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Studies on factors affecting the pathogenesis of *Alternaria solani* (Ell. and Mart.) Jones and Grout on Tomato *(Solanum lycopersicum* Mill*.)*

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Abstract

Development of disease is essential to study the various aspects of pathogen for the management of disease. Hence, the present study was conducted to ascertain the effect of inoculum load, age of pathogen culture, sub-culturing and host age on the development of early blight of tomato. Five conidial concentrations *viz.*, $5x10^3$, $1x10^4$, $5x10^4$, $1x10^5$ and $5x10^5$ conidia/ml were evaluated. *Alternaria solani* inoculum load of $5x10^5$ spores/ml gave maximum disease severity (34.60%) with shortest incubation period of 3.0 days followed by $1x10^5$ (31.53%) with 4.2 days of incubation period. The findings of the present study suggested that 14 days old culture of *A. solani* as compared to 7, 21, 28 and 35 days old was most virulent with highest mean disease severity (27.47%) and shortest incubation period (3.6 days). The shortest incubation period (3.0 days) was observed with first sub-culture as compared to 2^{nd}, 3^{rd}, 4^{th} and 5^{th} subcultures, which resulted in highest mean disease severity (31.13%). The results for the second sub-culture were also found to be at par. This indicated that the pathogen was most virulent up to second sub-culturing and that successive sub-cultures reduced the inoculum potential. The disease was observed more on the mature plants as compared to younger plant. It was also found that tomato plants of 50-60 days after sowing are most susceptible to early blight infection with maximum disease severity of 37.80 per cent and host plant most likely exhibited only seedling resistance against the necrotrophic pathogen *A. solani.*

Key words: Artificial inoculation, tomato, *Alternaria solani*, pathogenicity

India is one of the largest tomato growing countries in the world, occupying the first position in area and second position in production after China. Tomato is the second most important vegetable crop in the world after potato and accounts for nearly 32 per cent of the total vegetables produced in the country (Anonymous 2020). In spite of these achievements, the last couple of years, the low production of tomato in India has consequently resulted in drastic decrease in its per capita availability and has compelled India to import large quantity of tomatoes (Adhikari *et al.* 2017). The main cause of low productivity is due to various diseases and pests associated with solanaceous crops (Chauhan *et al.* 2020). Early blight is one of the important diseases of tomato responsible for average yield loss of 10 to 70 per cent in different parts of Northern India depending upon the severity (Shinde *et al.* 2018). Due to the lack of availability of the sources of resistance against *Alternaria solani*, early blight is considered the most damaging and widespread fungal disease of tomatoes (Sharma *et al.* 2021). Since early blight is increasingly destructive, disease management strategies need to be developed. The first step in any of the resistance breeding programme is to rapidly screen all the available genetic stocks, including the local land races, improved cuttings and exotic germplasms using empirical techniques in glass houses, or by field tests. For a successful screening, artificial inoculation of plants at susceptible growth stage with an adequate amount of inoculum is necessary. The artificial inoculation of host is necessary to obtain a more uniform disease; moreover, establishment of disease by artificial inoculation is also essential for studies of various aspects of plant pathology, including epidemiology, etiology, disease resistance, host-parasite interaction, and disease control (Thakur and Banyal 2022). Jie *et al*. (2009) also reported that the

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artificial inoculation method provided a foundational understanding of ecological enrichment to control banana wilt disease in future. In this report, the effect of age of pathogen culture, sub-culturing, inoculum load and host age on the development of early blight of tomato was ascertained with the objective to standardize these factors for pathogenesis studies of *A. solani* on tomato.

