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Evaluation of two protocols developed for *Xanthomonas albilineas* molecular detection in sugarcane

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Abstract

Leaf scald disease caused by *Xanthomonas albilineans* (Ashby) Dowson is one of the most devastating diseases of sugarcane which has a direct impact on production and manufacturing quality. Currently, molecular tests have been proposed for the detection of this phytopathogen. In this work, two PCR's protocols developed by Pan *et al.* in 1997 and 1999 were evaluated, with primers that amplify a fragment of the ITS region between the ribosomal genes 16S and 23S. The search of *X. albilineans* was done from the juice obtained of 35 asymptomatic and symptomatic plants of sugarcane cultivated in localities of the provinces of Misiones and Corrientes, Argentina. *X. albilineans* was detected in only one of the symptomatic samples but in any of the asymptomatic ones. The protocol developed in 1999 was the one that gave the best results. It is important to emphasize the detection of this phytopathogen in the province of Misiones.

Keywords

Phytopathogen, Molecular Detection, Sugarcane Diseases, Leaf Scald, *Xanthomonas*

1. Introduction

Sugarcane is considered a natural renewable resource. Sugarcane industrialization leads to a growing range of end-use products and intermediate materials that expand its economic significance [3], [6].

The sugar sector has a great importance in the agroindustrial argentine production. In the last 20 years, sugar production in the country has increased overcoming in average 2.3 million tons in 2006-2010 [1].

In Argentina, 23 sugarcane mills have been installed, 15 of which are located in the province of Tucumán, 3 in Jujuy, 2 in Salta, 2 in Santa Fe and 1 in Misiones [1].

The total area planted with sugarcane exceeds 344,000 hectares, of which Tucumán represents 71 %, 19% Jujuy, 9% Salta and the rest is divided between Misiones and Santa Fe [1]. The annual sugar consumption is 40.62 kg/capita, while the world average is 22.31 kg/capita [21].

Currently, there is interest in the province of Misiones, where organic sugar is produced, to seek an increase in sugarcane production to spread cultivation to new rural areas, and to create an industry in terms of productivity able to compete with the costs of large sugarcane mills of Argentina and the Mercosur countries [21].

Limiting factors for sugarcane production are the effects of diseases caused by phytopathogens that affect the development of plants and produce low quality industrial raw materials. Of particular impact is the reduced sucrose content and purity of the juice, which results in poor recovery of sugar in the sugarcane mill [14].

One of the most important bacterial diseases and of difficult eradication is the leaf scald, whose etiologic agent is

Xanthomonas albilineans (Ashby) Dowson [7].

The disease is characterized by chronic and acute symptoms including: pencil-line leaf streaks, chlorotic leaf streaks either with or without necrosis, and reddish discoloration of vascular bundles at the nodes of the stalk [18].

But, sugarcane plants infected with *X. albilineans* may display similar symptoms to those found in plants infected with other microorganisms [23], which emphasizes the importance of diagnostic methods that eliminate the bias caused by the manifestation of visual symptoms.

Leaf scald is mainly transmitted by infected sets, cutting tools and agricultural machinery, constituting the phases of latency and eclipse, a key factor in the spread [18]. Furthermore, vegetative propagation of sugarcane and the continuity of a plantation for several years in the same area, make the surveillance of the disease less feasible [8].

At present no chemical control is used. The main alternatives for leaf scald control are the use of resistant varieties and certified planting material, resulting indispensable the availability of effective diagnostic methods. Moreover, it is necessary to control the introduction and spread of new strains of bacteria [11], [17].

Economic consequences can be severe when the disease occurs first in a region or when a new strain of the pathogen infects an area already contaminated by another.

The indirect damage caused by the disease is related to the high cost of replanting the devastated areas, production of healthy material (tissue culture plants and thermotherapy) and selection of resistant varieties, as well as the inability to raise a new variety due to its susceptibility [9], [12], [19].

Current methods for *Xanthomonas albilineans* (*X. albilineans*) detection are based on bacteriological culture conventional techniques on general plating or semiselective media, serological techniques using monoclonal or polyclonal antisera, and molecular biology techniques [2], [22].

The present study was conducted to evaluate two PCR protocols for *X. albilineans* detection from juice obtained from sugarcane plants grown in the provinces of Misiones and Corrientes, Argentina.

2. Material and Methods

2.1. Samples Collection

From May to August 2011, 35 asymptomatic sugarcane plants and sugarcane plants that showed some symptoms of leaf scald (loss of apical dominance in lateral sprouting and/or reddish dots in nodes or internodes) were collected from the surrounding countryside of the cities of Santa Ana, San Vicente, San Javier, Cerro Azul, Itacaruaré and Panambí in the province of Misiones and Bella Vista in the province of Corrientes.

Sampling was conducted at random and from each plant the lower third of the stalk was obtained and was transported to the laboratory in sterile bags.

