REVIEW

Characterization and diagnosis of Pseudomonas fuscovaginae *Miyajima, Tanii and Akita, causal agent of the Brown Sheath Rot in rice*

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ABSTRACT

Brown sheath rot is a bacterial disease that affects rice crops virtually in all regions of production in the world, but its incidence in Cuba has not been recorded yet. Its main causal agent is the species *Pseudomonas fuscovaginae*, although in some countries other species producing the symptoms are reported, including *Pseudomonas syringae* pv. *syringae*. Although *P. fuscovaginae* is registered in group A1 of the List of Quarantine Plagues of the Republic of Cuba, it has been reported in the Central America and Caribbean region, and therefore poses a threat to the country. Therefore, the aim of this review is to provide updated information on the characterization and effective diagnosis of P. *fuscovaginae*. Symptoms can be detected at different stages of the crop cycle. They are seen as brown necrotic spots in the sheaths, poor panicle emergence, grain staining and sterility, although this symptomatology may vary between different geographic regions. The strains of *P. fuscovaginae* have biochemical-physiological variability, even in the eight tests taken as distinctive of the species. They also have antigenic and molecular variability. Therefore, its diagnosis is complex and requires a polyphasic approach combining biochemical and physiological tests, other phenotypic tests, pathogenicity testing, immunodiagnosis and molecular diagnosis with specific primers or conserved gene sequencing (*rDNA 16S*, *rpoB*, *rpoD*, *gyrB*, among others) and accessory, individual or concatenated genes.

Keywords: biochemical-physiological characterization, molecular characterization, polyphasic diagnosis

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RESUMEN

Caracterización y diagnóstico de *Pseudomonas fuscovaginae* **Miyajima, Tanii y Akita, agente causal de la Pudrición parda de la vaina de arroz.** La Pudrición parda de la vaina es una enfermedad bacteriana que afecta el cultivo del arroz prácticamente en todas las regiones de producción en el mundo, pero no se ha registrado su incidencia en Cuba. Su principal agente causal es la especie *Pseudomonas fuscovaginae*, aunque en algunos países se informan otras especies que producen los síntomas, entre ellas *Pseudomonas syringae* pv. *syringae*. A pesar de que *P. fuscovaginae* se registra dentro del grupo A1 de la Lista de Plagas Cuarentenarias de la República de Cuba, se ha informado en la región centroamericana y caribeña, por lo que representa una amenaza para el país. Por tales razones, el propósito de este artículo de revisión es proporcionar una actualización sobre la caracterización y el diagnóstico eficaz de *P. fuscovaginae*. Los síntomas se pueden detectar en diferentes estadios del ciclo del cultivo. Estos se observan como manchas necróticas pardas en las vainas, pobre emergencia de la panícula, manchado de los granos y esterilidad, aunque esta sintomatología puede variar entre distintas regiones geográficas. Las cepas de *P. fuscovaginae* presentan variabilidad bioquímico-fisiológica, inclusive en las ocho pruebas tomadas como distintivas de la especie. También presentan variabilidad antigénica y molecular. Por lo tanto, su diagnóstico es complejo y se requiere un enfoque polifásico que combine pruebas bioquímico-fisiológicas, otras pruebas fenotípicas, ensayo de patogenicidad, inmunodiagnóstico y diagnóstico molecular con cebadores específicos o secuenciación de genes conservados (*ADNr 16S*, *rpoB*, *rpoD*, *gyrB*, entre otros) y genes accesorios, individuales o concatenados.

Palabras clave: caracterización bioquímico-fisiológica, caracterización molecular, diagnóstico polifásico

*I*ntroduction

Rice (*Oryza sativa* L.) provides staple food for more than half the world's population. [1]. Cuba is one of the main consumers of rice in Latin America, with an annual per capita consumption over 70 kg [2], and although the crop becomes more and more important, the average agricultural yield is approximately 3 ton/ha, much lower than the world average. Many factors influence this, among which are the effects caused by pests, which, under certain environmental conditions, are the most important limiting factors in the exploitation of this cereal [3, 4].

Brown sheath rot and Bacterial blight are considered, together with Piriculariosis, and Sheath blight, the most economically important diseases in rice cultivation worldwide. Sheath rot usually leads to another disease of great importance, grain spot, and although previously symptoms in the sheaths were mainly attributed the fungus *Sarocladium oryzae Sawada* [5], further studies indicated that the etiology of brown rot, in different geographic areas, may also be bacterial, mainly caused by fluorescent *Pseudomonas* [6-8].

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This pathogen poses a potential risk for Cuba, since its main causal agent could become an exotic species or introduced involuntarily, depending on the combined incidence of three main factors: i) its distribution worldwide by encompassing temperate climate regions as initially described, the tropics and the Central American and Caribbean region; ii) its transmission by seeds; and iii) the exchange of rice germplasm with countries where it is present [9].

In this sense, the aim of this work was to provide information on the characterization and effective diagnosis of *P. fuscovaginae*, to contribute to the phytosanitary surveillance of the crop through the prevention or early detection of the disease.

