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Validation of Screening Technique for Cotton Bacterial Blight Resistance under Controlled Condition

A. Sampath Kumar[1*], K. Eraivan Arutkani Aiyanathan[2], S. Nakkeeran[1] and S. Manickam[3]

[1]Department of Plant Pathology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, Coimbatore, 641003, India.
[2]Agricultural College and Research Institute, Tamil Nadu Agricultural University, Killikulum, Vallanadu, 628 252, India.
[3]ICAR-Central Institute for Cotton Research Regional Station, Maruthamalai Road, Coimbatore, 641003, India.

Authors' contributions

This work was part of PhD of thesis submitted by AS to Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore. All authors read and approved the final manuscript.

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ABSTRACT

Seven different methods of artificial inoculation such as 1. Carborundum injury, 2. Pin prick injury 3. Sand paper injury, 4. Syringe inoculation on lower surface of leaf without needle, 5. Syringe inoculation of veins on lower surface of leaf with needle, 6. Tooth picks inoculation on collar region and 7. Pressurized spray inoculation were evaluated to find out the efficient and precise screening method for cotton bacterial blight caused by *Xanthomonas citri* pv. *malvacearum* under controlled conditions (plant growth chamber). Inoculated seedlings were incubated at 28°C, 90% RH and 3000 LUX light intensity during day time and 22°C, 90% RH and absence of light during night time for symptom development. Among them, pin prick injury recorded maximum PDI (64.25) in 20-24 days post inoculation followed by sand paper injury (56.50 PDI) in 23-27 days post inoculation on 20 day

**Corresponding author: E-mail: sampath000@gmail.com;*

old LRA 5166 cotton seedlings compared to other methods. Both these methods developed all types of symptoms. Initial symptom of water soaked lesion was appeared in 7-8 days post inoculation in pin prick injury while it was 9-10 days in sand paper injury.

Keywords: Artificial inoculation methods; screening technique; cotton; Xanthomonas citri pv. *malvacearum.*

1. INTRODUCTION

Cotton the "white gold" contributes to 35.0% of the global fabric needs and 60.0% of clothing in India [1]. More than 10.0 million farmers are cultivating cotton and 30 million people are engaged in cotton related activities in India. It is cultivated around 12.58 million hectares (2019-20) with a production of 360 lakh bales and productivity of 486.33 kg/ha [2]. India is the only country in the world that cultivates all four cultivable *Gossypium* species namely *Gossypium arboreum* and *G. herbaceum* (Asian cotton), *G. barbadense* (Egyptian cotton) and *G. hirsutum* (American upland cotton) besides hybrid cotton [3]. Eleven states in India are growing cotton with 40% irrigated and 60% rainfed conditions. Among the cotton diseases, bacterial blight caused by *Xanthomonas citri* pv. *malvacearum* is a major disease prevailing entire cotton growing regions of India. The disease occurs almost all cotton growing areas of the world. Yield losses have been estimated from 10 to 30% and may exceed 50% in Asia and Africa [4]. Cotton bacterial blight caused by *X. citri* pv. *malvacearum* was the most widespread and destructive disease causing yield losses ranging from 5 to 35% [5]. Cotton fields infected with bacterial blight have been found to show as much as 80% yield loss in certain areas [6]. In India, bacterial blight of cotton has been recorded in all cotton-growing regions every year with 30% yield loss caused by different *Xcm* races [7]. Out of 25 diseases known to occur in cotton, the bacterial blight is the most wide spread and destructive disease reported to cause yield losses of about 10 – 30 per cent [8]. Jagtap et al. [9] conducted survey on cotton bacterial blight incidence in Marathwada region of Maharashtra and found the average PDI of 51.12 per cent among six districts surveyed. They recorded highest disease incidence in Parbhani district (67%) followed by Hingoli (63%), Nanded (58%) and Latur (54%). The lowest was recorded in Jalna district (36%).

It is essential to screen large number of germplasm lines to identify resistance sources. Efficient and precise screening methods are mandatory to identify the resistance sources as well as speed up the breeding programmes to deploy resistance under field conditions. Though several methods are employed in artificial screening, the precise screening method is the need of the hour. Relative humidity and temperature are crucial parameters for infection of bacterial pathogen in plants. By considering all the above points, the study was conducted to find out the best method for precise inoculation of *X. citri* pv. *malvacearum* in cotton seedlings and efficient screening of cotton genotypes for identification of disease resistance sources.

