IDENTIFICATION OF XANTHOMONAS BACTERIA USING PCR

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ABSTRACT

In plant-pathogen interactions, pathogens aim to overcome host defense responses while plants employ a battery of responses to limit pathogen growth and thus disease. In this "arms race" between hosts and pathogens, phytopathogens have evolved different molecular mechanisms that mediate adhesion to the host cell, colonization and dissemination and also different strategies to prolong their survival in the host. In citrus canker as well as in other plant-pathogen interactions, bacteria attachment to the host surface is determinant for the invasion of the tissue and depends on specific adhesins that are anchored in the bacterial outer membrane. The current study includes collection of 12 samples from the different areas of Dehradun (U.K). DNA was collected from all the sample and further subjected for different molecular parameters. DNA was isolated by Genetics sure DNA isolation kit which was utilized as template for Xanthomonas DNA quantification in the bench top PCR. Out of 12 specimens Xanthomonas DNA was quantified in samples by the utilization of Bench top PCR .The 20-mer oligonucleotides XACF and XACR were tested for X. a. pv. citri. As expected, a 561 bp DNA fragment was amplified. To check the specificity of the primers, a large collection of other microorganisms, including Xanthomonas species and their pathovars, were tested in PCR assay with primers XACF and XACR. None of the other Xanthomonas strains and reference microorganisms reacted with the primers; only X. a. pv. citri showed a single amplified DNA fragment . The primer pair, XACF and XACR, amplified a 561 bp DNA fragment from X. a. pv. citri, when 50 ng DNA was used as the template under optimized conditions (50 ml PCR reaction, containing 1.5 mM MgCl2, 0.2 mM of each dNTP, 10 pM of each primer, 2 units of Taq polymerase, and 5 ml of 10× PCR buffer). Similar conditions yielded reproducible results in the Perkin Elmer 9600 thermal cyclers. Taq polymerase enzymes supplied by different manufacturers also yielded similar PCR results.

INTRODUCTION

Xanthomonas is a large genus of gram-negative bacteria that cause disease in hundreds of plant hosts including many economically important crops. The disease is of great economic mportance because it causes severe yield loss and fruit quality reduction *(Jones et al., 1991; Cui et al., 2005)*. Five races, designated as T1 through T5 (T1 in X. euvesicatoria, T2 in X. vesicatoria, T3, T4, and T5 in X. perforans) have been defined by their virulence on tomato plants *(Astua-Monge et al., 2000; Jones et al., 2005).* Infected seeds, volunteer crop plants and diseased plant debris may serve as inoculum sources of the

disease The organism can be disseminated by rain and/or sprinkler irrigation droplets driven by the wind within fields and from nearby fields. The resence of the pathogen in infected seeds and disease transmission from these infected seeds has been demonstrated *(Zhao et al., 2007; Quezado-Duval and Lopes, 2010).* Accurate identification of the pathogen is the basic requirement for effective disease management *(Narayanasamy, 2011).* Identification and classification of plant pathogenic bacteria are historically based on phenotypic characteristics such as symptoms caused, presence of specific antigens for serology analysis (ELISA), biochemical characteristics, substrate utilization profiles (BIOLOG), fatty acid composition (FAME) and multilocus enzyme electrophoresis profiles (MLEE) *(Louws et al., 1999).* Biochemical tests are labor intensive and time consuming while serological methods have high development costs and at times less sensitive. The development of the polymerase chain reaction (PCR) by Kary Mullis in 1984 revolutionized molecular biology. It became instrumental in diverse applications due to its high sensitivity and versatility for nucleic acid amplification from any organism, including human, animal and plant pathogens *(Schaad and Frederick, 2002).* The value of PCR in phytodiagnostics was first described by Henson and French (1993), and has since been utilized for the detection of nematodes, fungi, bacteria, viruses, viroids and phytoplasmas, including pathogens of tomato *(Cuppels et al., 2006; Park et al., 2009).* In the present study, phytopathogenic X. Perforans were isolated from both suspected plant materials from farmer's field and seeds from different seed agencies, Karnataka, India, which infect tomato and the isolates, were confirmed by biochemical, hypersensitive response and pathogenicity test. These isolates were further subjecting to molecular diagnostic methods by a PCR using 16S rRNA and species specific primer for confirmation.

