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THE BACTERIAL WILT ORGANISM: THE CROP NEMESIS WITH A NEW TWIST

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ABSTRACT

Ralstonia solanacearum causes the destructive bacterial wilt of many crops including banana, tomato, tobacco, potato, eggplant, ginger and peanuts. It has remained a major nemesis of agricultural crops despite decades of research spent on it. For the past few years, however, a new twist has been introduced in the hope of better understanding the causal organism. This twist is through molecular approaches.

In the late 1990s, the "bugtok" disease which causes hardening of cooking bananas was suspected to be caused by *R. solanacearum* but its relationship with the causal bacterium in wilt of Cavendish bananas (Moko) was not clear. Molecular probing of the genome of 127 strains of bugtok and Moko causing strains using different probes and through DNA amplification by polymerase chain reaction (PCR) showed that this group of strains is of only one type, and thus is monomorphic. A similar study conducted fonr years later yielded almost the same result. Another approach was taken to study genetic diversity and that was to clone a repetitive element through the construction of a partial genomic library. Using this cloned rep-element for hybridization, it was shown that the group was indeed monomorphic with just an additional type, but which was not exclusive to either bugtok or Moko. Thus, it was concluded that the causal organisms of bugtok and Moko are one and the same type. In the former, the bacterium infects the plant through the inflorescence while in the latter, it does so through the roots. In the course of this study, it was established that only the banana strains harbor the rep-element. Thus, the flanking regions of the rep-element were sequenced, and primers synthesized for PCR. Indeed, the banana strains can be differentiated from the other strains by PCR. This constitutes a fast and efficient method of differentiating the diverse strains. Nonetheless, since PCR requires skill and a special machine, a more user-friendly method, the monoclonal antibody (MAB) technology, was developed. Using the PCR product as the immunogen, a MAB-based technique specific only to *R. solanacearum* banana strains was developed. However, the sensitivity of this MAB technique was lower than that of PCR.

Since banana strains are almost monomorphic, in contrast to tomato strains which are polymorphic, the rate of development of polymorphism was determined in a tomato strain as affected by host genotype. Through a series of PCR experiments utilizing a set of tomato varieties with different levels of resistance to bacterial wilt, a substrain, which had undergone changes in genotype and virulence, was isolated. This substrain was eventually able to cause 92% and 100% wilting of plants of the resistant cultivars C108 and 508, respectively. In contrast, the wild type strain induced wilting in only 8.3% - 16% in the resistant cultivars. The mutant strain was conclusively shown to break down the resistance of the tomato cultivars. This type of strain can be used as tester in breeding for durable disease resistance.

Other studies conducted by Opina and Natural on the genetic diversities of tomato and potato strains indicated a polymorphic nature. A PCR technique to detect the species *R. solanacearum* was devised by Opina and collaborators. Natural and colleagues also devised a technique for the selection of bacterial wilt free potato seed pieces. Balatero and colleagues utilized marker-aided selection in hastening the process of breeding for resistant varieties. Laurena *et al* have studied the basis of bacterial wilt resistance in tomatoes.

All of these studies in the Philippines have contributed to a better understanding of the pathogen and a more effective management of the bacterial wilt.

INTRODUCTION

The bacterial wilt organism now named *Ralstonia solanacearum* (Smith) Yabuuchi *et al.*, 1995 was previously known as *Burkholderia solanacearum* and as *Pseudomonas solanacearum* E. F. Smith in earlier years (Yabuuchu *et al.* 1995). It causes the destructive bacterial wilt of many solanaceous and non-solanaceous crops. The occurrence of *P. solanacearum* is a limiting factor in the growth of susceptible crops in warm temperate, semitropical and tropical zones of the world. It is a destructive disease due to its widespread occurrence and broad host range, as well as to the existence of different strains, their genetic variability and ability to survive in non-host plants. Yield loss estimates ranged from 15-90% (Zehr, 1969: Hayward and Hartman, 1994). All sorts of control measures have been proposed like rotation and fallow, nematode and weed control, stubble destruction, biological control, solarization, control of spread of water, minimal tillage, soil amendments and plant resistance (Hayward and Hartman, 1994). The use of host resistance remains the most important when used in combination with other control measures. To produce more durable resistant varieties, the biology and the genetics of the organism need to be understood. To achieve this, a new twist through molecular approaches has been tried and has yielded useful information.

This paper focuses on the work done by the author and her collaborators using molecular and biotechnology approaches with mention of some other recent molecular studies done in the Philippines on the bacterial wilt organism.