2. MATERIALS AND METHODS

2.1 Pathogen Isolation and Identification

Cotton bacterial blight pathogen, *X. citri* pv. *malvacearum* was isolated from field collected samples showing typical bacterial blight symptoms. Pathogen was isolated on nutrient agar medium through streaking of ooze out from infected tissues collected in sterile water. The colonies appeared on Nutrient Agar (NA) medium 72 h after incubation. Isolated cultures grow well on NA medium and produced dull to pale yellow, round, convex and mucoid colonies with glistening. Pathogen was confirmed through morphological and molecular techniques using PCR amplification of housekeeping genes. Pathogenicity was proved on susceptible cultivar LRA5166.

2.2 Inoculum Preparation

Highly virulent isolate MNSu isolated from Nagpur region of Maharashtra was used for artificial inoculation purposes. The *X. citri* pv. *malvacearum* pathogen pellet was obtained by centrifugation of 24 hours old culture multiplied in nutrient broth. The bacterial cells were dispersed in sterile distilled water and inoculated on 20 days old LRA 5166 cotton seedlings raised in pro trays using coco pith as growing medium by different inoculation methods. The concentration of pathogen inoculums was adjusted to $2x10^9$ CFU/ml.

2.3 Methods of Inoculation

2.3.1 Carborundum injury

Carborundum powder (400 mesh size) was sprinkled over the cotton leaves and swabbed using fingers to create mild pore or damage to leaves. Then pathogen was sprayed over the leaves [10].

2.3.2 Pin prick injury

Pin prick injury was made on cotton leaves using fine and sterilized needles for mild damage to leaves [11] and the pathogen was inoculated by spraying over the leaves (Fig. 1e).

2.3.3 Sand paper injury

The cotton leaves were gently pressed in both the surfaces using the sand paper (60 mesh) folds to cause mild injury to leaves [12] Inoculum was sprayed over the leaves (Fig. 1d).

2.3.4 Syringe inoculation on lower surface of leaf without needle

Sterile hypodermic syringe (24G X1", 0.55 x 25 mm -2.5 ml size) without needle was used. Pathogen inoculums was drawn in the barrel through needle hub and inoculated on leaf lamina through gentle pressure [13] on lower surface of the leaves (Fig. 1b).

2.3.5 Syringe inoculation of veins on lower surface of leaf with needle

Sterile hypodermic syringe (24G X1", 0.55 x 25 mm -2.5 ml size) with needle was used for inoculation. Pathogen inoculums was drawn through needle and inoculated into veins present in the lower surface of leaves [14] through needle injection (Fig. 1c).

2.3.6 Tooth picks inoculation on collar region of the seedlings

The pathogen was inoculated using toothpicks as per the procedure of Thaxton and El-Zik [15] with slight modifications. Toothpicks were dipped in the inoculums and the sharp tip was used to pierce the collar region of the cotton seedlings (Fig. 1f).

2.3.7 Pressurized spray inoculation

Atomized spray with high pressure was used to spray the inoculums over the seedlings up to saturation [16].

The inoculated seedlings were covered with polythene bag for 24 hours to create moisture in order to facilitate the bacterial infection in the leaves. Seedlings were incubated in plant growth chamber at 28°C, 90% RH and 3000 LUX light intensity during day time and 22°C, 90% RH and absence of light during night time (Labtech - LGC 5101, Daihan Labtech India Pvt. Ltd) in the Department of Plant Pathology (Fig. 1a) for symptom expression. Plants were examined for the appearance of lesions from 5 to 35 days of post- inoculation. Three replications were maintained for each method of inoculation. The Percent Disease Index (PDI) was calculated according to Sheoraj [17] using 0 to 4 disease rating scale

$$\text{Per cent Disease Index (PDI)} = \frac{\text{Sum of all numerical ratings}}{\text{Total number of leaves observed}} \times \frac{100}{\text{Maximum grade}}$$

Fig. 1a. Incubation of *X. citri* pv. *malvacearum* inoculated LRA 5166 cotton seedlings in plant growth chamber for symptom development

Fig. 1b. Syringe inoculation of pathogen without needle on lower surface of LRA 5166 seedling

Fig. 1c. Syringe inoculation of pathogen with needle on lower surface of leaves of LRA 5166 seedlings

Fig. 1d. Sand paper injury on both surface of leaves of LRA 5166 seedlings

Fig. 1e. Pin prick injury on upper surface of leaves of LRA 5166 seedlings

Fig. 1f. Tooth picks inoculation of pathogen on collar region of LRA 5166 seedlings

