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Standardization of methods for pathogenicity of pomegranate bacterial blight caused by *Xanthomonas axonopodis* pv. *punicae*

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Abstract
The pathogenicity test of *Xanthomonas axonopodis* pv. *punicae* was performed with three different methods viz. Pin-prick method, Infiltration method and Spray inoculation method. Data revealed that amongst three different methods, infiltration method was found most suitable for development of symptoms which had taken 7 days for symptoms development. This method was followed by pin prick and spray inoculation method method which had taken 12 and 15.87 days, respectively for initiation of symptoms. Morphological characters of different *Xap* isolates were studied on nutrient agar media. Five isolates collected from different locations differed in respect of size of colony, shape of colony and colour of bacterial colony. Isolates *Xap*-1 and *Xap*-4 developed medium to large bacterial colonies. The isolates *Xap*-1 and *Xap*-3 developed small to medium sized bacterial colonies, whereas, *Xap*-5 produced small bacterial colonies. *Xap*-1 and *Xap*-3 produced small circular colonies, whereas, *Xap*-2 and *Xap*-4 produced circular to irregular bacterial colonies and *Xap*-5 produced circular bacterial colonies. *Xap*-1 and *Xap*-4 showed yellowish bacterial colonies, whereas, *Xap*-2 and *Xap*-5 showed light yellow coloured colonies and *Xap*-3 showed yellow coloured bacterial colonies.

Keywords: Pomegranate, bacterial blight, pathogenicity

Introduction
Pomegranate (*Punica granatum* L.) is a favourite table fruit in tropical and sub-tropical regions of the world which belongs to family *Punicae*. Pomegranate is being cultivated on an area of 193 thousand ha in India with production of 2198 thousand metric tonnes (Anonymous, 2016). Bacterial blight caused by *Xanthomonas axonopodis* pv. *punicae* is one of the most important disease of Pomegranate. Bacterial blight infection results in appearance of water soaked oily spot symptoms on leaves, stems and fruits which consequently decreases fruit production and market value. Considering the importance of disease the present studies were planned to standardize different methods for pathogenicity of bacterial blight caused by *Xanthomonas axonopodis* pv. *punicae.*

Material and Methods
An experiment was conducted in the department of plant pathology, College of Agriculture, Latur to proving the pathogenicity test with different methods and morphological characterization of *Xanthomonas axonopodis* pv. *punicae.*

Isolation and purification
The Bacterial blight (oily spot) affected leaves, fruits and stems showing typical symptoms of disease were collected from the pomegranate orchards and brought to the laboratory for further studies. The diseased leaves and fruits samples were rinsed in running tap water to wash adhered soil particles. The small bits (2.5 mm) of infected leaves and fruits were cut with sterilized blade and disinfected with 0.1 percent aqueous solution of mercuric chloride ($HgCl_2$) for two minutes. Then these surface sterilized bits were washed by giving three changes of sterile distilled water to remove traces of mercuric chloride. The pieces of sterilized leaves and fruits were placed on blotter paper for absorbing the water drops and it was dried for few minutes. These bits were then placed in sterile test tube containing 2.5 ml sterile distilled water and crushed with the help of sterilized glass rod and loopful of bacterial suspension was streaked on cooled Nutrient Agar Petri dishes under aseptic condition. These plates were incubated in the incubator for 72 to 96 hrs at 28$\pm$2 ^{0}C temperature. After three days typical bacterial colonies having yellow colour were noticed in all Petri dishes. The well developed bacterial colony free from contaminant was transferred on another Nutrient Agar plates and slants. Through frequent sub culturing, pathogen was purified and maintained on agar slants.

Pathogenicity

The pathogenicity test was performed with pure culture of the bacterial pathogen isolated from diseased samples affected with *X. axanopodis* pv. *punicae.* Three different methods viz. Pin-prick method, Infiltration method and Spray inoculation method were used for confirming the pathogenicity of the causal bacterium.

