African Journal of Biotechnology Vol. 9 (54), pp. 9092-9095, 29 December, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

Review

Molecular and biochemical characterization of *Xanthomonas axonopodis* pv. *citri* pathotypes

Khalid Hussain[1,2], Khalid Nawaz[2], Abdul Majeed[2], Ikram-ul-Haq[3], Feng Lin[1]*, Kazim Ali[4], Shahid Afghan[4], Farah Khan[5], Abdul Ghani[6] and Ghulam Raza[7]

[1]Crop Biotechnology, Shenyang Agricultural University, Liaoning–China.
[2]Department of Botany, University of Gujrat (UOG), Pakistan.
[3]Institute of Biotechnology and Genetic Engineering, University of Sindh, Jamshoro Pakistan.
[4]Shakarganj Sugar Research Institute (SSRI), Jhang, Pakistan.
[5]Department of Botany, Lahore College for Women University, Lahore, Pakistan.
[6]Department of Biological Sciences, University of Sargodha, Sargodha, Pakistan.
[7]Nuclear Institute of Agriculture (NIA) Tandojam, Sindh-Pakistan.

Accepted 17 December, 2010

Prevalence of citrus bacterial canker caused by *Xanthomonas axonopodis* pv. *citri* in citrus groves is the major impediment and limiting factor in successful citrus production. Severity varies among different species, varieties and prevailing climatic conditions. Despite extensive studies on the biology, epidemiology and management of this disease, there is still little known about the role of different biocontrol agents for management of this disease. Traditional management of *X. axonopodis* pv *citri* is brought about by chemicals which have become complicated through the development of chemical resistance, and as such, it is hazardous for health. It is necessary to identify the pathotypes of *X. axonopodis* pv. *citri* through biochemical and molecular characterization and to determine the role of different biocontrol agents (antibiotics and plant extracts), in order to find out a safer way for controlling citrus canker as disease severity results in defoliation, dieback, premature fruit drop and blemished fruit that consequently decrease fruit production and market value.

Key words: Citrus, bacterial canker, *Xanthomonas*.

INTRODUCTION

Citrus is a delectable, juicy and seedless fruit having great nutritional significance (Khan et al., 1992). Additionally, it possesses enormous therapeutic qualities (Chaudhry et al., 1992). Although citrus crop is kept in great esteem, yet its present status is threatened by a number of problems, including low production caused by diseases. Citrus plant is attacked by a number of diseases like citrus canker, gummosis, citrus decline, CTV, greening, etc. Citrus canker is caused by the bacterium "*Xanthomonas campestris* pv. *citri*" that is probably the worst enemy to citrus plants (Sahi et al., 2007). *X. campestris* pv. *citri* is a rod shaped gram negative bacterium with single polar flagellum. The growth of this bacterium is obligatorily aerobic, while maximum temperature for growth is 35 to 39°C (Mehrotra, 1980; Whiteside et al., 1988).

Citrus canker disease is of regular occurrence on several citrus cultivars in varying degrees of incidence depending on the climatic conditions. The bacterium, *Xanthomonas*, causes different symptoms ranging from pustules to necrotic lesions consisting of erumpent corky tissue surrounded by water soaked tissues and yellow halo on leaves, stems and fruits (Zekri et al., 2005; Graham et al., 2004; Schubert and Sun, 2003; Burning and Gabriel, 2003; Das, 2003; Bergamin-Filho et al., 2000). As such, disease severity on susceptible variety results in defoliation, dieback, premature fruit drop and blemished fruit, which consequently decrease fruit production and market value (Zekri et al., 2005; Stall and Seymour, 1983).

There are many types of citrus canker disease caused by various pathovars and variants of the bacterium *Xanthomonas axonopodis* (Graham et al., 2004). Due to

*Corresponding author. E-mail: fenglinsn@126.com.

symptoms similarity, the separation of these bacterium forms is very difficult based on host range, cultural and physiological characteristics, bacteriophage sensitivity (Civerolo, 1984), serology (Alvarez et al., 1991), plasmid fingerprints (Pruvost et al., 1992), DNA-DNA homology (Egel et al., 1991) and by various RFLP and polymerase chain reaction (PCR) analyses (Cubero and Graham, 2002; Hartung, 1992; Hartung et al., 1989; 1993; 1996; Verniere et al., 1998). All cultivars of citrus are susceptible to canker, but grapefruit, Mexican lime and lemon are highly susceptible, whereas sour orange and sweet orange are moderately susceptible. Mandarins are moderately resistant (Gottwald et al., 2002) and all young above-ground tissues of citrus are susceptible to *X. axonopodis*. In fact, bacterial pathogen is transmitted into the plant tissues through natural openings (stomata) and mechanical injuries (wounds). However, as pathogen enters into the plant lesions, the colour changes into brown, and as such, water soaked margin, surrounded by a chlorotic halo, appears.

