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M.C. Ravikumar, Heminder Singh & R.H. Garampalli

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Comparative evaluation of long-term storage techniques on viability and virulence of *Alternaria solani*

M.C. Ravikumar [1], Heminder Singh [1], R.H. Garampalli *

Department of Studies in Botany, Manasagangotri, University of Mysore, Mysuru 570006, India

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Abstract

The study was conducted to test the viability and pathogenicity of different forms of *Alternaria solani* inoculum stored at different conditions and time intervals for a period of 16 months. The aim was to confirm the survival period of pathogen in soil and to determine the simplest method to maintain inoculum in laboratory for further studies. The cultures stored at 5 °C for 2, 4, 8 and 16 months were pathogenic, while cultures stored at room temperature turned non-pathogenic after 4 months. All 6 successive sub-cultures retained viability and virulence. All the target-spots stored at 5 °C were viable after all time intervals as tested by culturing and subsequent spray of harvested mycelial suspension, but at the room temperature (RT), 16 months old inoculum lost viability and virulence. The powdered tissue samples of target-spots stored at 5 °C were found pathogenic by spray method after all the intervals of the time but, at RT 16 month old sample lost virulence. In inoculation by soil splashing, powdered tissue stored in paper bag at 5 °C retained pathogenicity till 8 months, while at RT, the inoculum stored in paper bags and the inoculum mixed in soil lost pathogenicity after 4 months.

Keywords: *Alternaria solani*; Paper bag; Room temperature; Viability; Virulence; PEBI

1. Introduction

Tomato (*Lycopersicon esculentum* Mill), one of the most remunerable and widely grown commercial vegetable crops in the world, belongs to the family Solanaceae. The phyto-pathogenic fungus *Alternaria solani* causes early blight of tomato which is one of the most catastrophic diseases of the world causing heavy economic loss to tomato growers [1]. Due to its broad host range, extreme variability in pathogenic isolates was observed and due to prolonged active phase of the disease cycle, it is very difficult to manage the disease [2–6]. *A. solani* has the ability to infect tomato and potato plants under both dry and wet conditions [7] and it can grow over a wide range of temperatures (4–36 °C) [8]. In field conditions, the inoculum survives as conidia, chlamydospores and mycelium on plant debris for several months and acts as primary source of infection for next season crops [9–14]. *A. solani* (Mysore pathovar)

* Corresponding author. Tel.: +91 821 2419757/8; fax: +91 8212419759.
E-mail addresses: mcruom@gmail.com (M.C. Ravikumar), rednimeh75@gmail.com (H. Singh), rajkumarhg@gmail.com (R.H. Garampalli).
[1] Tel.: +91 821 2419758.
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lack sporulation ability on artificial medium compelling to conduct greenhouse experiments by spraying mycelial suspension [2,15]. Therefore, the present work was carried out to test the viability and pathogenicity of the different forms of inoculum by storing in different environmental conditions at different time intervals with the twin objectives of confirming the survival period of pathogen in soil similar to field conditions and to determine the suitable methods to maintain viability of inoculum for long term storage in laboratory conditions.

2. Materials and methods

2.1. Collection and storage of inoculum

The source of inoculum was from the target spots infected leaves and petioles of tomato plants from the fields of Krishnarajanagara Taluk of Mysore District in Karnataka state, India during the month of October–November 2012. Infected part of the leaves, petioles and stems were cut by scissor and shade dried. Part of the collected diseased sample was used to culture the causal organism on Potato Dextrose Agar (PDA) in Petri plates as described below in Section 2.2.1. The rest of the inoculum samples were stored in 3 different forms simulating the probable survival of inoculums in the natural environmental condition. Firstly, the sample was stored in the form of dried infected target spots, secondly, in the form of powdered target spots by grinding the sample to fine powder. In third method, the causal organism was stored in the form of mycelial culture obtained from infected parts of the target spot. All the samples were stored at 2 different environmental conditions at 5 °C and room temperature (RT). The viability and virulence of the pathogen was assessed at 4 different time periods starting from 0 day for fresh inoculum and 4 months, 8 months and 16 months for dried and powdered inoculum by spray and splashing inoculation method. The culture obtained after inoculation on PDA was also stored at different environmental condition as stated above. The 7–10 days old cultures were considered as fresh culture and other cultures were stored for 2, 4, 6 and 8 months successively under two different condition. Further, the *A. solani* culture was sub cultured consecutively for six times to assess the virulence of the pathogen after successive subculture. The inoculum was tested for its viability by culturing on PDA in Petri plates and the pathogenicity was confirmed by 2 different inoculation methods as described below.