Brown sheath rot of rice: etiology, taxonomical classification, distribution, bioecology, symptomatology and damages

Pseudomonas fuscovaginae (ex Tanii, Miyajima and Akita, 1976) Miyajima, Tanii and Akita (1983) was reported as the causal agent of brown sheath rot of rice for the first time in 1976 in northern Japan [10]. After its omission in the List of Approved Bacterial Names of 1980, and in accordance with the International Code of Bacterial Nomenclature in force at that time, it was proposed again in 1983 [11]. It is classified within the Kingdom Bacteria, Phylum *Proteobacteria*, Class *Gammaproteobacteria*, Order *Pseudomonadales*, Family *Pseudomonadaceae*, Genus *Pseudomonas*, Species *Pseudomonas fuscovaginae* (ex Tanii, Miyajima and Akita, 1976) Miyajima, Tanii and Akita (1983) [12].

Since 1985 it was reported in rice in Burundi [13], later in Latin America [14] and Madagascar [6]; as well as in sorghum and maize in Burundi [15], and wheat in Mexico [16]. It has spread to practically all regions where rice is planted, and it is also reported in the Philippines, Indonesia, Nepal, China, Iran, Brazil, Malaysia and Australia [6, 9, 17-22].

The bacterial species *Pseudomonas syringae* pv. *syringae* van Hall was also identified as causal agent of rice sheath rot in Hungary, China, Australia, Chile [9] and Iran [23, 24]. Additionally, biochemical, serological, pathogenic and molecular evidence, as well as the study of fatty acid profiles, corroborated the existence of other species of *Pseudomonas* causing this disease in the Philippines, Indonesia and Cambodia [25, 26]. Symptoms of brown sheath rot can be detected from the earliest stages of the crop cycle, and infected young plants often die. When infection occurs in the last growth stages, paniculation, filling and grain quality are affected, the rice plant population may become chlorotic, and in the final stages of infection, the entire sheath becomes necrotic [22, 27]. However, typical symptoms of the disease are expressed in the panicle development or booting stage, which is the most susceptible to disease in host cereals [28].

Cottyn [29] characterized the most common symptomatology as brown necrotic spots on the sheath of the flag leaf, which varied in length, or as necrosis spread on the sheaths, poor panicle emergence, sterility, unfilled grains and kernel spotting (total or with only small brown spots); although Adorada *et al*. state that these last symptoms manifest themselves only in severely affected panicles [27]. Other authors suggest that *P. fuscovaginae* may cause reddish-brown necrosis, 2-5 mm wide, along the sheath, as well as extended water spots and necrosis with poorly defined margins on the panicles, which generate spotted glumes prior to emergency [14].

Although there are discrepancies in the symptomatology described by different authors, this shows common patterns and the differences could be related to the region being analyzed. Hence Cottyn *et al*. [17] claimed that typical symptoms of bacterial or brown sheath rot described by Tanii *et al*. [10], were not observed in rice from the tropics.

The greatest impact of this bacterium on crop yield is due to the fact that it causes sterility of the grain, which affects not only the yield, but also the quality of the grain (unfilled grains, kernel spotting, low quality grains) and its subsequent use as seed [29]. Due to their incidence, rice crop yield losses of up to 58 % were recorded in Japan [30], 72.2 % in Indonesia and 100 % in Madagascar [22]. In Italy, an average value of crop losses of 1.1 % was estimated, due to grain sterility and damage at harvest time of more than 30% of spotted grains, when susceptible cultivars were used [27]. According to Cottyn [29], considering the large surface used for rice production in the world, even a conservative estimate of 1-5 % annual losses could be translated into tons of rice and billions of dollars in loss of earnings to farmers. This situation exacerbates if we take into account seed transmission [31].

In Cuba, until the year 2017, the presence of the bacterium *P. fuscovaginae* had not been reported, from samplings carried out in the national territory, nor in the results of bacterial diagnosis in imported vegetable material [32]. That is why this species continues to be included in group A1 of the List of Quarantine Pests of the Republic of Cuba [33].

Characterization of *P. fuscovaginae*

Culture, cellular morphology, and biochemical-physiological characterization

The species *P. fuscovaginae* is Gram-negative, aerobic, catalase-positive and produces visible fluorescent pigments that diffuse in iron-deficient culture medium, such as the B medium of King *et al*. (KB) [34]. The cells have a morphology of rounded ends bacilli, with dimensions of 0.5-0.8 × 2.0-3.5 µm, which are found in simple form or in pairs, moving through one to four polar flagella and do not form spores [11].

Colonies in nutrient agar (NA), incubated at 28 °C, for 4 to 5 days, reach a diameter of 3 to 5 mm. They are circular, white or creamy-white to pale-brown, smooth, shiny, translucent, elevated and butyrous [11, 35]. In KB medium, between 24 and 48 h of growth, some strains form convex, circular white-creamy colonies, with smooth edges; they have an average diameter of 2 mm at 5 days; while others are relatively flat and circular, with more irregular edges and diameter of 2-5 mm at 5 days. Crystals can also be observed in the medium under bacterial growth after 24 to 48 h, whose size, shape and number vary between strains [7].

The species *P. fuscovaginae* does not produce levan in NA medium with 5 % sucrose. It has oxidative

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metabolism of glucose in Hugh-Leifson's oxidation-fermentation medium. Moreover, it has a positive reaction to Kovac oxidase, to the synthesis of arginine dihydrolase (ADH); to the hydrolysis of Tween 80, gelatin and starch, and to margarine lipolysis and peptonization. Nevertheless, denitrification tests, methyl red, Voges-Proskauer, synthesis of phenylalanine deaminase and urease, production of hydrogen sulphide, formation of 2-ketogluconate and indole, and hydrolysis of esculin and arbutine are negative. It does not require organic factors for its growth, which is optimal at 28 ºC, and does not occur at 37 ºC.