Materials and Methods

Alternaria solani (Berk.) Sacc. culture was derivedfrom diseased tomato leaf samples using standard leaf bit methodology (Dhingra and Sinclair 1985)at CSK HPKV Palampur. Fungal culture was purified by single spore method (Choi *et al.* 1999) and the isolate was maintained on potato dextrose agar (PDA) in the Department of Plant Pathology. To enhance the sporulation of *A. solani*on PDA medium, the plates were exposed to UV light exposure for 20 second after 4-5 days of incubation and then placed back in the incubator (Aragaki 1961; Yadav *et al*. 2015). A conidial suspension was prepared by scraping mycelia and spores from plates of actively growing fungal cultures into autoclaved water and filtering the suspension through four layers of cheese cloth to remove most of the mycelia. The filtered spore suspension was centrifuged at 2000 x g for 5 min and re suspended in deionized water. This centrifugation was repeated till a clear spore suspension was obtained. *Alternaria solani* conidia being long beaked, tend to clog together among themselves and with bits of media and mycelium. This prevented accurate calculation of the spore concentration. Therefore, a clear spore suspension was preferred. After the final wash, supernatant was discarded and spores were re suspended in water containing 0.05% Tween-20.

Inoculum load

Different inoculum concentrations (spores/ml) were evaluated to standardize the optimum inoculum concentration required for successful infection and development of early blight symptoms on tomato plants. For this, two-month-oldseedlings of susceptible tomato hybrid Avtar were spray inoculated with different conidial concentrations *viz.*, $5x10^3$, $1x10^4$, $5x10^4$, $1x10^5$ and $5x10^5$ conidia/ml. Simultaneously, control treatment by spraying sterilized water was also maintained. The inoculated plants were placed in net-house by maintaining RH > 90% for 48 hours at temperature 25-30°C and thereafter the pots were watered daily until the development of disease symptoms. Five plants were taken for each replication and the treatments were replicated thrice in CRD. The incubation period was recorded for each treatment and per cent disease index (PDI) was recorded using rating scale given by Pandey *et al.* (2003). The PDI values were expressed using the formula given by McKinney (1923). Disease progression was measured by calculating AUDPC and apparent rate of infection (r) as per logistic equation given by Vander plank (1963).

$$\text{PDI} = \sum \frac{Severity\ grade \times Number\ of\ leaves}{Maximum\ grade \times Total\ number\ of\ leaves\ scored} \times 100$$

$$\text{AUDPC} = \sum_{i=1}^{n-1} \frac{y_i + y_{i+1}}{2} \times (t_{i+1} - t_i)$$

Where, y_i= Disease severity at the i^{th} observation
t_i = time (in days) at the i^{th} observation
n = total number of observations.

$$r = \frac{2.303}{t_2 - t_1} \log 10 \frac{x_2(1 - x_1)}{x_1(1 - x_2)}$$

Where,
r = apparent infection rate per day
t_2-t_1= time interval between first and last observation
x_1 & x_2= proportion of leaf area covered by lesion at t_1 and t_2 time intervals, respectively
(1-x_1) & (1-x_2) = proportion of healthy leaf area at t_1 and t_2 time intervals, respectively

Age of culture

The isolated culture of *A. solani* from the infected tomato leaves on PDA was maintained in the laboratory for different time durations *viz.,* 7 days old culture, up to 35 days. The pathogenicity tests were carried out in polyhouse condition by spraying mycelial suspension ($1x10^5$ conidia/ml) from 7, 14, 21, 28 and 35-days old culture on two-months old seedlings of susceptible tomato hybrid Avtar grown in pots. The inoculated plants were placed in net-house by maintaining RH > 90% for 48 hours at temperature 25-30°C and thereafter the pots were watered daily until the development of disease symptoms. Five plants were taken for each replication and the treatments were replicated thrice in CRD.Observation on incubation period was recorded for each treatment.

Per cent Disease Index (PDI) was recorded at weekly interval. The AUDPC and infection rate were calculated as described earlier.