3. RESULTS AND DISCUSSION

The breeding for disease resistance is the highly significant method and long term solution for disease resistance. Precise and rapid artificial screening method is the core activity in resistance breeding. In Australia, resistance to bacterial blight is a mandate for all commercial cotton varieties [18]. In the present experiment, significant differences were observed among various inoculation methods for symptom expression and Percent disease index (PDI) of cotton bacterial blight (Table 1). Among them, pin prick injury recorded maximum PDI (64.25) in 20-24 days post inoculation followed by sand paper injury (56.50 PDI) in 23-27 days post inoculation compared to other methods. Both these methods developed all types of symptoms like angular leaf spot, petiole blight, vein blight and black arm (Fig. 2). Initial symptom of water soaked lesion was appeared in 7-8 days in pin prick injury while it was 9-10 days in sand paper injury. Syringe inoculation with (33.50 PDI) or without (41.60 PDI) needle developed medium range of disease incidence and produced angular leaf spot, petiole blight and vein blight symptoms only. Black arm symptom did not appear in these methods. Initial symptoms appeared in 10-14 days and advanced symptoms in 24-30 days. Though tooth pick inoculation developed all types of symptoms, the PDI (32.75) was low and time taken for initial (10-14 days) and advanced (26-30 days) symptom expression was bit late. Pressurized spray inoculation developed lowest PDI (25.10) and time taken for initial (18-20) and advanced (34-37) symptom expression was too long. Carborundum injury caused less disease incidence (27.50 PDI) with fewer symptoms and delayed expression.

Other workers have also tried different methods and reported. High-pressure sprayers and carborundrum as an abrasive material were used to deliver the plant pathogenic bacteria into the plants for pathogenicity and virulence testing [10,16]. Salah Eddin et al. [12] tested eight different methods of artificial inoculation on 30 days old susceptible cotton hybrid TCB 209. They reported that sandpaper inoculation method recorded the maximum disease incidence of 85 per cent followed by pressurized spraying (75 per cent) and hypodermic syringe (70 per cent) methods. Symptoms were expressed 15-21 days after inoculation. Our study revealed that, pin prick injury performed well followed by sand paper injury. But pressurized spray recorded lowest PDI. Alexander [19] used the tooth picks dipped with bacterial inoculum to scratch the lower surface of leaves of cotton seedlings for artificial inoculation. This study supported the requirement minor injuries for bacterial infection. Dizon and Reyes [10] mixed the carborundum along with the bacterial suspension of *X. citri* pv. *malvacerum* and sprayed over the cotton plants for pathogenicity and virulence study. Pkania [20] used the syringe infiltration method for pathogenicity study of *X. citri* pv. *malvacearum* in cotton without needle. He inoculated the pathogen on lower surface of the leaves with gentle pressure. Likewise, Mahmood and Hussain [21] found that hypodermic inoculation was performing well to screen cotton against bacterial blight compared to other methods like scratching, hand rubbing or spraying. Kangatharalingam et al. [22] developed a technique to inoculate cotton leaves uniformly and gently in the internal phyllosphere from the upper surface. They designed a custom-made inoculation apparatus to immerse a circular area

of the adaxial surface of a leaf in inoculum for 90 seconds. In susceptible leaves, uniformly distributed water-soaked spots were observed 7 to 8 days after inoculation. On contrary to our results, Sharma et al. [23] evaluated nine different inoculation methods for *X. axonopodis* pv. *punicae* on pomegranate, and reported that spraying of inoculum on whole plant without pin prick injury yielded highly reproducible symptoms with disease incidence of 71.0% and severity of 55.5% within 21 days under optimal temperature and humidity conditions and up to 39 days under less favourable conditions. The symptoms mimicked the natural symptoms observed in orchards.

Our results were supported by Patel et al. [24], where they have inoculated Pseudomonas fuscovaginae in rice plants with pin prick injury for artificial inoculation to identify the virulence loci in the bacteria. Manmohan et al. [25] tried four different inoculation techniques for bacterial leaf streak disease of maize caused by Acidovorax avenae subsp. avenae. They found that whorl prick inoculation method was the most effective in producing disease symptoms followed by pin prick inoculation. Injury caused during pin prick method might have facilitated the quick entry of pathogen in the plants and faster expression of symptoms compared to all other methods in the present study. In general the bacterial pathogens in plants usually spread more during the monsoon period. The heavy wind coupled with rain splash effectively transmits the bacterium and spreads the disease. Wind aberrations causes minor injuries to plant parts through scratches and rain splashes helps to infection of bacterium in passive way. In conclusion pin prick injury followed by spray inoculation was found to be the efficient and best method for artificial screening as it developed maximum PDI with all types of symptoms including early expression of initial and advanced symptoms.