In order to avoid cross-contamination and reduce the number of saprophytic microorganisms, the cuttings were washed with anionic detergent, disinfected with alcohol 70° and rinsed with sterile distilled water. Subsequently, a portion of the stalk of approximately 2 cm above the second or third node was sectioned with a knife. This portion was subjected to positive pressure, with clamp, in order to perform the extraction of 2 mL of juice. The juice was collected in sterile eppendorf and was preserved in a freezer at -20 °C until extraction of nucleic acids by boiling according to the protocol proposed by [15].

2.2. Molecular Assays

X. albilineans detection was performed by PCR using two protocols with primers amplifying a fragment of the ITS region between the ribosomal genes 16S and 23S.

The first protocol used ALA4 / L1 primers and generated an expected amplification product of 360 bp [15], while the second protocol used the PGBL1 / PGBL2 primers with an expected product of 288 bp [16].

The sequences of these primers are as follows (5′→3′): L1 (CAAGGCATCCACCGT); ALA4 (CCCGACTGGCTC CACCACTG); PGBL1 (CTTTGGGTCTGTAGCTCAGG); and PGBL2 (GCCTCAAGGTCATATTCAGC). All primers were synthesized by Operon Molecules for Life (USA) and used according to company specifications.

All amplifications were carried out in a final volume of 25 µl containing buffer PCR 1X [200 mM *Tris*-HCl, 0.1 mM EDTA, 1.0 mM DTT, 50 % glycerol (v /v)] (Invitrogen, USA), 0.2 mM dNTPmix, 1 µM of each primer, and 1 unit of *Taq* polymerase (Invitrogen,,USA). The concentration of $MgCl_2$ (Invitrogen, USA) was 2 mM and 3.5 mM for Ala4/L1 and PGBL1 / PGBL2 primers, respectively.

In the first protocol [15], reactions were run for 35 cycles, each consisting of 30 s at 94 °C, 45 s at 54 °C, and 1min s at 72 °C, with initial denaturation of 3 min at 94 °C and final extension of 10 min at 72 °C, while in the second protocol [16], reactions were run for 40 cycles, each consisting of 30 s at 94 °C, 45 s at 56 °C, and 1min s at 72 °C, with initial denaturation of 3 min at 94 °C and final extension of 10 min at 72 °C.

PCR assays were performed with a PTC-0150™ thermocycler (MJ Research, Watertown, MA, USA). Each amplified PCR product was electrophoresed (5V/cm) on a 2.0% agarose gel (Biodynamics®), stained with ethidium bromide, and visualized on a UV transilluminator.

PCR optimization was performed using *X. albilineans* genomic DNA as a positive control. DNA was quantified using TM Qubit fluorometer (Invitrogen).

The fragments obtained by PCR amplification were confirmed by sequencing (Macrogen Inc., South Corea). The sequences were analyzed by alignment with reference sequences from Genbank using the CodonCode AlignerTM program.

3. Results

The first protocol tested, based on the primer pair ALA4 / L1, generated amplification products of 310 bp in four

samples and a 360 bp fragment of the 35 samples studied, the latter coming from the province of Misiones.

In the second protocol tested (PGBL1/PGBL2 primers), only one positive sample (~290bp) was obtained; this corresponded to the same sample with a fragment of 360pb that was obtained with the first protocol.

Figures 1 (first protocol) and 2 (second protocol) show 9 PCR products obtained out of 35 sugarcane juice samples studied.

For sample 7, the sequences of the PCR products obtained by both protocols showed a 100% identity with *X. albilineans* strains deposited in GenBank, whereas the sequences corresponding to the four products obtained from 310 bp (first protocol) had 100% identity with *Pseudomonas fluorescens*.

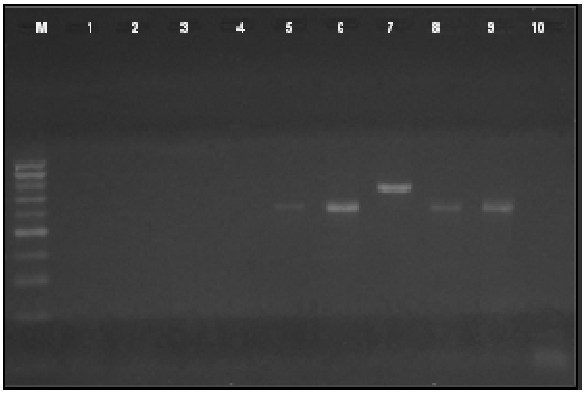

Fig. 1. *PCR products obtained with primers L1 and ALA4*

M: 50 bp molecular weight marker; lanes 1 to 9: samples tested; lanes 5, 6, 8 and 9: nonspecific products; lane 7: 360bp expected product corresponding to *X. albilineans*; 10: negative control.