This bacterium produces acids from the glucose, arabinose, rhamnose and mannitol metabolism; but not from maltose, sucrose, raffinose, inulin, salicin, dextrin, adonitol, erythritol, inositol, dulcitol, α-methylglucoside. It grows by using as sole carbon source citrate, malonate, succinate, urate, acetate, β-alanine, L-valine, L-lysine, L-arabinose, trehalose; although it does not use tartrate, hippurate, 2-ketogluconate, inositol, sorbitol, adonitol or polygalacturonic acid. Also, it does not grow in nutrient broth supplemented with sodium chloride (5 %), does not produce soft rotting in potato, nor produces pits in polypectate gel [6, 11, 12, 36].

Some characteristics change between the strains, for instance, the response to the hypersensitivity reaction (HR) in tobacco leaves, considered as positive and typical of this species by some authors [11, 37], negative for some strains [7, 38], and variable for others [13]. Variations have been also documented among strains from Latin America, Asia, Africa and Australia, when analyzing the production of acids from xylose, mannose, lactose, trehalose, sorbitol, inositol, the generation of levan starting from sucrose, the reaction of the yolk, the tyrosinase synthesis and growth in nutrient broth supplemented with potassium chloride [11, 14, 39]. Despite the variability in the production of acids from trehalose, some authors consider it as one of the characteristics that differentiate *P. fuscovaginae* from other fluorescent and ADH-positive species [7, 36].

Mechanisms involved in pathogenicity

Miyajima [11] described the colonization steps of the tissues of the flag leaf sheath, from the presence of bacterial cellular groups in the epidermis of the adaxial side of the rice flag leaf sheath, which penetrate into the tissues of the host through open stomata and multiply in the substomatic cavity. During the onset of symptoms, *P. fuscovaginae* cells are found in the intercellular spaces of the substomatic parenchyma and lysinogen parenchyma, where great bacterial populations can be observed.

Jaunet *et al*. explained the pathogenicity of five strains of *P. fuscovaginae*, expressed as the capacity of multiplication of the agent inside the plant, through the general model of independent action; i.e., that the probability of multiplication of bacterial cells within the host, is independent from the dose of inoculum, and they established a high pathogenicity is characterized by a high efficiency of low doses of inoculum. They claimed that in the *P. fuscovaginae*-rice interaction, there is a variable virulence between strains and they suggested studying it thoroughly in a higher number of bacterial strains and rice cultivars, and comparing this variability with the genetic diversity of the pathogen.

Contradictorily to the assumption that the appearance of the sheath rotting disease was related to low temperatures, in regions with temperate or high-altitud tropical climate, low temperatures acted negatively on the pathogenicity process because they reduced the probability of multiplication of *P. fuscovaginae* inside the rice plant [30].

On the other hand, this species produces phytotoxic metabolites that induce necrosis in the sheaths and inhibition of panicle emergence, characteristic of the disease. They are associated with seven peptide substances of less than 5000 Da, where syringotoxins and fuscopeptins A and B stand out. For the latter, it was determined, among others aspects, their biological activity and conformations at molecular level [40, 41].

Phytotoxic metabolites known generally as lipodepsipeptides (LDPs) also caused glume spotting and reproduced symptoms frequently observed in plants infected naturally, in particular, poor panicle emergence due to inhibition of stem elongation during paniculation, although not always a correlation existed between the severity of this poor emergence and yield reduction, with disease incidence [28].

Besides, the rice plant was sensitive to toxins in all grow stages and the kernel spots they induced spread to untreated tissues. Nonetheless, these compounds are not host-specific. LDPs cause the death of host cells or affect their functions by interrupting ion transport through the plasmalemma [28].

More recent researches report the presence of loci associated with virulence in *P. fuscovaginae*. Among them are those closely related to functions involved in virulence, such as the PilZ protein of type IV pilus biogenesis, the T6SS machinery (Type VI secretion system) and the syringopeptin sintetase; as well as others related to metabolic functions, although they are also indispensable for virulence, like arginine biosynthesis, ethylene production, protein transport, and sulfur metabolism [42].

Quorum Sensing systems of *P. fuscovaginae*

Quorum Sensing (QS) systems are an important mechanism of intercellular communication, within the complex signaling systems used by phytopathogenic bacteria to regulate the expression of virulence genes at cellular and population level. Different types of molecules such as N-acyl Homoserine Lactones (AHL), fatty acids and small proteins, mediate this mechanism. The AHL-mediated signaling systems, dependent on the LuxI and LuxR protein families, play critical roles in the virulence of a wide range of Gram-negative bacteria [42].

P. fuscovaginae has two AHL-QS systems, and requires both to develop its pathogenicity mechanisms and for plant infection models [43]. These systems, named PfvI/R and PsI/R, are negatively regulated by the repressor proteins RsaM and RsaL, intergenically located between the *luxI* and *luxR* families [44]. This species also conserves proteins called LuxR 'solos' (PfvR1 and PfvR2), due to the absence of the LuxI/R AHL-QS canon system [42, 45].

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Molecular characterization of *P. fuscovaginae*

In a study of the genetic diversity of *P. fuscovaginae* and other *Pseudomonas* isolated from diseased rice plants, by restriction fragment length polymorphism (RFLP) analysis of the 16S rDNA gene amplified by Polymerase Chain Reaction (PCR), distinctive restriction patterns were observed for *P. fuscovaginae* with four of the nine restriction enzymes evaluated (*Hin*FI, *Nde*II, *Msp*I, *Dde*I), and a restriction site (*Hin*fI 935) present only in the isolates of this species. Variability was also obtained between the strains, which were grouped into four haplotypes that included most isolates in the first two, and one in each of the remaining, where the latter was a reference strain from Japan [25].

