

Original Research Article

<https://doi.org/10.20546/ijcmas.2018.704.017>

Pathogenicity Test and Convenient Inoculation Method of *Xanthomonas axonopodis* pv. *vignaeradiatae* Caused Leaf Spot of Green gram

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ABSTRACT

Green gram bacterial leaf spot pathogen *X. axonopodis* pv. *vignaeradiatae* was isolated from diseased samples collected from different locations of Udaipur district. The pathogenicity was proved on one month old green gram plants of cv. SML-668 and isolate Xav-02 (Bhatewar) was found more virulent with 4 days incubation period and require 12 days for disease development. Initially dark brown water soaked spots were found on cotyledons and then appeared on leaves and stem of seedlings. On leaves, irregular dark brown raised necrotic spots were developed with shot holes. Further, elongated dark brown spots with cracks were detected on stems. Whereas, dark brown necrotic spots were developed on pods and infected pods had small, shrivelled, deformed and discolored seeds. Thus diseased plants had poor standing without mortality. To find out most convenient and efficient method for disease production five different artificial inoculation methods were used such as (1) Spraying of bacterial suspension once, (2) Spraying of bacterial suspension twice, (3) Spraying of bacterial suspension thrice, (4) Rubbing bacterial suspension with carborundum powder and (5) Spraying of bacterial suspension after multi needle pricking. The spraying of bacterial suspension thrice method gave quicker symptoms expression in potted plants as well as field conditions, respectively.

Keywords

Bacterial suspension,
Green gram, Artificial
inoculation,
Pathogenicity, Spray

Article Info

Accepted:
04 March 2018
Available Online:
10 April 2018

Introduction

Green gram [*Vigna radiata* (L.) Wilczek] is one of the important pulse crops in India, which has been cultivated since ancient times. It is widely cultivated throughout the Asia, including India, Pakistan, Bangladesh, Sri Lanka and south China. Green gram is well suited to a large number of cropping system and a popular cereal-based diets due to easily digestible, rich in protein (25-28%), oil (1.0-1.5%), fiber (3.5-4.5%), ash (4.5-5.5%) and carbohydrates (62- 65%) on dry weight basis

(Singh *et al.*, 2010). It not only plays an important role in human diet but also in improving the soil fertility by fixing the atmospheric nitrogen (Muhammad *et al.*, 2004).

Green gram plant suffers from two bacterial disease namely, bacterial leaf spot and halo blight (*Pseudomonas syringae* pv. *phaseolicola*). Out of these two diseases the former is economically more important and widespread in India (Thind, 2012). Bacterial leaf spot pathogen *Xanthomonas axonopodis*

pv. *vignaeradiatae* produces symptoms on leaves, stems, pods and seeds. Initially small water-soaked spots are observed on leaves and these spots enlarge and the Centre turns necrotic and brown. The lesion is surrounded by a narrow band of bright yellow tissue, stem infection is less common and begins as a water-soaked spot, which becomes reddish-brown and usually without chlorosis. Stem girdling may develop at the cotyledonary node (Patel and Jindal, 1972; Chand, 1986).

Borah *et al.*, (2000) were collected diseased leaves of green gram plants, from the experimental fields of IARI, New Delhi. Isolation was done on modified Sucrose Peptone Agar (SPA) media at 28±2°C and purified colony was maintained on Yeast Glucose Chalk Agar (YGCA) slants at 5-10°C in the refrigerator. Dutta *et al.*, (2005) cultured the leaf spot of green gram bacterium on nutrient agar (NA) and pure culture maintained on yeast glucose chalk agar media (YGCA). Raghuwanshi *et al.*, (2013) studied on the isolation and obtained pure culture of four isolates of the *X. axonopodis* pv. *punicae* on nutrient agar medium amended with sucrose from infected leaf, stem and fruit samples of pomegranate. Parthasarathy *et al.*, (2014) isolate and purified bacterial leaf blight of rice (*X. oryzae* pv. *oryzae*) on nutrient agar medium plates.