2.2. Viability and virulence test of inoculum

Inoculum samples were tested for viability and virulence by four methods as mentioned below.

2.2.1. In vitro *viability test and* in vivo *pathogenicity test of target spot cultures*

The inoculum samples were stored at two different temperature, 5 °C and RT for different time intervals; 2nd day after inoculum collection, 4 months, 8 months and 16 months. The 'target spots' on infected leaves and petiole samples of tomato plants stored at both 5 °C and at RT were surface sterilized with 70% ethanol, 0.1% mercuric chloride, and washed with double distilled water 5–6 times and cultured on PDA to isolate the pathogen. All the samples were tested for their viability by culturing on PDA media *in vitro* condition. The plates were incubated for 7 days at 22 °C under 12HL:12HD conditions. The percent viability of target-spots was recorded as percentage of target-spots producing pathogen colony in Petri plates (Table 1). Cultured fungus was observed under calibrated microscope and identified based on morphological characters of mycelium.

Table 1
Tests for viability of target-spot tissues (leaf and petiole) stored at 5 °C and RT[a] (by culturing on PDA media).

Storage period	Storage condition	Total no. of target spots cultured/plate		Average percentage of viability	
		Leaf spots	Petiole spots	Leaf spots (%)	Petiole spots (%)
The day after inoculum collection	–	10	6	100 ± 0	100 ± 0.0
After 4 months	5 °C	10	6	93.4 ± 3.3	94.4 ± 5.5
	RT	10	6	86.7 ± 3.3	88.6 ± 5.5
After 8 months	5 °C	10	6	76.7 ± 8.8	77.7 ± 5.5
	RT	10	6	50.0 ± 5.7	55.5 ± 5.5
After 16 months	5 °C	10	6	63.4 ± 8.8	50.0 ± 9.5
	RT	10	6	0.0	0.0

[a] RT = room temperature.

Mycelial suspension of cultures was tested for their pathogenicity by spray method in greenhouse. Petri plate containing 9 days old culture was flooded with sterilized double distilled water and gently scraped with spatula. The suspension thus obtained was collected in 30 mL test tube and vertexed to make small segments of the mycelium. Each mycelium segment was considered as single spore as it formed individual colony. This mycelia suspension was standardized using heamocytometer to 10,000 mycelial CFU/mL. Tween-twenty (1 drop/L) was added as an adjuvant. Pots containing one-month old plants (6 plants/pot) were used for treatment. All the treatments were maintained in triplicate. Control plants were sprayed with 30 mL DW/pot. Pots were covered with transparent polythene bags to maintain moisture for 24 h. Sufficient moisture was maintained after 24 h by using wet cloths. Symptoms started appearing after 2–3 days of inoculation as necrotic spots. After 15 days of plant growth from all the treatments, leaf showing target spots symptoms were excised and placed on PDA medium to re-isolate, culture and observe the spores under calibrated compound microscope to confirm the pathogen. All the experiment, both for viability and pathogenicity tests, were conducted twice for each storage period and mean of the readings was used for calculations.

2.2.2. Target-spot powder pathogenicity tests by spray and splashing methods

The 'target-spots' of infected portions of tomato plants stored at 5 °C and RT for different intervals of time were tested for their virulence by spray method. The 'target-spot' tissue from the infected tomato leaves was grind into fine powder by mortar and pestle. This fine inoculum powder (250 mg/treatment) was dissolved in distilled water, shaken well and filtered by using muslin cloth to block the entry of debris. Distilled water containing inoculum was poured into test tube; volume was made up to 100 mL. Control consisted only 100 mL distilled water. Basic experiment design remained similar to Section 2.2.1.

The inoculum samples stored in paper bags at 5 °C and RT were tested for their pathogenicity by splashing method by mixing 250 mg of powdered target-spot leaf tissue in top 5 cm layer of burlap bag soil. Six-month old plants raised in poly-house were transplanted into each burlap bag with 30 cm diameter. Watering was done twice a day for 2 days by sprinkling from top of the plants to simulate splashing effect. Basic experiment design remained similar to Section 2.2.1.

2.2.3. Pathogenicity tests in target-spot powder mixed soil by splashing method

The target-spot powder samples were mixed in the soil at the rate of 250 mg inoculum in 100 g soil and stored in poly-house with occasional watering (simulating virtual-field conditions). The tests were conducted by mixing the inoculum in top 5 cm layer of burlap bag soil and transplanting the poly-house raised 1-month old plant. Rest of the experiment was maintained according to splashing test as mentioned in Section 2.2.2.