Pathogen sub-culturing

To investigate the effect of sub-culturing of pathogen on the development of early blight on tomato, the first isolated and purified colony of *A. solani* grown on PDA for 7 days was designated as the first-generation culture. For subsequent sub-culturing, mycelial plug from the center of each colony was used to establish a new growth on a Petri plate with PDA. Five plates were prepared at each sub-culturing after every 7 days up to 5 generations. The pathogenicity tests were carried out in polyhouse condition by spraying conidial suspension ($1xl0^5$ conidia/ml) from 1^{st}, 2^{nd}, 3^{rd},4^{th} and 5^{th} generation pathogen culture on two-month-oldseedlings of susceptible tomato hybrid Avtar grown in pots. Five plants were taken for each replication and each treatment was replicated thrice in pots along with an uninoculated control. The inoculated plants were placed in net-house by maintaining RH > 90% for 48 hours at temperature 25-30°C and thereafter the pots were watered daily until the development of disease symptoms. Observations on incubation period and per cent disease index (PDI) were recorded. The AUDPC and infection rate were calculated as described earlier.

Host age

Tomato seeds of susceptible hybrid Avtar sown (five seeds/pot) in pots were spray inoculated at 30, 40, 50 and 60 days after sowing. For this staggered sowing was done at 10 days interval up to 60 days. Plants were grown in controlled condition. The pathogenicity tests were carried out in polyhouse condition by spraying mycelial suspension ($1xl0^5$ conidia/ml). Five plants were taken for each replication and each treatment was replicated thrice in pots along with an uninoculated control. The inoculated plants were placed in net-house by maintaining RH > 90% for 48 hours at temperature 25-30°C and thereafter the pots were watered daily until the development of disease symptoms. Incubation period and per cent disease index (PDI) were recorded and AUDPC and infection rate were calculated as described earlier.

Results and Discussion

Inoculum load

The effect of different inoculum levels on disease development was studied and the data has been presented in Table 1. Significant variation in incubation period, disease severity, AUDPC and rate of infection was recorded at different inoculum loads. Inoculum load of $5x10^5$ spores/ml gave maximum disease severity (34.60%) with shortest incubation period of 3.0±0.71 days followed by $1x10^5$ (31.53%) with 4.2±0.45 days of incubation period. However, minimum disease severity (26.93%) was observed at inoculum level $5x10^3$ after 6.8±0.84 days of incubation period. The disease progress was also significantly high at $5x10^5$ spores/ml inoculum load which was evident by highest AUDPC (485.80) and apparent infection rate (0.07). This was followed by $1x10^5$ conidial concentrations with 448.00 AUDPC and 0.06 rate of infection. Significantly lowest disease severity was observed at $5x10^3$spores/ml with minimum AUDPC and apparent infection rate *i.e*. 377.30 and 0.01, respectively. The standardization of inoculum load helped determine that spore concentration between $1x10^5$ and $5x10^5$spores/ml can initiate the infection for successful disease development with minimum incubation period. Therefore, for all further experimentations inoculum load of $1x10^5$ spores/ml was used.

Table 1. Effect of inoculum load of *Alternaria solani* on the development of early blight of tomato

Inoculum load (spores/ml)	Incubation period days	Disease severity (%) after disease appearance				AUDPC	Apparent rate of infection (r/day)
		7	14	21	Mean		
$5x10^3$	6.8±0.84	18.20	27.00	35.60	26.93	377.30	0.01
$1x10^4$	5.4±0.55	21.80	30.00	36.20	29.33	413.00	0.02
$5x10^4$	4.4±0.55	20.00	31.20	38.20	29.80	422.10	0.04
$1x10^5$	4.2±0.45	21.20	33.40	40.00	31.53	448.00	0.06
$5x10^5$	3.0±0.71	23.40	35.00	45.40	34.60	485.80	0.07
LSD (0.05)	-	0.69	1.23	1.89	-	3.89	0.02