A suitable, convenient and ensured method of inoculation is very important for various field and green house studies. Bacterium enters inside the leaves and stem mainly through stomata, lenticels and wounds. Among artificial methods of inoculation, spraying bacterial suspension under pressure on leaf surface causes visible water soaking lesions on the leaves in field conditions. Srivastava and Rao (1963) observed that surface irrigated summer grown green gram crops were totally free from bacterial leaf spot pathogen. Such seed lots from summer grown green gram

crops played an important role in the integrated disease management for this disease. Shah (1988) studied bacterial blight of cowpea and found spray inoculation twice at interval of 24 h as best and most suitable for the development of disease on cowpea plants in pots and fields. Shukla *et al.*, (1995) observed surface injury with help of multi needle as best method for development of citrus canker disease in citrus plants. Gena *et al.*, (2008) while working with different methods for artificial inoculation of bacterial blight pathogen on cowpea plants. They found that two sprays at 24 h interval as most effective followed by abrasion with carborundum powder, with incubation period of 5-6 days and 6-7 days, respectively. Maji and Nath (2015), described that carborundum abrasion method was most convenient and efficient method of inoculation for producing black rot of cabbage disease caused by *X. campestris* pv. *campestris* on potted plants.

Materials and Methods

Collection of diseased samples

Samples of naturally infected green gram plants were collected from different villages of Udaipur district during *Kharif*, 2013 and 2014. The infected aerial parts of the diseased samples were carefully placed in polythene bags, properly tagged and brought to the laboratory. Samples were thoroughly washed with sterile distilled water. To confirm the presence of bacterium, ooze tests were performed regularly from different plant parts of green gram.

Isolation of the pathogen

Infected plant parts such as leaf, stem, pods and seeds showing typical symptoms of leaf spot were taken for isolation of causal bacterium. For isolation, infected portions of plants were cut, surface sterilized with 0.1

percent mercuric chloride (HgCl₂) solution for two minutes and rinsed thoroughly thrice with sterile distilled water. The diseased bits were then transferred individually into a few drops of sterile water on a sterilized glass slide under aseptic conditions. The diseased bits were given a cut with sharp sterilized blade. The bits were left for one minute to allow bacterial ooze to come out in water. A charged needle with ooze was streaked on nutrient agar Petri plates. Three Petri plates were streaked at a time, without recharging the needle loop.

Purification of the pathogen

This procedure was repeated several times using fresh sets of nutrient agar plates each time. The inoculated plates were incubated at 28±2°C for 48 h in an inverted position. The suspected bacterial colonies were picked up with the help of sterilized inoculated loop and further streaked on the surface of yeast extract glucose chalk agar (YGCA) medium. The inoculated plates were incubated at 28±2°C for 48 to 72 h and obtained bright yellow coloured bacterial colonies. The purified bacterial colonies were streaked on YGCA slants and stored at 5°C in refrigerator and also in sterile distilled water tubes, by suspending 2-3 loops full of the bacterial culture for further studies.

Pathogenicity tests

Pathogenicity tests were conducted by inoculating one month old green gram plants raised in 25 cm earthen pots by spraying the bacterial suspension thrice at 24 h intervals. A fresh 72 h old bacterial culture, grown on YGCA media was used for inoculations on the plants. The culture was harvested in 10 ml sterile water diluted to a concentration of 2.5×10⁸ cfu/ml and used immediately. The suspension was sprayed on plants with hand atomizer twice at 24 h interval. Suitable controls were maintained using only distilled water in place of inoculum suspension. The

forty inoculated plants were kept in cage house under high humid condition for 48 h then under natural cage house conditions. Uninoculated plants served as control. The plants were watered at frequent interval and regularly observed for the appearance of disease symptoms. Then pathogen was again reisolated from newly inoculated plants and compared with the original culture.

Inoculation methods

The following five different inoculation methods were tried to find out most convenient and efficient method for disease production *viz.*, (i) Spraying of bacterial suspension once, (ii) Spraying of bacterial suspension twice, (iii) Spraying of bacterial suspension thrice, (iv) Rubbing bacterial suspension with carborundum powder and (v) Spraying of bacterial suspension after multineedle pricking.

