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Research Article

Effect of Plant Extract on Cytomorphological Alteration in *Alternaria solani*

Surbhi Mehta* and Kanika Sharma

Microbial Research Laboratory, Department of Botany, Mohanlal Sukhadia University, Udaipur (Rajasthan), India

***Corresponding Author:** Surbhi Mehta, Microbial Research Laboratory, Department of Botany, Mohanlal Sukhadia University, Udaipur (Rajasthan), India.

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Abstract

Effect of Plant Extract on Cytomorphological Alteration in *Alternaria solani*

The present study is based on the evaluation of antifungal potential of plant extract against plant pathogenic fungi. *Eucalyptus globulous* Labill. Leaf extract was prepared by reflux method. Petroleum ether extract was assayed for estimation of Minimum Inhibitory Concentration and Minimum Fungicidal Concentration.MIC and MFC was done by two fold serial dilution method against *Alternaria solani*. Various cytomorphological changes like mycelia width, conidial size, condiophore structure etc. after the treatment with the extract concentrations. 0.019mg/ml to 10mg/ml of Petroleum ether extract was assayed and MIC was found to be 2.5 mg/ml for *Alternaria solani* and MFC for this fungus was observed at 5 mg/ml. Mycelium width of *Alternaria solani* increased up to 69.52% at 1.25 mg/ml concentration of the extract and conidia size of the *Alternaria solani* was reduced up to 72.17% at 1.25mg/ml (sub MIC) concentration of the petroleum ether extract. The inhibition of conidia and mycelia formation was observed at MIC of the extract *i.e.* 2.5mg/ml. Scanning electron microscopic study was also showed the changes in *Alternaria solani* structure so it can be concluded that Plant extracts naturally and significantly inhibit the conidial size and mycelia growth hence could be a safer alternative to chemical fungicides to control plant diseases.

Keywords: Reflux Method; MIC; MFC; Cytomorphological Changes; Chemical Fungicide

Introduction

Growth and reproduction of microbes is usually denoted by change in total population rather than an increase in size or mass of an individual organism. In the sustainable control of plant diseases there is a strong need to control the growth of microorganisms or their reproductive structures.

Alternaria solani causes blight disease which belongs to the sub-division Deuteromycotina, class Hyphomycetes, family Dematiaceae. *A. solani* can be survived as saprophytes as well as weak parasites. The mycelium is made up of septate, branched, light brown hyphae which turn darker with age [1]. The reason behind hyphal growth is the continued and coordinated expansion of a series of fungal cell tips into a linear or complex structure. The genus is listed in filamentous fungi which have obclavate or beaked, pigmented conidia with transverse and longitudinal septa (Muriform). The spores of *A. solani* occur commonly in the air and also in soil. Perfect stage of *Alternaria* species belongs to the *Pleospora* of loculoascomycetes [2].

The characteristic feature of hyphal growth is sustained polarized growth. Sporulation is the characteristics features of fungi. In the spore formation procedure there large numbers of mitotically derived spores or conidia are produced [3-5]. *Alternaria solani* reproduce asexually by means of conidia and these conidia or spores are the primary agent for infecting host plant. In the petri plates

visible inhibition of fungal hyphae as well as affected reproductive structure is found and can be measured and calculated as % mycelial growth inhibition [6].

It has been reported that under favourable condition there is increase in conidial production of *Alternaria* species [7-11]. *In vitro* sporulation is affected by nutrition, light spectrum, and temperature. For instance production of sporulation of *Alternaria spp*., the V8 juice medium, PDA (potato-dextrose-agar) and media with parts or extracts of plants are used in protocols to maintain viability of the colony after subcultures [12,13].

Secondary metabolites are the powerful agents present in the plants which are helpful in defence mechanism against plant pathogens [14]. The antimicrobial nature of plant extracts is because of these secondary metabolites and various workers have reported that nature of plant extract in their study [15-19]. The secondary metabolites induces various morphological and cytological changes in the microorganisms including fungi and bacteria by molecular action [20]. These molecules of secondary metabolites are target specific in nature and their biochemical and molecular targets are mainly proteins such as receptors and enzymes. Hence, all morphological/cytomorphological alterations may be related to the effect of secondary metabolites on cell wall synthesis, changing in membrane permeability, thickening of cell wall by binding to the receptors as well as disruption of cell membrane [21].

In the present study MIC and MFC of petroleum ether extract of *Eucalyptus globulus* leaf was determined and the effect of different concentrations ranging up to MIC on cytomorphological parameters like mycelium width and conidial size of test fungus has been studied.