BACTERIAL WILT IN BANANA

R. solanacearum causes two different diseases in banana, Moko (Roperos and Magnaye, 1991) and bugtok (Soguilon *et al.*, 1994). Moko disease in banana was first reported to have occurred in Giant Cavendish plantations in 1969 and later in Davao del Norte during the same year. Moko and bugtok diseases differ in the type of symptoms they exhibit. The symptoms of Moko include wilting, stunting and death of the plants. On the other hand, the symptoms of bugtok are essentially confined to the floral raceme, foliar symptoms being rare or absent. Fruits of affected plants are discolored red, brown or black starting from the core and progressing towards the whole pulp of the fruit. It is usually a dry rot. In mature plants, bracts covering the male bud "puso" are dry, slightly rolled up and loosely attached (Soguilon *et al.*,1994).

The question raised in the 1990s was whether the causal organisms of the bugtok disease of fruits of cooking bananas and that of Moko of Giant Cavendish banana (Soguillon *et al.*, 1994) are the same. It was necessary to establish the relationship of this strain to the other banana strains causing Moko or bacterial wilt of bananas and to other foreign strains infecting *Musa* spp. At that time, foreign scientists were coming to the Philippines to resolve the problem. As a challenge to do something about the problem, we embarked on genetic diversity and analysis studies of the Philippine banana strains of *R. solanacearum* with funding from PCASTRD and ACIAR

We collected Moko and banana strains from different parts of Mindanao where the disease was known to be rampant. We did pathogenicity tests, with both Moko and bugtok strains causing wilting and death of most of the Dwarf Cavendish plantlets. They also caused wilting of tomato plants, but the tomato strains did not effect wilting of the Giant Cavendish plants (Ilagan, 1996).

Biovar testing revealed that Moko and bugtok strains belonged to biovar 1 based on utilization of sugars and sugar alcohols (Magistrado and Raymundo, 1996). Genetic analysis by restriction fragment length polymorphism (RFLP) and polymerase chain reaction (PCR) was then done to establish relatedness among the strains.

Restriction fragment length polymorphisms (RFLP) and Polymerase Chain Reaction

RFLP was done using initially six probes (as cited in Raymundo *et al.*, 1999). This method consists of isolating the DNA of the different strains, digesting the DNA with the restriction enzyme, *Eco* R1, running digested DNA in a gel, transferring the separated fragments to a nylon membrane through Southern blot transfer, then hybridizing the DNA bands in the nylon membrane with the different probes (Raymundo *et al.*, 1999). In an experiment using probes (labeled DNA) consisting of DNA fragments which harbor the genes for extracellular polysaccharides (E36, O7, Q50), endoglucanase (pHE3), tryptophan biosynthesis

strain, no polymorphism was observed among the banana strains.

Polymerase chain reaction using ERIC, REP, and BOX primers (rep-PCR) was also used to analyze the same set of strains. These primers amplify the different repetitive elements present in the genome of the bacteria. No polymorphism was likewise detected among the banana strains.

The monomorphicity of the banana organism was confirmed a few years later with a new set of isolates (Lavina, 2000).

The above result provided evidence that the Moko and bugtok causing organisms are one and the same type of strain.

Cloning of a Repetitive Element from a Philippine Banana Strain

In an effort to search for a probe that would detect polymorphism among the strains of *R. solanacearum* (Raymundo *et al.*, 1998), a repetitive element was cloned from a banana strain. A partial genomic library was constructed using banana strain No. BuD3 by digesting the genomic DNA to completion, ligating to Bam H1-digested pBluescript vector and transforming to competent *Escherichia coli* DH5-a cells. Colony blot analysis was done and a clone harbored (pM114) in which a repetitive element was identified (Raymundo and Ilagan, 1999).

A total of 97 Philippine banana-infecting strains were analyzed and all harbored the repetitive element. Ninety one of the strains exhibited the same hybridization pattern typified by Strain No. BuD4). Strain No. Bu22b had an extra topmost hybridization band with four other strains sharing the same pattern. One Moko isolate P1-118 had the same pattern as Bu22b except for an extra copy of the repetitive element which hybridized with a fragment of about 10 kb in size. The different hybridization patterns were not exclusive to either Moko or bugtok isolate.

Several other non-banana strains consisting of 39 vegetable, eight tobacco, four mulberry and four ginger strains were also analyzed. Most of the strains did not show any hybridization. A few, however, exhibited a few hybridization bands. By restriction mapping, pM114 was deduced to be 2.28 kb.