The bacterium suspension (2.5×10⁸cfu/ml) was used as inoculum for spraying. The experiment was done using one month old 40 green gram plants raised in 25 cm earthen pots (10 plants in each pot) in cage house and 120 green gram plants (30 plants in each row) in field for each inoculation method, with hand atomizer. These methods are described as under,

Spraying of bacterial suspension once, twice and thrice (Klement, 1968)

The pathogen usually enters the tissue through stomata, lenticels, hydathodes this method of inoculation is the most natural. The plants were kept in humid condition prior to inoculation to allow the stomata to open and to create high intercellular humidity in the tissues around the natural openings. The bacterial suspension (2.5×10⁸cfu/ml) was sprayed on green gram plants with hand atomizer. The inoculated plants were kept under high humid

condition for 48 h by covering them with polyethylene bags and then left as such under natural condition. In double and triple spray inoculation methods, consecutive two and three spray were applied at 24 h intervals, respectively.

Rubbing bacterial suspension with carborundum powder (Leben *et al.*, 1969)

The plants were inoculated by bacterial suspension with the help of cotton swab on both the surface of leaves. The cotton swab were soaked in inoculum carried some carborundum powder (300 mesh) for making gentle injury and then applied inoculum simultaneously.

Spraying of inoculum after multineedle pricking (Starr and Dyes, 1965)

The leaves were injured with the help of multineedle prepared by fixing 8-10 fine pins with the help of a rubber band tightly.

The injury to the leaves was gentle so that it did not tear or perforate leaf surface. The bacterial suspension was then atomized on the leaf surface.

Suitable controls were maintained in each case using distilled water in place of inoculum suspension. The plants were inoculated by spray inoculation respective methods and the potted plants were replaced again in humid chamber in green house (RH 78-91%, and 28 ±2 ° C temp. for 48 h) and later kept under natural conditions.

The plants were watered at regular interval and frequently observed for disease development. The pathogen was reisolated from inoculated plants as described earlier and then compared with the original culture. Similar results were obtained repeatedly and in this way Koch's postulates were proved.

Results and Discussion

Collection of diseased samples

During survey's, infected plant parts shown typical symptoms of bacterial leaf spot of green gram were collected from naturally infected field of different locations of Udaipur district during *Kharif* 2013 and 2014. The disease samples were collected from various infected parts like leaf, stem and pods.

Isolation and purification of the bacterium

The bacterial leaf spot of green gram causal organism *X. axonopodis* pv. *vignaeradiatae* was isolated from infected leaves and stem of the green gram plant showing typical symptoms of bacterial leaf spot disease. Isolation was done by adapting poured plate method as explained in material and methods. Well separated colonies of each isolate were picked up and streaked on nutrient agar (NA) medium and incubated at 28±2°C for 72 h.

The pure colonies obtained were again purified on yeast extract glucose chalk agar (YGCA) media slants by streaking and then incubated at 28±2°C for 72 h and stored at 5°C in refrigerator and also in sterile distilled water tubes, by suspending 2-3 loops full of the bacterial culture for further studies. The pathogen isolated from each location was designated as an 'isolate' viz., Xav-01 to Xav-10. The cultures were renewed by sub-culturing once in a fortnight on YGCA media slants and such cultures did not show any variation in cultural characters even after several generations of sub-culturing (Table 1 and Fig. 2).

Proving pathogenicity

Koch's postulates were followed to prove pathogenic nature of *X. axonopodis* pv. *vignaeradiatae* isolates.

Table.1 Isolates of *Xanthomonas axonopodis* pv. *vignaeradiatae* collected from different green gram growing areas of Udaipur district

Locations	Tehsil	Variety	Plant part used for isolation	Isolate designation
Mavli	Mavli	RMG-62	Leaf	Xav-01
Bhatewar	Vallabh Nagar	Local	Leaf	Xav-02
Sarjana	Vallabh Nagar	GM-4	Leaf	Xav-03
Jaswantgarh	Gogunda	SML-668	Stem	Xav-04
Semar	Gogunda	Local	Leaf	Xav-05
Phalasiya	Jhadol	RMG-268	Leaf	Xav-06
Jhadol	Jhadol	RMG-62	Leaf	Xav-07
Mandwa	Kotra	Local	Leaf	Xav-08
Saradi	Salumber	SML-668	Stem	Xav-09
Sundara	Kherwara	Local	Leaf	Xav-10

Table.2 Pathogenic variability among different isolates of *Xanthomonas axonopodis* pv. *vignaeradiatae* tested on potted green gram plants in cage house

