) followed by garlic bulb extract (46.13, 32.29%) and *Pseudomonas florescence* (68.33, 64.78%) at 48 and 72hours. Among the systemic fungicides Tebuconazole was found best in an inhibition of spore germination and mycelia growth (Rajeswar Rao, 2010 and Thejakumar and Devappa, 2016). Other fungicides Mancozeb exhibited good reduction of mycelial growth than Bordeaux mixture. Adodo (2009) observed that neem oil reduced the incidence of leaf spot of groundnut in both lab and field condition. Several workers reported the inhibitory effect of garlic bulb extract on the mycelial growth of *Alternaria tenuis, Alternaria solani* (Babu *et al.,* 2007) and *Alternaria brassicae* (Shivpuri, 1997). The inhibitory effect of the garlic bulb on the mycelial growth and conidial germination of *Alternaria alternate in vitro* might be due to presence of some antifungal compounds viz., diallyl-di-sulphide and allicin containing sulphur has strong toxic properties against various fungi. Neem oil has been found effective for inhibiting the mycelial growth. The similar results also reported by Babu *et al.,* (2007), It is due to presence of antifungal compound like monoterpenes and Azadirachtin.

Table 1 : Effect of bioagents, Plant extract and fungicides on the cultural characteristic of *Alternaria alternata-*

Treatment	Mycelial Growth	Sporulation	Shape of Conidia	Colour of conidia	Transverse septa	Longitudinal Septa
Tebuconazole 250 EC @ 0.02%	Slightly raised & media Fluffy	**++++**	Circular	Dark brown	**6-9**	**2-3**
Bordeaux mixture @5000 ppm	Raised & Fluffy	**+++**	Circular	Light brown	3-5	2-3
Mancozeb 75 WP @ 0.3%	Flat & velvety	**++++**	Circular	Light black with whitish	1-4	0-3
Nimbicidine @ 2.0%	Raised & fluffy	++++	Circular	Dark grey to black	1-5	0-2
Garlic bulb extract @ 10%	Raised & fluffy	+++	Sub circular	Whitish grey	1-6	0-3
Tulsi leaf extract @ 10%	Medium fluffy	++++	Circular	Black	1-5	0-2
Trichoderma @ 2.0%	Medium fluffy	++++	Circular	Whitish black	2-4	0-2
Pseudomonas @ 2.0%	Fluffy cottony	+++	Circular	Whitish black	1-5	0-2
Trichoderma + *Pseudomonas* @ 2.0%	Fluffy Medium	++++	Circular	Whitish grey	1-6	0-3
Control	Medium Fluffy	+++	Circular	Whitish black	1-5	0-2

Table 2 : Efficacy of bioagents, Plant extract and fungicides on the mycelial growth inhibition of *Alternaria alternata-*

Treatments		Dose (%)	Radial Growth (mm)		% Inhibition	
			PDA		48 hrs	72 hrs
			48 hrs	72 hrs		
T1	Tebuconazole 250EC	0.02%	0.00	0.00	100.00	100.00
T2	Bordeaux mixture	5000 ppm	5.00	6.00	81.20	93.3
T3	Mancozeb	0.3%	22.34	22.94	79.46	74.51
T4	Nimbicidine	2.0%	30.33	30.96	73.13	65.6
T5	Garlic bulb extract	10%	55.33	55.72	46.13	38.08
T6	Tulsi leaf extract	10%	65.66	65.94	38.52	26.73
T7	*Trichodermaharzianum*	2.0%	45.65	45.96	80.09	48.93
T8	*Pseudomonasflurescens*	2.0%	50.43	51.12	68.33	43.2
T9	*Trichoderma+ Pseudomonas*	2.0%	52.22	52.84	71.88	43.51
T10	Control		90.00	90.00	00	00
	CD at 5%		0.058	0.075	0.087	0.306

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