The use of the cloned repetitive element as a probe showed that the banana strains of *R. solanacearum* have very low level of polymorphism. This was evident in the same hybridization pattern in 91 of the strains, with the remaining six differing only by the presence of an extra copy of the element. The Moko strains were likewise not differentiated from the bugtok strains. While the strains were obtained from different parts of Mindanao and the Visayas, the fact that all the strains from either Giant Cavendish or cooking bananas, which are generally asexually propagated, may have contributed to the low level of polymorphism or monomorphicity.

Due to the distinct difference in hybridization patterns, pM114 can be a very useful probe to distinguish the banana group of strains. As non-banana strains do not attack banana plants (Ilagan, 1996), it is important to have a means of

not attack banana plants (Ilagan, 1996), it is important to have a means of differentiating the two groups of strains of *R. solanacearum*. The cloned element has likewise a great potential in the detection and monitoring of the organism in epidemiological studies.

Analysis of Genetic Variation of a Population of Banana Infecting Strains of *Ralstonia solanacearum*

The 97 Philippine strains studied earlier were analyzed together with 24 banana-infecting strains from other countries. RFLP using the same set of probes and rep-PCR were also utilized. (Raymundo *et al.*, 1998; Ilagan, 1996).

E36 (pMO030536) resolved three to four fragments ranging from a little less than 4 kb to 10 kb. Four bands were exhibited by Philippine bugtok and Moko isolates. These isolates showed no polymorphism and did not differ from the *Heliconia* strains H290 and H412. Other foreign strains of banana, e.g.. B413, an sfr strain, which is an insect-transmitted Moko isolate (Buddenhagen, 1985) differed in their banding patterns, although they shared two similar bands with the Philippine banana isolates.

Probe O7 (pMO031507), which harbors the EPS region 2, resolved only one homologous fragment, while Q50 (pMO031750), that harbors EPS region 3, did not give any hybridization signal. With probe Z1217 from the ginger strain ZO4 library, Philippine bugtok and Moko isolates, together with the foreign banana strain A4522 from Honduras, exhibited exactly similar banding patterns and hence they were considered as one haplotype. *Heliconia* strains H290 and H412 from Costa Rica differed by one band whose size was about 4.5 kb. H290 had a fingerprint similar to those of the Philippine banana isolates when hybridized with EPS probes. Philippine banana isolates also differed from the sfr strain, B413, from Venezuela. Plantain strains Pl415, Pl414 and Pl416 from Peru and Pl483 from Colombia exhibited similar fingerprints. Banana strain B465 from Costa Rica had banding patterns similar those of the plantain strains.

Probing with pHE3 revealed six different banding patterns and resolved two to seven fragments. The banding patterns observed for bugtok and Moko isolates were identical but different from the foreign banana isolates A4521 and A4519, plantain strains Pl415 and Pl416 and *Heliconia* strain H412 from Costa Rica.

The probe pT161 failed to distinguish strains infecting *Musa* and *Heliconia* families regardless of the source of the strains. Only one homologous band was resolved using the probe pT161.

Using the cloned repetitive element, it was found that the rest of the foreign banana strains harbored the repetitive element except for strains B539, B521, B522 and B413, although they showed different banding patterns. The sfr strain B413 had only two faint copies of the element, while strain B521 had a single copy and B522 bad none. Except for two extra upper bands (16 and 20 kb), Honduras strain A4522 had a pattern similar to those of typical Moko and bugtok isolates.

Heliconia strain H290 had almost the same pattern except for one 3.7 kb and one 16 kb extra band. *Heliconia* strains H290 and H412 from Costa Rica had 26 and 24 bands, respectively.

The probe pM114 was able to detect differences among the Philippine Moko and bugtok isolates, but did not differentiate the two strains. This probe also revealed more haplotypes among strains infecting the *Musa* and *Heliconia* families compared to the EPS, pZ1217 and pHE3 probes.

PCR analysis using the REP primers yielded multiple distinct DNA products. The use of REP primers showed two of the Philippine strains diverging from the dominant haplotype by the presence of three additional bands. The rest were monomorphic. Comparison between B413, an sfr strain, from Venezuela, and Philippine strains of bugtok and Moko revealed a big difference in the REP-PCR pattern. The A4519 banana strain, which was isolated from Honduras, was very similar to Philippine bugtok and Moko isolates, except for one band. *Heliconia* strains from Costa Rica (H290, H291 and H412) did not differ much from each other except for an extra band present in H412. Representative foreign banana strains from Guyana, Grenada, Venezuela, Costa Rica and Honduras were also polymorphic as exemplified by B539, A4519, B522, B521, B466, B465 and B417. This was similar to plantain strains Pl414, Pl415 and Pl484 which were from Colombia, Peru and Costa Rica, respectively.

