J.Mycopathol.Res. 62(2) : 361-368, 2024, ISSN : 0971-3719 (Print), 2583-6315 (Online) 10.57023/JMycR.62.2.2024.361

Effect of inoculum load on disease establishment in early blight of tomato

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Received : 30.03.2024 Accepted : 25.05.2024 Published : 24.06.2024

Inspite of various previous studies on host pathogen interactions, reports of the effects of inoculum load on host symptom development and disease manifestation, is limited. The goal of this research was to study the impact of different inoculum density on disease progression during infection of tomato by *Alternaria solani* (Sorauer, 1896). Disease severity was evaluated subsequent to inoculating tomato leaves with two different spore concentrations of *A. solani*. Although the fungus was able to establish itself on the host surface with both of the concentration of inoculums, the trend of the establishment was distinctly different. When higher inoculum load was used there was rapid establishment of the fungus in the first phase of infection, i.e. within 24 hours post inoculation. There was almost sixty fold increase in fungal load, then followed by less increase in the subsequent days. In the lower inoculum load, the fungus established more slowly in the first two days increasing more sharply on the third day post inoculum. Overall, the development of lesions on leaves with the high inoculums, covered almost half of the leaves at the end of first day post inoculation, compared to only one tenth of the leaves in the case of the low inoculum. Infection with lower conidial concentration resulted in minimal necrosis in the leaves of tomato plants and the tissue integrity was more or less maintained as observed under compound microscope after Trypan blue staining. Scanning electron microscopy showed differential hyphal behaviour depending on inoculum load. SEM revealed that leaves inoculated with higher concentration of spores resulted in the entire leaf surfaces being covered by extensive hyphal mats within three days of infection compared to scanty hyphae on leaves with lower inoculum load. The higher disease index in leaves infected with greater spore concentrations corroborated these observations. Given the significant effect of inoculum load on disease progression, the monitoring of inoculum load in the field is essential for assessing the potential threat of a disease at its nascent stage, facilitating timely interventions to reduce its dissemination.

Keywords: *Alternaria*, disease index, early blight, inoculum, necrosis, tomato

INTRODUCTION

During early infection stages, whether the pathogen will be successfully establishing itself on the host is dependent on the inoculum load (Khamari*et al.* 2019). The progression of diseases caused by fungi, depends on various factors, among which environmental factors (Nazarov *et al.* 2020; Singh *et al.* 2023; Lin *et al.* 2023) and availability of inoculum in the vicinity of plants (Garbelotto *et al.* 2020, Lin *et al.* 2023) are the critical ones.

Most previous studies have mainly focused on proposing disease forecast models as an aid for prediction of epidemics where the disease incidence is often predicted based on the weather patterns or the local climate (Shah *et al*. 2019; Farigoule *et al.* 2022). Some studies correlated cropping seasons, extent of disease occurrence and different agro-climatic conditions with disease progression (Farigoule *et al.* 2022; Kalyankumar *et al.* 2022). Also a few reports are available on various plant disease protection measures (Nazarov *et al.*2020, Van der Heyden *et al.*2021) that influence phytopathogenic spread across fields.

There exist only a few reports on the effect of inoculum load on diseases progression. Khamari

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et al. (2019) studied the effect of inoculum load of the phytophathogen *Macrophomina phaseolina* on the germination percentage of sesame seeds, seed rot and seedling mortality. Increase in the amount of inoculum largely reduced the germination of sesame seeds, increased rotting of seeds with enhanced seedling mortality. Additionally, for vector borne pathogens the spread of disease was found to be dependent on the vector abundance (Gruber *et al*. 2013) which again is directly related to inoculum load. Host defense response was found to vary seasonally in *Arabidopsis halleri* during Turnip Mosaic Virus infection (Honjo *et al*. 2020). In 2019, Saini and his co-workers have shown that the disease initiation in Indian gooseberry (*Emblica officinalis*) is accelerated when the spore concentration of *Penicillium islandicum* is gradually increased and a minimum threshold of inoculum is required for successful infection. Dutta *et al*. (2012), worked on the effect of exposure of water melon seeds to different inoculum loads, on transmission of bacterial fruit blotch in seedlings and spatiotemporal spread of the disease in water melon. In the case of *Phytophthora ramorum* infection, it was observed that different zoospore concentrations have resulted in differential host responses and symptom development in numerous ornamental plants (Garbelotto *et al*. 2020). Thus, lower inoculum load, below a certain threshold limit, in the environment is not likely to cause any disease, on the other hand, the presence of higher inoculum load might cause the disease.