As a result of cosmopolitan occurrence of citrus canker, different aspects of the disease have been potentially addressed and adequately researched in various parts of the world, thereby generating substantial information on the biology and management of the disease. As such, the local conditions only limited the work that has been done on the identification of pathotypes epidemiology for proper management (Akhtar et al., 1996). It has become mandatory to substitute the conventional method of disease management (chemical control) with safer and environment-friendly management strategies (biological and genetic control). This would lead to the alleviation of the dependence on the chemicals that will be ecofriendly for the environment.

BIOCHEMICAL CHARACTERIZATION

Biochemical analysis of canker infected leaves showed a remarkable decrease in amino acid contents in infected leaves as compared to healthy leaves. Additionally, more pronounced increase in total phenols was also observed in *Citrus reticulata* (Kishore and Chand, 1972; 1975). Biochemical, physiological characteristics and metabolic profiles of *X. campestris* pv. *Citri* strains, associated with A, B, C, D and E (CBSD) forms of citrus bacterial canker disease were grouped into three by means of hydrolyses of gelatin, casein and tolerance to NaCl. A and CBS strain, associated with CBCD, can be separated from B, C and D by their ability to utilize maltose, starch and glycogen. As such, C strains possess quite some unique characteristics of utilizing D α -alanine and L- serine and differ from strains B and D, but have very similar metabolic profiles (Verniere et al., 1991).

After artificial inoculation with all the pathotypes of *X. axonopodis* pv. *citri* strains, isolated from Mexican lime, C*itrus aurantiifolia* produced typical erumpent bacterial canker lesions. Strains from this group also hydrolysed gelatin and casein and have been grown in the presence of 3% NaCl like the typical *X. axonopodis* pv. citri pathotype A (Verniere et al.,1998).

MOLECULAR CHARACTERIZATION

An internal standard is employed for the detection of citrus bacterial canker by PCR to ensure the quality of the DNA extraction which properly requisite the existence of amplification reaction. The ratio of PCR products from the internal standard and bacterial target is used to estimate the initial bacterial concentration in citrus tissues with lesions (Cubero et al., 2001).

Phenotypical characterization and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of *X. axonopodis* pv. *citri* strains in Southern Iran showed slight differences in soluble protein profiles among the strains. Moreover, superoxide dismutase (SOD) and esterase (EST) binding patterns were distinct on the basis of isozymic analysis. Asiatic (A) and a typical Asiatic (aA) forms were classified from these strains by the host range specificity and phenotypic characters, and as such, the negligible restriction patterns were presented by DNA finger printing analysis using *Eco*RI between these two groups (Mohammadi et al., 2001).

A comparison among 73 *X. axonopodis* strains, isolated from the affected citrus trees in Southern Iran by amplified fragment length polymorphism (AFLP) fingerprinting, revealed five clusters. These included: (1) strains of group C, (2) strains of groups B and D, (3) strains of pathotype E, (4) strains of pathotype A together with the main group of Iranian strains and (5) seven strains from Iran which constituted a separate cluster. Later, the seven strains were considered as new pathotypes due to their dissimilarity with the other pathotypes (Khodakaramian and Swings, 2002).

Molecular diagnostic procedures can be used for the detection and identification of exotic bacteria isolated from citrus leaves in kerikeri as produced via canker-like symptoms caused by *Elsinoe fawcettii*, different from *X. axonopodis* pv. citri (*Xac*). The polymerase chain reaction (PCR) analyses of plant tissue and bacterial colonies were performed due to its specificity and sensitivity of Xac detection (Taylor et al., 2002). A wide range of physiological, biochemical, serological, molecular and pathogenic variation was found among strains of bacteria associated with citrus canker. However, a better understanding of the pathogenic specialization and proper identification of *Xac* strains are needed (Das, 2003).

