Diagnosis and Characterization of the Citrus Canker Pathogen (*Xanthomonas axonopodis* pv. *citri*) Strains in Ethiopia

Eshetu Derso[1] *and C. Verniere*[2]
[1]*Ethiopian Institute of Agricultural Research (EIAR),*
Debre Zeit research center P.O. Box 32, Debre Zeit, Ethiopia, Fax: 00251116461251
[2] *CIRAD-Université de la Réunion, UMR PVBMT, Saint Pierre, La Réunion, F-97410 France*

Abstract

Citrus canker, caused by *Xanthomonas axonopodis* pv. *citri*, is a very destructive disease that is listed as a quarantine organism in many countries. Occurrence and distribution of the disease in the Rift Valley of Ethiopia had been reported. Studies were conducted with the objective of monitoring recent spread of the disease in the country, identifying, characterizing and determining the genetic diversity of 20 Ethiopian strains on the basis of their pathogenecity, biochemical, physiological, and molecular properties. Diagnosis was carried out using polymerase chain reaction (PCR). Pathogenecity tests, biochemical and physiological characterizations, insertion sequence ligation mediated polymerase chain reaction (IS-LM- PCR) and multilocus variable number of tandom repeat analysis (MLVA) were carried out following standard procedures established for same. Citrus canker was observed to spread to central and northern parts of the country. Pathogenecity tests indicated canker symptoms on Mexican lime (*Citrus aurantifolia*) and alemow (*Citrus macrophylla*) leaves and not on sweet orange (*Citrus sinensis*) and grapefruit (*Citrus paradisi*). The expected 492-bp amplification products were produced with DNAs of all Ethiopian strains. In the biochemical and physiological characterization, all strains showed similar reactions. All four primers in the IS-LM-PCR did not indicate satisfactory polymorphism within the strains. On the other hand, MLVA analysis indicated polymorphism between the tested Ethiopian strains and those from international collections. On the basis of pathogenecity, biochemical and physiological, tests, ISLM-PCR, and MLVA analysis results, the Ethiopian strains were grouped into a cluster comprising of *X. citri* pv. *citri* pathotype A* and is epidemiologically important on Mexican lime and alemow.

Introduction

Citrus canker is the most important and a very destructive disease, caused by an invasive bacterial plant pathogen species known as *Xanthomonas axonopodis* pv. *citri*, listed as a quarantine organism in many countries. It is believed that four pathotypes of the citrus canker pathogen (A, A*, B, and C) occur around the world and are induced by variants of the same causal agent. These pathotypes are primarily distinguished by their geographic origin and their host range (Pruvost *et al.*, 1992). Asian or A type is the most common and the most damaging of the canker strains and is pathogenic to virtually all cultivated members of the family Rutiace (Schubert and Miller, 2000).

Minor genetic variation of citrus canker strains has been detected in the A strains in Florida and other citrus growing regions of the world (Gottwald *et al.*, 2002) that could help to determine identification of their origin when introduced into new locations. Vernier and co-workers (1998) also designated some isolates of the Asian strain with a host range restricted naturally to Key/ Mexican lime (*C. aurantifolia*) and alemow (*C. macrophylla*) as A* strains.

Citrus canker is believed to have originated in the tropical areas of Asia, particularly in South China, India and Indonesia. It probably was disseminated from the islands of the Pacific Ocean to Japan and from Japan to countries such as the United States and South Africa (Stall and Seymour, 1983). In South America, citrus canker was first reported in Brazil in 1957. It subsequently spread to Uruguay, Paraguay, and Argentina. In spite of attempts to eradicate, the disease has become permanently established there. In recent years, it has spread to islands in the Indian Ocean, the Middle East and Africa (Eshetu *et al.*, 2004, Balestra *et al.*, 2008).