Isolates	Incubation period (Days)	Days required for development of superficial spot on leaves	Days required for development of brown, rough spot on leaves	Days required for development of necrotic, corky patches on leaves
Xav-01	06	08	10	15
Xav-02	04	06	08	12
Xav-03	06	07	09	14
Xav-04	06	09	11	15
Xav-05	04	07	10	13
Xav-06	06	08	11	15
Xav-07	04	06	09	14
Xav-08	07	10	13	16
Xav-09	08	10	13	15
Xav-10	07	10	12	16

Table.3 Different methods of inoculation to find out a suitable method of producing bacterial leaf spot disease in potted and field plants by using bacterial suspension (2.5×10^8 cfu/ml)

Inoculation Methods	Potted Plants			Field Plants			Incubation periods (Days)
	Number of inoculated plants	Number of infected plants	Infection (%)	Number of inoculated plants	Number of infected plants	Infection (%)	
Spraying of bacterial suspension once.	40	11	27.50 (31.619)*	120	39	32.50 (34.747)	8-10
Spraying of bacterial suspension twice	40	15	37.50 (37.977)	120	48	40.00 (39.400)	6-7
Spraying of bacterial suspension thrice	40	27	67.50 (55.256)	120	82	68.33 (55.769)	4-5
Rubbing bacterial suspension with carborundum powder	40	21	52.50 (46.435)	120	69	57.50 (49.320)	4-5
Spraying of bacterial suspension after multineedle pricking	40	19	47.50 (43.566)	120	61	50.83(45.478)	5-6
SEm±			0.7264			1.4128	
CD at 5%			2.2383			4.3531	
CV%			3.38			6.29	

* Figures in the parenthesis are arc sin transformed value

Fig.1 Pathogenic variability among different isolates of *Xanthomonas axonopodis* pv. *vignaeradiatae* tested on potted green gram plants in cage house