Despite existing literature on the aetiology of plant diseases, substantial knowledge gaps persist, impeding a comprehensive understanding of the correlation between inoculum load and disease severity and their consequential impact on symptom development and host defense strategies. To date, no comprehensive studies have been conducted to delineate the sequential progression of lesion development induced by varying inoculum loads; a critical aspect thus remains unexplored in tomato plants. Similarly, the amount of hyphal load development subsequent to inoculation with different spore or conidial concentrations over time-course has not been documented. The development of symptoms, particularly the extent of tissue necrosis at successive time intervals following exposure to high and low inoculum loads, remains inadequately characterized. This knowledge gap hampers a holistic comprehension disease progression study and understanding the relation between the inoculum load and severity of the disease.

In this study, *Alternaria solani*, the causal organism of Early Blight disease, was selected as the pathogen, and tomato (*Lycopersicon esculentum*) as the host. The primary objective of this research was to determine whether the conidial concentration of *A. solani* exerts any influence on the severity of the disease. Additionally, we aimed to investigate potential impacts of inoculum load on host and assess its role in symptom development within the host.

MATERIALS AND METHODS

Fungal Material

The fungus causing early blight disease of tomato, *Alternaria solani* (Sorauer, 1896) [Indian Type Culture Collection, Indian Agricultural Research Institute (ITCC No. 4632)] was maintained as pure culture according to Ray *et al*. (2015). Potato dextrose broth and potato dextrose agar plates and slants were used for culture and maintained at 28°C.

Growth and maintenance of plants

Tomato seeds of the Pusa Ruby variety (PR), obtained from Sutton Pvt. Ltd., India, were grown in commercial soilrite according to our previous publications; Ray *et al*. (2015), Koley *et al*. (2022). This soilrite consists of a blend of horticulture grade perlite, Irish peat moss, and exfoliated vermiculite in a precise ratio of 1:1:1. The plants were grown within plant growth convirons at 28°C.

Preparation of spore suspensions and inoculation of tomato

For inoculation of plants, 15 day old *A. solani* culture with spores, was used. The black masses of spores were scraped out of the petridishes and were suspended in 20ml sterile distilled water in a conical flasks. The number of spores per unit

volume was counted using haemocytometer under a compound microscope (Leica) and adjusted to 2×10^6 spores/ml and 8 $x10^6$ spores/ml.10µl spore suspension droplet was placed at the centre of 3 week old tomato leaves and were incubated in 28°C for 24, 48 and 72 hrs. Water was used in control sets.

Assay of disease progression and calculation of disease index

To evaluate early blight disease after infection with two spore concentrations, $2X10^6$ spores/ml and $8X10^6$ spores/ml, the disease progression in inoculated leaves was measured in terms of percentage of area of necrosis in each leaf. The disease progression was assayed according to Koley *et al*. (2022). The area of necrotic regions developed on leaves were measured and were represented as the percentage of necrotic area against the area of the whole leaf.

Study of necrotic lesion development

Detached leaves of three weeks old plants, with leaf length 2.5-3.5 cm were placed in petriplates containing beds of wet filterpaper and were drop inoculated with two spore concentration viz. 2×10^6 spores/ml and $8x10^6$ spores/ml separately. After incubation the length of the necrotic lesions were measured at an interval of 24 hrs, upto 72 hrs post infection. The experiments were repeated thrice, with at least 3 experimental sets each.

Cell viability assay using Trypan Blue Staining

Trypan blue was used to assay the viability of the leaf cells after infection with different spore concentrations according to our previously established protocol (Ray *et al*. 2015 and Koley *et al*. 2022) and were viewed under Olympus BX-51 microscope.