ERIC primers when used in polymerase chain reaction yielded more amplified fragments but fewer haplotypes than the REP primers. Strains shared numerous bands in common although they differed in the banding patterns. Bugtok and Moko strains exhibited exactly similar genomic fingerprints. The sfr strain B413 appeared distinct from the Philippine bugtok and Moko strains. Plantain strains Pl415 and Pl491 were very much alike, as well as Pl418 and Pl416. Banana strains B539 and B541 from Grenada, B521 from Guyana and A4519 from Honduras showed identical genomic fingerprints. The three *Heliconia* strains were slightly different from each other with strain H412 having three prominent fragments with a size range of approximately 3 to 6 kb. Some plantain strains also differed from each other to some degree.

With the BOX primers, majority of the Philippine banana strains showed no polymorphism. Some, however, differed by a few bands as exemplified by 1687, BuD2 and BSFI. *Heliconia* strains from Costa Rica (H290, H291 and H412) differed from each other by the presence of an extra band in H412. Representative foreign banana strains from Guyana, Grenada, Venezuela, Costa Rica and Honduras were also polymorphic as exemplified by B539, A4519, B522, B521, B466, B465 and B417. Similar results were obtained in plantain strains Pl414, pl415 and Pl484 from Colombia, Peru and Costa Rica, respectively. There were strains which shared exactly similar bands as exemplified by plantain strains Pl418 and Pl415 from Peru and Honduras.

Composite RFLP and PCR analysis yielded a dendrogram showing the relationship of the different banana and *Heliconia* strains. At 65% similarity level,

this group of strains was divided into six clusters. Cluster A contained seven haplotypes which included the representative Philippine bugtok strains BuD2, 1689, 1687, *Heliconia* strains H290 and H412 from Costa Rica, banana strains B417 from Honduras and B492 from Costa Rica. All plantain strains regardless of the place of isolation, belonged to one cluster. Cluster B contained banana strains B465 and B466 from Costa Rica. Cluster C contained five haplotypes which included the sfr strain B413 from Venezuela and banana strains B521 and B522 from Guyana and B539 and B540 from Grenada.

Bugtok and Moko strains were confirmed to be monomorphic based on the different probes used except pM114 which harbors the repetitive element. The use of pM114 resulted in three distinct haplotypes among Philippine banana strains, although these strains would elicit different symptomatologies. In the rep-PCR method, Philippine banana isolates also showed differences to a certain extent.

The dendrogram obtained from a composite analysis of several probes and the PCR data gives a picture of the relationships of the strains because of the greater number of loci examined. Results indicated that there is substantial genetic diversity in the population of banana-infecting strains of *R. solanacearum*. This may also suggest that they may have evolved from a common ancestral progenitor. Observed relationships of strains isolated from different geographical locations might be due to the introduction or movement of strains as affected by human and climatic factors.

With the use of RFLP and PCR analyses, the Philippine banana strains were confirmed to be homogeneous except for some strains. It was also shown that these strains shared the same lineage with other foreign banana strains. The apparent high homogeneity of Philippine banana strains is probably related to the limited host species from which the strains were isolated. The bugtok strains appeared to be Moko strains transmitted to cooking bananas by insects through the inflorescence. These strains are definitely different from the strains from other countries, especially the sfr strain. It is therefore important to the banana industry in the Philippines that care to ensure that the sfr strain which caused an epidemic in Venezuela (Buddenhagen, 1985) should not be introduced in the country.

POLYMERASE CHAIN REACTION BASED DETECTION OF BANANA ISOLATES

In 1997, Opina *et al.* designed a PCR based technique for the differentiation of the *Ralstonia solanacearum* strains. We wanted to develop a PCR-based method to specifically detect *R. solanacearum* banana strains based on the cloned repetitive element shown to be specific for Philippine banana strains. Primers were designed based on the sequence of the first 200 bases at each end of the repetitive element present in pBluescript (pM114). The sequences of the primers were:

M114F, 5' - TGGTCGCCGCTTTCGTCATCC - 3' and M114R, 5' - TTGGAGGTGTGCGCCATCAAC - 3'.
(Ilagan *et al.*, 2001).

The two primers M114F and M114R, at the annealing temperature of 65°C, amplified a 2.28 kb fragment. All 96 bugtok and Moko strains tested yielded the expected PCR product. The specificity of the primers to the Philippine banana strains was confirmed by the non-amplification of the non-banana strains. The only exception was the *Heliconia* strain, which shares the same lineage as the banana strains in the Philippines (Ilagan, 1996). No amplification was observed from the DNA from *Pseudomonas*, *Xanthomonas* species, *Escherichia coli* and other bacteria.

