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Morpho-cultural studies and *in vitro* assessment of fungal sensitivity to different concentrations of chitosan against *Alternaria solani*

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Abstract
The studies revealed that *A. solani* were found responsible for causing early blight of tomato. The morphological characteristics of *Alternaria solani* were studied. The mycelium width about 2.97 to 4.19 µm and the length of conidia was about 150 to 300 µm and 14 to 18 µm thickness. The conidia were having long and thick beak. The research studies revealed that all the ten culture media tested, encouraged better growth of *A. solani*. The different cultural media *viz*., PDA, Oat Meal agar were most suitable media for *A. solani* and exhibited maximum radial mycelial growth i.e., 83.67 mm and 62.67 mm respectively.
In vitro assessment of chitosan against *Alternaria solani* on PDA showed most promising results. Chitosan @ 500 ppm inhibited highest mycelial growth whereas minimum mycelial growth inhibition was found at 50 ppm concentration. Increase in concentration of chitosan increases mycelial growth inhibition of test pathogen.

Keywords: Tomato, early blight, chitosan, Alternaria

Introduction
Tomato (*Lycopersicon esculentum* Mill.) is one of the world's most profitable and extensively cultivated vegetable. It is a short-lived, small, annual perennial crop belonging to the family Solanaceae. Tomato plants suffer with large number of biotic stresses including insect pests and diseases from the time of emergence to till harvest. Tomatoes suffer with various diseases cited by fungi, bacteria, viruses, nematodes *etc*. (Mark *et al*., 2006) [1]. More than 200 diseases have been reported to infect tomato in the world (Atherton and Rudich, 1986) [2]. Large number of fungal diseases such as early blight (*Alternaria solani*), Late blight (*Phytophthora infestans*), Septoria leaf blight (*Septoria lycopersici*), Powdery mildew (*Oidiopsis taurica*), Fusarium wilt (*Fusarium oxysporum f. sp. lycopersici*), Collar rot (*Sclerotium rolfsii*) and Damping off (*Pythium sp*.) are causing severe losses in tomato. Among the fungal diseases, early blight caused by *Alternaria solani* is one of the most important and frequent occurring disease of the crop nation and worldwide (Jones and Grout, 1897) [3]. The pathogen *A. solani* belongs to class Deuteromycetes, order Moniliales, family Dematiacea, genus *Alternaria* and species *solani* (Jones and Grout, 1897) [3]. Chitosan is an organic natural biopolymer modified from chitin, which is the main structural component of squid pens, cell walls of some fungi and shrimp as well as crab shells. (Suchada *et al*., 2010) [4]. Chitin is the second most abundant polymer in nature after cellulose (Cohen-Kupiec and Chet, 1998) [5]. Chitinases have been implicated in plant resistance against fungal pathogens because of their inducible nature and *in vitro* antifungal activities (Taira *et al*., 2002) [6]. It is environmentally safe and non-toxic to higher organisms (Kumar, 2000) [7]. It is natural compound hence, there is no harm to environment and due to antifungal activity, it will be helpful to manage early blight of tomato. Present investigation was carried out with the objectives *viz*., isolation, morphological and cultural characteristics, *in vitro* evaluation of chitosan against *A. solani*.

Material and Methods
Lab experiments was carried out on Morpho-cultural studies and *in vitro* evaluation of different concentrations of chitosan against *Alternaria solani* was conducted in the laboratories under department of Plant Pathology, Pune during 2020- 2021. The material used and the methods followed are described in this chapter.
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Early blight leaf samples of tomato were collected from field of College of Agriculture, Pune and the pathogen *Alternaria solani* was isolated from the naturally infected tomato plants showing typical symptoms of the disease. The infected portions of the leaves along with some healthy tissue were cut into small pieces. These pieces were surface sterilized with 0.1 per cent mercuric chloride solution for 30 seconds then washed thoroughly in sterile distilled water thrice to remove traces of mercuric chloride, if any, and then transferred aseptically to sterilized potato dextrose agar (PDA) plates. They were incubated at 27±1 °C and checked after every 24 hr for the growth of the fungus. The fungus was identified based on the morphological characteristics. Later, a bit of the fungal growth was transferred to PDA plates. The pure culture of the fungus was obtained by following hyphal tip culture under aseptic conditions.

1. Morphological characteristics of *A. solani*

Morphological characters of the pathogen, *A. solani* infecting the tomato, were studied by collection of the infected leaves showing characteristics early blight disease symptoms, gently by tapping the infected leaves in drop of distilled water by sterilized needle on clean glass slide. The morphological characters *viz*., mycelial width, size of conidia, length of beak, number of transverse and longitudinal septa were recorded. The size of conidia was measured using ocular micrometer (calibrated using stage micro meter) under the compound microscope at 100X magnification.