The economic importance of citrus canker stems from the fall of heavily infected fruits. Another aspect lies in the fact that citrus fruits produced in canker–endemic regions are placed under strict international regulation of plant quarantine (Goto, 1992) and hence, restricting the region's market. Severe infections lead to defoliation, die back and premature fruit drop, reducing yields by as much as 50%. In addition to tree debilitation, losses occur in quality and quantity and unmarketability of blemished fruits for fresh consumption (Schubert and Miller, 2000). It also results in devastating socioeconomic and political impacts.

The symptom of citrus canker may vary on different varieties and under different weather conditions. It is characterized by the formation of circular, water soaked lesions that become raised and blister-like, growing into white or yellow spongy pustules that then darken and thicken into a light tan to brown corky canker which is rough to the touch. An essential diagnostic symptom is tissue hyperplasia, i.e., unusual growth (Gabriel, 2001). The bacteria over seasons in leaf, twig, and fruit canker lesions. Plants of the genus *Citrus* are known to be susceptible to citrus canker in varying degrees.

Management of citrus canker is very difficult and there is no cure for it. Eradication and the subsequent restriction on importation of citrus fruits from regions where citrus canker is endemic, comprises a typical example of regulatory control (Goto, 1992).

In Ethiopia, the occurrence of the disease was reported in the Rift Valley (Eshetu *et al.*, 2004). However, there was lack of quantitative information on host range and genetic variability of *X. axonopodis* pv. *citri* in the country.

Hence, studies were conducted with the objective of monitoring the disease spread, identifying, characterizing and determining the genetic diversity of the Ethiopian strains on the basis of their pathogenicity, biochemical, physiological, and molecular properties.

In this paper, the use of PCR, pathogenecity, biochemical and physiological tests, and insertion sequence ligation mediated-PCR (IS-LM-PCR) and multiple locus variable number tandem repeat analysis (MLVA) typing systems to diagnose and characterize the Ethiopian *X. axonopodis* pv. *citri* strains are described.

Materials and Methods

Monitoring of citrus canker

Thirteen locations in two regional states were assessed for the occurrence and intensity of citrus canker on Mexican lime between October to November 2009. In Amhara and Oromia regions, the monitoring process was undertaken in backyard orchards belonging to farmers and at nursery sites and research centers. Monitoring methodology for occurrence and distribution was carried out following previously reported procedures by Eshetu (2006). Disease assessment was carried out randomly on forty representative attached leaves and 50 intact fruits per tree. Sample sizes were determined from similar studies conducted on Phaeoramularia leaf and fruit spot of citrus (Seif and Hillocks, 1999).

Disease incidence and severity data on leaves and fruits were arcsine transformed prior to analysis by descriptive statistics using Excel 2003 program (IMI, 1995). Precision of citrus canker incidence

and severity were determined using coefficient of variation (CV) and standard error (Nutter, 2001).

Sample collection and pathogen isolation

Leaves showing canker symptoms were collected from Mexican lime (*C. aurantifolia*) in the Rift Valley at five locations viz., Melka Werer, Metehara, Awara Melka, Merti Jeju, Nura Era, and were designated as MW, MH, AM, MJ and NE respectively. Ten different leaflets from each site were washed for 10 minutes in running tap water, surface sterilized by dipping in 70% ethanol for one minute, and rinsed twice with sterile distilled water (SDW). Individual lesions were punched with a cork borer and separated from the leaves, finely chopped and placed in sterile Petri plates and then were put into McCartney bottles containing five ml sterilized distilled water and left for 15 minutes. From each sample, 0.1 ml aliquots of the wash suspension were taken in a pippettor and streaked out on Petri plates containing the selective medium yeast dextrose chalk agar (YDCA). Petri plates were incubated at 30°C for 48 hr (Schoulties *et al.*, 1987).

Inoculum preparation

Bacterial isolate preparation was carried out with slight modification of the methodology by Egel and co-workers (1991). Bacterial strains from the 20 isolates were cultured for 14 hr in nutrient glucose broth, harvested by centrifugation at 8,000 g for 15 minutes and after centrifugation of the cell suspension, bacterial pellets were re-suspended in sterile tap water. Bacterial population was adjusted turbidimetrically to 10^8 colony-forming units per ml (0.18 absorbance at 590 nm, measured using Spectrophotometer Pharmacia NOVASPEC^R II) and appropriate dilutions were made. Final populations were determined by plating on nutrient glucose agar (Difco nutrient agar amended with 1% glucose) (Egel *et al.*, 1991).