Preparation of samples for Scanning Electron Microscopy

Leaves were meticulously prepared for scanning electron microscopy SEM according to our published protocol (Koley *et al*. 2022). The prepared samples were analyzed using a scanning electron microscope (Carl Zeiss EVO 18, Germany).

Statistical analysis

All the data were statistically analyzed using software according to our previous publications (Chowdhury *et al*. 2017a,b). Each experiment was done in a completely randomized design (CRD) with three independent experiments having three replicates each and values represented as the standard error mean ±SEM. The data were subjected to one-way analysis of variance (ANOVA) with different letters indicating significant differences between treatments at $p<0.05$, according to Duncan's multiple range test (DMRT), using a software package, SPSS version 16, 2007.

RESULTS

Effect of differential inoculum loads on disease progression

The step-wise development of lesions was systematically investigated and analysed through the measurement of the extent of tissue necrosis in tomato leaves subsequent to exposure to high and low spore concentrations. The progression of necrotic lesions was monitored over a period of 72 hours. Although both spore concentrations exhibited a progressive augmentation in necrotic lesion size with advancing hours post-infection, there was a distinct difference in the rate of increase in necrotic lesions. With higher inoculum load, there was rapid increase in the formation of necrotic lesions within the first phase of infection, i.e. within 24 hours post inoculation. There was almost fifty fold increase in lesion area, then followed by less increase in the subsequent days. In the lower inoculum load ($2X10^6$ spore concentration), the lesion development was slow in the first two days increasing more sharply on the third day post inoculum (Fig.1A).

Initially, a necrotic rim developed surrounding the inoculum spot, while the leaves mostly retained its green colour, indicating the unaltered integrity of leaf cells. At 72 hours post-inoculation, the necrotic lesions gradually expanded, covering a small leaf area of 38 mm^2 **(**Fig.1, A**.-** Middle panel**)**, which is 2.4 times less than the necrotic area in leaves infected with $8X10^6$ spores. On the other hand, from as early as 24 hours after infection,