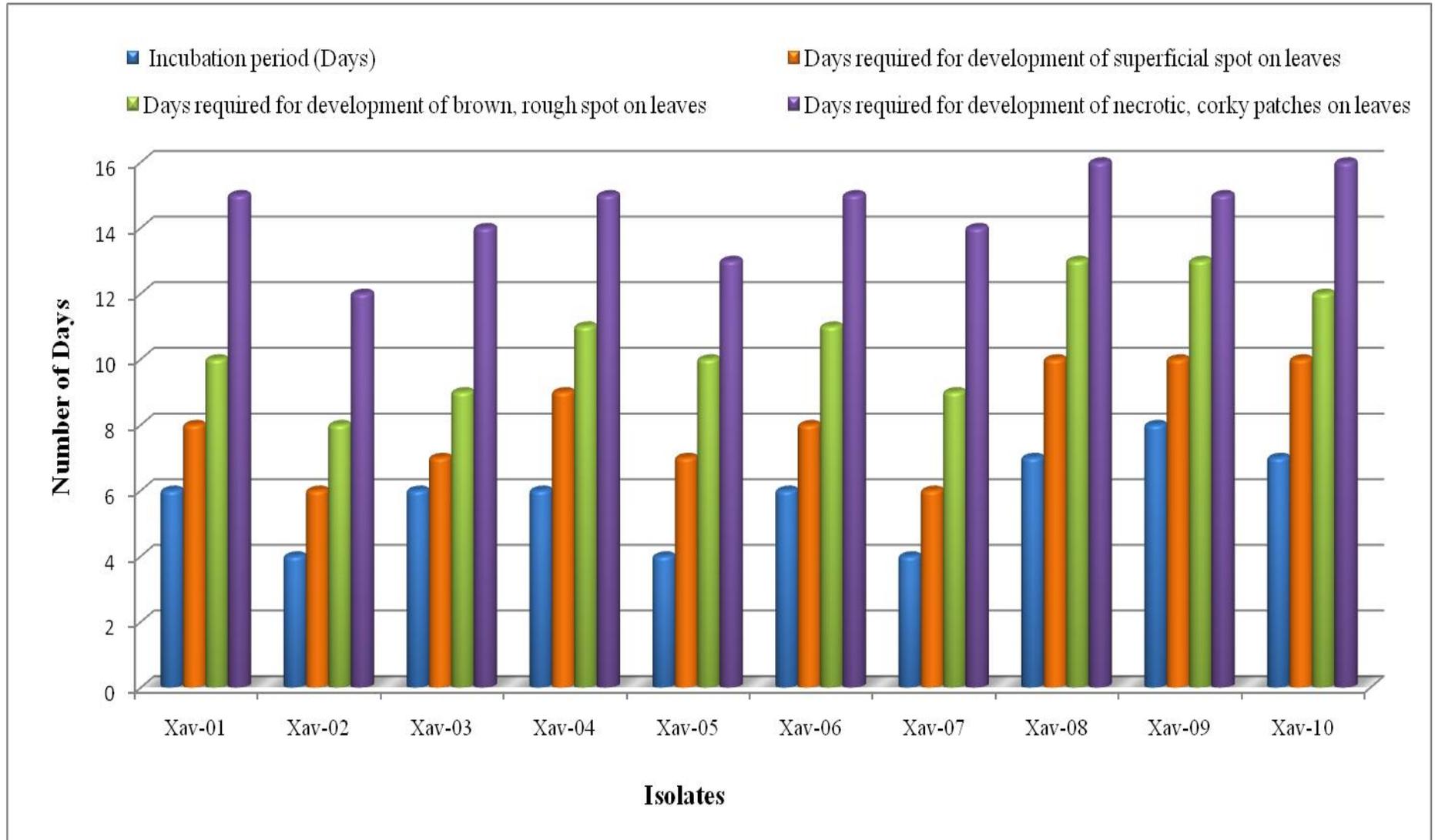


Fig.2 Isolates of *Xanthomonas axonopodis* pv. *vignaeradiatae* collected from different