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Inoculation Technique, Storage Period and Spore Concentration of *Alternaria solani* on Tomato for Early Blight Development

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Abstract Effect of inoculation technique, inoculum concentration and storage period were evaluated on the development of early blight disease on tomato in relation to host susceptibility under controlled environmental conditions. Four inoculation methods were used (spray inoculation, root dip inoculation, droplet inoculation and soil inoculation) with 10^4/ml spore concentration for better disease development on two susceptible varieties (Co-3 and Arka Vikash), in which the droplet method gave maximum PDI (85.19 and 81.48%). The droplet method gave better discrimination of resistance level at a range of conidial densities followed by commonly used spray inoculation methods. When culture of *Alternaria solani* was grown for 30 days on sorghum grains it gave minimum incubation period and maximum number of lesions on both susceptible varieties of tomato as compared to other storage period. Spore concentration 10×10^3 was better for disease development on both susceptible varieties.Mycelium growth in *A. solani* was recorded maximum at 12 days after inoculation on both potato dextrose agar and potato dextrose broth media.

Keywords Inoculation technique, Storage period, Spore concentration, *Alternaria solani*, Early blight.

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Introduction

Early blight of tomato (*Lycopersicon esculentum*), caused by *Alternaria solani*, is economically the most important disease of tomato in USA, Australia, Israel, UK and India, where significant reductions in yield (35—78%) during field survey have been observed. Being the worlds second most cultivated crop, with a production estimated at 150 million tones and acreage of 5.2 million hectares, the tomato is an indispensable vegetable crop world over and of course, for India. China is the worlds largest producer of the tomato (48.1 mt) followed by India (19.5 mt). Turkey, Italy, Iran, Egypt, Brazil, Spain, Mexico and Russia are also significant producers [1].

This disease, which in severe cases can lead to complete defoliation, is most damaging on tomato

Solanum lycopersicum L. [2] in regions with heavy rainfall, high humidity and fairly high temperatures (24—29°C). Field evaluations can identity sources of resistance but the major drawbacks are the lengthy duration of the tests, uncontrollable environmental conditions necessary for infection and the presence of other foliar pathogens [3]. The EB lesions resulting from spray inoculation are scattered on the leaves; this requires an observer to estimate the combined area of all lesions on all leaflets as a percentage of the total leaf area. Another disadvantage of the spray inoculation method is that the inoculum may not be uniformly distributed on the leaves. Furthermore, the method is not sensitive enough to discriminate moderately resistant from susceptible plants. An alternative method to obtain more precise and reliable disease readings is offered by a method in which individual droplets of fungal inoculum suspension are inoculated on leaflets.

A simple, computerized forecasting system, fast, based on monitoring of various environmental parameters, has been developed in Pennsylvania, USA, to determine periods when environmental conditions are favorable for the development of *A. solani* on tomato and to provide an efficient fungicide application schedule. However, *A. solani* has the ability to grow over a wide range of temperatures (4-36°C) and is reported to infect tomato and potato plants under both dry and wet conditions. Biological parameters such as primary inoculum level, host susceptibility and physiological age of the host were not included in the fast forecaster, which could explain why this system was not very successful in suppressing early blight of tomato in locations other than Pennsylvania, or on other hosts (potato). Most knowledge of early blight of tomatoes is based on field observations of the disease and pathogen under natural infections. Data derived from field studies with natural infections are variable, due to uncontrolled inoculum concentration and weather parameters, whereas studies conducted in controlled environments with artificial inoculations remove much of this variability. This paper describes an investigation of how *A. solani* inoculum level, storage period, culture growth phase and inoculation technique effect development of early blight on tomato plants in controlled environments.

Materials and Methods

Effect of inoculation technique

The fungus survives on crop, crop debris, seed and soil. Significant correlations were observed between two susceptible tomato varieties (Co-3 and ArkaVikash) that have been grown under glasshouse conditions using four inoculation methods. six weeks after germination seedlings were inoculated with different inoculation methods viz. spray inoculation, root dip inoculation, droplet inoculation and soil inoculation that were used with conidial suspension (10^4/ml). The 30 days old grain based inoculum grown on sorghum grains was used for inoculation. After inoculation the temperature 25 ± 2°C and humidity 90—100% were maintained in poly house with the help of humidifier. The inoculated plants were regularly examined for appearance of symptoms starting from 24 h after inoculation. The data on PDI were recorded on five different times at 7 days intervals i.e. 7, 14, 21, 28 and 35 days after inoculation (DAI). Disease severity was scored on a ten-point scale (0-9) as described by Ghosh et al.[4].

The percentage disease index (PDI) and area under disease progress curve (AUDPC) were calculated as follows

$$PDI = \frac{\text{Sum of all ratings} \times 100}{\text{Total no. of observations} \times \text{maximum rating scale}}$$

Percent Disease Index (PDI) was worked out by using formula given below .

$$AUDPC = \sum_{i=1}^{n-1} \{[X_{i+1} + X_i) / 2] * (ti + 1 - ti)\}$$

Where :

√ X_i is the disease index expressed as a proportion at the i^{th} observation.