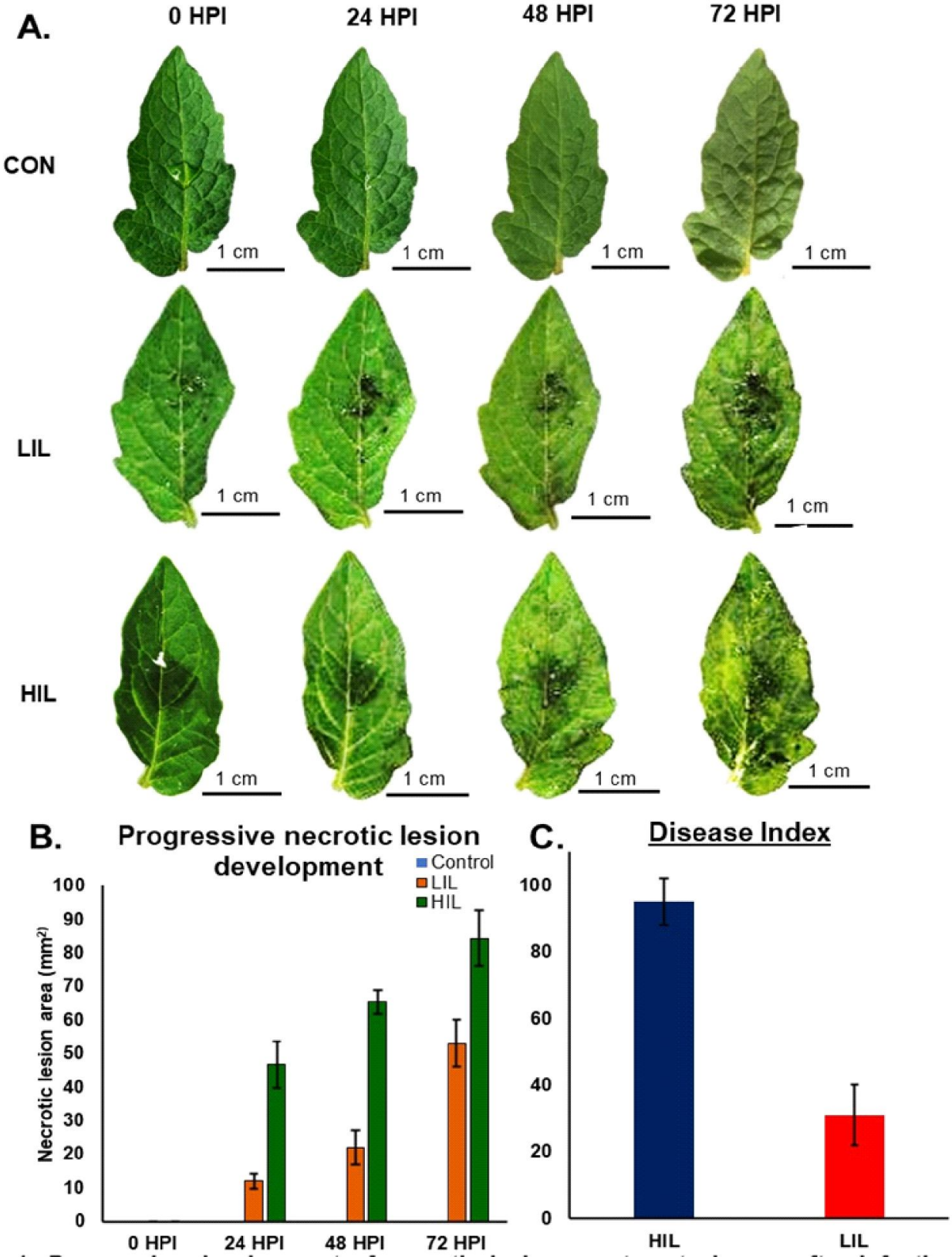

Fig. 1: Progressive development of necrotic lesions on tomato leaves after infection with two different inoculum loads [Higher inoculum load (HIL) 8 x 10^6/ml and lower inoculum load (LIL) 2 x 10^6/ml. A. - Development of necrotic lesion and its gradual increase with increasing hours post infection (HPI) after infection with two different spore concentrations B.-Comparison of areas of necrotic lesions over a time period after infection. C. Disease indices for the two different concentrations of inocula

the manifestation of these lesions was markedly conspicuous in leaves subjected to a higher spore concentration, with necrotic areas developing at various parts of the leaf including the tip and margins (Fig.1A Lower panel). In the latter case, almost the entire leaf underwent necrosis by 72 hours post-inoculation (hpi). Throughout all observed time-points, the percentage of necrotic leaf area remained consistently less in leaves infected with a lower spore concentration which was half that of the necrotic lesion area developed in the leaves with higher spore concentration (Fig.1B).

The calculated disease index corroborated these above observations. The DI was almost three-folds higher in the higher inoculum load compared to the lower inoculum load.

Comparison of development of host symptoms upon infection with different inoculum loads

Whether or not a necrotrophic pathogen will establish itself on or within a host, is dependent upon its ability to induce host cell death (Chowdhury *et al*. 2017a). Being a necrotrophic pathogen, *Alternaria solani* induces the disruption of tissue integrity and a reduction in cell viability. In this study, the outcomes were different for hosts infected with different concentrations of spores. Overall, a progressive decline in cellular viability was observed over successive infection hours for both the leaves (Fig.2 A), however for all time points examined the amount of non-viable cells was more than double in the leaves exposed to high inoculum load as compared to the leaves infected with lower spore concentration (Fig.2B.). Notably, in leaves infected with a higher spore concentration, large and prominent blue patches were evident from as early as 24 hours post-infection and almost 47% of total leaf area was necrosed (Fig.2 A-lower panel)and by the end of the 72-hour exposure period, 91% of the leaf tissue lost its integrity (Fig.2 B). On the other hand, leaves exposed to a lower inoculum generally maintained viability throughout the study duration. Initially, only a limited number of cells lost viability which are represented by small blue dot like patches (Fig. 2A-middle panel). By the end of 72-hrs post inoculation, there was an increase in both the number and size of blue patches, although the magnitude remained significantly lower compared to the leaves exposed to higher spore concentrations at all experimental time points.