Pathogenecity test using detached leaves

Plant materials

Fruits of sweet orange (*C. sinensis*) Mexican lime (*C. aurantifolia*), grapefruit (*C. paradisi*) and alemow (*C. macrophylla)* were obtained from Melkassa Agricultural Research Center of the Ethiopian Institute of Agricultural Research (EIAR). Seeds from the respective fruits were extracted and washed with clean water and surface-dried in a cool place on a clean screen surface under shade. The seeds were planted in open furrows about three cm deep in the soil in plastic boxes. Seedlings were grown in glasshouse with ambient temperature and relative humidity ranging from 25 to 30°C and 60 to 100%, respectively. They were then transferred to larger containers at four leaf stage. All test cultivars when pencil-sized were bud grafted on sour orange (*C. aurantium*) that was produced from seed. Plants were maintained in 5 l (10 × 10 × 35 cm) plastic pots in soil mixture containing sand, clay and chicken dung (3:1:1 v/v) (Alves *et al.*, 2004).

Inoculation

Two thirds to fully expanded immature leaves from seedlings of the test cultivars; Mexican lime (*C. aurantifolia*), alemow (*C. macrophylla*), grapefruit (*C. paradis*) and sweet orange (*C. sinensis*) grown in the greenhouse, were detached and separated. Leaflets were washed for 10 minutes in running tap water, surface sterilized by dipping in 70% ethanol for one minute, rinsed twice with sterile distilled water, and placed with their abaxial surface exposed in 100 mm-diameter plastic Petri plates each containing 20 ml of 1.5% water agar amended with 100 mg/l benzimidazole (Fox, 1993). Four detached leaves from each test cultivar were punctured with a 26 gauge syringe needle (Egel et *al.*, 1991) on three to five spots, depending on leaf size, on both sides of the midrib. Ten µl of a bacterial suspension containing 10^8 cfu/ml were placed on each wound site. Non-inoculated controls were treated with an equal volume of sterile distilled water. Petri plates with inoculated detached leaves were placed in a growth chamber at 30°C and incubated under fluorescent lights (60 micronE sec/min m/2) for 14 hr photoperiods for nine days to induce optimal symptom expression (Verniere *et al.*, 1998).

Disease assessment

Nine days after inoculation, the inoculated leaflets were examined using 10x magnification under stereo microscope fitted with ocular micrometer. Lesions were recorded as "present" or "absent" and

their sizes determined by measuring the diameter and taking their means for the respective cultivar (data not presented) and lesions were photographed. To confirm presence of the pathogen, re-isolations were made. Sample lesions on inoculated leaves were excised and grown on selective media, yeast dextrose chalk agar (YDCA). The detached leaf assay was repeated once (Broadbent *et al.*, 1992). The design was completely randomized design (CRD) with four replicated Petri plates each containing a single leaf. Variation in lesion size were compared by ANOVA analysis, and means were compared using Tukey's HSD procedure (alpha = 0.05). ANOVA analysis and Tukey procedures were run using the statistical software package SAS V8 (Statistical Analysis Systems, Cary, NC).

Biochemical and physiological characterization

The 20 *X. axonopodis* pv. *citri* strains were compared as per the procedure of Eshetu (2006) on the basis of their, biochemical, physiological and metabolic properties. It includes, Gram Reaction using 3% KOH, Voges-Proskauer (VP) and Methyl Red Test (MR), Levan Production, Nitrate Reduction, Hydrogen Sulphide Production, Milk Proteolysis (Casein hydrolysis, Starch Hydrolysis, Sodium Chloride Tolerance, Oxidase Reaction Oxidative/Fermentative Growth Test, Gelatin Hydrolysis, Esculin (Aesculin) Hydrolysis and Esterase Activity (Tween Hydrolysis). Test results were recorded as "+"(positive response) or "-"(negative response). The experiment was repeated once (Broadbent *et al.*, 1992). The design was completely randomized design (CRD) with four replicated Petri plates/ beakers used for each determinative test and strain.