Materials and Methods

Extract preparation

Eucalyptus globulus Labill. leaves were shade dried at room temperature and finely ground in an electrical grinder. The ground material was passed through sieve No. 240 so as to obtain powder of mesh size 60, which was used to prepare extract. Reflux method of solvent extraction was used for separation of different organic constituents of dried and powdered leaf. This method involves continuous extraction of powdered dried plant material in soxhlet apparatus with petroleum ether extract of leaves. 40 gm dry leaf powder was kept in soxhlet extraction unit and extracted with 280 ml petroleum ether till soluble fractions were extracted [22,23].

Estimation of minimum inhibitory concentration (MIC) of petroleum ether extract of *E.globulus* leaves

Minimum inhibitory concentration (MIC) was determined by broth dilution method [24,25]. Potato dextrose broth (PDB) was used for determining inhibitory activity. 200 mg of the extract was dissolved in 10 ml of acetone to prepare stock solution of 20mg/ml. Two fold serial dilution method was used for the preparation of 10 mg/ml, 5 mg/ml, 2.5 mg/ml, 1.25 mg/ml, 0.625 mg/ml, 0.312 mg/ml, 0.156 mg/ml, 0.078 mg/ml, 0.039 mg/ml, 0.019 mg/ml. concentration from the stock solution and subsequently autoclaved. The final concentration was serially diluted with sterile potato dextrose broth medium to attain final concentration 1000 µg/ml, 500µg/ml, 250µg/ml, 125µg/ml, 62.5µg/ml, 31.2µg/ml, 15.6µg/ml, 7.8µg/ml, 3.9µg/ml, 1.9µg/ml, 0.9µg/ml and 0.04µg/ml. All these tubes were than respectively inoculated with 10 µl of spore suspension (1×10^6 spores/ml) of *Alternaria solani* and incubated at 27 ± 2°C for 72 h. One tube containing extract free autoclaved medium was used as control. Three replicates of each concentration were maintained and experiment was repeated thrice.

Estimation of MFC of petroleum ether extract of *E. globulus* leaves

A loopful of fungal biomass from each tube containing 9ml broth medium and MIC as well as all concentrations were streaked onto the extract free medium PDA slants and incubated at 27 ± 2°C for 72 h. Presence or absence of growth was observed after respective incubation time. Appearance of growth indicates that the extract concentration is just fungistatic and absence of growth indicates that extract concentration is fungicidal.

Effect of extract on morphology of test fungi

Effect of petroleum ether extract on various morphological and cytological parameters of *Alternaria solani* was studied. Test fungus was treated with increasing concentrations of the extract till MIC. A small fungal biomass consisting of mycelium and spores were removed from each tube and microscopic examination was done after staining with cotton blue and mounting in lactophenol. Changes in mycelium width, conidia size and number of conidia were also observed with the help of Olympus trinocular research microscope BX-51 and analysed by ocular micrometer using microscope. Conidia/spore counting was done by haemocytometer.

Scanning electron microscopic study

Fungal colonies of those treated with petroleum ether extract, were prepared for scanning electron microscopy. In SEM preparation, the samples were fixed 1 cm sample holder coated with carbon supported film and put the sample in vacuum chamber for SEM analysis (SICART Anand, Gujrat).

Results and Discussion

MIC and MFC are presented in table 1 (Figure 1) 0.019mg/ml to 10mg/ml of PE extract was assayed and MIC was found to be 2.5 mg/ml for *Alternaria solani* and MFC for this fungus was observed at 5 mg/ml.

S. No.	Test pathogen	MIC (mg/ml)	MFC (mg/ml)
1.	*Alternaria solani*	2.5	5

Table 1: MIC and MFC of Petroleum Ether Fraction of *Eucalyptus globulus* Leaf Extract.

Result of study of effect of petroleum ether extract of *E. globulus* leaf on morphology and reproduction of *A. solani* are presented in table 2 and 3 and figure 2 and 3. Luxuriant growth of test fungus was observed in control. Thick mycelial mat, circular cottony dark black brown growth in control showed presence of dark, unbranched conidiophores broader than vegetative hyphae, bearing chains of conidia which are dark brown, transversely and longitudinal septa having distinct beak (Figure 2A). Sequential decrease in conidial size and increase in mycelia width was observed with increasing concentration of the extract till MIC (Figure 2 and 3) At MIC complete inhibition of growth was observed. Damaged conidia with reduced size and number were observed in treated culture (Figure 2A-C).

Figure 1: Morphological Alterations in *Alternaria solani* Due to Treatment with Petroleum ether Extract on Different Concentrations.