Primers for rep-PCR with BOX and ERIC elements are used for separation and differentiation of strains' type within the same pathotype. *Xanthomonas* strains causing canker in Florida was evaluated by this methodology and it relates these strains to isolates in a worldwide collection (Graham et al., 2004). Citrus bacterial canker

(CBC) is caused by at least two groups of phylogenetically distinct *Xanthomonas citri* strains. Therefore, accurate, fast and reliable detection of this bacterium is of much importance as it has been reported thrice in Florida during the last 15 years. Fast, sensitive and reliable real-time polymerase chain reaction (PCR) assay along with designed primers have been developed to detect all canker-causing strains keeping in view its importance both in terms of specificity and sensitivity (Mavrodieva et al., 2004).

The presence of citrus canker on key/Mexican lime (*C. aurantiifolia*) and alemew (*Citrus macrophylla*) trees had been reported long before its detection in South Florida. Colonies, isolated from different infected portions, resembled the Asiatic group of *X. axonopodis* pv *citri* (Xac-A) strains, in terms of growth characters on nutrient agar plates. Xac-A specific monoclonal antibody A1, using enzyme linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) based assays, could not detect this bacterium. These strains can easily be identified and differentiated from *Xac*-A and *Xac*-A* using ELISA, PCR- based tests, pulsed- field gel electrophoresis of genomic DNA and host specificity (Sun et al., 2004).

An integrated approach was used for the detection and comparison of *X. axonopodis* pv. *citri* (*Xac*) from imported citrus fruits that include bacterial isolations, three conventional polymerase chain reaction (PCR) protocols and real time PCR with SYBR green or a taqman probe in canker lesions. By real time PCR, using Taqman probe, bacterium lesions were relatively detected more as compared to SYBR green and conventional PCR. Real time PCR with Taqman probe is the most sensitive and fastest screening method of *Xac* on fresh fruit samples (Golmohammadi et al., 2007).

Management of citrus bacterial canker, *X. axonopodis* pv *citri*, could be done by adopting different ways that were earlier mentioned in the literature. Diffusates, from various plants such as forest trees, shrubs, herbs, fruit seeds, etc. and from various parts of *Phyllanthus emblica, Accacia nilotica, Sapindus mukorossis* and *Terminakia chebula* which exhibited an inhibition zone of 4.83 to 6 mm at 50 g/liter, appeared to be the most effective diffusate against *X. campestris* pv. *citri*. These diffusates of higher plants having increased antimicrobial activity could be used for managing citrus canker disease as possessing both protective and curative actions (Akhtar et al., 1997).

Different types of Thai herbal extracted by 95% ethyl alcohol at 100,000 ppm concentration were tested by paper disc diffusion method on double layered NGA against *X. axonopodis* pv. *citri* and citrus bacterial canker. Guava leaf, beleric myrobalan fruit, pomegranate fruit peel, nut gall fruit and myrobalan wood fruit had more pronounced effect on the inhibition of bacteria, on culture media. While tested at different concentrations of 1,000,000, 50,000, 10,000, 5000 and 1000 ppm, guava leaf could inhibit growth up to 50,000 ppm as compared to 10,000 ppm by myrobalan wood fruit extract, whereas beleric myrobalan fruit, pomegranate fruit peel and nut gall peel extracts caused inhibition at all concentrations (Vudhivanic, 2003).

Anti-bacterial activity assays of extracted powdered leaf and pod material of *Caesalpinia coriaria* (Jacq.) Willd with water and successively amid different solvents such as petroleum ether, benzene, chloroform, methanol and ethanol suggested that it is a potential plant for the management of phytopathogenic *Xanthomonas* pathovars of tomato, French bean and cotton. Aqueous pod extract showed significantly increased activity by cup diffusion method, while a comparison of the inhibitory activity of the extracts with the antibiotics bacterimycin 2000 and streptocycline revealed that methanol and ethanol extract of both leaf and pod and aqueous extract of pod were significantly higher than that of the antibiotics tested (Mohana and Raveesha, 2006).

Biological control of citrus bacterial canker, *X. axonopodis* pv. *citri*, was carried out by pseudomonas strains (Putida and fluorescent) *in vitro* and in green house. On *in vitro* based evaluations, strains having high potential for inhibition along with antagonistic activities were selected against *X. citri* for green house evaluation as the disease was reduced by the selection between 23.8 and 64% (Khodakaramian et al., 2008).