Molecular Characterization

PCR based diagnosis of *X. axonopodis* pv. *citri* strains

A total of 20 bacterial test strains were used in this study. Single colonies were subcultured on LPGA (Yeast extract 7 g /l, glucose 7 g/l and agar 18 g/l; propiconazole 20 mg/l) plates for 24 hr at 28°C. These subcultures were used to inoculate four ml of YP broth (yeast extract,7 g/l; peptone,7g/l;pH 7.2) tubes, which were incubated at 28°C on an orbital shaker for 16-18 hr. These suspensions were used for DNA extraction using the DNeasy tissue kit (Qiagen, Courtaboef, France) following the manufacurere's instructions. DNA concentrations were estimated by spectrophotometer (TKO 100 Fluorometer, Hoefer, and San Francisco) and amplified using Thermocycler (Mastercycler 5330 Applied Bioscience). PCR products were separated and visualized by gel electrophoresis (Liu *et al.*, 2003). PCR was used to check the identity of these isolates by using *X. citri* pv. *citri* strain FDC12 from New Zealand as a reference strain and sterilized distilled water (SDW) as the template for the negative control. Primers used were XAC4 and XAC7 (PE Applied Biosystems, France). PCR fingerprint results were converted to binary form (0 = absence or negative; 1 = presence or positive) as described by Nei and Kumar (2000).

Genetic diversity (polymorphism) analysis

Twenty strains collected from the Rift Valley between January and February 2008 were used both in the IS-LM- PCR and MLVA. Fifteen representative *X. axonopodis* pv. *citri* A and A* strains originating from various countries were also included in the MLVA.

IS-LM-PCR fingerprinting and MLVA analysis were performed as described by Leo and co-workers (2004). To test the reproducibility of both techniques, two independent DNA extractions were used for all strains, with CFBP 2525 as a reference strain in each experiment. Representatives of pathotype A, and A* strains were also included in the MLVA analysis. Primers IS1GF (blue), IS1D3 (green), IS2D3 (red) and IS3IRF (blue) were used in both tests. Amplified PCR products were separated and visualized by capillary electrophoresis using genetic analyzer (ABI PRISM-3130xl). Results were analyzed by GeneMarker® 4.0 software. The presence and absence of fragments were scored as a binary matrix and analyzed. The size of each fragment in the range of 50 – 500 base pairs (bp) was determined. Fragments above a threshold set to 500 relative fluorescence units were scored. Only fragments detected for both DNA replicates were scored as positive in the data matrix. Dice similarity indices were used to construct weighted

neighbor-joining trees from a pooled data set (Saiton and Nei, 1987). The robustness of the tree was assessed by boot strap (1000 re-samplings). The diagnosis and molecular characterization studies were conducted at Université de la Réunion, Saint Pierre, France.

Results

Monitoring citrus canker

The data in Table 1 shows the occurrence and distribution of citrus canker in the north and central parts of the country on Mexican lime. In Oromia region, it was recorded at Melkassa and Debre Zeit agricultural research centers on experimental plots, while in the Amhara region it was recorded on farmers' orchards in Shewa Robit and at a testing site belonging to Sirinka research center at Jari. High disease incidence on leaves, 75%, and severity, 45%, were observed at Jari followed by Shoa Robit and Melkassa. Relatively low disease incidence, 40%, and severity, 25%, were observed at Debre Zeit (Table 1).