A: Mycelium, Conidia and Conidiophores of *Alternaria solani* (Control at 400 x)

B: 1 Normal Mycelium (at 400 x)

2 Mycelium showing increased width (at 400 x)

C: 1 Normal Conidia (at 400 x)

2 Conidia showing decreased size (at 400 x)

Figure 2: Scanning Electron Microscopic Study showing morphological alterations in *Alternaria solani* Due to treatment with Petroleum ether extract.

A: Normal Mycelium

B: Mycelium showing increased width

C: Normal Conidia

D: Conidia showing decreased size.

S. No.	Extract Concentration (mg/ml)	Mycelium width (µm) ± SD	% Increase in Mycelium width
1.	Control	1.78 ± 0.04	-
2.	2.5	NF	-
3.	1.25	5.94 ± 0.05	69.52
4.	0.625	5.15 ± 0.03	65.36
5.	0.312	4.55 ± 0.02	60.32
6.	0.156	3.67 ± 0.03	51.49
7.	0.078	3.53 ± 0.04	49.82
8.	0.039	2.54 ± 0.05	29.92
9.	0.019	2.26 ± 0.05	21.23

Table 2: Effect of Different Concentrations of Petroleum Ether Extract of *E. globulus* Leaf on Mycelium Width of *Alternaria solani.*

S. No.	Extract concentration (mg/ml)	Conidia size (µm) (L×W)	Conidia Size (µm) ± SD (Area)	%Reduction in Conidia Size
1.	Control	56.66×39.62	2244.86 ± 0.18	-
2.	2.5	NF	NF	-
3.	1.25	36.79×16.98	624.69 ± 0.17	72.17
4.	0.625	39.6×19.81	784.47 ± 0.12	65.05
5.	0.312	42.45×.22.64	961.06 ± 0.18	57.18
6.	0.156	45.28×28.33	1282.78 ± 0.09	42.85
7.	0.078	48.11×31.13	1497.66 ± 0.15	33.28
8.	0.039	50.94×33.96	1729.92 ± 0.14	22.93
9.	0.019	53.77×36.62	1969.05 ± 0.12	12.28

Table 3: Effect of Different Concentrations of Petroleum Ether Extract of *E. globulus* Leaf on Conidia size of *Alternaria solani*.

Effect of petroleum ether extract of *E. globulus* leaf on growth and reproduction of test fungus

Effect of petroleum ether extract of *E. globulus* leaf on mycelial width and conidial size of *Alternaria solani* is presented in table 2 and 3 and Figure 1(B and C). Mycelium width of *Alternaria solani* increased up to 69.52% at 1.25 mg/ml concentration of the extract. Conidia size of the *Alternaria solani* was reduced up to 72.17% at 1.25mg/ml (sub MIC) concentration of the petroleum ether extract. The inhibition of conidia and mycelia formation was observed at MIC of the extract *i.e.* 2.5mg/ml (Figure 3 and 4).

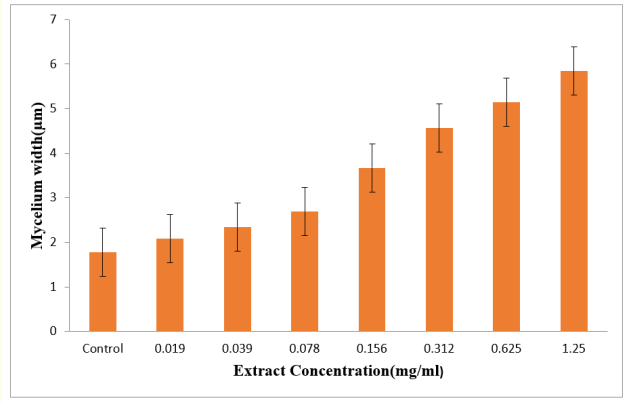

Figure 3: Effect of Different Concentrations of Petroleum ether Extract of Eucalyptus globulus Leaf Extract on Mycelium Width of *Alternaria solani*.

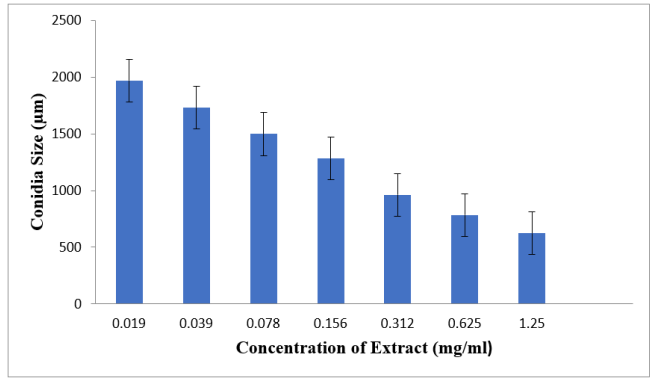

Figure 4: Effect of Different Concentrations of Petroleum ether Extract of Eucalyptus globulus Leaf Extract on Conidia Size of *Alternaria solani.*