In addition, by amplification of the intergenic spacer region (16S-23S rDNA), a broad polymorphism was evidenced among species transmitted by rice seeds (*Acidovorax avenae* subsp. *avenae*, *Burkholderia glumae*, *P. fuscovaginae*, *P. syringae* pv. *syringae*, *Xanthomonas oryzae* pv. *oryzae*, *X. oryzae* pv. *oryzicola*), but very low intraspecies polymorphism [46].

Regarding the classic methods most used in genetic variability studies of bacteria, one of them comprises genomic profiling by rep-PCR, which differentiate various genomic regions located between the REP sequences (Repetitive palindromic extragenic sequences of 35-40 bp), ERIC (Enterobacteria repetitive intergenic consensus sequences, from 124 to 127 bp) and BOX elements (154 bp-long) [47, 48]. In the case of *P. fuscovaginae*, research based on the BOX-PCR technique confirmed intra-specific polymorphic patterns for strains isolated from rice seeds in the Philippines, which share multiple bands of equal mobility [49]. However, two groups were established with 38 % similarity when comparing isolates of *P. fuscovaginae* from this region to reference strains from Japan and Burundi, as well as other fluorescent species (*P. syringae* pv. *syringae*, *P. putida* bv. A). The first comprises isolates from the tropics, while the second comprises the reference strains [50].

Trends in the identification of phytopathogenic bacteria, with emphasis on *P. fuscovaginae*

Characterization is a key element in bacterial systems, and despite the development of new methodologies, both these and the 'traditional' ones are fundamental to determine if a strain belongs to a known taxon or constitutes a new one [51].

At present, bacterial identification addresses a polyphasic approach, *i.e*. it combines three types of methods: phenotypic, genotypic and phylogenetics) [52]. Phenotypic analysis examines the morphological, physiological and chemical characteristics of the cell, such as: morphology, motility, physiology, metabolism, types and proportions of fatty acids present in the lipids of the cytoplasmic membrane, chemical composition of the cell wall, pigment production and other chemotaxonomic markers [52]. Genotypic analysis considers the characteristics of the genome, including DNA-DNA hybridization, guanine-cytosine ratio, DNA profiles: restriction patterns (e.g RFLP) and PCR-based genetic fingerprinting using 'Ribotyping' (rDNA RFLP), rep-PCR, Random Amplification of Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP) [52, 53]. Likewise, phylogenetic relationships of bacteria are determined by the analysis of the sequences of genes coding for: 5S, 16S and 23S rRNAs; Tu Elongation Factor, ATPase β-subunit, RecA, the DNA-gyrase protein, and other functional genes [51, 52, 54].

For the genus *Pseudomonas*, phylogenetic analyses of *rpoB*, *rpoD*, *gyrB* genes, either independent or concatenated, are also performed [55, 56]; while for *P. fuscovaginae*, multilocus sequence analysis (MLSA) technique was recently used by the concatenated sequences of 10 conserved genes (*acsA*, *aroE*, *dnaE*, *guaA*, *gyrB*, *mutL*, *ppsA*, *pyrC*, *recA* and *rpoB*) and complete genomes of this species were explored and compared with those of other Pseudomonas species related to the disease [57].

Trends in the identification of phytopathogenic bacteria by genotypic methods also include a specific branch, based on central genome analysis (16S rRNA, genes involved in replication-transcription and other essential functions), and another interspecific, based on the analysis of genome accessory genes (virulence genes, membrane proteins and excretory systems, regulatory genes) [58]. For *Pseudomonas* species causing sheath rotting, recent researches indicate that both *P. fuscovaginae* and the strains that classify as similar to it (*P. fuscovaginae*-like) appear to have an open pangenoma, where each isolate represents a very different lineage that carries its own arsenal of accessory genes [57].

For the genus *Pseudomonas*, diagnosis using serological and molecular techniques is not well-established yet. There is high homology in central genome, but also high variability in virulence genes among the members of this genus. Hence, a combination of phenotypic and genotypic methods is used as essential components in the polyphase taxonomy of numerous bacterial species, including *P. fuscovaginae*.

Phenotypic methods used in the identification of *P. fuscovaginae*

Since the 1980's, pathogenicity tests, serological tests and the biochemical profile were used as basic tests for the diagnosis of bacterial sheath rot disease [7, 18, 59]. Nevertheless, none of the methods alone allows a reliable diagnosis, but the combination of at least 2 of them [36].

Selective and semi-selective media

Also, from the 80's two semi-selective culture media and one selective were used for the detection of *P. fuscovaginae*. The semi-selective medium (KBS) is based on a modification of King's B medium, with the inclusion of casamino acids and five antibiotics [60].

Zeigler and Álvarez [61] propose a differential medium with the addition of bromothymol blue and arginine to the basal medium of Ayers et al., which allows distinguishing four species (*A. avenae*, *B. glumae*, *P. fuscovaginae* and *P. syringae* pv. *syringae*) related to sheath rot and kernel spotting.

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Other authors developed a selective medium where *P. fuscovaginae* produces, after four to five days of incubation, round, smooth, high, translucent, beige-to-cream colonies with production of green pigments at the center of some of them that begin to lose their color from the eight day on [62].