CONCLUSION

It was concluded that biochemical and molecular characterization of *X. axonopodis* pv. *citri* is necessary for the identification and control measures of citrus canker disease. However, disease severity results in defoliation, dieback, premature fruit drop and blemished fruit which consequently decrease fruit production and market value.

REFERENCES

Alvarez AM, Benedict AA, Mizumoto CY, Pollard LW, Civerola EL (1991). Analysis of *Xanthomonas campestris* pv, *citri* and *X. c. citrumelo* with monoclonal antibodies. Phytopathology, 81: 857:865.

Akhtar MA, Bhatti MHR, Aslam M (1996). Characterization of *Xanthomonas campestris* pv, *citri* strains. Pak. J. Phytopathol. 8(1): 5-10.

Akhtar MA, Rahber-Bhatti MH, Aslam M (1997). Antibacterial activity of plant diffusate against *Xanthomonas campestris* pv. *citri*. Int. J. Pest Manage. 43(2): 149-153.

Bergamin-Filho A, Amorim L, Laranjeria L, Gottwald TR (2000). Epidemiology of citrus canker in Brazil with and with out the Asian citrus leafminer. In: Proceedings of the International citrus canker Research Workshop, Ft. Pierce, Florida.

Burning AM, Gabriel DW (2003). *Xanthomonas citri*: breaking the surface. Mol. Plant Pathol. 4(3): 141-157.

Civerolo EL (1984). Bacterial canker disease of citrus. J Rio Grande Valley Hort. Assoc. 37: 127-146.

Cubero J, Graham JH, Gottwald TR (2001). Quantitative PCR method for diagnosis of citrus bacterial canker. Appl. Environ. Microbiol. 67(6): 2849- 2852.

Cubero J, Graham JH (2002). Genetic relationship among worldwide strains of *Xanthomonas* causing canker in citrus species and design of new primers for their identification by PCR. Appl. Environ.