PCR-based techniques have been demonstrated to be sensitive, efficient and cost-effective for detection and identification of microorganisms. While other studies focused on detection of *R. solanacearum* in general or on those specific to white potatoes, this study was the first report of a sensitive PCR-based technique specific for detection and diagnosis of Philippine banana strains. This technique should be useful for fast diagnosis of the disease.

To make this technique applicable to direct detection of the bacterium from the soil, investigations were done on methods of direct DNA isolation. A number of protocols for isolation of genomic DNA from soil inoculated with known concentrations of *R. solanacearum* were used in the study (Lavina, 2000) DNA recovered was used as templates for PCR using M114 primers. Among the methods, only the protocol of Audy (1996) and MoBio kit for isolating DNA were able to produce the M114 amplicons consistently. The use of combined extraction buffer for chemical lysis, bead beater for homogenization and Wizard^{O} spin filter for purification produced good amplification. The most sensitive method for detection in soil using PCR was the method of Audy (1996) with the sensitivity level of 3.75 x 10^6 CFU/g. DNA amplification was present only from DNA extracted from the pellet indicating that the DNA was concentrated in the bottom of the tube after centrifugation. The use of extraction buffer yielded more PCR products than the use of TNPE buffer. Using the MoBio kit (MoBio Laboratories, Inc.), DNA was successfully isolated from soil samples and produced the desired 2.28 Kb fragment when used in PCR with M114 primers. In our method for isolation of DNA from soil, the modification using a combination of extraction buffer, bead beating and purification by spin filter produced DNA and an M114 amplicon was discerned when used in PCR. The best conditions for isolation of DNA from soil samples included the use of extraction buffer for chemical lysis, bead beater for homogenization and Wizard^{R} spin filter for purification. Detection of *R. solanacearum* directly from soil was shown to be possible; however, sensitivity level was only 10^5/g, thus further studies are needed to improve the method.

MONOCLONAL ANTIBODY-BASED DETECTION METHOD AND APPLICATION

PCR is a very sensitive technique but it requires special skills and machine. Thus, efforts were exerted to develop a more user friendly technique in the form of monoclonal antibody (MAB) based on the cloned repetitive element specific to *R. solanacearum* banana strains only (Exconde *et al.*, 2000). In this approach, the PCR product of the repetitive element, pM114, was the source of the immunogen. This is in contrast to the conventional method, which utilizes whole bacterial cells or other bacterial antigens in producing antibodies for the detection of a plant pathogen. Since DNA is a large non-immunogenic molecule which can only elicit an antibody response in an immunized animal when it is bound non-covalently to an immunocarrier, the amplified DNA product was conjugated to methylated bovine serum albumin (MBSA). The PCR product was injected into a mouse, and the resulting antibody producing spleen cells of the mouse were obtained and fused with myeloma cells generating monoclonal antibody-producing hybridoma cells. Since the immunogen was conjugated with MBSA, it was necessary to screen and eliminate hybridomas that reacted to MBSA. After a series of purification and screening, 24 monoclones were obtained. One of the five clones that were successfully mass produced was further studied.

Results showed that the MAB produced was specific to the banana strains. All non-banana isolates, other bacterial genera and all other species of *Pseudomonas* tested in this study, except *P. maltophila*, gave negative reactions based on their absorbance values which were lower than the calculated threshold values.

The sensitivity level of detection of the banana antibodies using different cell populations of the organism in pure culture and in inoculated soil was observed initially at 1×10^7 cells/ml. However, the detection limit was improved further to 1×10^5 cells/ml when the cells were lysed or solubilized with SDS.

The banana antibodies were applied in the detection of *R. solanacearum* from field collected banana, non-banana and soil samples (Exconde and Raymundo, 2000). A large proportion of the banana samples screened showed positive detection of the wilt organism, especially those collected from the Mindanao area. Screening of soil samples showed varied distances where the organism was detected. The antibodies were also applied in indexing 208 banana plants from Cavite, Laguna and from the orchard nursery of the Department of Horticulture, UPLB where 14% of the plants showed positive reactions.

The important role of the developed monoclonal antibody was shown in its usefulness in detecting the organism in banana tissues that have not yet shown symptoms. This is especially true in cases of bananas to be used as source of mother plants for tissue culture. It is extremely important that the mother plant be free from the bacterial wilt organism. Otherwise, the resulting tissue cultured plantlets that will be used to establish farms and plantations will be the eventual source of inoculum for disease spread.