Table 1. Occurrence and distribution of citrus canker in the north and central parts of Ethiopia (2009)

Location	Altitude (masl)	Host	Crop age (year)	Mean* incidence and severity on leaves		Mean* incidence and severity on fruits	
				Incidence (%)	Severity (%)	Incidence (%)	Severity **
Shoa Robit	1570	Mexican. lime	10	63ab (45-81)	40a	21ab (10-32)	2 (1-3)***
Jari	1710	Mexican. lime	9	75a (50-90)	45a	25a (15-35)	2.5 (2-3)
Melkassa	1560	Mexican. lime	2	55b (40-75)	30ab	20a	1.4 (1-2)
Debre Zeit	1950	Mexican. lime	3	40bc (35-60)	25b	10b	1 (0-2)
Mean	-	-	-	54.6	33.06	16.8	1.58
CV (%)	-	-	-	48.12	28.1	34.00	-
SEM	-	-	-	9.4	4.8	2.8	-

Means within a column followed by the same letters are not statistically different at $p \leq 0.05$

* Mean of five trees, ** 0-4 score scale, where 0=0%, 1=1-10%, 2=11-25, 3 = 26 - 50 %, 4 = >50 % (Seif and Hillocks, 1999).

*** Values in parenthesis are ranges of citrus canker incidence and severity.

Detached leaf test (pathogenecity test)

In the detached leaf tests, less erumpent and non-spreading lesions were formed and *X. axonopodis* pv. *citri* strains were consistently recovered from inoculated detached leaves of Mexican lime (*C. aurantifolia*) and alemow (*C. macrophylla)* at the end of both experiments. Typical symptoms developed one week after inoculation. Lesions were less erumpent and did not spread, and water-soaking and necrosis were absent after 10 days. No symptoms were observed on grapefruit, sweet orange and the control (Table 2).

Biochemical and Physiological Characterization

The biochemical and physiological characterization of strains from the 20 representative isolates showed similar reactions to the standard determinative tests. They were negative to the nitrate reduction, methyl red test, fermentative growth test and oxidase reaction, while they showed positive reactions to the rest of the tests (Table 3).

Table 2. Pathogenicity tests of *Xanthomonas axonopodis* pv. *citri* strains from Ethiopia on citrus cultivars using detached leaf tests

Citrus cultivar	Lesion	Recovery of *X. axonopodis* pv. *citri*
Mexican lime (*C. aurantifolia*)	Present	Present
Alemow (*C. macrophylla*)	Present	Present
Sweet orange (*C. sinensis*)	Absent	Absent
Grapefruit (*C. grandis*)	Absent	Absent
Control (SDW)	Absent	Absent

Table 3. Biochemical and physiological characteristics of *Xanthomonas axonopodis* pv. *citri* strains from Ethiopia

Determinative test	Reaction
Gram stain using 3% KOH	+
Levan formation	+
Nitrate reduction	-
Voges- Proskauer and methyl red test	+/ -
Hydrogen sulphide production	+
Casein hydrolysis	+
Starch hydrolysis	+
Sodium chloride tolerance	+
Oxidative/ Fermentative growth test	+/ -
Gelatin hydrolysis	+
Aesculin hydrolysis	+
Tween hydrolysis	+
Oxidase reaction	-

Molecular Characterization

PCR based diagnosis

The two primer mixes were able to generate amplification products from the 20 studied isolates. The expected DNA fragments having bp size 492 that were similar to the standard check FDC12 were observed at all lanes where the strains were inoculated. On the other hand, no DNA fragments were amplified on lanes where the untreated control viz. sterile distilled water (SDW) was inoculated. Two gels were used to visualize PCR products and were placed side by side. On the first gel, the untreated control (T) was on lane 5 while on the second gel it was shown on lane 21 (Fig.1).