Microbiol.68: 1257-1264.
Das AK (2003). Citrus canker-A review. J. Appl. Hort. 5(1): 52-60.
Egel DS, Graham JH, Stall RE (1991). Genomic relatedness of *Xanthomonas campestris* strains causing diseases of citrus. Appl. Environ. Microbol. 57: 2724-2730.
Gottwald TR, Graham JH, Schubert TS (2002). Citrus canker the pathogen and its impact. Online. Plant health progress.doi:10.1094/ PHP-2002-0812-01-RV.<http://plantmangementnetwork.org/pub/phb/ review/citrucanker/>.
Golmohammadi M, Cubero J, Penalver J, Quesada JM, Lopez MM, Llop M (2007). Diagnosis of Xanthomonas axonopodis pv *citri*, causal agent of citrus canker, in commercial fruits by isolation and PCR based methods. J. Appl. Microbiol. 103: 2309-2315.
Graham JH, Gottwald TR, Cubero J, Achor DS (2004). *Xanthomonas axonopodis* pv. *citri*: factors affecting successful eradication of citrus canker. Mol. Plant Pathol. 5(1):1-15.
Hartung JS, Pruvost OP, Villemot I, Alvarez A (1996). Rapid and sensitive colorimetric detection of *Xanthomonos axonoppodis* pv. *Citri* by immunocapture and nested-polymerase chain reaction. Phytopathology, 87: 95-101.
Hartung JS, Civerolo EL (1989). Restriction fragment length polymorphism distinguish *Xanthomonos campestris* pv. *Citri*. in: Proc. 7th Int. Conf. Plant Path. Bacteria, Akademiai Kiado, Budapest, Hungary. pp. 503-508
Hartung JS, Daniel JF, Pruvost OP (1993). Detection of *Xanthomonos campestris* pv. *Citri* by the polymerase chain reaction. Appl. Environ. Microbiol. 59: 1143-148.
Hartung JS (1992). Plasmid-based hybridisation probes for detection and identification of *Xanthomonos campestris* pv. *Citri*. Plant Dis. 76: 889-893.
Khan MM, Khan MA, Inam-ul Haq M, Ahmad R, Aziz I (1992). Incidence of citrus canker caused by X. campestris pv. *citri* orchard in Faisalabad District. In: Proceed. 1st Inter. sem. citriculture in Pakistan. Dec. 2-5. University of Agriculture Faisalabad. pp. 311-314.
Khodakaramian G, Swings J (2002). AFLP Fingerprinting of the strains of *Xanthomonas axonopodis* inducing citrus canker disease in southern Iran. J. Phytopathol. 150: 227-231.
Khodakaramian G, Heydari A, Balestra GM (2008). Evaluation of Pseudomonas bacterial isolates in biological control of citrus bacterial canker disease. Int. J. Agric. Res. 3(4): 268-272.
Kishore V, Chand J (1972). Citrus canker in Haryana. Haryana Agric. Univ. J. Res. 27: 124-127.
Kishore V, Chand JN (1975). Resistance of citrus to citrus canker caused by *Xanthomonas citri*-analysis of phenols and sugars. Indian Phytopath. 28: 46-50.
Mehrotra RS (1980). Bacteria and Bacterial Diseases. Plant Pathology. Tata McGraw–Hill pub. Co. Ltd. New Delhi. pp. 636-638.
Mavrodieva V, Levy L, Gabriel DW (2004). Improved sampling methods for real-time polymerase chain reaction diagnosis of citrus canker from field samples. Phytopathology, 94: 61-68.
Mohammadi M, Mirzaee MR, Rahimian H (2001). Physiological and biochemical characterization of Iranian strains of *Xanthomonas axonopodis* pv. *citri*, the causal agent of citrus bacterial canker disease. 149: 65-75.
Mohana DC, Raveesha KA (2006). Anti-bacterial activity of *Caesalpinia coriaria* (Jacq.) Willd. against plant pathogenic *Xanthomonas* pathovars: an eco-friendly approach. J. Agric. Tech. 2(2): 317-327.
Pruvost O, Hartung JH, Civerolo EL, Dubois C, Perrier X (1992). Plasmid DNA fingerprints distinguish pathotypes of *Xanthomonas campestris* pv. *citri*, the causal agent of citrus bacterial canker disease. Phytopathology. 82: 485-490.
Sahi ST, Ghazanfar MU, Afzal M, Rasheed A, Habib A (2007). Incidence of citrus canker disease caused by Xanthomonas campestris pv. *citri* (Hasse) dows on kinnow (*Citrus reticulata*) and its chemotherapy. Pak. J. Bot. 39(4): 1319-1327.
Schubert TS, Sun X (2003). Bacterial citrus canker. Plant Pathol. Circular No. 377. Fl. Dep. Agric. and Cons. Services. Division. Plant industry. pp. 1-6.
Stall RE, Senymour CP (1983). Canker, a threat to citrus in the Gulf-coast States. Plant Dis. 67: 581-585.
Sun X, Stall RE, Jones JB, Cubero J, Gottwald TR, Graham JH, Dixon WN, Schubert TS, Chaloux PH, Stromberg VK, Lacy GH, Sutton BD (2004). Detection and Characterization of a new strain of Citrus Canker Bacteria from Key/ Maxican Lime and Alemow in South Florida. Plant Dis. 88: 1179-1188.
Taylor RK, Tyson JL, Fullerton RA, Hale CN (2002). Molecular detection of exotic phytopathogenic bacteria: A case study involving canker like symptoms on citrus. New Zealand Plant Prot. 55: 53-57.
Verniere C, Devaux M, Pruvost O, Couteau A and Luisett J (1991). Studies on the biochemical and physiological variation among strains of *Xanthomonas campestris* pv. *citri.* the causal agent of citrus bacterial canker disease. Fruits, 46(2): 160-170.
Verniere C, Hartung JS, Pruvost OP, Civerolo EL, Alvarez AM, Maestri P, Luisetti J (1998). Characterization of phenotypically distinct strains of *Xanthomonas axonopodis* pv. *citri* from Southwest Asia. Eur. J. Plant Pathol. 104: 477-487.
Vudhivanic S (2003). Efficacy of Thai herbal extracts for growth inhibition of *Xanthomonas axonopodis* pv. *citri*, the bacterial canker of citrus. pp. 1-8.
Whiteside JO, Garney SM, Timmer LW (1988). Compendium of citrus diseases. The American Phytopathological Society. p. 80.
Zekri M, Chamberlain H, Timmer P, Roberts P, Muchove R (2005). Field identification of citrus canker symptoms and decontamination procedures. Uni. Florida. IFAS extension.