√ t_i is the time (days after planting) at the i^{th} observations.

And n is the total number of observations.

Description of disease scale (0-9)

Sl. No.	0-9 Scale	Percent leaf area infected
1	0	No.infection
2	1	0 – 10
3	2	10 – 20
4	3	20 – 30
5	4	30 – 40
6	5	40 – 50
7	6	50 – 60
8	7	60 – 70
9	8	70 – 80
10	9	80 – 100

Effect on storage of inoculum and its longevity

The polypropylene bags containing inoculum of *A. solani* were stored at 25 ± 2°C in the incubator and after every 30 days interval i.e.0, 30,60,90 and 120 days after inoculation they were checked for viability and pathogenicity of inoculum that was tested on 42 days old susceptible varieties of tomato (Co-3 and Arka Vikash), with conidial suspension of 10^4/ml . Control plants were sprayed with sterile distilled water. The experiment was conducted in pot condition with five replications. Number of lesions per leaflet was recorded 15 days after inoculation.

Effect of inoculum concentration on susceptible tomato varieties

Two susceptible varieties (Co-3 and Arka Vikash) of tomato were taken for experiment. Six weeks after germination of tomato plants they were inoculated with different inoculum concentrations viz. 0×10^3, 1×10^3 2×10^3, 5×10^3 and 10×10^3 in pot condition. There were five replications for each varieties / conidial concentration combination. Control plants were sprayed with sterille distilled water. The 30 days old culture was used for inoculation from sorghum grains. After inoculation the temperature 25 ± 2°C and humidity 90–100% were maintained in poly house with the help of humidifier. Number of lesions per leaflet were recorded after 15 days of inoculation.

Effect on growth phase of the pathogen

The growth phase study was conducted on potato dextrose agar (PDA) and potato dextrose broth (PDB). 20 ml PDA in each petri plates and 25 ml PDB in each 100 ml flasks were poured. Poured petri plates were inoculated with 5 mm disc of *A. solani* culture and flasks were sterilized at 121°C for 20 minutes at 15 Ib psi pressure and then inoculated with 5 mm disc of *A. solani* culture. The inoculated petri plates and flasks were incubated at 25 ±2°C. a set of petri plates and flasks with three replications were observed starting from first day up to full growth. The culture was filtered through Whatman No. 1 filter paper. Before filtering, the filter papers were dried to a constant weight by drying in hot air oven at 50°C. The mycelial mat on the filter paper was thoroughly washed with distilled water dried in hot air oven at 50°C. The filter paper with mycelial mat was weighed in a digital electronic balance. The weight of dry mycelial mat was recorded and the data were statistically analyzed and maximum growth period was determined.

Table 1. Standardization of inoculation technique for development of *A. solani* on tomato plants.

Techniques	Co-3 IP (Days)	Co-3 Final PDI value	Co-3 AUDPC	Arka Vikash IP (Days)	Arka Vikash Final PDI value	Arka Vikash AUDPC
Spray inoculation	5	74.07	1309.26	4	74.07	1374.07
Root dip inoculation	8	37.04	596.30	8	37.04	570.37
Droplet inoculation	4	85.19	1620.37	4	81.48	1542.59
Soil inoculation	8	25.93	427.78	7	29.63	479.63
CD (1%)		8.53			6.13	
SE (m)		2.818			3.718	
SE (d)		3.575			4.175	
CV		12.544			10.434	

Table 2. Storage effect of inoculums on spore viability and inoculums quality. *IP = Incubation period.

Storage period	Spore g^{-1} (10^3)	IP* Co-3	IP* Arka Vikash	Number of lesion 15 DAI Co-3	Number of lesion 15 DAI Arka Vikash
0	0	0	0	0.00	0.00
30	8.4	5	6	14.50	13.25
60	4.7	7	8	9.50	10.00
90	2.8	10	11	7.00	7.00
120	0.8	14	13	4.50	4.25
	CD (1%)			1.416	1.075
	SE (m)			0.465	0.354
	SE (d)			0.658	0.500
	CV			13.112	10.248

Table 3. Effect of spore concentration of *A. solani* on tomato for early blight development. * IP=Incubation period.

Spore density (10^3/ml)	IP* Co-3	IP* Arka Vikash	Number of lesion 15 DAI Co-3	Number of lesion 15 DAI Arka Vikash
0	0	0	0.00	0.00
1	12	13	4.00	3.50
2	10	11	5.75	4.75
5	7	8	13.25	12.25
10	5	6	15.25	13.75
	CD (1%)		2.230	2.050
	SE (m)		0.733	0.674
	SE (d)		1.037	0.953
	CV		19.167	19.676

Results and Discussion

Standardization of inoculation technique

Four inoculation methods were used (spray inoculation, root dip inoculation, droplet inoculation and soil inoculation) with spore concentration 10^4 spore/ml for better disease development on two susceptible varieties (Co-3 and Arka Vikash) (Table 1) . The droplet method gave maximum PDI (85.19 and 81.48%) followed by spray inoculation method (same 74.07 %) on both susceptible varieties, respectively. The incubation period was also minimum in droplet method (4 days in both varieties) followed by spray inoculation method (5 and 4 days) on both susceptible varieties (Co-3 and Arka Vikash), respectively. The droplet inoculation method is simple to apply and allows an objective evaluation of EB severity. The method has been used to evaluate EB resistance components.