However, 176 out of 204 isolates obtained in the Philippines grew in KBS medium, suggested as semi-selective, and 44 were tentatively identified as *P. fuscovaginae*, by the use of the differential medium with arginine. Only the medium described by Miyajima was selective, and allowed identifying three strains of this species, with the typical colonies previously described. Yet, eight other strains identified as *P. fuscovaginae* by the commercial kit Biolog (Biolog Inc., Hayward, CA, USA) were excluded by this method [18].

Pathogenicity tests

Verification of Koch postulates is performed in the early development stages of the crop (in 21-day-old plants) and in the early panicle emergence stage [17, 39]. The more common inoculation methods are spraying or injection of 100 µL of a bacterial suspension (10^6-10^9 c.f.u./mL) between the sheaths of at least 10 rice plants, 21 days after germination, at a distance of 5 cm on the soil surface, or on the adaxial side of the flag leaf sheath of plants in the early panicle emergence stage (65 days after sowing). Inoculation through puncture has also been used. The inoculated plants were kept under controlled conditions (greenhouses), with average daytime temperatures of 31-34 °C and nocturnal temperatures of 19-24 °C, and high relative humidity (80-90 %) during the first 24-48 hours after inoculation [27, 30, 36].

Not only are the typical sheath rot symptoms assessed, but also kernel spotting in the ripening stage. The symptoms observed in the sheaths consist of necrosis, which begins as small brown spots around the inoculation point, subsequently joining to form indefinite spots on the sheaths and sometimes the stems, and which extends along the sheaths, continuing in some plants, by the central nerve of the younger leaves [14, 17, 27, 59].

Biochemical tests

The distinctive biochemical profile is limited to eight tests: presence of cytochrome oxidase C, and ADH, production of acids from trehalose; as well as a negative reaction to the production of levan, 2-ketogluconate and acids from inositol, sucrose and sorbitol [36].

However, there is a diversity of criteria for the differentiation of *P. fuscovaginae* from other species of this genus which are ADH and oxidase positive. While most combine production tests of 2-ketogluconate and acid from trehalose and inositol [11, 13, 36], they also use other tests, such as the production of acids from sucrose and sorbitol, production of levan, use of inositol, sorbitol and 2-ketogluconate, among others [11, 59]. Although the production of acids from trehalose is one of the characteristics used as distinctive of the species, the authors who described it found small variations in its responses with the studied strains [11].

In turn, regarding the non-production of 2-ketogluconate as one of the tests considered main in the differentiation of *P. fuscovaginae* from other fluorescent *Pseudomonas*; eleven strains isolated in the Philippines were reported from samples of rice sheat rot and kernel spotting, 2-ketogluconate producers, identified as *P. fuscovaginae* [18]. Something similar happened with strains from Australia, Nepal, Japan and Colombia [39].

All of the above-mentioned indicates that these eight biochemical-physiological tests are not sufficient for the differentiation of the *P. fuscovaginae* species from other fluorescent *Pseudomonas* associated with the symptom [63].

Since the 1990s, biochemical characterization has been carried out mainly through the commercial kits API 20 NE (bioMérieux, France) and Biolog GN2 (Biolog Inc., Hayward, CA, USA) [7, 18]. Other groups also use the commercial kit API 50 CH (bioMérieux, France) [38].

Siderotyping

Another important taxonomic criterion among phenotypic methods is the production of siderophores, which is generally genus-specific and can also be species-specific. In this sense, the production of pyoverdines by fluorescent *Pseudomonas* is emphasized, which species specificity is due to the variability of the peptide part of the molecule [64]. Pyroverdines are characterized by being fluorescent under ultraviolet light. They consist of a quinoline chromophore, associated with a peptide chain of 6-12 amino acids and a side chain consisting of a dicarboxylic acid. The peptide chain between strains and species is variable, about 50 have been described, and 106 have been predicted. The catechol of the chromophore and two amino acids (β-hydroxyaspartic or hydroxamic acids derivatives from ornithine) participate in the chelation of iron [65].

At the same time, the strains that produce the same pyoverdines are grouped in siderovars. Knowing the siderovars of a species, pyroverdines can be used as specific tools for its identification, by comparison with a reference or with a general database compiling the characteristics of all the pyoverdines [64, 65]. *P. fuscovaginae* and *P. asplenii* produce an atypical pyoverdine that forms a complex with the Fe^{3+} ion, related to the presence of 2 β-hydroxyaspartic acids as iron ligands, whereas in typical pyroverdines it is always based on ornithine. The presence of these ligands in the pyoverdines of *P. fuscovaginae*, *P.syringae* and *P. cichorii* influences the color and spectral characteristics of the chelates-Fe(III) in a pH range from 3 to 7, which can be detected using spectrophotometric assays such as isoelectric focusing electrophoresis (IEF), mass spectrometry (MS) and mainly HPLC [66, 67].

Fatty acid profiles

The composition of fatty acids of bacteria varies from one species to another, in chains length and the presence or absence of unsaturated groups, rings, branched chains or hydroxyl groups. Therefore, the fatty acid profile of a particular bacterium may be useful in diagnosis. For this, fatty acids are extracted from cellular hydrolysates of bacterial cultures and are chemically modified to form their corresponding methyl esters. These volatile derivatives are then identified by gas chromatography. Subsequently, the types and amounts of fatty acids in the chromatogram

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of the bacteria under study are compared with a database containing the profiles of thousands of reference bacterial strains grown under the same conditions, and the strain with the highest similarity to the bacteria analyzed by computer programs is selected [52].