2.2.4. Viability and pathogenicity test of cultures and sub-cultures

Pathogen cultures isolated from the infected tomato leaves on PDA were maintained in the laboratory under different conditions; at 5 °C and RT for different durations of time viz. fresh (7–10 days old cultures), 2 months, 4 months, 8 months and 16 months. The pathogenicity tests were conducted in poly-house condition by spraying mycelial suspension as mentioned in Section 2.2.1. Further, the virulence was tested for successive six subcultures at 10,000 mycelial CFU/mL in poly-house condition by spray method.

2.3. Calculation of disease index

Evaluation of early blight symptoms was done after 9 days of inoculation. Early blight severity on each tested tomato plant leaf was recorded on a scale of 0–5, where 0 = no visible lesions on leaf; 1 = up to 10% leaf area affected; 2 = 11–25%; 3 = 26–50%; 4 = 51–75% and 5 = more than 75% leaf area affected [16]. Percentage of early blight index (PEBI) for each plant was calculated from disease scale by using the following formula [15].

$$\text{PEBI} = \frac{\text{Sum of all ratings}}{\text{No. of sampled leaves} \times \text{maximumdiseasescale}} \times 100$$

2.4. Statistical analysis

Data were subjected to one-way analysis of variance (ANOVA), followed by Tukey's post-test at $p \leq 0.05$ level of significance using Graphpad prism 5 software.

3. Results

The experimental results revealed that, the fresh leaf and petiole target-spot tissue samples showed 100% viability (fungal growth) on PDA culture plates and the mycelia suspensions culture showed virulence in poly-house experiments (Table 1). While, all samples generally showed gradual decline in percent viability as

Table 2
Tests of viability and pathogenicity of stored (at 5 °C and RT[a]) target spottissues, cultures and successive sub cultures by spray and splashing methods.

Inoculum type	Test	Method	Temperature									
			5 °C					RT				
			0 Day	2 M	4 M	8 M	16 M	0 Day	2 M	4 M	8 M	16 M
Target spot	Viability	Culturing	+		+	+	+	+		+	+	−
	Pathogenicity	Spray of culture suspension from target spots	+		+	+	+	+		+	+	−
Target spot powder	Pathogenicity	Spray of powder suspension	+		+	+	+	+		+	+	−
	Pathogenicity	Powder splashing (from soil)	+		+	+	−	+		+	−	−
Target spot powder in soil	Pathogenicity	Powder splashing (from soil)						+		+	+	−
Stored cultures	Viability and Pathogenicity	Suspension spray	+	+	+	+	+	+	+	−	−	−
Successive subcultures	Viability and Pathogenicity	I subculture suspension spray						+				
	Viability and Pathogenicity	II subculture suspension spray						+				
	Viability and Pathogenicity	III subculture suspension spray						+				
	Viability and Pathogenicity	IV subculture suspension spray						+				
	Viability and Pathogenicity	V subculture suspension spray						+				
	Viability and Pathogenicity	VI subculture suspension spray						+				

[a] RT = room temperature; + = positive result in viability/pathogenicity test; − = negative result in viability/pathogenicity test; empty column indicates experiment not done.

the storage time increased from 4 months to 16 months. The decline was more rapid in case of samples stored at room temperature where, the viability dropped to 0% at 16 months storage time. Whereas, the samples stored at 5 °C, 63.4% of leaf-spot tissue samples and 50% of petiole tissue samples remained viable.

The results of spray and splashing of powdered target-spot tissue showed that the samples which stored at 5 °C remained virulent during all the time intervals; fresh tissue sample and after 4, 8, and 16 months of storage time and caused initiation of early blight symptoms as early as 36 h after inoculation. In the sample stored at RT treatment, virulence was observed only until 8 months storage of the leaf-spot tissue and subsequently lost its virulence when tested after 16 months. For splashing method inoculation, the ability to cause disease in the stored tissue sample lasted for 8 months at 5 °C storage and only 4 months at RT storage. The experiment conducted to test the survival period of pathogen in soil by mixing and storing the powdered leaf-spot tissue in soil revealed that, the inoculum was virulent enough to cause early blight symptoms on tomato plants in the case of fresh sample and also by the sample stored for 4 and 8 months. However, the virulence was lost in the sample stored for 16 months (Tables 2 and 3).

The results of the experiments carried out to test the pathogenicity of the pure pathogen cultures which were stored at 5 °C and RT for different time intervals; fresh (7–10 days old) culture, 2 months, 4 months, 8 months and 16 months old culture showed that, the cultures stored at 5°C were retained virulence after all the time intervals. Whereas, in case of the culture stored at RT, the virulence was observed only in fresh (7–10 days old) culture and 2 months old cultures and lost the virulence in 4 months, 8 months and 16 months old cultures (Table 1). The successive subcultures obtained from the first fresh culture till subsequent six subcultures

Table 3
Percentage of early blight index (PEBI).