REVIEW OF LITERATURE

The *Xanthomonas* genus has been subject of numerous taxonomic and phylogenetic studies and was first described as *Bacterium vesicatorium* as a pathogen of pepper and tomato in 1921. Dowson later reclassified the bacterium as *Xanthomonas campestris* and proposed the genus *Xanthomonas*. *Xanthomonas* was first described as a monotypic genus and further research resulted in the division into two groups, A and B. Later work using DNA:DNA hybridization has served as a framework for the general *Xanthomonas* species classification. Other tools, including multilocus sequence analysis and amplified fragment-length polymorphism, have been used for classification within clades. While previous research has illustrated the complexity of the *Xanthomonas* genus, recent research appears to have resulted in a clearer picture. More recently, genome-wide analysis of multiple *Xanthomonas* strains mostly supports the previous phylogenies. To prevent infections, limiting the introduction of the bacteria is key. Some resistant cultivars of certain plant species are available as this may be the most economical means for controlling this disease. For chemical control, preventative applications are best to reduce the potential for bacterial development. Copper-containing products offer some protection along with field-grade antibiotics such as oxy tetracycline, which is labeled for use on some food crops in the United States. Curative applications of chemical pesticides may slow or reduce the spread of the bacterium, but will not cure already diseased plants. It is important to consult chemical pesticide labels when attempting to control bacterial diseases, as different *Xanthomonas* species can have different responses to these applications. Over-reliance on chemical control methods can also result in the selection of resistant isolates, so these applications should be considered a last resort.

MATERIAL AND METHODS

The purpose of a PCR (Polymerase Chain Reaction) is to make a huge number of copies of a gene. This is necessary to have enough starting template for sequencing. The cycling reactions. There are three major steps in a PCR, which are repeated for 30 or

40 cycles. This is done on an automated cycler, which can heat and cool the tubes with the reaction mixture in a very short time. Denaturation at 94°C.During the denaturation, the double strand melts open to single stranded DNA, all enzymatic reactions stop (for example: the extension from a previous cycle).Annealing at 54°C:The primers are jiggling around, caused by the Brownian motion. Ionic bonds are constantly formed and broken between the single stranded primer and the single stranded template. The more stable bonds last a little bit longer (primers that fit exactly) and on that little piece of double stranded DNA (template and primer), the polymerase can attach and starts copying the template. Once there are a few bases built in, the ionic bond is so strong between the template and the primer, that it does not break anymore. Eextension at 72°C :This is the ideal working temperature for the polymerase. The primers, where there are a few bases built in, already have a stronger ionic attraction to the template than the forces breaking these attractions. Primers that are on positions with no exact match, get loose again (because of the higher temperature) and don't give an extension of the fragment. The bases (complementary to the template) are coupled to the primer on the 3' side (the polymerase adds dNTP's from 5' to 3', reading the template from 3' to 5' side, bases are added complementary to the template.