Since the 1970's and 1980's, fatty acid methyl esters (FAME) patterns have been used to differentiate species of *Pseudomonas* and groups of species. It has been reported that the presence of hydroxyl, cyclopropane and branched chain acids is characteristic of groups and species of this genus [68].

Commercial assessment systems for the identification of species of the genus *Pseudomonas*, based on FAME, Sherlock Microbial Identification System (MIS) (MIDI Inc. Newark, DE, USA) have been used since the 90's. The presence of three types of hydroxy acids (2-hydroxy, 3-hydroxy and iso-branched-3-hydroxy) allowed discriminating six groups of *Pseudomonas* strains. Not only unique profiles for infraespecific taxa (subspecies, biovars, pathovars) are found, but also good correlations between clusters made with fatty acid data and clusters based on DNA-DNA and DNA-rRNA hybridization results [68]. However, Vancanneyt *et al*. [70] concluded that the fatty acid content of the main species did not allow discriminating species within the different groups.

Slabbinck *et al*. [68] also evaluated the possibilities of the analysis of fatty acid methyl esters (FAME) for the identification of phytopathogenic species within the genus *Pseudomonas*; specifically identification at the species level and discrimination of the group of phytopathogenic species from those that are not. For these authors, differentiating between phytopathogenic Pseudomonas species is difficult; although they consider that some species can be identified by the use of 'random trees'. They claim that there is a statistical relationship between some fatty acids and pathogenesis in plants.

The use of the FAME microbial identification system together with other phenotypic and genotypic methods has allowed identifying *Pseudomonas* species present in rice seeds [49]. In previous studies this system allowed including numerous fluorescent *Pseudomonas* in a heterogeneous group and differentiating them from species of the same genus, like *P. syringae* and *P. aeruginosa* (Schroeter) Migula; and at the same time from *B. glumae* and *B. plantarii* species. Despite this, it was not sufficient for the differentiation of most of the fluorescent *Pseudomonas* isolated from rice plants with symptoms of sheath rot and kernel spotting [18]. In turn, strains identified as *P. fuscovaginae*, which caused these diseases in the maturation stage of rice in Australia, and 12 reference strains of the same species, could not be identified by FAME-MIS analysis [39].

Cell total protein content

Also among the phenotypic characters used for characterization, there is the total protein composition of cells. In this sense, a numerical analysis of electrophoretic fingerprints of cellular proteins of 210 reference strains of the genus *Pseudomonas*, and of other genera previously included in it (*Acidovorax*, *Burkholderia*, *Brevundimonas*, *Comamonas*, *Ralstonia* and *Hydrogenophaga*) was performed. These data demonstrated that protein profiles are species- rather than genus-specific.

Strain patterns of the species *P. aeruginosa*, *P. agarici*, *P. alcaligenes*, *P. amygdali*, *P. caricapapayae*, *P. chlororaphis*, *P. cichorii*, *P. coronafaciens*, *P. corrugata*, *P. ficuserectae*, *P. fragi*, *P. mendocina*, *P. pertucinogena*, *P. tolaasii* and *P. viridiflava*, were uniform and different. Whereas, there was a significant protein electrophoretic heterogeneity within the species *P. fluorescens Migula*, *P. marginalis*, *P. pseudoalcaligenes*, *P. putida*, *P. stanieri* and *P. stutzeri. Type strains of the species P. lundensis*, *P. meliae*, *P. mucidolens*, *P. oleovorans*, *P. resinovorans*, *P. synxantha* and *P. taetrolens*, occupied separate positions. Likewise, three strains of *P. syringae* coincided in the same group. However, representative strains of the species *P. asplenii* and *P. fuscovaginae* showed similar profiles [70].

Another technique used for classification and identification is matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI TOF MS), based on a characteristic protein profile of the strain. With this technique it is possible to differentiate some species of *Pseudomonas*, but it locates in the same group, without marked differences, strains of *P. fuscovaginae*, *P. syringae*, *P. marginalis*, *P. fluorescens*, *P. asplenii*, and *P. tolaasii*, among others [71].

Immunochemical methods used in the identification of *P. fuscovaginae*

Immunodiagnosis using agglutination and precipitation techniques

In the first decades of the study of the disease, inmunodiagnosis was one of the essential components of the diagnosis of sheath rot. The principal immunochemical methods were agglutination, either in slides, in tubes or in microELISA plates, and to a lesser extent precipitation techniques, such as double immunodiffusion, described by Ouchterlony and Nilsson [72].

Some of the anti-*P. fuscovaginae* antisera, obtained by various methods, reacted with strains from different regions. As the HMB266 anti-strain antiserum, with six strains from Japan, 1 from Burundi and 4 from Madagascar; and in another research, this same antiserum had reacted with three reference strains from Japan, three strains from Burundi, 16 strains collected in Gisha, four from Murongwe and three from unknown geographic origin, supplied by the International Rice Research Institute IRRI), Philippines. Nevertheless, they did not recognize other strains of the species, such as BCE32 and 532, from Colombia [13, 36]. In turn, other antisera only cross-reacted with few reference strains or strains native to some regions (such as anti-GR2 antiserum, with four strains from Madagascar, two from Japan and one from Burundi) [7, 36]. Another antiserum (anti-strain PDDCC 5940, from Japan), reacted by agglutination on micro-ELISA plates with reference strains of *P. fuscovaginae* but only with 75 % of strains from Latin America, China, Madagascar, Nepal, Philippines, Sierra Leone, Thailand and Turkey [59].

