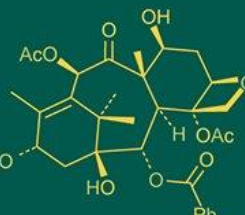


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Comparative studies of different inoculation methods of *Xanthomonas axonopodis* pv. *citri* Causing citrus canker in acid lime

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Abstract

Citrus canker, caused by *Xanthomonas citri* subsp. *citri*, is a major bacterial disease that severely affects citrus production worldwide. Efficient artificial inoculation methods are essential for pathogenicity studies and evaluation of disease management strategies. This study compared different inoculation methods for inducing citrus canker in acid lime (*Citrus aurantifolia*) under attached and detached leaf conditions. Five methods, namely leaf injection (leaf infiltration), pin-prick, needleless leaf injection, leaf clipping, and spray inoculation, were evaluated in attached leaves, while pin-prick, leaf clipping, and spray inoculation were assessed in detached leaves. Symptom development was monitored for 21 days after inoculation. In the attached leaf assay, leaf injection produced the earliest and most severe canker symptoms, followed by pin-prick and leaf clipping, whereas spray inoculation failed to induce symptoms. In the detached leaf assay, the pin-prick method initiated symptoms as early as four days after inoculation and resulted in the most rapid and consistent disease development. The superior performance of leaf injection and pin-prick inoculation was attributed to direct pathogen entry through wounded tissues, facilitating rapid bacterial colonization. The study identifies leaf injection for attached leaves and pin-prick inoculation for detached leaves as reliable, reproducible, and efficient methods for citrus canker pathogenicity studies and disease management research.

Keywords: *Xanthomonas citri* subsp. *Citri*, Citrus canker, Acid lime, Inoculation methods, Pathogenicity assay

1. Introduction

Citrus is the third most popular fruit in India, behind mango and banana, and canker is one of the main obstacles to its growth. Although the tree is affected by various diseases like citrus greening, quick decline, citrus slow decline, citrus wither tip, and citrus gummosis, citrus canker caused by *Xanthomonas axonopodis* pv. *citri* is one of the most threatening citrus diseases (Das, 2003) [1]. This disease has a serious economic impact on citrus production worldwide (Jalan *et al.*, 2013) [4]. It causes yield losses of up to 16-23 percent (Naqvi *et al.*, 2022) [5].

Infection of plants by bacteria can occur in multiple ways. Infection is generally considered to be passive, *i.e.*, accidental, although a few cases of plant chemo-attractants have been reported. Bacteria can be sucked into a plant through natural plant openings such as stomata, hydathodes or lenticels. They can enter through abrasions or wounds on leaves, stems or roots or through placement by specific feeding insects (Vidaver & Lambrecht, 2004) [7]. The bacteria causing citrus canker enters the leaves through stomata or through wounds caused by weather damage or insects, such as citrus leaf miner *Phyllocnistis citrella* (Graham *et al.*, 2004) [3]. Symptoms of the disease include leaf spot, fruit rind blemishing, defoliation, shoot dieback, and fruit drop in favorable environmental conditions conducive to pathogen proliferation (Das, 2003) [1]. Considering the importance of the pathogen and disease the present studies were conducted to compare the different inoculation methods of *Xanthomonas axonopodis* pv. *citri* causing citrus canker in acid lime.

2. Materials and Methods

The experiment entitled "Comparative studies of different inoculation methods of *Xanthomonas axonopodis* pv. *citri* causing citrus canker in acid lime" was carried out during

2021-2022 to 2022-2023 at the Department of Plant Pathology, College of Agriculture, Parbhani, VNMKV Parbhani.

Time taken for appearance of differentiating canker symptoms on leaves of the acid lime, when inoculated by different methods in both attached leaf and detached leaf were tested and observations were recorded till 21st day of inoculation and the methods used were as follows.

1. Leaf dip method

The leaf tip (2-3cm from tip) was cut with the scissors that are dipped into inoculum. Care was taken that before every cut scissor was immersed into the inoculum.

2. Spray

Inoculum was sprayed using hand sprayer.

3. Leaf injection /leaf infiltration

The bacterial inoculum was loaded into the injection syringe. Care was taken that no air bubble was present while sucking the inoculum through the needle. Then the needle of the syringe (containing inoculum) was placed on the lower surface of the leaf, particularly in the interveinal region. The leaf was held firmly by providing support on the opposite side at the point of injection (mouth of the needle) with the index finger, while the thumb was placed from the lower side above the mouth of the needle. The content (inoculum) in the injection was then infiltrated slowly by pressing the syringe with the other thumb. Care was taken to avoid the bigger wound/hole or injury at the point of injection. Three injection infiltrations were performed on lower side of the midvein. Three leaves were inoculated per plant. The inoculated shoots were covered with a plastic bag for 24 h to maintain high humidity conducive for bacterial growth in the leaves. Developments of symptoms on leaves were evaluated periodically up to 21 days post inoculation.