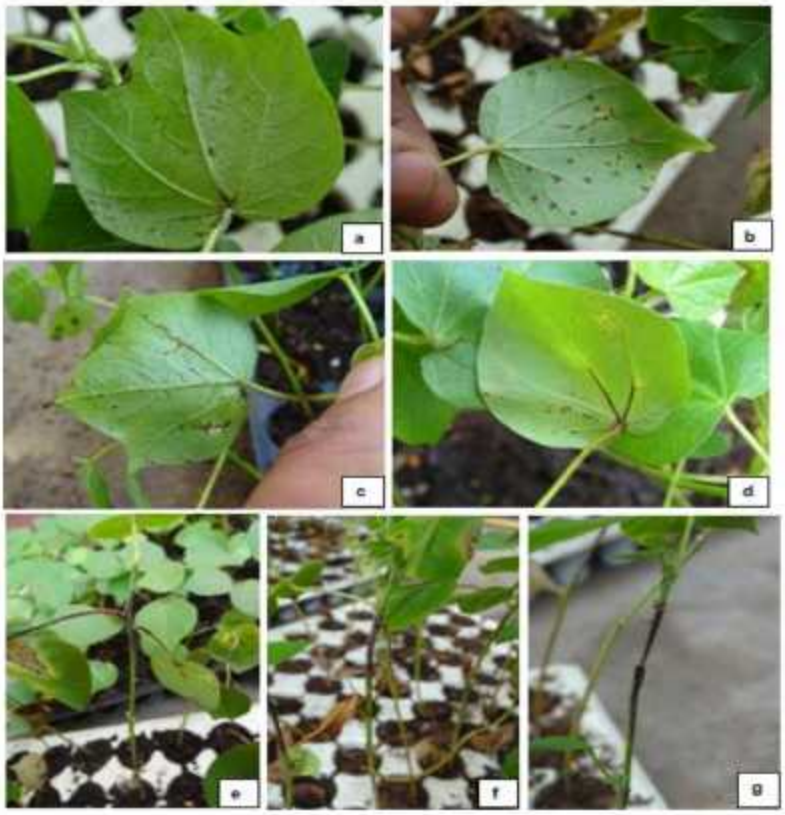

Fig. 2. Various types of symptom development in LRA 5166 seedlings inoculated with *X. citri* pv. *malvacearum* using different inoculation methods a) Water soaked lesions on lower surface of leaf b) Angular leaf spot on lower surface of leaf c) and d) Vein blight symptoms on lower surface of the leaves e) Petiole blight symptom f) and g) Black arm or stem blight symptoms

Table 1. Influence of different types of inoculation method on days taken for symptom expression after inoculation, types of symptoms observed and Percent disease index (PDI)

S.no	Inoculation method	Days taken for initial symptom expression	Days taken for advanced symptom expression	Types of symptom observed	Percent Disease Index (PDI)
1	Carborundum injury	14-16	26-30	ALS	24.50
2	Pin prick injury	7-8	20-24	ALS, PB, VB, BA	64.25
3	Sand paper injury	9-10	23-27	ALS, PB, VB, BA	56.50
4	Syringe inoculation on lower surface of leaf without needle	10-12	24-28	ALS, PB, VB	41.60
5	Syringe inoculation of veins on lower surface of leaf with needle	12-14	26-30	ALS, VB	33.50
6	Tooth picks inoculation on collar region of the seedlings	12-14	26-30	ALS, PB, VB, BA	32.75
7	Pressurized spray inoculation	18-20	34-37	ALS, VB	25.10
8	Absolute control (Water spray)	-	-	-	-

ALS-Angular leaf spot; PB-Petiole blight; VB-Vein blight; BA-Black arm

4. CONCLUSION

Among seven different inoculation methods, pinpricking of leaves followed by spray inoculation was found to be very effective in screening of cotton seedlings against bacterial blight under controlled conditions with 28°C, 90% RH and 3000 LUX light intensity during day time and 22°C, 90% RH and absence of light during night time in plant growth chamber.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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