Comparison of Scanning Electron Micrographs showing hyphal load on leaf surfaces after infection with different inoculum concentrations

In the preceding experiments, notable differences were observed in both the development of necrotic lesions by *A. solani* and differential extent of symptom development, in the two amounts of inoculum loads. Consequently, the behaviour of the fungal hyphae was next observed under Scanning Electron Microscopy (SEM) to find if there is any correlation between the fungal behaviour with the symptoms on host. Evidently, in leaves infected with $8X10^6$/ml spores, almost half of the leaf surface was covered with the pathogen hyphae and the entire leaf became swiftly enveloped by the fungus within the 72-hr span, indicating rapid establishment of the fungal hyphae (Fig.3 Aii.). On the other hand, in the case of infection with lower spore concentration, even after 72 hrs of infection significantly scantier hyphal growth was observed on the leaf surfaces (Fig. 3 Ai.). Therefore, the percentage of leaf area covered by hyphae was significantly lower, a difference of 25% compared to the leaf inoculated with $8X10^6$/ml spores, across all observed time points (Fig. 3 C).

The progression of plant diseases is dependent on critical determinants, namely the nature of the host, the pathogenicity of the causal organism and favourable environmental conditions (Honjo *et al*. 2020). Previous reports from our laboratory, have revealed that host factors affect disease progression based on differential behaviours of pathogens on the surfaces and within the tissues of resistant and susceptible hosts, leading to differential host responses (Ray *et al*. 2015; Basu *et al*. 2016; Chowdhury *et al*. 2017a). The present study demonstrates that disease manifestation is not solely dependent on the host factors and climatic conditions but is also influenced significantly by how quickly the pathogen can establish itself on the host, which again is dependent on the inoculum load. It is further

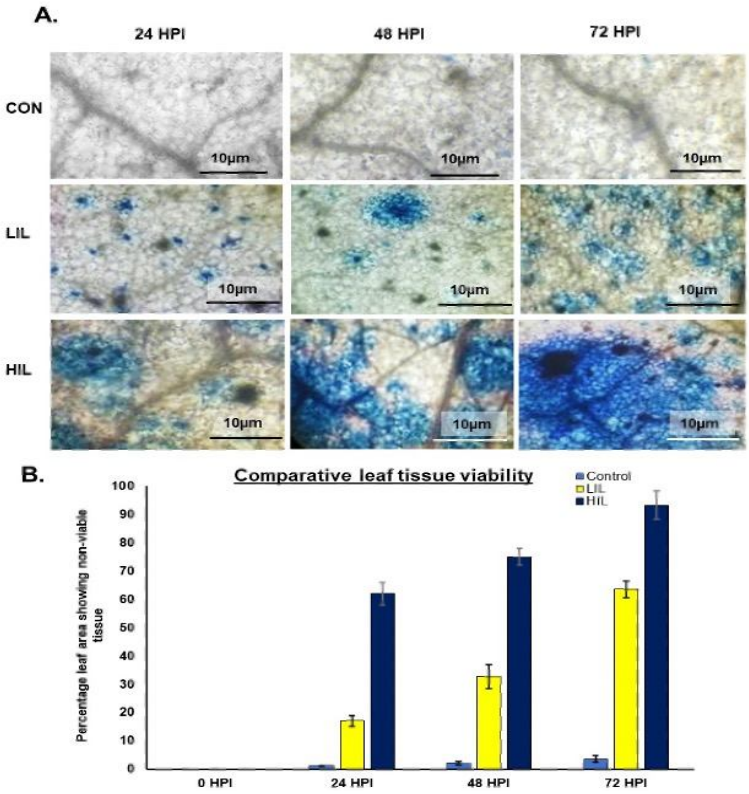

Fig. 2. : Comparison of cell viability in tomato leaves of Pusa Ruby (PR) variety of tomato after infection with *Alternaria solani* spores of two different concentration A. Bright field microscopy showing necrotic areas in the leaf tissues at different time points after infection. B. Comparison of percentage of tissue necrosis at different time points in the leaves for two different concentration of spores.