2. Cultural characteristics of *A. solani*

The fungal cultures were isolated and grown in different media in order to study its growth characters and ability to sporulate. The composition of the following media is mentioned in Appendices. The different media used for these studies are as listed below,

1. Sabouraud's agar
2. Czapeck's dox agar
3. Malt extract agar
4. Asthma and Hawker's Agar
5. Waksman Agar
6. Glucose Peptone Agar
7. Oat meal agar
8. Richard's agar
9. Hansen Agar
10. PDA

Standard method was used for preparation of media under laboratory conditions and sterilized plates in triplicate for each medium was poured with equal quantity (20 ml) of media. The plates were inoculated with uniform discs of mycelium of seven days old culture of *A. solani* which was removed by cork borer and were placed at the centre of plates. The plates were incubated at 28±1 ˚C temperature in an incubator and observations on mean colony diameter, colour, sporulation and growth characters were recorded for seven days after inoculation.

3. *In vitro* evaluation of chitosan against *A. solani* on PDA

In vitro assessment of fungal sensitivity to chitosan was studied by poison food technique (Nene and Thapliyal, 1984) [8]. The chitosan, commercial name "Vasant Urja" was provided by Vasantdada Sugar Institute, Pune.

The different concentrations of the chitosan were added and mixed thoroughly with Potato dextrose agar medium (PDA) in conical flasks to obtain desired concentrations. Medium without chitosan served as control. After solidifying of the medium, 5 mm diameter agar disc of test fungus was cut from 7-8 days old culture of *A. solani* by using sterilized cork borer and placed in the centre of the petri-plates and plates were incubated at 28± 2 ^{0}C in inverted position. The untreated plates were fully covered with the test fungus, the radial mycelial growth is measured in all the plates. The mycelial colony diameter was measured in both the directions and average was recorded. Per cent mycelial growth inhibition in treated plate was calculated by using the formula given by Vincent (1947) [9].

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

Where

I = Per cent inhibition of fungal growth.
C = Growth of fungus colony diameter (mm) in control.
T = Growth of fungus colony diameter (mm) in treatment.

Table 1: Treatment detail of Chitosan and its concentrations on PDA

Sr. No.	Chemical name	Trade name	Conc. Used	Manufacturer
1.	Control	-	-	-
2.	Chitosan	Vasant Urja	50 ppm, 75 ppm, 100 ppm, 125 ppm, 150 ppm, 200 ppm, 250 ppm, 500 ppm.	VSI, Pune.

Result and Discussion

1. Morphological characters of *A. solani*

The temporary slide mounts were prepared in lactophenol with spores of pathogen. Then, they were observed under high power (100X) microscope and major using ocular micrometer. The morphological characters of *A. solani* depicted below.

The colony growth showed cream to greyish black colour, with thin to dense mycelial mat having little aerial growth. The mycelium was septate and dark coloured, conidia were brown in colour with long beaked, having both transverse and longitudinal septa (Table 2).

The mycelium is septate, 2.97 - 4.19 µm in width. Conidia were solitary straight and ellipsoidal tapering to beak, 150-300 µm in length and 14-18 µm thick. Long as well asthick beak was observed. The number of transverse and longitudinal septa in the range of 3-10 and 0-2, respectively.

The description of this fungus agreed with the description given for *A. solani* by Ellis and Martin (1882) who observed that conidia were solitary straight, ellipsoidal tapering to beak, length 150-300 µm and 15-20 µm thick, with 8-10 transverse and 0-4 longitudinal septa. The studies on the morphological characters of the isolated *Alternaria* sp. showed its close identity as described by earlier workers Singh (1987) [13], Ellis and Gibson (1975) [11] and Neergard (1945) (Table 2).

Table 2: Morphological characters of *A. solani* causing early blight disease of tomato

Structure	Characters	Size
Colony	Cream to greyish black, with thin to dense mycelial mat having little aerial growth	-
Mycelium	Septate and dark coloured	Width 2.97 - 4.19 µm
Conidia	Long beaked, dark coloured, both transverse and longitudinal septa	Length 150 - 300 µm Thick 14 - 18 µm

2. Cultural studies

2.1 Growth characters on different media

Cultural characteristics *viz*., mycelial growth, colony diameter and sporulation of *A. solani* were studied *in vitro* using ten different culture media and the results obtained are presented in Table 3.

