

Review article

DIAGNOSTIC DEVELOPMENT FOR WILD FIRE SURVEILLANCE

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ABSTRACT: Information about the characterization and diagnostic of wild fire was compiled. This bacterial disease, exotic for Cuba, is caused by *Pseudomonas syringae* pv. *tabaci*. The species *Pseudomonas syringae* is traditionally divided into pathovars depending on the host range and symptoms. For its study, different phenotypic and genotypic methods are used. Such methods allow the traditional taxonomic confirmation of this pathovar within the species. Serological and molecular techniques such as: ELISA tests, immunofluorescence, polymerase chain reaction (PCR) are also used for the characterization and detection of the bacterium. Such techniques are very useful and sensible for the diagnostic.

(Key words: *Pseudomonas syringae* pv. *tabaci*; REP-PCR; pathogenicity; serology)

DESARROLLO DEL DIAGNÓSTICO PARA LA VIGILANCIA DEL FUEGO SALVAJE

RESUMEN: En el presente trabajo se recopiló información acerca de la caracterización y diagnóstico del fuego salvaje causado por *Pseudomonas syringae* pv. *tabaci* que es una enfermedad bacteriana, exótica para Cuba. La especie *Pseudomonas syringae* tradicionalmente se divide en patovares en dependencia del rango de hospedantes y los síntomas. Para su estudio se utilizan diferentes métodos fenotípicos y genotípicos, los cuales permiten la confirmación taxonómica tradicional de este patovar, dentro de la especie. Las técnicas serológicas y moleculares también se utilizan para la caracterización y detección de la bacteria, entre ellas las pruebas de ELISA, inmunofluorescencia, reacción en cadena de la polimerasa (PCR), que resultan útiles y sensibles para el diagnóstico.

(Palabras clave: *Pseudomonas syringae* pv. *tabaci*; REP-PCR; patogeneidad, serología)

INTRODUCTION

Wild fire is considered one of the most aggressive diseases affecting tobacco and other economically important crops such as soybean and bean (19, 20). This disease was first reported in North Carolina in 1917. Nowadays, it is present in USA, Mexico and Brazil, among other countries. Its long-distance dissemination is mainly by seeds, where the bacterium can survive for two years. In plantations, it disseminates by water (rain or irrigation), and mechanically by agricultural instruments. Its control is difficult and expensive and losses of 100% of the yield have been reported (1). In Brazil, (18) the presence of the bacterium was reported in bean, thus confirming, based on the pathogenicity reaction and the nutritional characteristics, that isolates inducing symptoms in *Phaseolus* constituted a different group within the pathovar. Later on, (26) the behavior

of different bean crops against *P. syringae* pv. *tabaci* was studied and the totality of the varieties was shown to be susceptible to the disease. Recently in this same country, the bacterium was reported affecting *Carica papaya* plants (5).

The present work is aimed at describing the main characteristics of the bacterial diseases known as wild fire, and exposing the advances and possibilities of its diagnostic, due to the great interest for our agriculture for being an exotic disease for Cuba.

CHARACTERISTICS OF THE CAUSAL AGENT

The disease is caused by *P. syringae* pv. *tabaci*, an aerobic, Gram negative rod, which is motile by the presence of flagella, optimum growth temperature

ranges from 24 to 28°C with a maximum of 38°C. Among the most important characteristics of this species are levan production and the presence of a fluorescent green pigment when it is cultured in King B medium and observed with ultraviolet light. For the taxonomic classification, the main morphological, biochemical and physiological features are studied using the classical methods and differentiation media. In such media, the enzymatic activity and the capacity to use carbohydrates, amino acids and alcohols with the production of acid and gas are expressed. Nowadays, "multi test" (tables and informatic programs) systems are commercialized, and systems commercialized by the French Firm Biomereux (among them API and Biolog), based on these characteristics, are used (20).

There are still many questions concerning the classification of the genus *Pseudomonas* with relation to the different species. This genus heterogeneity between the fluorescent and non-fluorescent groups had marked a relative distance between them. *P. syringae* belongs to the first group and it is, based on pathogenicity, divided into more than 40 pathovars. For this reason in 1980, the International Committee on Bacteria Systematic carried out a re-classification and reduced the number of the species accepted (from 100 to 23). Many of the strains previously classified as different species formed groups within *P. syringae*, based on tests initially designed for their classification and molecular tests, among them the rRNA:DNA ratio among strains determined by hybridization (6, 20). In other works carried out with *P. syringae*, the degree of similarity or differentiation among the pathovars *atofaciens*, *coronafaciens*, *garcae*, *glycinea*, *helianthi*, *lachrymans*, *maculicola*, *phaseolicola*, *pisi*, *syringae*, *tabaci*, *tomate* and *cichorii* was evaluated by LOPAT tests, analyses of fatty acids by gas chromatography, Biolog and API (4, 6, 14).