In studies on epidemiology and control of the disease, the developed monoclonal antibody is vital. The MAB was used to compare the effectiveness of three methods of disposal of Moko infected plants: intact burning and fumigation with vasamid, chopping and burning before exposure to sun, and intact burning alone and exposure to the sun (Raymundo *et al.*, 1999). The study demonstrated that selective media were not sensitive enough to detect the organism at lower levels. On the other hand, PCR, which is extremely sensitive can not be used with soil samples because of inhibitory substances in soil. The use of a very specific monoclonal antibody appears to be the only feasible alternative. Without the use of the monoclonal antibody, it would not have been possible to show that treatment involving burning and application of a soil fumigant is necessary to reduce if not totally eliminate the organism. The results further showed that the organism remains in the soil if the soil is not fumigated, althougb the infected plants were burned.

With the development of this diagnostic monoclonal antibody, rapid detection of the wilt organism will be facilitated. This is needed in the light of increased international trade and movement of plant propagative materials such as tubers, seeds, rhizomes and other planting materials. Hence, the availability of a sensitive detection reagent is very critical in discerning the presence or absence of a pathogen. Proper diagnosis of the wilt organism through the use of this detection reagent can prevent or minimize crop damage through early detection and appropriate control measures.

This study combines molecular and serological approaches in producing a detection method for a plant pathogen. With the use of ELISA-MAB, the bacterial wilt organism can be monitored for determination of the efficiency of a particular treatment in epidemiological studies prior to quarantine and management of the disease under field conditions.

Sequence Analysis of the Cloned Repetitive Element

The cloned repetitive element was sequenced to determine its significance in the organism and to design primers for a more robust and rapid PCR. Analysis of the sequence revealed the presence of two insertion sequences (IS) with terminal direct and inverted repeats (Bagsic-Opulencia *et al.*, 2001). ISs are mobile genetic elements which can insert multiple sites in a target molecule and cause mutation. They are sometimes associated with pathogenicity and virulence functions in plant pathogens.

Primers based on the internal region of the IS amplified a 217 bp product after 2 hr PCR compared to 8 hrs using the other set of primers. The new primer set detected not only the Philippine banana strains and members of RFLP 24 but also *Heliconia* strain from Colombia, *Pothos* strains from Hawaii, anthurium strains, and banana strains from Colombia and Peru (RFLP 25). A distantly related potato strain from Peru was also detected indicating that the ISs are more widely distributed than previously known. The PCR primers are useful in monitoring movement of the ISs within a population of *R. solanacearum* to gain better understanding of the regional dispersal of this pathogen.

The IS element also encoded for the enzyme transposase required for its mobility. A153 bp region is also found downstream of the sequence. It is significantly homologous (61% identity) to the ATP-binding sub-unit of the abc transporter for rtx toxin in *Xylella fastidiosa* which causes citrus variegated chlorosis in orange trees. The rtx (repeats-in-toxin) toxin family is a large group of multi-domain pore-forming exotoxins produced by a variety of pathogenic gram (-) bacteria with haemolytic, leukotoxic, cyclolytic and leukocyte-stimulating activities. They are secreted from the bacteria and, after processing are inserted into the membrane of host cells where they cause cell rupture (Braun and Focareta, 1991).

The activity of rtx toxins depends on an activator and an associated abc transporter system. The abc transporters perform export functions to facilitate translocation of the toxin across the host cell membrane. The abc transporter system requires energy in the form of ATP to facilitate translocation. The proteins making-up the dedicated export system exhibit a bighly conserved motif called the ATP-binding cassette (abc) which hinds the ATP (Fath and Kolter, 1993).

BACTERIAL WILT IN TOMATO

Genetic Diversity of Philippine Tomato Strains

Opina *et al.* (2000) have used random amplified polymorphic DNA (RAPD) markers and rep primers in PCR in optimized conditions to study genetic variability of tomato strains. Analysis of 83 isolates resolved 71 haplotypes with 11 clusters, five clusters composed of strains from Mindanao, three of Luzon strains and three of a mixture of isolates from Luzon and Mindanao. The origins of the strains were correlated to the clustering of the isolates. This tomato population was shown to be highly diverse within and between biovars and origin.

Effect of Host on Genetic Variability of a Tomato Strain

Because of the great diversity of the tomato strains compared to the banana strains, a study was done to determine how fast polymorphism develops in the tomato strain and to establish the effect of host genotype on the diversity of the organism (Orlina *et al.*, 2001).