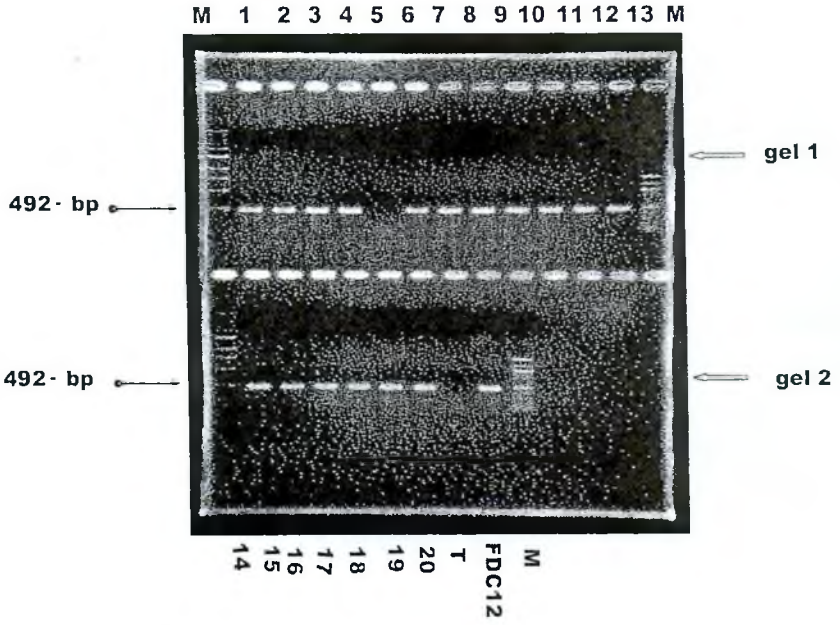

Fig.1 = Polymerase chain reaction (PCR) based diagnosis of *Xanthomonas axonopodis* pv. *citri* strains from Ethiopia. M= Molecular marker, FDC12 = positive control, T = template for the negative control

Genetic diversity (polymorphism) analysis

X. axonopodis pv. citri strains collected from the Rift Valley regions and designated as MW, MH, AM, MJ and NE were not differentiated into genetically different entities and showed low boot strap values (Fig.2). The analysis was based on 14 variable number of tandom repeats (VNTRs) and was performed using insertion sequence ligation mediated polymerase chain reaction (ISLM-PCR) Manhattan distance / Neighbor Joining - bootstrap = 1000).

It appeared that the strains showed no polymorphism within the group. On the other hand, MLVA analysis grouped all of the Ethiopian *X. axonopodis* pv. *citri* strains in a cluster only comprised of *X. axonopodis* pv. *citri*, pathotype A* (Fig.3). Thus, the test clearly indicated existence of genetic variability, polymorphism, between the Ethiopian strains and the rest of the strains from international collections. Moreover, the strains indicated very high boot strap values which can corroborate their genetic diversity.

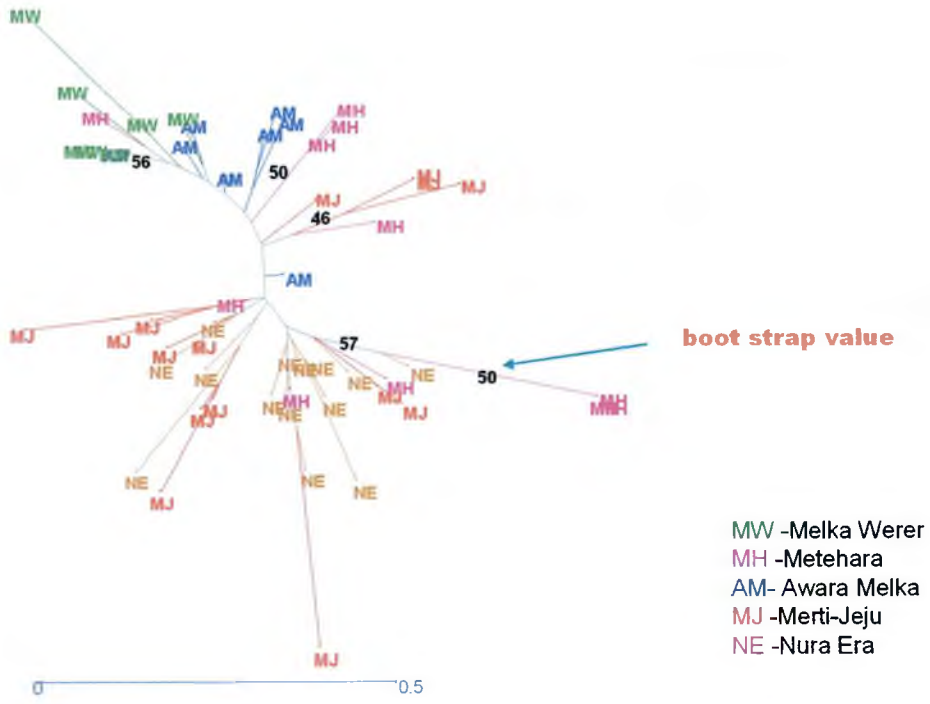

Fig .2 = Genetic diversity analysis within *Xanthomonas axonopodis* pv. citri strains from Ethiopia using IS-LM-PCR