SYMPTOMATOLOGY

The disease is very variable regarding the aggressiveness level. It is mainly transmitted by contaminated seeds, where it can survive for two years. This is the primary route of propagation. Weeds, grass residues or other native hosts can serve as inoculum sources (1); its control is difficult and expensive. *P. syringae* pv. *tabaci* penetrates into the plant through the stomata and affects it in any of its stage, it multiplies in the leaf intracellular spaces producing a toxin which causes necrosis, from the edge to the center. These lesions are observed as grayish yellow greasy spots (from 0, 5 to 1cm diameter). The center of the lesions turns brown, and there is a yellowish green halo surrounding it. In a few days, the lesions can reach a diameter of 2 or

3cm; and large and irregular adjacent lesions are formed causing the death of the leaves (3, 11, 18).

TABTOXINS

The majority of the phytopathogen bacteria produces one or several kinds of substances (phytotoxins, phytohormones, exopolysaccharides, siderophora), known as virulence factors, which take part directly or indirectly in the pathogenesis processes (3). Almost all the bacterial toxins are metabolism secondary products. They are very harmful for the plant, and they are also active at very low concentrations. Until now, five different toxins, chemically defined, have been described (coronatine, phaseolotoxin, tabtoxin, siringomicine and tagetitoxin). They are produced by different *P. syringae* pathovars that are able to induce chlorosis or necrosis in the plant. There are mutant strains which do not produce any of these toxins; some microorganisms have been reported to inactivate tabtoxins through their enzymes (4, 22, 28).

The action mechanisms of phytotoxins are diverse. They can affect cell permeability, inactivate enzymes and act as anti-metabolites inhibiting the plant essential growth factors. A clear example of a phytotoxin blocking the amino acid synthesis is the phaseolotoxin produced by *P. syringae* (21). *P. syringae* pv. *tabaci* produces a toxin known as tabtoxin, which is responsible for the formation of the plant chlorotic halo. The molecule of the toxin segregated by the bacterium does not have biological activity, but by plant peptidases, it is transformed into a bioactive component called tabtoxin- β -lactamic that irreversibly inactivates the glutamine synthetase enzyme under light conditions (3, 21). This leads to a decrease of glutamine levels and an accumulation of ammonium in plant tissues, causing photosynthesis to uncouple, a selective destruction of the thylakoid membranes and a reduction of the defense capacity (13).

IMPORTANCE OF THE DISEASE FOR CUBA AND DIAGNOSTIC DEVELOPMENT FOR ITS SURVEILLANCE

Wild fire is an exotic disease for Cuba according to the Resolution 335 (2004). It is registered in group A-1, where the pests of economic importance not being reported in the country are listed, and the entry of strains is absolutely prohibited (8).

Cuba is probably the only tobacco producer country where the pathogen has not been reported, possibly due to the limited introduction of seeds (24). For this reason, it is important for plant protection laboratories to

rely on quick and sensible diagnostic kits for the screening of seed and plant samples coming from abroad, and in this way to avoid the introduction of the disease. At present, several methods such as taxonomic, molecular or serological techniques and pathogenicity tests are used for wild fire diagnostic (2, 15, 20).

PATHOGENICITY

In the last years, new plant species (soybean, bean, cucumber, pea and more recently papaya) have been reported as susceptible to *P. syringae* pv. *tabaci* (5, 17, 18, 19, 25). Regarding pathogenicity, the flagellin present in the flagella of this species has been demonstrated to behave as a strong elicitor and to induce the delayed hypersensitivity reaction in non-host plants. In mutant strains, where flagellin is not produced, motility is reduced, and they lose their capacity to produce HR and to cause the disease in tobacco, besides the previously described tabtoxin effects on the plant (15, 22).

Pathogenicity was comparatively studied including Koch's basic principles and the delayed hypersensitivity reaction of *P. syringae* pv. *tabaci* isolates from new reports in the plants: *Carica papaya*, *Aster* sp., *Celosia* sp. and *Cucumis sativus* obtained in IBC (5), with respect to *P. syringae* pv. *tabaci* strains isolated from tobacco. All the *P. syringae* pv. *tabaci* strains studied proved to be pathogenic for tobacco, bean and soybean, regardless of their origin, including strains from papaya, aster and cucumber crops. This indicates that the disease in the field can spread from crop to crop (2). These results have a practical application in crop rotation. In countries like Cuba, where this disease is exotic, this aspect has a great importance from the phytopathological surveillance and quarantine point of view, since bean and soybean seeds are imported. Besides, it should be taken into account that weeds serve as inoculum reservoir for pathogens allowing their survival for long periods of time and hindering their elimination in crop areas. Consequently, the selective elimination of natural hosts in affected areas can contribute to control the pathogen population in an integrated management program (25, 26, 27).