On the other hand, by means of double immunodiffusion assays, there was no evidence of a reaction between the anti-*P. fuscovaginae* A, antiserum from

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Japan and 11 strains of *P. fuscovaginae* from the Philippines, attributed to the antigenic variability between Japanese and Philippines strains [18].

Immunofluorescence

Rott *et al*. [7], by using indirect immunofluorescence, found specificity when using the anti-HMB266 antiserum (1/640 dilution), which did not react exclusively with reference strains BCE32 and 532 from Colombia. Although anti-GR2 antiserum (1/2000 dilution) reacted with the same strains of *P. fuscovaginae* tested with the above antiserum, it showed a weakly positive reaction with strains of *P. marginalis*; and at a lower dilution (1/200) did not allow differentiation since it reacted with all strains assessed, identified as *P. fuscovaginae*, *P. fluorescens* and *P. marginalis*.

In general, there is variability in the serological properties of *P. fuscovaginae*, and this allows grouping the strains taking into account the antiserum and the technique used [7]. Admittedly, a positive reaction, regardless of the technique used, complements information for the identification of *P. fuscovaginae*, a negative reaction should not be considered as excluding [18, 36].

As for the antigenic relationships between species associated with rice cultivation, there was a cross-reaction, by the agglutination technique, between pathogenic bacteria, particularly between fluorescent and non-fluorescent species, but with very little reciprocity between both types of species [59]. In turn, bacterial strains not pathogenic to rice were unable to react with antisera obtained against pathogenic species [59], with the exception of anti-GR2 antiserum at low dilutions [7].

On the 90's, research stressed on the need to increase specificity through other techniques and the use of monoclonal antibodies [36].

Diagnostic kits based on the ELISA technique

For the identification of *P. fuscovaginae* there are commercial diagnostic kits based on variants of the ELISA technique, such as double-antibody 'sandwich' ELISA (ELISA-DAS) and indirect ELISA using monoclonal antibodies [73, 74].

Nucleic acid-based methods

In the last two decades of the XX century, polyphasic taxonomic studies have played a crucial role on improving the classification of *Pseudomonas*, with methods for the analysis of microorganisms at molecular level [55, 75].

Through the DNA-DNA and DNA-rRNA hybridization techniques the genus was divided into 5 groups called rRNA I to V [55]. Subsequently, with the 16S rRNA (rrs) gene sequencing many species were transferred to other genera in the subclasses of Proteobacteria: *Alpha* (e.g. *Aminobacter*, *Brevundimonas*, *Devosia*, *Methylobacterium*, *Oligotropha*, *Sphingomonas*, *Zavarzinia*), Beta (*Acidovorax*, *Burkholderia*, *Comamonas*, *Hydrogenophaga*, *Ralstonia*, *Telluria*, *Vogesella*) and Gammaproteobacteria (*Chryseomonas*, *Flavimonas*, *Halomonas*, *Herbaspirillum*, *Marinobacter*, *Pseudoalteromonas*, *Pseudomonas sensu stricto*), or between subclasses Beta and Gammaproteobacterium (*Stenotrophomonas*) [12, 56, 76].

The genus *Pseudomonas sensu stricto* contains all species that correspond to the rRNA group I, such as fluorescent *Pseudomonas* and other related bacteria. Although the taxonomy of this genus has progressed slowly due to the sequencing of rRNA (for a crude phylogeny) and DNA-DNA hybridization (to delimit species), species-level identification is often very complex. Excellent phenotypic systems do not allow discriminating species within the complex subgroups of *P. fluorescens*, *P. putida* or *P. syringae*. Siderotyping, on the other hand, does not allow for greater differentiation, and ribotyping, although providing a high resolution in the separation of DNA hybridization groups, has serious disadvantages with regard to gene sequencing [55].

Conserved gene sequencing. Genome sequencing

The gene that has been used the most for taxonomic criteria, based on its nucleotide sequence, is *rrs*, but its high degree of conservation, which confers advantages for its universality, simultaneously leads to a smaller number of information sites on its sequence, and its usefulness is questioned due to its heterogeneity. Therefore, this frequently does not allow revealing a precise and statistically supported phylogeny at species level. So it is traditionally used to attribute a genus to a particular bacterial strain, but the resolution is too low to locate it at species level [56, 75].

Therefore, in order to obtain a more precise phylogeny within the genus *Pseudomonas*, other gene sequences are used, such as the genes coding for conserved proteins, mainly *rpoB*, *gyrB*, *rpoD*, and, to a lesser extent, other genes coding for structural or functional proteins, such as *oprI*, *oprF*, *atpD*, *carA* and *recA* [55, 56, 77-81].

The *gyrB* gene codes for the β-subunit of DNA gyrase protein, responsible for the negative supercoiling of DNA during replication; the *rpoD* codes for the sigma 70 subunit of RNA polymerase, while *rpoB* codes para la β-subunit of RNA polymerase. This last gene is highly conserved and a copy thereof is present in all bacteria because of its essential role in cellular metabolism. Several authors postulate the *rpoB* gene as a good candidate for phylogenetic analysis and identification of bacteria from clinical samples, and more comprehensively, hundreds of species of the genus Pseudomonas sensu stricto, as well as some of the genera that previously were part of *Pseudomonas sensu lato*, i.e., *Brevundimonas*, *Ralstonia*, *Comamonas* and *Burkholderia* [56, 82]. Concomitantly, due to its discriminatory power, the *rpoB* gene has been used for specific detection and for phylogenetic analysis of different bacterial groups [56, 76, 83, 84].