4. Pin prick

The inoculum was applied on the micro wounds created using pin pads.

5. Needleless Leaf injection

Immature leaves (75% expanded) on greenhouse seedlings of acid lime of the same type used for detached leaf assay were inoculated using needleless syringe. Test bacterial inoculum (as described under detached leaf assay) was infiltrated by pressing needleless syringe to produce a zone of water-soaked tissue about 6mm in diameter. Three injection infiltrations were performed on each side of the midvein. Three leaves were inoculated per plant. The inoculated shoots were covered with a plastic bag for 24 h to maintain high humidity conducive for bacterial growth in the leaves. Developments of symptoms on leaves were evaluated periodically up to 21 days post inoculation.

3. Results and Discussion

A. Attached leaf assay

In the attached leaf test, five methods were used: leaf injection or leaf infiltration, pinprick method, needleless leaf injection method, leaf clipping method, and spray method.

- 1. Leaf injection or leaf infiltration method:** Observations were recorded from the next day of inoculation, and they were moderate around the 14th

day. It had severe and differentiating symptoms on the 21st day.

- 2. Pin-prick method:** After inoculation, on the 7th day, the weak symptom appeared and continued to be weak until the 14th day, but it was observed to be a strong canker symptom on the 21st day.
- 3. Needleless leaf injection method:** The initial weak canker symptom was observed on the 14th day after inoculation, whereas it was moderate on the 21st day.
- 4. Leaf clipping method:** The observations were on par with the observations of the pin-prick method. After inoculation, on the 7th day, the weak symptom appeared and continued to be weak until the 14th day, but it was observed to be a moderate canker symptom on the 21st day.
- 5. Spray inoculation method:** There were no symptoms after the spray inoculation.

The above-mentioned results revealed that the leaf injection/leaf infiltration method was able to produce the desired canker symptom early as compared to other methods, while the spray inoculation method failed to produce any canker symptom. First symptoms appeared on lower sides of the leaves as irregular water-soaked spots. Later, such spots appeared raised from the upper side of the leaves and they were rough to touch with yellow haloes.

B. Detached leaf assay

In the detached leaf test, three methods were used: the pinprick method, the leaf clipping method, and the spray inoculation method.

- 1. Pin-prick method:** The weak level of canker lesions was initiated from the 4th day to the 6th day, whereas on the 7th day, there were moderately water-soaked canker lesions. Strong canker lesions were observed on the 14th day, and the same was observed until the 21st day.
- 2. Leaf clipping method:** Weak canker symptoms were observed from the 5th day to the 7th day, and they were moderate on the 14th, 21st, and so on.
- 3. Spray inoculation method:** No symptoms developed until the 21st day, and so on.

The above-mentioned results revealed that the pinprick method was able to produce the desired canker symptom early (4th day) as compared to other methods, whereas the spray inoculation method failed to produce any canker symptom.

From the ongoing results it has been inferred that the leaf injection method and pinprick method of inoculation convincingly reproduced the early symptoms on the leaf, which indicates that both the inoculation methods are efficient. This may be due to direct entry point created by the inoculation methods to the passively entering bacteria.

The present study demonstrated that the development and severity of citrus canker symptoms were greatly influenced by the method of inoculation. Among the attached leaf inoculation techniques, leaf injection (leaf infiltration) produced the earliest and most severe symptoms, followed by the pin-prick and leaf clipping methods, whereas spray inoculation failed to induce disease symptoms. Similarly, in the detached leaf assay, the pin-prick method resulted in the earliest symptom development, while no symptoms were observed with spray inoculation. The rapid symptom