Methods

a. Pin-prick method

Lower surface of leaves was pricked with sterilized needle in multiple (5-10 numbers) points. Bacterial inoculum ($2x10^8$ cfu/ml population) was placed on the pricked area by placing a drop of cell suspension and through cotton swab which was pre soaked in inoculum.

b. Infiltration Method

Bacterial inoculum was taken in disposable injection syringe of 0.5ml capacity. Care was taken that no air bubble was trapped in the suspension. The needle of syringe was placed on lower surface of the leaf, particularly at interveinal region and gently inoculum was injected. The leaf was firmly hold by providing support on the opposite side at the point of injection (mouth of the needle) with the help of index finger (left hand), while the thumb (left hand) was placed on the lower side above the mouth of the needle. The inoculum was infiltrated slowly by pressing the syringe with the thumb (right hand). Bigger wounds were avoided at the point of injection. Plants were incubated in humid condition. After infiltration, plants were periodically monitored and observed for hypersensitive and susceptible reaction. The hypersensitive reaction appeared within 8-24 h of infiltration, while susceptible reaction required 72-96 h depending on environmental factors, host stage, pathogens virulence level, etc. Same procedure was adopted for twigs, stem and even fruits.

c. Spray Inoculation Method

Healthy susceptible host plants were sprayed on the foliage with the help of hand sprayer. Sprayed plants were incubated in humid condition and turgidity of leaf was maintained with the help of humidifier.

Re-isolation

The pathogen was re-isolated from the artificially inoculated plant under poly house condition. The isolations yielded a yellow culture of *Xanthomonas axonopodis* pv. *punicae.* on nutrient agar medium.

Results and Discussion

The pathogenicity of *Xanthomonas axonopodis* pv. *punicae* was established on young and healthy plants of susceptible pomegranate cv. Bhagawa grown in earthen pots under controlled conditions in polyhouse. The plants treated with sterile distilled water served as control. Both inoculated and controlled plants were kept in polyhouse for development of typical symptoms of the diseases. The symptoms of the disease developed within 7 to 15 days after inoculation on leaves. First symptoms appeared on lower sides of the leaves as irregular water soaked spots. Later on such spots appeared raised from the upper side of the leaves and they were rough to touch with yellow haloes. Subsequently they became brown in colour and coalesced to form blighting appearance and defoliation of the leaves. The control plants were free from infection.

Standardization of Inoculation Methods

The data revealed that amongst three different methods, infiltration method was found most suitable for development of symptoms which had taken 7 days for symptoms development. This method was followed by pin prick method which had taken 12 days for initiation of symptoms and spray inoculation method had taken maximum days for production of symptoms i.e 15.87 days. Maximum number of lesions i.e 3 lesions on leaves of pomegranate were observed in the plants inoculated with infiltration method followed by pin prick method and spray inoculation method. After re-isolation maximum number of colony forming units of *Xap* were observed in case of infiltration method followed by pin prick method and spray inoculation method. In all the three methods adopted for inoculation of the pathogen, infiltration method was found very successful for establishment of pathogen inside the host. The symptoms were also found to be appeared by spray inoculation method and pin prick method on lower surface of the leaves. The re-isolation attempted from artificially infected diseased plant tissues on Nutrient Agar consistently yielded *X. axonopodis* pv. *punicae*, thus fulfilling Koch's postulates and association of *X. axonopodis* pv. *punicae* with pomegranate was confirmed. The results of present study on isolation, identification and pathogenicity of the bacterium *X. axonopodis* pv. *punicae* correlates with reports of Hingorani and Singh (1959) [4], Chand and Kishun (1991) [3], Raghuwanshi *et al.*, (2013) [5] studied the pathogenicity test on Bhagawa variety of pomegranate inoculated with four isolates showed typical symptoms of bacterial blight disease when incubated under glasshouse conditions. Control plants inoculated with distilled water did not showed any symptoms. Similarly, Bora and Kataki (2014) [5] reported that the pathogenicity test which was done by spraying 48 hour old bacterial culture suspension (2×10^8 cfu/ml) on pinpricked 40 days old healthy pomegranate leaves. Infection was occurred within 23 days of inoculation and produced identical symptoms observed on original pomegranate plant. The organism was re-isolated from artificially inoculated plant which yielded an organism similar to one used in the inoculation experiments. This satisfied all the conditions required to establish that the disease was bacterial borne disease which was caused by the pathogen *Xanthomonas axonopodis* pv. *punicae.*