DISCUSSION

Development of specific primers and DNA probes for identification and detection has been reported for a number of plant pathogenic bacteria (*Hartung et al., 1993; Leite et al., 1994; Rasmussen and Reeves, 1992*) including xanthomonads. This study found hrpW to be a useful gene for detecting X. a. pv. citri with PCR assay. It has been revealed that hrpW has domains similar to harpins and pectate lyases and can elicit the plant hypersensitive response and bind to pectate (*Charkowski et al., 1998*). This study revealed that nucleotide and amino acid sequences of hrpW were highly variable among Gram-negative plant pathogenic bacteria such as *Xanthomonas* and *Pseudomonas*. In comparison of nucleotide sequences, hrpW from X. a. pv. citri is 43.9% similar to that of *Xanthomonas campestris pv. campestris*, while 17.2% similar to that of Pseudomonas syrinage pv. tomato . Successful detection of the pathogens using PCR techniques depends upon the specificity of primers. In this study, the specificity and sensitivity of PCR assays were evaluated through the detection of the pathogen in naturally infected plants. PCR conditions such as primers, template, concentration of Mg2+ (*Bassam et al., 1992*), thermocyclers, and thermostable polymerase origin (*Schierwater and Ender, 1993*) have been shown to affect amplification. In this study, all these parameters were optimized to avoid artifacts and to ensure reproducibility of amplification. Consistent results of amplification of a 561 bp fragment from X. a. pv. citri by XACF and XACR were also obtained by using various PCR machines and different Taq polymerase enzymes supplied by various manufacturers.

CONCLUSION

In plant-pathogen interactions, pathogens aim to overcome host defense responses while plants employ a battery of responses to limit pathogen growth and thus disease. In this “arms race” between hosts and pathogens, phytopathogens have evolved different molecular mechanisms that mediate adhesion to the host cell, colonization and dissemination and also different strategies to prolong their survival in the host. Xac has several different molecular mechanisms to infect citrus plants being an interestingly system to study plant-pathogen interactions. The knowledge in citrus canker pathogen-host interaction is critically needed in order to provide new tools for developing sustainable and economically viable control measures. Moreover, understanding the bacterial mechanisms by which plant pathogens attack their hosts is central to the study of plant pathology.In citrus canker as well as in other plant-pathogen interactions, bacteria attachment to the host surface is determinant for the invasion of the tissue and depends on specific adhesins that are anchored in

the bacterial outer membrane. Once attached to the host, Xac delivers pathogenicity effector proteins inside the plant cell trough the type III secretion system that modulate the plant basal defense response for the benefit of the pathogen. In order to prolong its survival in the plant, Xac must form biofilms that allow further colonization, process in which XacFhaB adhesin and EPS are involved. Finally, Xac has a new strategy to manipulate host responses that involves XacPNP, a gene probably acquiredin a horizontal gene transfer event, that modulates host homeostasis through mimicking the role of PNPs, improvinghost photosynthesis and thus leading to a more healthy tissue during the infection. This regulation of the host status serves to suit Xac biotrophic lifestyle and to prolong Xac survival. In conclusion,

RESULTS

The current study includes collection of 12 samples from the different areas of Dehradun (U.K). DNA was collected from all the sample and further subjected for different molecular parameters. DNA was isolated by Genetics sure DNA isolation kit which was utilized as template for *Xanthomonas* DNA quantification in the bench top PCR.Out of 12 specimens *Xanthomonas* DNA was quantified in samples by the utilization of Bench top PCR .The 20-mer oligonucleotides XACF and XACR were tested for X. a. pv. citri. As expected, a 561 bp DNA fragment was amplified. To check the specificity of the primers, a large collection of other microorganisms, including *Xanthomonas* species and their pathovars, were tested in PCR assay with primers XACF and XACR. None of the other *Xanthomonas* strains and reference microorganisms reacted with the primers; only X. a. pv. citri showed a single amplified DNA fragment . The primer pair, XACF and XACR, amplified a 561 bp DNA fragment from X. a. pv. citri, when 50 ng DNA was used as the template under optimized conditions (50 ml PCR reaction, containing 1.5 mM MgCl2, 0.2 mM of each dNTP, 10 pM of each primer, 2 units of Taq polymerase, and 5 ml of 10× PCR buffer). Similar conditions yielded reproducible results in the Perkin Elmer 9600 thermal cyclers . Taq polymerase enzymes supplied by different manufacturers also yielded similar PCR results.

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