green gram growing areas of Udaipur district

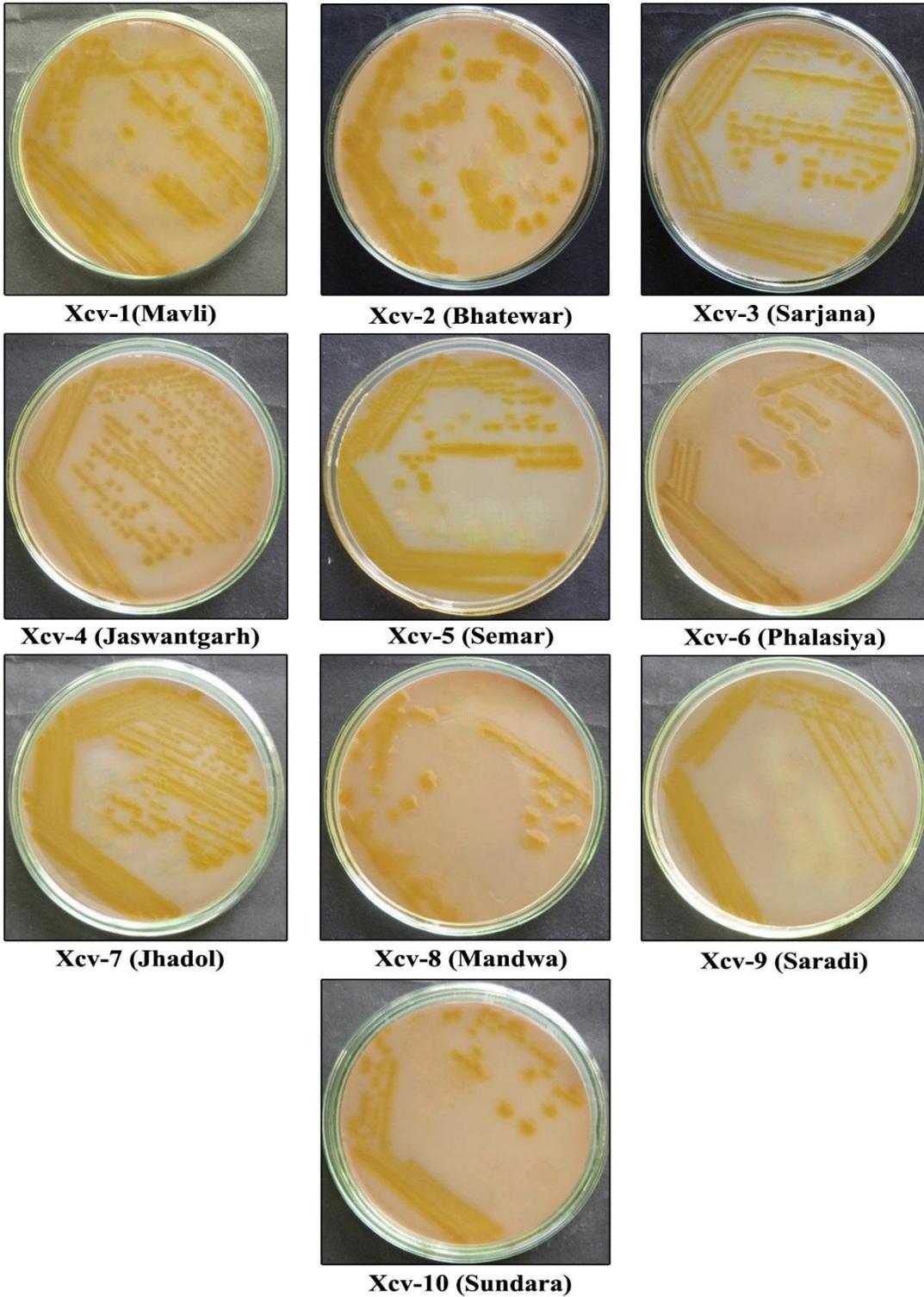
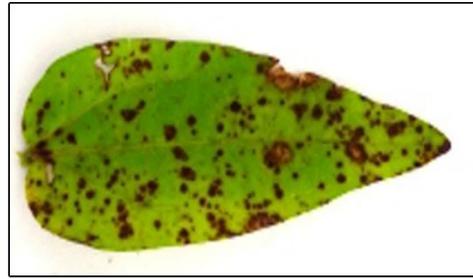