Out of ten *R. solanacearum* strains inoculated to tomato cultivars with different levels of resistance to bacterial wilt, four strains were found more virulent based on their ability to cause a greater number of wilted plants including resistant cultivars. The genomic DNA of these four strains were subjected to rep-PCR and among the four, T280 and T523 showed variability. The rest of the study, however, was focused on the most virulent strain, T523.

REP-PCR of strain T523 produced 31 bands ranging from 0.2 Kb to over 6 Kb in size. The fingerprint of a representative of the reisolates from this strain produced 31 bands with about the same range of sizes, but with the mutant strain having an extra 0.3 Kb and a missing 0.7 Kb fragment.

The wild type strain (T523), a reisolate from a L-180 (moderately resistant) plant, which has the same fingerprint as the wild type (T523-726), and the mutant isolate (T523-731) were inoculated individually to the same set of tomato cultivars used in the initial virulence test. Wilting of the susceptible plants (Yellow Plum and L-390) was observed four days after inoculation (DAI) in plants inoculated with the mutant isolate T523-731. The wild type strain T523 and the other isolate T523-726 were able to cause wilting of bacterial wilt-susceptible plants seven days after inoculation.

At ten DAI, the mutant isolate T523-731 was able to breakdown the resistance of of resistant cultivars C108 and 508 causing wilting percentages of 92% -100%. In comparison, the parent strain T523 and isolate T523-726 elicited 8.33% and 16.6% wilting of C108 and 508, respectively.

The mutant isolate was able to cause 100% wilting of resistant cultivars C108 and 508, with an average of 68% for all of the resistant cultivars. The wilting incidence of moderately resistant cultivars inoculated with the mutant isolate was at 74%. The wild type strain and strain (T523-726), another strain with same fingerprint as the wild type strain but which was resisolated from an susceptible plant, were able to cause wilting of the resistant cultivars 3% - 4%.

Wilted and non-wilted plants were collected one month after inoculation . DNA isolation was done on three isolates/plant/variety/strain using 12 plants, seven varieties and three isolates to a total of 542 DNA samples. The genomic fingerprints of the reisolates were determined by REP-PCR, which showed that the haplotype of all isolates obtained from susceptible cultivars remained the same. In contrast, changes in DNA fingerprints of isolates were discerned from moderately resistant and resistant plants.

About 3-6% of the T523-731 reisolates from the resistant Improved Pope and L-180-1 shifted back to the parent or the wild type's haplotype. Some reisolates of the mutant T523-731 were observed to exhibit another haplotype. This was observed from Hawaii 7996 reisolates and is characterized by an additional band (0.2 Kb) on the T523-731. These reisolates were gathered from healthy or asymptomatic Hawaii 7996 plants. Four haplotypes of strain T523-731 were observed on the reisolates from the second virulence test as compared to two haplotypes after the first virulence test.

It was also noted that two DNA types were observed from different isolates obtained from the same plant. This is true for the resistant cultivars (Hawaii 7996, C108 and 508) and a moderately resistant cultivar (L-180). This could mean that variability within a plant could be induced or brought about by the resistance of the plant to the pathogen. These changes in the DNA type of the mutant isolate can be due to the fact that pathogen populations may exhibit a high degree of differentiation as a result of adaptation to specific host species and that after the pathogen has specialized on that particular host, selection may result in further genetic differentiation of the pathogen population (Leung *et al.*, 1993).

The substrain T523-731, therefore, may have pre-existed in the population at a low level and that host selection favored the increase in the frequency of these more virulent components of the strain when they were introduced to plants with resistance genes and were grown on a larger scale. Its virulence can be said to be conditioned by the genes for resistance in the host as evidenced by the enhanced virulence of the reisolates of this strain obtained from moderately resistant and resistant cultivars.

The use of varieties with major resistance genes has been one of the most successful means of controlling plant diseases. The resistance is effective until strains of the pathogen to which it does not confer resistance become established (Flor, 1971). Cultivars Yellow Plum and L-390 wilted as expected since they do not contain any resistance gene to bacterial wilt. The moderately resistant and resistant cultivars, on the other hand, were able to survive because of their resistance to *R. solanacearum*.

While most plant-pathogen interactions can be easily explained by the gene-for-gene concept, resistance to bacterial wilt in tomato does not seem to fit the model; rather it is multigenic with partial resistance and also highly complex (Wang *et al.*, 1998). Resistance in tomato was described earlier to be inherited as a polygenic character, with several loci playing a quantitative role in the ability of a plant to withstand pathogen attack and later on to be controlled by partially dominant resistance genes, which was particularly evident in the early stages of infection (Acosta, 1964). According to Balatero *et al.* (2000), resistance genes exhibited partial to complete dominance based on the performance of the F1, F2, F3, and backcross generations in their genetic analysis of the different populations derived from crosses involving tomato lines with different sources of resistance to *R. solanacearum*.