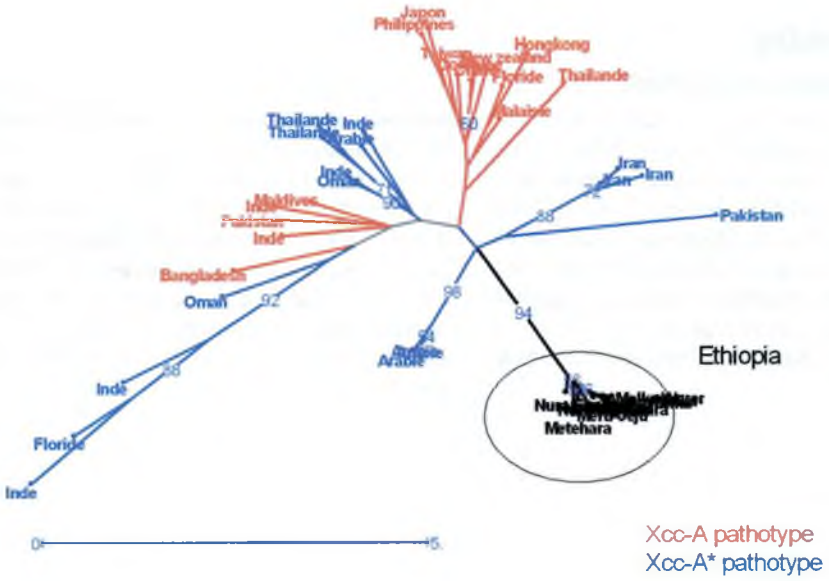

Fig. 3 = Neighbor joining tree of Xanthomonas axonopodis pv. citri strains from Ethiopia and international collections (14 minisatelites (MLVA)/ Manhatten distance)

Discussion

Previous studies in the Rift Valley of Ethiopia indicated that citrus canker occurred at elevations ranging from 800-1200 masl and was mostly thought to be a low land disease (Eshetu *et al.*, 2007). However, in the current study it was recorded at elevations ranging from 1560 masl at Melkassa to 1950 at Debre Zeit. Hence, elevation seems to play minor role in citrus canker development. On the other hand, Agrios (2005) reported that the disease is more severe in areas in which the periods of high rainfall coincide with the periods of high mean temperature.

The use of pathogenecity, biochemical and physiological tests, PCR, insertion sequence ligation mediated-PCR (IS-LM-PCR) and multiple locus variable number tandem repeat analysis (MLVA) typing systems allowed to diagnose and evaluate diversity of *X. axonopodis* pv. *citri* strains causing citrus canker in Ethiopia and to relate them to worldwide collections in order to establish the genetic variability between the strains.

Confirmation on strains of the causal agent, *X. axonopodis pv. citri,* was sought by application of appropriate techniques viz. isolation, purification and characterization following the procedure of Strange (2003). To seek gross comparisons among the strains pathogenicity tests, which distinguish between the strains ability to infect host cultivars, were conducted on seedlings of the four citrus cultivars. In the current study, the detached leaf assay was utilized as a method to determine the reaction of the test citrus cultivars to the citrus canker pathogen, *X. axonopodis* pv. *citri* strains. The technique allowed cultivar X strain specificity to be detected, such that each of the test citrus cultivar showed a consistent, stable and repeatable specific reaction towards the representative strains. The assay allowed rapid and accurate evaluation of the test cultivars. Citrus canker lesions on artificially inoculated leaves in the laboratory were generally similar but not always identical to naturally occurring lesions in the field. This might be due to as yet unknown specific environmental requirements for infection and symptom development (Rodriguez *et al.*, 1985). The search for identity has been narrowed to the genus *Xanthomonas* using biochemical and physiological

characterization that detected no differences in their metabolism.