The results are in conformity with other workers. Coffey *et al.* (1975) reported that early blight severity gradually increased on young tomato plants with increase in *A. solani* conidial concentration from $5x10^3$ to $8x10^4$spores/ml. A positive correlation between inoculum concentration and symptom development has also been demonstrated for other *Alternaria* species by Christ and Maczuga (1989) and Vloutoglou (1994). Vloutoglou and Kalogerakis (2000) reported that as the inoculum concentration of *A. solani* increased from $6x10^3$ to $11x10^3$conidia/ml, the percentage of tomato leaf area affected and defoliation increased linearly. Bhardwaj (2018) observed $6x10^4$ as the ideal inoculum concentration for rapid advancement of *Alternaria porri*on garlic, while inoculum concentration below $2x10^4$ noticeably delayed the infection process. Adequate amount of inoculum is a pre-requisite for initiation of symptoms and disease progress as this is likely to be proportional to the amount of pathogenicity factors required for initial host invasion.

Age of pathogen culture

To study the effect of age of pathogen culture on the development of early blight of tomato, an experiment was conducted in pots with 7, 14, 21, 28 and 35-days old pathogen culture maintained on PDA. The data presented in Table 2 revealed that 14 days old culture was most virulent with highest mean disease severity (27.47%) and shortest incubation period (3.6±0.89 days) along with maximum AUDPC and infection rate of 378.70 and 0.038 r/day, respectively. The difference in incubation period was statistically insignificant in plants inoculated with 21 to 35 days old cultures. Minimum disease severity (13.87%), AUDPC (200.20) as well as infection rate (0.018) was recorded with 35 days old pathogen culture. The disease severity obtained with 7 (23.27%) and 21 (23.53%) days old culture was statistically at par with each other. Pathogen culture older than 14 days indicated successively reduced virulence as evident from delayed disease appearance and slow disease progress with low terminal disease severity. The pathogen virulence was also observed less with 7 days old culture, indicating that about 2 weeks old pathogen culture is most suitable for development of disease and sporulation. The rate of sporulation and growth rate significantly decreased after 14 days pertaining to the depleting nutrients in the culture media

Vloutoglou and Kalogerakis (2000) studied the effects of age of culture, inoculum concentration, wetness duration and plant age on development of early blight and on shedding of leaves in tomato plants and found that all these factors could affect the disease development. Koley and Mahapatra (2015) reported that maximum growth of *A. solani* was observed at 8 days after inoculation however, the growth rate consistently decreased after 3 days of inoculation. The sporulation rate also declined with increase in the age of culture. Similar observations were recorded for other fungal pathogens by Sennoi *et al.* (2013) and Anand (2019).

Pathogen sub-culturing

An experiment was conducted *in vivo* to study the effect of sub-culturing of *A. solani* on the development of early blight of tomato and the data recorded are

Table 2. Effect of age of culture of *Alternaria solani* on the development of early blight of tomato

Age of culture (days)	Incubation period (days)	Disease severity (%) days after disease appearance				AUDPC	Apparent rate of infection (r/day)
		7	14	21	Mean		
7	4.8±0.45	16.80	20.20	32.80	23.27	326.20	0.031
14	3.6±0.89	20.20	25.80	36.40	27.47	378.70	0.038
21	5.4±1.00	16.40	22.20	32.00	23.53	319.20	0.027
28	5.7±0.71	8.60	16.40	27.00	17.33	239.40	0.026
35	6.6±0.55	7.80	15.60	18.20	13.87	200.20	0.018
LSD (0.05)	-	2.09	1.55	2.92	-	4.10	0.002

presented in Table 3. The shortest incubation period (3.0±0.71 days) was observed with first sub-culture which also resulted in highest mean disease severity (31.13 per cent) with maximum AUDPC and infection rate of 437.50 and 0.051 r/day, respectively. The 2nd subculture was found to be statistically at par with 1st subculture resulting in 30.47 per cent disease severity with AUDPC and infection rate of 432.60 and 0.054 r/day, respectively. The incubation period gradually increased with subsequent sub-culturing of the pathogen and it was recorded to be maximum (7.6±0.55 days) for the 5th sub-culture. The conidial production and rate of conidial germination was reduced on sub-culturing and this indicated that the pathogen was highly virulent up to second sub-culturing and that successive sub-cultures reduced the inoculum potential leading to delayed disease appearance and lower disease progress and terminal disease severity.