The level of genetic variability of a plant pathogen determines its ability to survive and adapt to its changing environmental conditions, in this case the presence of novel resistance genes in the host tomato plants. The increased virulence of *R. solanacearum* strain T523 and the apparent increase in its genetic variability showed that it was able to adapt to the pressure exerted by the resistant host. The resistance gene(s) in the cultivars C108, 508 and Hawaii 7996 became ineffective and this seems to be due to the response the *R. solanacearum* strains to the selective pressure exerted by the resistant cultivars themselves.

This study shows that pathogen populations, indeed, are genetically variable and that they respond to selection pressures.

BACTERIAL WILT IN POTATO

Using rep-PCR, Lando and Natural (2000) showed that bacterial wilt causing strains in potato were highly diverse, similar to tomato strains but in contrast to banana strains. Out of 74 potato strains from Benguet which were analyzed by rep-

PCR, four clusters resulted. This indicates the high genetic variability of this group of strains.

Another study done on potato strains was on the comparison of techniques to detect *R. solanacearum* in potato (Villa and Natural, 1999). The efficiency of the use of modified selective medium (mod SM), PCR and ELISA was compared in detecting *R. solanacearum* in potato tubers. Modified SM with chlorothalonil was found to decrease plating recovery to only 40.65%. Chlorothalonil was found to make the medium more selective for *R. solanacearum*. Seven primer pairs specific to different biovars of *R. solanacearum*, developed in the laboratory of Professor J. Timmis, University of Adelaide, Australia were tested on isolated DNA and boiled cells of *R. solanacearum* of potato. Primer pair 759F/760R was able to detect 37 out of 61 isolates tested, sensitive up to 1 cell per reaction, and also detected biovars 1, 2 and 3. In ELISA, four of the six antisera tested on *R. solanacearum* gave positive reaction to at least 2.6×10^4 cells. Mod SM, PCR, and ELISA were able to detect *R. solanacearum* in potato tubers and tuber washing. PCR was the most expensive relative to Mod SM and ELISA; however, the sensitivity and rapidity of the test time offset the cost, making PCR a very promising technique for *R. solanacearum* detection.

OTHER STUDIES

Cell wall modification was studied as an inducible structural defense response of resistant and susceptible cultivars of tomato to the bacterial wilt pathogen (Laurena, 1993). Extensin was 25 to 45 % higher in resistant cultivars. Likewise, lignin was increased, wall-bound proanthocyanins were higher in the root cell and in the stem cell walls of resistant cultivars compared to those of tbe susceptible cultivars. Tensile strength of infected susceptible cultivars decreased and stem tissues were heavily macerated with great quantities of slime. In contrast, infected stem tissues of resistant cultivars has still cell coherence and slime was not evident. The cell wall of resistant mato plants was less vulnerable to enzymatic digestion.

Balatero *et al.* (2001) worked on the development of molecular markers for tomato breeding for bacterial wilt resistance. They utilized three PCR-based techniques to identify markers that could be tightly linked to resistance factors: amplified fragment length polymorphism (AFLP), microsatellite or simple sequence repeats (SSR) and resistance gene analogs(RGA). Using these techniques, they constructed a linkage map consisting of 80 markers for Hawaii 7996 X Wva700 mapping population using F6 recombinant inbred lines (RIL) which were screened for resistance against two virulent *R. solanacearum* strains under greenhouse conditions. Seven AFLP and RGA marker clustered in at least two genomic regions putatively associated with resistance in both strains. Strain specific marker-trait associations that clustered in three other linkage groups indicating strain-specific type of resistance were also observed. This result emphasizes the need for a good understanding of the genetics of the pathogen. The development of these PCR-

bacterial wilt resistance in tomato.

CONCLUSION

Ralstonia solanacearum, nemesis of many crops is still here with us, but we have come a long way since it was discovered in the Philippines. With molecular approaches, we have come to understand the organism better. We now know that except for banana strains, the others are highly diverse. This implies that varieties bred by using a limited number of strains from certain localities may be broken down by other strains from other areas. We have shown that evolution and selection towards virulence can be induced by host resistance genes. The struggle between the pathogen and the scientists, therefore, continues. Luckily, we now have a more efficient method, marker-assisted breeding, to produce resistant varieties, allowing us to move faster and perhaps a few steps ahead of the pathogen.

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