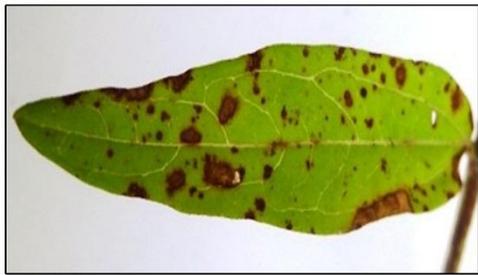
Fig.3 Pathogenic variability of different isolates of *Xanthomonas axonopodis* pv. *vignaeradiatae* tested on potted green gram plants in cage house



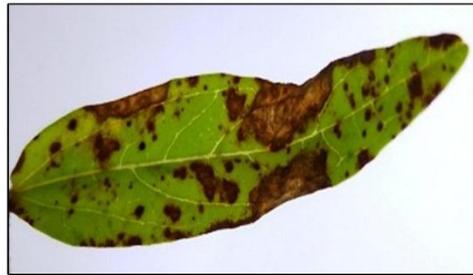
Xcv-1



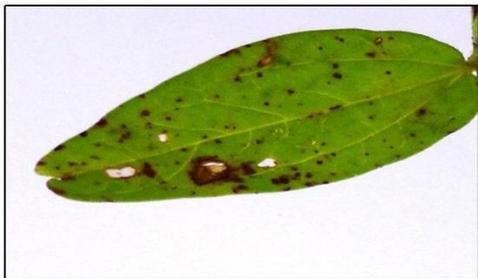
Xcv-2



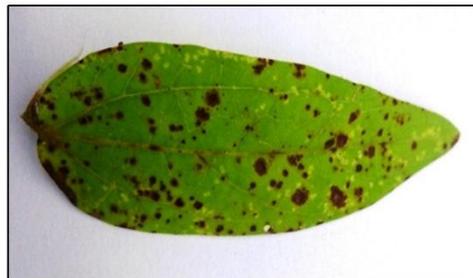
Xcv-3



Xcv-4



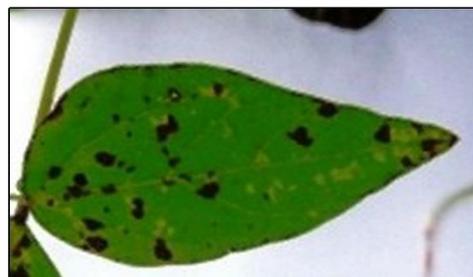
Xcv-5



Xcv-6



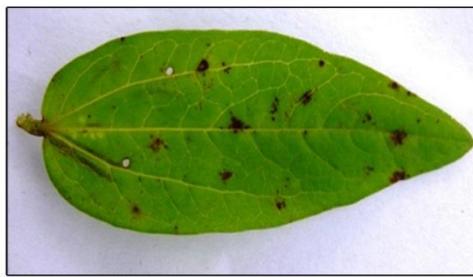
Xcv-7



Xcv-8



Xcv-9



Xcv-10

Fig.4 Typical symptoms of leaf spot disease on cotyledons, stem, pod and leaves of green gram plants



Dark brown colour water soaked spot on cotyledons



Dark brown lesions on stem



Chocolate necrotic patches in vascular region



Dark brown spots on pods



Deformed pods



Shot hole and brown spots



Brown spots with yellow margin



Corky symptoms on old leaves

Fig.5 Different methods of inoculation to find out a suitable method of producing bacterial leaf spot disease



Spraying of bacterial suspension once



Spraying of bacterial suspension twice



Spraying of bacterial suspension thrice



Rubbing bacterial suspension with carborundum powder



Spraying of bacterial suspension after multineedle pricking



Uninoculated control

Pathogenicity of all ten isolates was confirmed on one month old susceptible green gram plants variety (SML-668) in cage house by spraying of bacterial suspension thrice 2.5×10^8 cfu/ml with atomizer. The initial disease symptom was characterized as the development of small water soaked lesions, which later on transformed into a necrotic corky lesion on leaves and stems, which can easily be compared with control plants showing no symptoms. However, each isolate required different incubation period for the development of water soaking reaction on leaves. Moreover, the isolates *viz.*, Bhatewar (Xav-2), Semar (Xav-05) and Jhadol (Xap-07) produced small water soaked lesions on the 4 days followed by isolates *viz.*, Mavli (Xav-01), Sarjana (Xav-03), Jaswantgarh (Xav-04) and Phalasiya (Xav-06) required about 6 days to produce similar small water soaked lesions. Whereas, isolates *viz.*, Mandwa (Xap-08) and Sundara (Xav-10) required about 7 days for production of small water soaked lesions and only Saradi (Xap-09) isolate required 8 days for production of small water soaked lesions. After 6 to 10 days of inoculation the spots were increased in size and turned brown yellow to dark coffee brown in colour which later developed into dry necrotic, corky patches on leaves. The development of necrotic, corky patches on leaves was faster when inoculated with isolates *viz.*, Xav-02 (started at about 12 days) followed by Xav-05 (13 days), Xav-03 (14 days) and Xav-07 (14 days). It clearly indicates that isolates *viz.*, Xav-02 (Bhatewar), was highly virulent and followed by Xav-05, Xav-03 and Xav-07. Reisolations were carried out from these symptoms for each isolate and comparisons were made with original culture to confirm the identity of the pathogen. The reisolated culture resembled the original mother culture and hence pathogenicity test was confirmed. Finally pure culture was maintained on yeast extract glucose chalk agar media on plates and slants

in refrigerator for further laboratory studies (Table 2, Fig. 1, 2 and 3).

Disease symptoms

Symptoms caused by bacterial leaf spot pathogen, *Xanthomonas axonopodis* pv. *vignaeradiatae* were observed on all above ground parts of the green gram plants. Cotyledons of seedlings raised from the infected seeds has dark brown water soaked spots, necrotic spots increased in size and later on the spots covered the young leaves. Longitudinal elliptical lesions extending from soil level to the growing tip developed on the stem. On trifoliate leaves, dark brown or chocolate brown irregular spots were appeared, spots were necrotic and covered the entire leaf surface. The shot hole symptoms in leaves were also observed in old spots and the superficial eruptions collapsed leaving depressions. Under severe foliar infection several necrotic spots were seen on leaves but never detected leaf blight phase. Lesions from petiole extended to the branches and stem. Entire stem had elongated dark chocolate brown lesions similar to symptoms of leaves. In severely infected plants, longitudinal slits or cankers/cracks were observed on stem. Infected cut stem also exhibited chocolate necrotic patches in vascular regions. Dark brown and raised spots/lesions on pods were seen. Such spots increased in size and the whole pod become necrotic and deformed. Such pods have small, discoloured, shrivelled and deformed seeds. All severely diseased plants were weak with poor standing but completely not killed (Fig. 4).