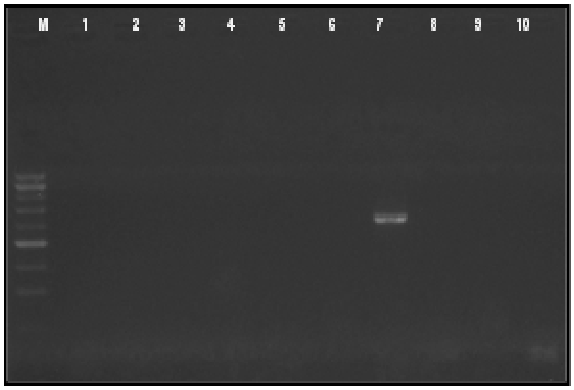

Fig. 2. *PCR products obtained with primers PGBL1 and PGBL2.*

M: 50 bp molecular weight marker; lanes 1 to 9: samples tested; lane 7: ~ 290bp product expected corresponding to X. albilineans; 10: negative control.

4. Discussion

Leaf scald is one of the diseases with the greatest impact on crops, causing yield losses, since it slows the growth of the plant, reduces the number of stalks, and decreases the quality of the juice extracted from sugarcane [20].

Damage caused by leaf scald in susceptible varieties of sugarcane can reach more than 90 % of crops, causing destruction in years or even months [9], [18].

Therefore, the identification of the etiologic agent is important to implement control measures and to development of resistant varieties, among other control methods [7].

Leaf scald sugarcane, can present different types of symptoms due to its systemic character [12].

Whatever the diagnosis method are used, an important aspect is whether the plants to be studied exhibit characteristic symptoms of the disease. However, lack of symptoms can result in latent infection, a very common leaf scald phase, which makes difficult the diagnosis of the disease and favors its dissemination [4], [5], [19].

Davis *et al.* [5] when comparing the performance of different methods for *X. albilineans* detection detected without difficulties the bacterium in symptomatic plants, but in asymptomatic ones, the frequency of detection depended on the method. These authors state that their results could be due to the small population size of the pathogen in asymptomatic plants ($1.1x10^5$ cells/mL) in contrast to the population size ($2.8x10^{10}$ cells/mL) in symptomatic ones.

In our study, *X. albilineans* was detected in only one of the symptomatic samples obtained in the province of Misiones. *X. albilineans* was detected in none of the asymptomatic samples. These results agree with those described by several authors, who have indicated that this phytopathogen is detected more frequently in symptomatic than in asymptomatic plants [4], [5], [12], [15].

It has been reported [23], that morphological and histopathological abnormalities similar to those produced by leaf scald can be produced by various factors such as damage caused by cold, iron deficiency, injuries due to insects or infections produced by other microorganisms This could explain the low detection obtained in symptomatic plants studied, which had only one or two symptoms of leaf scald.

Another factor to consider is the detection limit of both PCR protocols (1 pg of *X. albilineans* genomic DNA) [16].

Some authors [4] have informed that classical PCR would consistently detect *X. albilineans* only in samples containing at least 2×10^4 CFU /mL.

For the first PCR protocol studied in this work, the authors [15] reported that it was simple, sensitive and rapid working with both sugarcane juice obtained from diseased and healthy plants as strains of *X. albilineans*, *Xanthomonas* spp. (*X. oryzae*, *X. campestris* and *X. fragariae*), *Leifsonia xyli* subsp. *xyli* and saprophytic bacteria, although in the latter case some nonspecific reactions were observed. This was due to the fact that primer Ll is a universal primer and primer Ala4 has limited specificity [16].

Our results indicate that the non-specificity of this protocol would also apply to samples of juices, because amplification products of 310 bp, size close to the expected, were obtained in four samples, although the sequencing data showed belonging to *Pseudomonas fluorescens*.

Using the same protocol, a fragment of same size to the expected (360 bp) was obtained in one sample, which when sequenced showed 100% identity with *X. albilineans*.

Furthermore, in 1999, Pan *et al* [16] proposed a new

protocol based on a novel design of highly specific primers (PGBL1 and PGBL2). In this case, the amplification product expected for *X. albilineans* was 288 bp as reported by [10] and [13]. The authors [16] reported that it is very unlikely that other bacterial species can be amplified with PGBL1and PGBL2 primers because they were designed on the basis of multiple sequence alignment among sequences of *X. albilineans*.

In our study, only one positive sample without nonspecific bands in PCR assays was obtained with sugarcane juice using PGBL1 and PGBL2 primers. By sequencing, it was verified that the plant was infected with *X. albilineans*.

5. Conclusions

From the methods proposed for *X. albilineans* detection in sugarcane, molecular techniques are considered rapid, sensitive and specific tools.

ALA4 and L1 primers allowed the identification of *X. albilineans* to amplify a band of 360 bp specific for the bacteria, but they also amplified fragments of other saprophytic sugarcane bacteria as *P. fluorescens* with a size close to the expected (310 bp).

In contrast, only *X. albilineans* was detected using the second protocol and amplification products were not observed among any other bacterial strains.

Despite the detection of *X. albilineans* in only one of the 35 juice samples studied, our results demonstrate that the methodology used in molecular assays is effective, being the protocol described by Pan *et al*. in 1999 [16] the one which gives better results for its specificity.

Moreover, it is relevant to emphasize the detection of this phytopathogen in the province of Misiones, Argentina. Its presence in the province highlights the need for strong measures to improve sanitary controls in sugarcane.

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