In spite of the multiple advantages of using the rpoB gene sequence for the identification of bacterial species, multilocus sequence analysis (MLSA/MLST) is often recommended and frequently used, with a view to increasing the resolution of phylogenetic analyses, through the analysis of several conserved genes [51, 56, 80, 81, 84, 85].

Sequencing of *rrs* and *rpoB* genes from some reference strains of *P. fuscovaginae* from different regions (Japan, Burundi, Madagascar, Brazil, Nepal, and Colombia) has allowed for phylogenetic studies [55, 56].

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While sequencing these genes from native Australian isolates and comparing them with those from databases, has been an essential element for confirming the identification of strains within a set of characters addressed with a polyphasic approach. In this study, a higher resolution was achieved with the *rpoB* gene analysis, compared to *rrs*. The rrs gene located all Australian isolates within a group that contained strains of *P. fuscovaginae* and *P. asplenii*; while analysis of the rpoB gene sequence placed Australian isolates within a single group (formed, in turn, by closely related subgroups), which showed greater similarity to the reference strains of *P. fuscovaginae* than to *P. asplenii* [39].

The variability present in this study, agrees with the approaches of Jaunet *et al*. [25] regarding the presence of several haplotypes in the species *P. fuscovaginae*. Hence, the authors recommended the use of several methods for identification, including, during the last decade, the insertion of complete genomic sequences of their strains, to make use of all genes and to be able to carry out phylogenetic studies from different combinations (Stodart B; personal communication).

PCR-based diagnosis

Early efforts to diagnose sheath rot disease based on nucleic acid properties, and more specifically in PCR, were based on studies of genetic diversity between strains, through PCR-RFLP and BOX-PCR.

Jaunet et al. [25] discriminated *P. fuscovaginae* from other fluorescent *Pseudomonas* isolates from diseased rice plants by PCR-RFLP analysis of 16S rDNA genes, thereby forming a group that was separated from the other *Pseudomonas* species of group I of rRNA, and that was shaped, in turn, by four haplotypes. Whereas Cottyn *et al*. [50], by fingerprinting analysis by BOX-PCR, distinguished two groups of strains belonging to the species *P. fuscovaginae*, related to 38 % similarity, one included isolates from the Philippines, and the other isolates from Japan and Burundi. However, these techniques did not provide an effective diagnosis of the disease.

Research in Africa reported the combined molecular diagnosis of bacterial pathogenic rice species, including *P. fuscovaginae*, based on DNA fingerprinting obtained by the PCR technique and the use of a pair of primers for each species analyzed, PfF3 and PfR3 for *P. fuscovaginae*. They considered the presence of at least one amplified band as a positive result for the detection of the species, and together, they found the presence of three genotypes within *P. fuscovaginae*, with no apparent relation to the origin of strains [86].

However, these primers are not specific because they allow the amplification of a fragment of the expected size for the species *P. fuscovaginae*, in other species of the genus (saprophytes present in samples of rice plants, pathogenic of humans), and even in saprophytic or phytopathogenic bacterial species belonging to other genuses (Rivero-González D, unpublished data).

Diagnosis based on Loop-Mediated Isothermal Amplification (LAMP)

Loop-Mediated Isothermal Amplification (LAMP) is a rapid and sensitive technique, first developed by Notomi *et al*. [87]. It is less sensitive to inhibitors than PCR, and for this reason, it applies to a wide range of clinical and field samples [88]. In recent years, this technique has been used for the detection of human, animal and plant pathogens [89-92]. The availability of complete genomic sequences (or draft sequences) of phytopathogenic agents enables the rapid design of primers for assays such as LAMP, through a comparative genomic study aimed at the selection of target coding sequences, conserved for the species in question.

Recently, a selective and sensitive LAMP detection assay was developed based on a comparative analysis with draft genomic sequences of representative *P. fuscovaginae* strains. This assay, using a set of six primers named Pf8, allows the differentiation of *P. fuscovaginae* strains from a wide range of bacteria commonly isolated from rice and other plants, not only for genomic DNA samples purified from pure cultures, but also from living or heat-inactivated cells. Moreover, this novel technique detects bacteria in extracts or exudates from vegetal material of infected host plants [93].

Conclusions

The rice sheath rot is a disease of complex etiology and distributed worldwide, *P. fuscovaginae* its main causative agent, which continue in the List of Quarantine Pests of the Republic of Cuba. This species displays high metabolic versatility, genetic plasticity, and functional adaptation, and increased antigenic and molecular variability. Hence, any single technique is unable for reliable diagnosis unless in combination. For that purpose, a polyphasic approach including a positive assay for pathogenicity is required, including at least one positive result from a biochemical or immunochemical tests together with a sequence-specific amplification test (PCR-based, sequencing of conserved or accessory genes, either isolated or concatenated). In fact, it has been possible to detect the pathogen using this polyphasic approach, both in pure culture (obtained from diseased plants) and in infected sheath extracts, but it is very important to develop a detection system in seeds. All these methods will reinforce and certainly provide national pest quarantine and surveillance systems with more reliable analytical and diagnostic tools for the preservation of healthy crops.

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