Though each of the above methods is best suited for detecting and characterizing *X. axonopodis* pv. *citri* strains, combination of the tests provided accurate characterization (Nester *et al.*, 2004).

PCR-based methods for diagnosis of citrus canker have been described previously (Schubert *et al.*, 2001). These methods are based on primers designed to amplify a fragment in a DNA of *X. axonopodis* pv. *citri*. Biochemical and physiological tests and PCR diagnosis on the strains showed characteristics specific to the species, *X. axonopodis* pv. *citri*, and did not differentiate them into pathotypes (A or A*).

On the other hand, pathogenecity test, ISLM-PCR and MLVA analysis revealed that all 20 Ethiopian strains belong to pathotype A* and hence the methods indicated characteristics specific to the pathotype.

Low boot strap values were observed within the Ethiopian *X. axonopodis* pv. *citri* strains while high values were observed between the Ethiopian strains and those from international collections. Boot strap values usually indicate the probability of dissimilarity or polymorphism within and between strains. Values exceeding 70 % are expected to show genetic variability, while strains having probability levels below this figure are believed to be genetically similar (Nei and Kumar, 2000). Hence, in this study low boot strap values might confirm absence of polymorphism within the Ethiopian strains while high values for the international strains and the Ethiopian strains might indicate polymorphism or genetic diversity between the strains. MLVA analysis revealed considerable heterogeneity and amplified polymorphisms, rather than their homologies. It also analyzed the genetic structure and diversity among populations of strains in the international collections and those from Ethiopia. In addition, it detected variation at individual loci that might have resulted from differences in the number of tandem repeats (VNTR) which again could be thought to be caused by errors in DNA replication (Liu *et al.*, 2003). The result of this study show that MLVA is a valuable technique to characterize *X. axonopodis* pv. *citri* strains. It has also been successfully used by Leo and co-workers (2004) to characterize several different bacterial species and proven to be an excellent method with high resolution.

Conclusion

On the basis of pathogenecity, biochemical and physiological tests, PCR identification, ISLM-PCR and MLVA analysis, Ethiopian strains of *X. axanopodis* pv. *citri* were closely related to pathotype A* and not to pathotype A. This confirms the larger geographical distribution of pathotype A*, and to our knowledge, this is the first report of its presence on the African continent. This could allow studying the epidemiology of pathotype A* strains in a unique situation where they do not compete with pathotype A strains (Eshetu et. al., 2009). The molecular characterization of Ethiopian strains suggests that this introduction event is not related to the recent introduction of citrus canker in neighboring Somalia where *X. citri* pv. *citri* pathotype A was identified (Balestra *et al.*, 2008). The occurrence of pathotype A in Somalia is very alarming and we suggest that Ethiopia should prevent the introduction and establishment of this wide host range pathotype to avoid further negative impacts on citrus production. To make this a reality, efforts should be made to exclude the pathogen from entering the country through quarantine and establishment of task forces to regulate the introduction of citrus fruits or any other planting materials of citrus spp. from neighboring countries, especially from Somalia. On the other hand, large scale production of Mexican lime (*C. aurantifolia*) in Ethiopia requires special caution, in this case, production of lemon (*C. limetta*) could be considered as an alternative option.

Maintaining nursery sanitation should also be strictly practiced in large scale farms. Monitoring of pathotype A* and its distribution in the country should continue on a regular basis. Awareness creation seminars and trainings should be given at various levels in different regions of the country.

Finally, it is noteworthy that if the appropriate preventive measures indicated above are not properly pursued, it would only be a matter of time before pathotype A is introduced and wipeout the

entire citrus plant in Ethiopia, and then it would be too late to reverse the situation.

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