Pathogenic variability among different isolates of *Xap.*

Five different isolates collected from Latur and Osmanabad district were subjected to pathogenic variability amongst these isolates and data so obtained is presented in Table 2. Amongst five isolates *Xap* -4 isolate had more lesion size i.e 0.9 mm after 15 days of inoculation and 4.3 mm after 18 days of inoculation which showed water soaked circular to irregular dark brown spots with yellow halo symptoms followed by *Xap* -3 which showed 0.7 mm lesion size after 15 days and 4.1 mm after 18 days after inoculation showing water soaked circular to irregular, light brown spots. The *Xap*- 1 isolate showed less lesion size i.e 0.3 mm after 15 days and 3.5 mm after 18 days of inoculation.

Table 1: Standardization of Inoculation Methods for *Xap*.

Inoculation method	No. of days required for initiation of symptoms	No. of lesions observed after inoculation	CFU/ml observed after re-isolation
Pin-prick method	12	3	10×10^6
Infiltration Method	7	4	12×10^6
Spray Inoculation Method	15.87	2	8×10^6
SE+	0.505	0.277	
CD=0.01	1.496	0.820	

Table 2: Pathogenic variability among isolates of *Xap*.

Sr. No.	Isolate	Lesion size (mm)		Symptom appearance
		After 15 days	After 18 days	
1	*Xap*-1	0.3	3.6	Water soaked, circular to irregular, dark brown spots
2	*Xap*-2	0.2	3.8	Water soaked, irregular, light to dark brown spots
3	*Xap*-3	0.7	4.1	Water soaked, circular to irregular, light brown spots
4	*Xap*-4	0.9	4.3	Water soaked, circular to irregular, dark brown spots with yellow halo
5	*Xap*-5	0.7	3.9	Water soaked, circular to irregular, dark brown spots with yellow halo

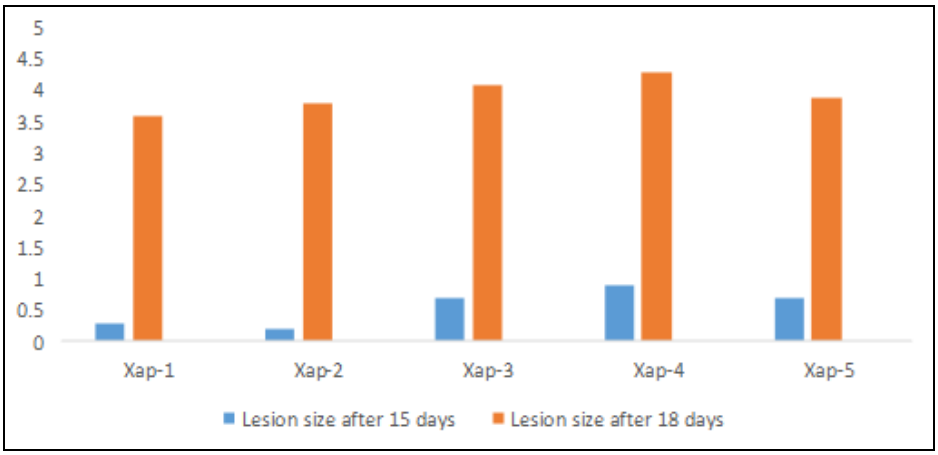

Fig 1: Pathogenic variability among isolates of *Xap*.

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