IMMUNOCHEMICAL DIAGNOSTIC

As the agricultural sciences develop, sensitive, reliable and quick diagnostic techniques, along with the observation of the symptoms and other elements, become more needed (4). The current biotechnology advances offer a considerable support in this respect. That is why it is asserted that success in prevention and phytopathological control depends, to a large extent,

on diagnostic quality. Immunoenzymatic assays, agglutination tests with latex, immunofluorescence and DOT-BLOT, among the antigen-antibody reaction based-techniques, are more used in phytopathology (2, 16, 20, 24). For the serological techniques, polyclonal antibodies, which recognize the totality of the strains within the pathovar, are used (2, 24), though the presence of lipopolysaccharides in *P. syringae* pv. *tabaci* has been notified (23), and these structures could be related to the presence of serogroups.

The direct agglutination on slides and plates and the latex test are the most used agglutination variants in phytopathology. The first one is used for the detection of numerous phytopathogen bacteria, while the second one can be from 100 to 1000 times more sensitive than the precipitation technique in tube. In it, the antibody is coupled to polystyrene particles, when the sensitized latex particles are confronted with the specific antigen; an aggregation of spheres is produced magnifying the immune reaction in this way (10).

For being an exotic disease for Cuba and due to the great importance of being prepared for its diagnostic, antigens and antibodies have been obtained and the serological techniques optimized (2, 24). This has allowed having available specific antibodies, serological techniques, indirect immunofluorescence, ELISA-DAS, analytical ultramicro system (AUMS) and agglutination with latex particles.

DIAGNOSTIC AND MOLECULAR CHARACTERIZATION

Wild fire diagnostic by molecular techniques uses PCR variants with primers amplifying for the tabtoxins produced by different *P. syringae* strains (5, 13). The characterization of bacterial diseases in plants aims at determining the clonal relationship existing among diverse isolates of the same species. This information is very important for the phytosanitary surveillance in exotic and quarantine diseases, since when outbreaks are produced, it allows determining the number of circulating clones, identifying the contamination or reservoir source and the transmission routes, and evaluating the efficacy of the control measures aimed at avoiding dissemination. The typification methods are classified into two big groups: the phenotypic group based on physiological and biochemical characteristics and the genotypic group based on DNA studies. The phenotypic methods of typification are less reproducible and have a lesser discrimination power than the genotypic methods. This is because the expression of a phenotypic character is the result of the interaction: genotype-environment and thus,

susceptible to be modified by variations of the environmental conditions (2, 4, 6).

In the last years, the advance in molecular biology has allowed developing efficient tools for characterization. One of these tools is Pulsed-Field Gel Electrophoresis (PFGE) used for bacteria and fungi typification. Such tool has a high discrimination power and an excellent reproducibility; however, it has the inconvenience of being very slow and laborious and, therefore, its daily use in the laboratory is little practical. That is the reason why the search of other methods, alternative to PFGE, is necessary (12, 13, 20). There are other typification techniques based on the amplification of nucleic acids by PCR. They are based on the amplification of genes or polymorphic DNA sequences and the electrophoretic separation of the amplification products. PCR techniques can be used together with other molecular methods such as enzymatic restriction, hybridization with specific probes or sequencing of nucleic acids (12).

REP-PCR is a typification method where the primers used are those hybridizing with the repeated or repetitive DNA sequences (rep sequences) that are distributed in the chromosome of many Enterobacteria and some Gram-positive bacteria and fungi. With such technique, the regions that separate the rep sequences are amplified. That is why, polymorphism results from the variability in the repetition of such sequences and the distance between contiguous copies caused by DNA insertions. Palindromic and extragenic repetitive sequences (REP sequences) and the extragenic repetitive consensus sequences of enterobacteria (ERIC sequences) are some of the most used rep sequences in epidemiological studies. REP-PCR is characterized by its simplicity, quickness and relative low cost, once a thermocycler is available (6, 9, 12, 14). The band patterns are often simple. REP-PCR has an acceptable discrimination power that can be increased with the use of fluorescent primers, though this makes the technique more expensive because an automatic DNA sequencer is needed for the analysis of the band patterns. Amplification of ERIC sequences is another typification technique used for studying the clonal reaction in diverse Gram-negative bacteria. DNA patterns, amplifying for ERIC-PCR are often less complex than those generated by REP-PCR. In a work carried out by the authors (2) using the REP-PCR technique, *P. syringae* pv. *tabaci* strains from tobacco were shown to be identical to the type isolate, also from tobacco, forming a first group. Bean, Cucumber and *C. papaya* isolates were similar among them for a second group; *Aster* and *Celosia* isolates formed a third group, while those from soybean were similar among them and different from the rest

forming a fourth group. On the other hand (5), the isolates from *C. papaya* plants causing leaf lesions were identified by biochemical, physiological, serological and molecular tests using the PCR-RLFP technique of the preserved region 16S-23S of the ribosomal DNA, allowing grouping these isolates within *P. syringae* pv. *tabaci*. REP-PCR technique has been used in the study of Gam-negative phytobacteria (e.g. *Pseudomonas savastanoi* pv. *phaseolicola*). REP elements are highly preserved and form stable structures in mRNA. It has been observed that REP sequences play an important role in chromosome organization in organisms lacking of nuclear structure (12, 13).

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