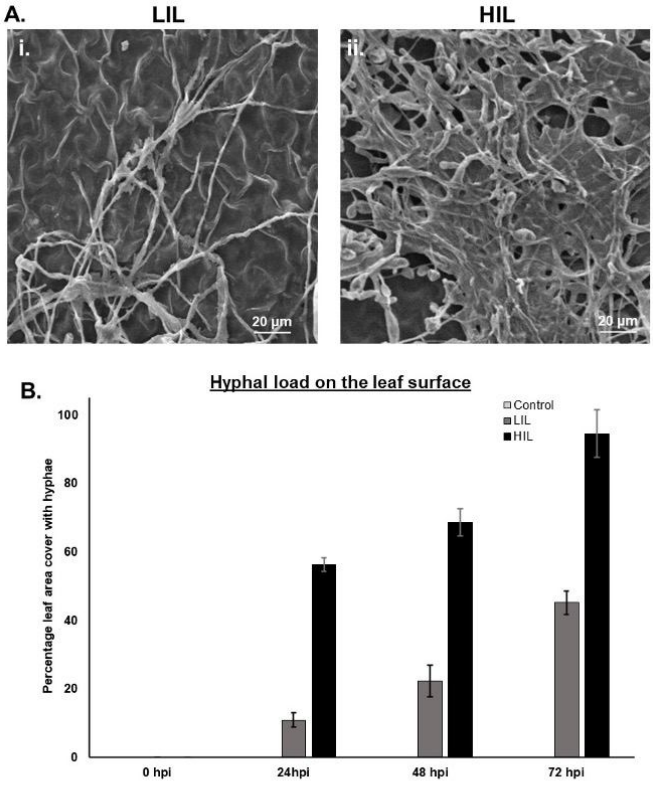

Fig. 3 : Comparison of hypal load under two different inoculum concentration. A. Scanning electron microscope image of tomato leaf surface showing differential hypal load after 72 hours of infection. B. Graphical representation of hypal load at successive time points post infection.

inferred that the severity of disease is directly correlated with the concentration of spores of *A. solani* that is available at the start of the infection. Results of inoculation with higher spore concentration showed robust disease progression and symptoms development within the first day post infection. Then subsequently the progression was less. By the end of the experiment, i.e. by third day, there is almost complete disruption of host tissue integrity. Conversely when the tomato leaves were inoculated with lower spore concentrations, the leaves were less affected even after 2 days of infection. It was only on the third day there was a sharp rise in disease development. The initial disease development brings about a large difference in the final outcome of the disease, which is reflected in the disease index. These observations clearly show that inoculum load has a primary role in determining the disease outcome. There are a few reports that corroborate the present findings. In a previous report by Matthews *et al*. (2023) involving a study with *Fusarium oxysporum* and ginger, the inoculum density was positively associated with symptoms like discoloration of leaves and yellowing of rhizomes; on the other hand, parameters like root length and rhizome weight was negatively associated. Saini *et al*. (2019), reported that in the blue mould rot caused by post-harvest pathogen *Penicillium islandicum* in Indian gooseberry, the inoculum load directly affected disease initiation. There was no disease incidence below a certain inoculum density, beyond which there was progressive increase in disease with increase in inoculum load. Similarly, Navas-Cortés *et al*. (2000) reported that the density of *Fusarium oxysporum* f. sp. *ciceris* chlamydospores per gram of soil affected the disease progression in chick pea cultivars that results in development of wilt symptoms. Due to the pivotal role of inoculum load on disease progression, the assessment of inoculum load in the field is of great importance for crop cultivation. Such studies are essential for assessing the potential threat of a disease at initial stage, facilitating timely interventions to reduce its impact.

ACKNOWLEDGEMENTS

All the authors are thankful to DST-PURSE, UGC-CAS, DST-FIST and DBT Builder for providing facilities and funding for carrying out of the research work. CD, AD and MC are thankful to UGC; MT is thankful to CSIR for fellowships.

DECLARATIONS

Conflict of Interest. Authors declare no conflict of interest.

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