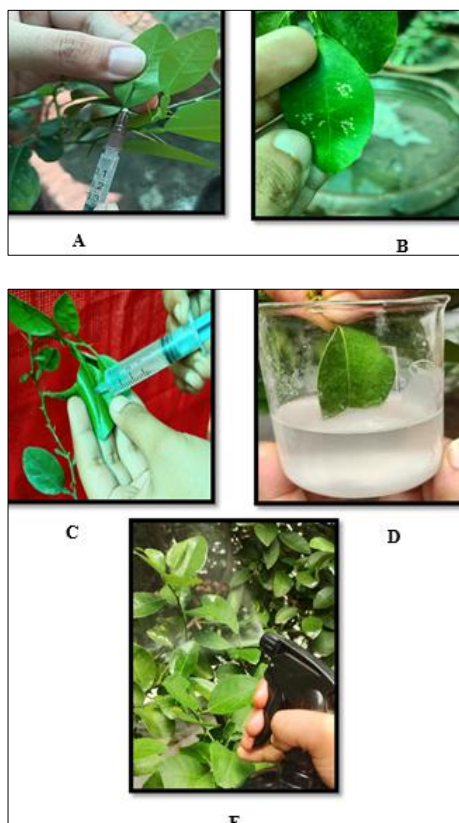
expression observed with leaf injection and pin-prick inoculation can be attributed to the direct introduction of *Xanthomonas citri* subsp. *citri* into the intercellular spaces and wounded tissues of the leaf. These methods bypass the natural barriers of the plant, allowing the pathogen to establish quickly, multiply within the apoplast, and produce virulence factors that cause cell hyperplasia, hypertrophy, and the characteristic raised canker lesions. In contrast, the leaf clipping method produced comparatively delayed symptoms because bacterial colonization was initially confined to the cut margins before spreading to the surrounding tissues. The absence of symptoms following spray inoculation may be explained by the inability of the bacterium to penetrate the intact cuticle and epidermis under the prevailing experimental conditions. Successful infection by *X. citri* subsp. *citri* through spray inoculation generally requires natural openings such as stomata or hydathodes, or wounds created by wind, thorns, or insect feeding, along

with prolonged periods of leaf wetness and high humidity. Since these conditions were not adequately available, bacterial entry and subsequent infection did not occur. Similar observations were reported by Graham and Leite (2004) ^[2], who demonstrated that wound-assisted inoculation methods provide rapid, consistent, and reproducible citrus canker infection for pathogenicity studies. Schubert *et al.* (2001) ^[6] also reported that *X. citri* subsp. *citri* primarily gains entry through stomata or wounds, after which it multiplies in the intercellular spaces, leading to the formation of typical corky canker lesions surrounded by chlorotic halos. Therefore, leaf injection and pin-prick inoculation methods are considered the most efficient, reliable, and reproducible techniques for pathogenicity testing, host–pathogen interaction studies, and evaluation of disease management strategies against citrus canker.

Table 1: Comparison of methods of inoculation of *Xanthomonas axonopodis* pv. *citri* in Acid lime

Sr. No.	Inoculation method	Time taken for symptom appearance (in days)								
		1 st	2 st	3 rd	4 th	5 th	6 th	7 th	14 th	21 st
Attached leaf										
1.	Leaf injection OR leaf infiltration.	-	-	-	-	-	+	+	++	+++
2.	Pinprick method	-	-	-	-	-	-	+	+	+++
3.	Needleless Leaf injection	-	-	-	-	-	-	-	+	++
4.	Leaf clipping	-	-	-	-	-	-	+	++	++
5.	Spray	-	-	-	-	-	-	-	-	-
Detached leaf										
1.	Pinprick	-	-	-	+	+	+	++	+++	+++
2.	Leaf clipping	-	-	-	-	+	+	+	++	++
3.	Spray	-	-	-	-	-	-	-	-	-

- No canker, + weak canker, ++moderate canker, +++ Strong canker (differentiating symptom)



A. Leaf injection method, B. Pinprick method
C. Needleless leaf injection method,
D. leaf clipping method, E. Spray method

Plate 1: Inoculation methods

4. Conclusion

The present study compared different inoculation techniques for establishing citrus canker caused by *Xanthomonas citri* subsp. *citri* under attached and detached leaf conditions. In the attached leaf assay, leaf injection (leaf infiltration) produced the earliest and most severe canker symptoms, followed by the pin-prick and leaf clipping methods, while spray inoculation failed to induce symptoms. In the detached leaf assay, the pin-prick method was the most effective, with symptom initiation as early as the fourth day after inoculation, followed by the leaf clipping method, whereas spray inoculation again failed to produce disease symptoms. The rapid and consistent symptom development observed with leaf injection and pin-prick methods was attributed to the direct introduction of the pathogen into wounded tissues, which facilitated bacterial entry, multiplication, and colonization. Conversely, the failure of spray inoculation was likely due to the inability of the bacterium to penetrate intact leaf surfaces in the absence of suitable natural openings or wounds. Overall, the study concluded that leaf injection (attached leaf assay) and pin-prick inoculation (detached leaf assay) are the most reliable, reproducible, and efficient methods for inducing citrus canker symptoms. These inoculation techniques can therefore be effectively employed for pathogenicity studies, host–pathogen interaction research, and evaluation of disease management strategies against citrus canker under controlled conditions.

5. Disclaimer (artificial intelligence)

Author(s) hereby declares that NO generative AI

technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

6. Competing Interests

Authors have declared that no competing interests exist.

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