2.2 Mycelial growth

The results revealed that all the culture media encourage better growth and variable sporulation of *A. solani*. The mean colony diameter recorded with all the test media ranged from 18.67 mm (Richards agar) to 83.67 mm (PDA).

Significantly highest mean colony diameter was recorded on PDA (83.67 mm). The next best culture media was Oat meal agar where significantly highest mean colony diameter (62.67 mm) was recorded. This was followed by Hansen Agar (58.00 mm), Waksman Agar (56.33mm), Czapeck's dox agar (53.00 mm), Glucose Peptone Agar (52.67 mm), Asthma and Hawker's Agar (43.00 mm), Sabouraud's agar (28.33 mm), Malt extract agar (24.67 mm). The least mean colony diameter was recorded on Richards agar medium (18.67 mm).

2.3 Growth characteristics

All the culture media exhibited a wide range of colony colour and morphology. The colony growth produced on all the culture media was mostly circular to irregular, profused, wooly and loose cottony.

The colony colour of *A. solani* was creamish to greyish black whereas colour of colonies produced varied from yellowish, dull brown to dark greyish with white centre, greyish with outer, brown hallow, olive green with regular or irregular periphery (Table 3).

2.4 Sporulation

All the ten cultures media were tested and exhibited a wide range of sporulation of the test pathogen. Potato Dextrose Agar and Oat meal agar recorded excellent (++++) sporulation. Good (+++) sporulation was recorded on Czapek's dox agar medium. Fair (++) sporulation was recorded on Waksman Agar, Glucose Peptone Agar, Subouraud's agar, Asthma and Hawker's Agar and Hansen Agar medium where as poor (+) sporulation was recorded on Malt extract agar and Richard's agar. (Table 3)

Table 3: Effect of various culture media on growth and sporulation of *A. solani*

Tr. No.	Name of media	Average colony diameter (mm)	Colony growth	Margin of colony	Sporulation
T_1	Sabouraud's agar	28.33	Yellowish, dull brown	Irregular	++
T_2	Czapeck's dox agar	53.00	Dark greyish with white centre	Smooth wavy margin	+++
T_3	Malt extract agar	24.67	Whitish yellow	Regular	+
T_4	Asthma and Hawker's Agar	43.00	Olive green	Regular	++
T_5	Waksman Agar	56.33	Greyish white	Regular	++
T_6	Glucose Peptone Agar	52.67	Greyish with outer brown hallow	Regular	++
T_7	Oat meal agar	62.67	Creamy white	Irregular	++++
T_8	Richards agar	18.67	Snow white	Regular	+
T_9	Hansen Agar	58.00	Light brown with white centre	Regular	++
T_{10}	PDA	83.67	Creamish to greyish black	Irregular	++++
S.E. (m) ±		1.27			
C.D at 1%		5.11			
CV (%)		4.57			

Where,
Excellent sporulation - ++++
Good sporulation - +++
Fair / moderate sporulation - ++
Poor / scanty sporulation - +

The similar findings are in conformity with the findings of earlier scientists Babu *et al*. (2000) [15], Arunakumar (2006) [14], Koley and Mahaptra (2015) [16], Shabana *et al*., (2015) [18], Najibullah *et al*., (2016) [17] and Prasanna *et al*., (2018) [19] where they reported that PDA media showed maximum mycelial growth and sporulation of *A. solani*.

3. *In vitro* evaluation of chitosan against *A. solani*

The effect of different concentrations of chitosan on growth of fungus was studied by poison food technique. It was observed that under absolute control treatment (T_9) the fungus (*A. solani*) grew profusely. On eighth day of inoculation, the mean colony diameter of fungus was 90 mm. The growth (in terms of colony diameter) of *A. solani* on media containing different concentrations of chitosan, showed that the media containing chitosan inhibited the mycelial growth of *A. solani*.

The results indicated that the degree of inhibition was dependent on the concentration of the chitosan in the fungal growth medium. (Table 4)

The media containing 500 ppm chitosan recorded the highest inhibitions of mycelial growth (89.63 per cent) with minimum colony diameters of 9.33 mm. This was followed by chitosan with concentration of 250 ppm, 200 ppm, 150 ppm, 125 ppm, 100 ppm, 75 ppm and 50 ppm with mycelial growth inhibition of 89.63, 79.63, 74.44, 66.30, 65.19, 57.78, 48.89 and 41.85 per cent, respectively. It was observed that with increase in the concentration of chitosan there was significant decrease in the mycelial growth and accordingly maximum inhibition was observed at highest concentration (500 ppm) than at lowest concentration (50 ppm).