Although sub-culturing is required to prolong the lifespan and/or increase the number of microbial cells in the culture, successive sub-culturing has been reported to affect the virulence, conidial yield, germination and stability of strains in various fungal pathogens (Bruslind 2021). The *in vitro* sporulation of *A. solani* requires special conditions and the conidial production tends to decrease after periodic sub-culturing (Yadav *et al.* 2015). Anand (2019) also recorded that young and early generation of pathogen culture gave maximum disease incidence of collar rot of cow pea and significant decrease in the disease incidence was observed with increase in age and sub-culturing of the pathogen.

Host age

To determine the most susceptible stage of tomato for infection of early blight, an experiment was conducted under pot condition. Susceptible tomato plants were inoculated with *A. solani* ($1x10^5$ spores/ml) at 30, 40, 50 and 60 days after sowing (DAS). Plant at 60 DAS was recorded as the most critical growth stage with highest disease severity of 37.80 per cent (Table 4). The disease progress was also significantly high at this stage which was evident by highest AUDPC (520.10) and apparent infection rate (0.06). This was followed by 50 days old plant with AUDPC and rate of infection *i.e.* 465.50 and 0.04, respectively.

Table 3. Effect of sub-culturing of *Alternaria solani* on the development of early blight of tomato

Sub-culture (SC)	Incubation period (days)	Disease severity (%) days after disease appearance				AUDPC	Apparent rate of infection (r/day)
		7	14	21	Mean		
SC1	3.0±0.71	22.00	31.60	39.80	31.13	437.50	0.051
SC2	4.2±0.45	21.80	32.20	37.40	30.47	432.60	0.054
SC3	4.4±0.55	16.40	27.80	33.40	25.87	368.90	0.046
SC4	5.4±0.55	12.40	20.40	29.40	20.73	289.10	0.033
SC5	7.6±0.55	12.20	19.00	24.00	18.40	259.70	0.033
LSD (0.05)	-	1.73	1.98	2.44	-	4.89	0.002

Table 4. Effect of host age on the development of early blight of tomato

Host age (DAS)	Incubation period (days)	Disease severity (%) after disease appearance				AUDPC	Apparent rate of infection (r/day)
		7	14	21	Mean		
30	4.0±0.71	13.60	26.60	39.20	26.47	371.00	0.03
40	4.6±0.55	16.80	30.40	42.60	29.93	420.70	0.04
50	4.0±0.71	22.00	32.00	47.00	33.67	465.50	0.04
60	4.6±0.89	26.00	35.20	52.20	37.80	520.10	0.06
LSD (0.05)	-	1.67	1.89	1.93	-	3.89	0.02

DAS- Days after sowing

Significantly lowest disease severity was observed in plants inoculated at the youngest stage (30 DAS) with minimum AUDPC and apparent infection rate *i.e.* 371.00 & 0.03, respectively. No significant difference in the incubation period was recorded between plants inoculated at 30 to 60 DAS. It was concluded that tomato plants of 50-60 DAS are most susceptible to early blight infection under suitable conditions. The host plant most likely exhibited only seedling resistance against the necrotrophic pathogen *A. solani.*

Pandey *et al.* (2003) observed that symptoms of early blight appear at 30-35 days after transplanting and the most critical stage was reported at 35 and 55 days of plant growth. Several workers have reported that older leaves are more susceptible to early blight than younger ones (Vennila *et al.* 2020). Kong *et al*. (1995) reported that the susceptibility of *Alternaria helianthi* in sunflower tissue increased with age, so that older leaves were more susceptible than young and expanding leaves. Similar observations on increased susceptibility of host with advanced growth stages have been reported in Alternaria blight on Indian mustardand *Alternaria porri* on onion and niger (Maniyar*et al*. 2018; Sharma and Ratnoo 2019).

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