Storage of inoculum and its efficiency

There was significant loss in the number of spores/gm of sorghum grains and inoculum efficiency when inoculum was stored for more than 30 days. But these grain based media were found best for long period storage of *A. solani* as compared to other artificial media. Significantly higher spore concentration was recorded at 8400/ gm of sorghum grains after 30 days incubation periods. (Table 2). When 30 days old sorghum grain culture was inoculated on two susceptible tomato varieties viz. Co-3 and Arka Vikash, 14.50 and 13.25 lesions/leaflet were recorded after 15 days of inoculation on both of them, respectively. Linear relationships were found between storage period and incubation period and between storage period and number of lesions/leaflets. No symptom developed and no defoliation was noted on uninoculated plants. Similar study was also done by Chand et al. [5] on *Cercosporacanescens* of mungbean and observed that the significant loss in the number of spores when inoculumwas stored for 60 days and inoculum efficiency was reduced from 16 lesions $leaflet^{-1}$ to 9 lesions $leaflet^{-1}$ on susceptible cultivar Kopergoan.

Standardization of inoculum concentration for early blight development

Under controlled conditions, all inoculated plants showed disease symptoms on leaves after inoculation, even with the lowest conidial concentration i. e. 1×10^3 conidia/ml (4.00 and 3.50 lesions/leaflets 15 DAI) on both susceptible varieties (Co-3 and Arka Vikash), respectively.Maximum number of lesions/leaflets were recorded with spore concentration of 10×10^3 i.e. 15.25 and 13.75 on two susceptible tomato varieties (Co-3 and Arka Vikash) which was not significantly different to spore concentration of 5×10^3 giving 13.25 and 12.25 lesions/leaflets (Table 3). Minimum incubation period of 5 and 6 days on Co-3 and Arka Vikash, respectively were noted with spore concentration 10×10^3. Linear relationships were found between inoculum concen-

Table 4. Effect of time on growth phase of *A. solani* on PDA and PDB incubate at 25 ± 2°C. * Average of three replications.

Days	Mycelial growth (12 DAI) PDA (mm)*	PDB (mg)*
1	0.00	0.00
2	7.66	18.00
3	13.67	36.66
4	21.33	50.33
5	29.00	100.66
6	39.33	116.66
7	45.66	136.33
8	50.00	166.66
9	55.33	207.00
10	67.66	304.00
11	77.66	431.66
12	87.66	556.00
CD (1%)	3.319	16.705
SE (m)	1.130	5.689
SE (d)	1.599	8.046
CV	4.746	5.568

tration and number of lesions/leaflets, and between inoculum concentration and incubation period. No symptoms developed and no defoliation was noted on water sprayed plants.

The effects of inoculum concentration on symptom development and defoliation of tomato plants in these controlled -environment experiments support the observations, who showed that early blight severity on young tomato plants increased asconidial concentration increased from 5×10^3 conidia/ml. A positive relationship between inoculum concentration and symptom development has also been demonstrated for other *Alternaria* species [6].

Effect on growth phase
of the pathogen

Maximum mycelial growth of 87.66 cm was recorded at 12 DAI on PDA medium. In liquid media the mycelial growth (mg) from inoculated flasks was harvested everyday starting from firstday after inoculation up to 12 DAI (Table 4). The dry mycelial weight was recorded and the results obtained were analyzed. Maximum growth was observed on 12th day of inoculation (556.00 mg).

Determination of optimum growth period is essenial to study the physiology of fungi. Maximum dry mycelial weight and growth in the present study was obtained on 12th day of inoculation, which is indicative of the optimum growth of the fungus.

Conclusion

In the droplet inoculation method leaflets of intact plants are inoculated with deoplets of *A. solani* conidial suspension and gave better results in comparison to other inoculation method. Its application to identify potential EB resistance sources in a collection of tomato accessions. Screening of large numbers of accessions in the glasshouse has never been conducted using the droplet inoculation metod, because this method was time taking and cumbersome to inoculate individual plants. Grain based inoculum on sorghum grains was found best for long period storage of *A. solani* compared to other artificial media. Its easy, cheap and non-chemical method for growth of *A. solani* and its long time storage. The early blight disease severity on young tomato plants increased as conidial concentration increased from 5×10^3 conidia/ml. A positive relationship between inoculum concentration and lesion/leaflets was observed.

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