Inoculum type	Pathogenicity test	Temperature									
		5 °C					RT[a]				
		0 Day	2 M	4 M	8 M	16 M	0 Day	2 M	4 M	8 M	16 M
Target spot	Culture suspension spray	85.1 ± 1.1***		81.6 ± 2.0***	79.8 ± 1.3***	73.2 ± 1.3***	86.7 ± 1.8***		79.2 ± 2.3***	72.4 ± 2.4***	
Target spot powder	Spray	82.5 ± 2.6***		77.5 ± 4.2***	74.6 ± 3.7***	69.4 ± 3.2***	82.9 ± 1.9***		70.3 ± 2.5***	61.9 ± 1.9***	
	Splashing	80.7 ± 2.2***		73.5 ± 3.8***	61.6 ± 3.2***	–	79.6 ± 2.2***		60.1 ± 2.0***	-	
Target spot powder in soil	Splashing						81.5 ± 2.1***		70.4 ± 2.1***	60.3 ± 1.6***	
Stored cultures	Suspension spray	83.4 ± 2.2***	82.7 ± 1.8***	81.3 ± 2.0***	80.9 ± 2.2***	78.6 ± 2.0***	82.5 ± 2.1***	79.2 ± 1.2***			
Successive subcultures	I subculture spray						85.5 ± 2.6***				
	II subculture spray						85.6 ± 2.9***				
	III subculture spray						81.3 ± 2.2***				
	IV subculture spray						84.0 ± 2.7***				
	V subculture spray						81.7 ± 1.8***				
	VI subculture spray						79.3 ± 2.1***				

[a] RT = room temperature.
*** Significance level at $p \leq 0.05$; Empty column indicates experiment not done.

retained the pathogenicity and were able to cause disease (Tables 2 and 3).

4. Discussion

A. solani, the causal agent of early blight on tomato, potato and other Solanaceous crops [4], has the ability to persist in the crop field for several months [11] to initiate infection [12]. In the present study, the pathogen samples stored in different forms, for different intervals of time were evaluated for their viability and pathogenicity. The pathogen failed to sporulate on PDA but, when sprayed as mycelial suspension, it was able to initiate early blight symptoms on tomato plants [2,15]. Our results corroborates with the earlier report, where viability and pathogenicity of *Alternaria carthami* was tested by keeping the diseased leaves in between the folds of blotting paper and found that the conidia of the pathogen was viable and virulent after 4 months and retained viability and virulence after 5 months as mycelium [13]. Studies also proved that, diseased plant debris stored under laboratory, refrigerator and in glass house condition for about 180 days and recovered at regular intervals of 20 days by isolation on media lost their percentage of recovery gradually [14].

The fresh culture and the successive subcultures were retained viability and pathogenic in the present study till six successive cultures. This was in contradiction to the earlier report that, all forms of inoculum stored at RT gradually lost the viability and pathogenicity [14]. The present study showed that, viability and pathogenicity of the inoculums, as target-spots powdered tissue or on culture plates stored at RT, revealed reduced pathogenicity more rapidly over time when compared to samples stored at 5 °C. All the subsequent cultures were virulent, which indicate that viability always associated with pathogenicity. The sample stored at 5 °C was found to be most effective method of inoculum storage than storing at RT. The pathogen cultures may be maintained at room temperature on PDA by repeated sub-culturing. In the present study, the most interesting observation came from comparison of room temperature storage of target-spots powdered tissue and pathogen culture on PDA. The target-spots powdered tissue retain pathogenicity for 8 months, cultures on PDA turn non-pathogenic beyond 2 month of storage. Thus, storage of inoculum as target-spot powdered tissue provides higher longevity than cultures stored on PDA at room temperature. It may also be found true for storage at 5 °C if the experiment is extended beyond 16 months. Therefore, it is concluded that among the simple storage techniques tested in the present experiment, storage as target-spots powdered tissue is the simplest and most effective method to maintain inoculum in laboratory with or without refrigerator facility. The study also confirms that the pathogen can survive in soil and on plant debris for up to 8 months which will serve as inoculums to initiate disease for next season crop. The present study results showed that, the pathogen is unable to cause disease after 16 months storage in soil, it may be suitable to go for crop rotation by giving a time interval of 1–2 years for effective management of early blight disease of tomato.

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