It was also noticed that, with the increase of chitosan concentration, the inhibition percentage of mycelia growth

was also increased.

The results are in consonance with those reported earlier by Stossel and Leuba (1984), Sudarshan *et al*., (1992) [20], Wang (1992) [25], Tsai *et al*., (1999) [24] and Rhoades and Roller (2000) [23] revealed that the minimal growth-inhibiting concentrations of chitosan varied between 10 and 5,000 ppm.

El-Hassni *et al*., (2004) [21] reported that chitosan exerts an inhibitory action on the hyphal growth of numerous pathogenic fungi, such as *Fusarium oxysporum*, *Botrytis cinerea*, *Monilina laxa*, *Alternaria alternata* and *Pythium aphanidermatum*.

Table 4: *In vitro* evaluation of chitosan against *A. solani*

Treatments	Treatment Name	Average colony diameter (mm)	Average inhibition Over Control (%)
T_1	Chitosan @ 50 ppm	52.33	41.85 (40.31)
T_2	Chitosan @ 75 ppm	46.00	48.89 (44.36)
T_3	Chitosan @ 100 ppm	38.00	57.78 (49.48)
T_4	Chitosan @ 125 ppm	31.33	65.19 (53.84)
T_5	Chitosan @ 150 ppm	30.33	66.30 (54.52)
T_6	Chitosan @ 200 ppm	23.00	74.44 (59.64)
T_7	Chitosan @ 250 ppm	18.33	79.63 (63.18)
T_8	Chitosan @ 500 ppm	9.33	89.63 (71.25)
T_9	Control	90.00	0.00 (0.00)
S.E (m) ±		0.68	0.41
CD at 1%		2.79	1.64
CV (%)		3.47	1.35

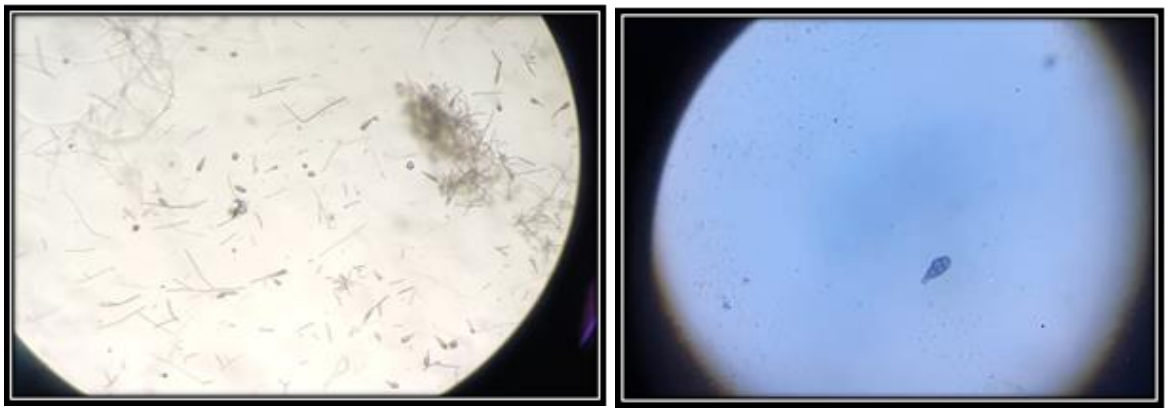

Fig 1: Microscopic morphology of *A. solani*

Fig 2: Cultural Variability *A. solani*

Media Used

T_1 - Sabouraud's agar
T_2 - Czapeck's dox agar
T_3 - Malt extract agar
T_4 - Asthma and Hawker's Agar
T_5 - Waksman Agar
T_6 - Glucose Peptone Agar
T_7 - Oat meal agar
T_8 - Richard's agar
T_9 - Hansen Agar
T_{10} - PDA

Fig 3: *In vitro* evaluation of Chitosan against *A. solani*

Treatments

T_1 - Chitosan @ 50 ppm
T_2 - Chitosan @ 75 ppm
T_3 - Chitosan @ 100 ppm
T_4 - Chitosan @ 125 ppm
T_5 - Chitosan @ 150 ppm
T_6 - Chitosan @ 200 ppm
T_7 - Chitosan @ 250 ppm
T_8 - Chitosan @ 500 ppm
T_9 - Control

Hernández-Lauzardo *et al*., (2011) [22] stated that recent studies of Chitosan-fungal cell interactions showed that the polymer penetrates the cell and cause intracellular affectations.

Gaikwad *et al*., (2019) [22] reported the effect of different concentrations of chitosan on growth of *A. porri* and stated that as the concentration of chitosan increases the inhibition percentage also increases.

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