Different methods of inoculation

Green gram plants were inoculated by five different methods *viz.*, spraying of bacterial suspension once, spraying of bacterial suspension twice, spraying of bacterial suspension thrice, rubbing bacterial

suspension with carborundum powder and spraying of bacterial suspension after multineedle pricking to find out suitable method of disease development for mass inoculation in potted plants as well as field plants. All inoculations were made on one month old green gram plants during *Khari* season in 2013, when general humidity conditions were very high (85-90%) and temperature ($28\pm 2^{\circ}\text{C}$) was suitable for disease development. The inoculated plants in pots and field were then observed daily for the appearance and development of bacterial leaf spots.

On the basis of appearance and development of disease observations, results revealed that green gram leaf spot pathogen could infect plants irrespective of the method used. The early expression of disease (4-5 days) was noted in both spraying of bacterial suspension thrice and rubbing bacterial suspension with carborundum powder followed by spraying of bacterial suspension after multineedle pricking (5-6 days), spraying of bacterial suspension twice (6-7 days) and spraying of bacterial suspension once (8-10 days) on potted plants as well as in field. It was noted that maximum disease (67.50 per cent) developed in triple sprays at 24 h interval followed by carborundum abrasion method (52.50 per cent), spraying after multineedle pricking (47.50 per cent), double spray (37.50 per cent) and single spray (27.50 per cent) in potted plants. Whereas, in field plants maximum disease (68.33 per cent) developed in triple sprays at 24 h interval followed by carborundum abrasion method (57.50 per cent), spraying after multineedle pricking (50.83 per cent), double spray (40.00 per cent) and single spray (32.50 per cent). Triple sprays at 24 h interval were found most convenient and efficient method of inoculation for producing disease on potted plants as well as in field plants (Table 3, Fig. 5).

Production of disease by artificial inoculation is an essential requirement to understand various aspects of leaf spot disease. Chand *et al.*, (1991) standardized the inoculation method for bacterial blight of pomegranate pathogen by inoculating the bacterial suspension (2×10^8 cfu/ml) on 40 days old leaves by pinprick, rubber block pressure, leaf cut and automization methods. They observed that, leaf cut was found superior, where they recorded 100 per cent infection covering 70 to 90 per cent leaf area within 21 days. The automization of bacterial suspension was found to induce lowest infection of 6 to 7.5 per cent with maximum incubation period of 17 to 40 days.

During present investigation, five inoculation methods *viz.*, spraying of bacterial suspension thrice, spraying of bacterial suspension twice at 24 h interval, spraying of bacterial suspension once, rubbing bacterial suspension with carborundum powder and spraying of bacterial suspension after multi needle pricking were tried to find out suitable and easy inoculation method for both pot and field plants. All the methods were able to produce disease on green gram plants but maximum disease was developed by triple sprays at 24 h interval and this method was proved to be convenient and suitable for producing disease in potted as well as in field plants. In support of the present findings, it was reported that the bacterial blight of cowpea incited by *X. campestris* pv. *vignicola* was more pronounced when the plants were spray inoculated twice at interval of 24 h as compared to carborundum abrasion method (Shah *et al.*, 1995).

However, the results of present findings were in disagreement from Allen *et al.*, (1981). He found that stem injection and leaf infiltration methods was reliable under greenhouse whereas, foliar sprays was most suitable for both greenhouse and fields. Further, Akhtar *et*

al., (2008) found that the clipping inoculation method to develop green gram blight disease is most effective method. According to Maji and Nath (2015) the carborundum abrasion method for producing black rot of cabbage disease on potted plants was most convenient and efficient methods of inoculation to develop bacterial disease.

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How to cite this article:

Jitendra Kumar and Anila Doshi. 2018. Pathogenicity Test and Convenient Inoculation Method of *Xanthomonas axonopodis* pv. *vignaeradiatae* Caused Leaf Spot of Green gram. *Int.J.Curr.Microbiol.App.Sci*. 7(04): 152-166. doi: <https://doi.org/10.20546/ijcmas.2018.704.017>