Scanning electron microscopic study showed some abnormalities in reproductive structure of *Alternaria solani* after treatment

with extracts. A gradual decrease in conidia size while swelling of hypha was observed due to treatment with extract At 1.25mg/ml concentration of petroleum ether extract, dichotomous branching in the conidiophores was observed and conidia were found directly attached to the mycelium (Figure 2). It is estimated that among the pathogens Fungi is a group of microorganisms which is responsible for damaging plants and hindering their growth [26]. Now days, chemical fungicides are being used to inhibit the growth of fungus, but these may develop resistance in fungal species exposed to them and also increase the risk of environmental pollution [27,28]. The use of herbal biocontrol agents is therefore suggested as an alternative way of controlling plant diseases [29].

Various antimicrobial secondary metabolites are responsible for producing morphological alterations in microorganisms. cell wall, cell membrane and cytoplasm of microorganism including metabolic activity, important enzymes as well as metabolites are the major target sites which is affected by antimicrobial agents [30]. Flavanoid, proanthyocunidins, tannins, anthraquinone, saponins and alkaloids have ability to inactivate microbial adhesions, enzymes, cell envelope, transport proteins etc. [31]. Therefore change in the morphology of hyphae could be due to the loss of integrity of the cell wall. Consequently plasma membrane permeability might be affected, which could explain the changes in morphology and size of mycelia.

Results suggested that petroleum ether extract of *E. globulus* leaf extract inhibits the growth of *A. solani* and this inhibition is concentration depended. When concentration of the extract increased, size of conidia decreased, while mycelium width was increased. Some abnormal structures were also observed in conidiophore and remarkable decrease the overall growth of the test fungi. According to results obtained in this study, concentration dependent plant extract inhibition of fungal growth may be due to increase in the concentration of active components on increasing concentration. Concentration dependent growth pattern of fungi is reported by some researchers [32-34].

The major changes in mycelium width and size of conidia were observed in the presence of secondary metabolites in plant extract. These secondary metabolites in the extract may inactivate the microbial adhesions, may change the permeability of the membrane allowing water to enter in and may get accumulated in the hyphae of test fungi that results in increase in the width of mycelium. Secondary metabolites have the ability to inactivate microbial adhesions by disrupting fungal membrane, fungal enzymes or fungal cell wall and fungal transport protein hence change in the fungal cell/hyphae morphology as well as other structure [35]. Some fungal pigments called melanin is formed by oxidative polymerization of phenolic compounds and synthesized during spore formation are natural products and associated with development of reproductive structures. Similarly dark brown pigment called [36-38].

Reported the remarkable effect of acetone fraction of *L. inermis* leaves and PE fraction of *E. citriodora* leaf, their mixture and essential oils on various cyto-morphological parameters *i.e.* mycelium width, conidial size, hyphal morphology, conidiophore size etc. of test fungi *A. flavus* and *A. Parasiticus.* The effect of plant extract on growth and reproduction of *P. aphanidermatum* and *P. myriotylum* is studied by some worker [39]. Inhibition of spore germination of *Colletrotrichum capsici Eucalyptus globulus* extracts has been reported [40].

The development of abnormal structures was also observed in place of reproductive structures of *Alternaria solani*. This could be because of active compound of secondary metabolites (terpenoid) present in the leaves of *E. Globulus* [41-43]. Terpenoids one of the most widely distributed has strong antimicrobial properties and it may cause a temporary change in the normal structure of conidia. Abnormal filamentation along with change in cytomorphology in plant pathogenic fungi has also been reported after treatment with plant extract [44]. Most of the cytomorphological changes were observed at and below sub MIC level. At MIC the fungistatic effect may be due to the inhibitory action of secondary metabolites present in the leaf extract of *E. globulus*.

Conclusion

Thus it can be concluded that plant extracts naturally and significantly inhibit the conidial size and mycelia growth hence could be a safer alternative to chemical fungicides which are used against plant disease early blight of tomato caused by *Alternaria solani.*

In the present study petroleum ether extract of *Eucalyptus globulus* leaf was found to strongly inhibit the mycelia growth and sporulation. It can be concluded by the observations that treatment with petroleum ether extract of *E. globulus* leads to the inhibition

of conidation, mycelial growth and morphological alterations in condiophore. These features are very advantageous in prevention of early blight of tomato which is caused by *Alternaria solani*.

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Conflicts of Interest

There are no conflicts of interest. As this is my original research work.

Ethics Approval

Not applicable.

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