Early Detection of Ratoon Stunting Diseases in Sugarcane Seeds Using Polymerase Chain Reaction Method

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Abstract. Sugarcane is a kind of plantation crop that can be used as a raw material for the sugar industry. The productivity of sugarcane can be influenced by several factors, including infection of pathogenic bacteria such as *Leifsonia xyli* f.sp. *xyli* (Lxx), the causal agent of Ratoon Stunting Disease (RSD) in sugarcane. The disease is one of the major diseases of sugarcane worldwide. RSD is symptomless unless the disease incidence is severe. The Polymerase Chain Reaction (PCR) method is generally known for detecting plant pathogens. However, there is still limited information on the detection of RSD using PCR. Therefore, our study aimed to detect the existence of RSD in sugarcane seeds using the PCR method. Three sugarcane varieties were used in this study, including AAS Agribun, AMS Agribun, and BQ (positive control of RSD). We did sample the stems and midribs of those three varieties at the age of 3 and 7 months. The results showed that *Lxx* was found both in the stem and midrib of sugarcane variety BQ at the age of 3 and 7 months. These results indicated that the PCR method can be used for early detection of RSD in sugarcane seeds.

1 Introduction

Sugarcane is a kind of plantation crop that can be used as a raw material for the sugar industry. The productivity of sugarcane can be influenced by several factors, including pathogenic bacterial infections such as *Leifsonia xyli* subsp. *xyli* (*Lxx*), the causal agent of Ratoon Stunting Disease (RSD) in sugarcane. This bacterium is gram-positive, straight rod-shaped and slightly curved cells (0.25-0.5 by 1-4 μM) [1]. The RSD was first discovered in 1944-1945 in sugarcane variety Q28 in Queensland, Australia. It was reported that almost all geographical areas of sugarcane plantations were infected by RSD [2]. The disease could cause an average yield loss of 5 to 50% in dry conditions in susceptible varieties [3]. RSD also results in shorter ratoons and varietal degeneration. The sustainable growth of China's sugar sector has been severely hampered by the expansion and infestation of RSD [4]. Sugarcane plants infected by *Lxx* bacteria is symptomless because it does not perform any typical external symptoms. The bacterium grows and develops within plant xylem tissue. By

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way of systemically infected stems or cuttings (sugarcane seeds) that are planted in new fields, the *Lxx* bacterium is spread from one field to another [5]. Disease dispersal occurs mechanically, especially at the time of harvest. To prevent the spread of RSD, the use of plant pathogen-free seeds and sanitation of sugarcane nurseries are very effective [6]. Duttamajumder stated that RSD disease is very contagious and spreads throughout the tissue, especially carried by infected seeds [7]. In addition, transmission can also occur through cutting tools that are used to harvest sugarcane stalks. It is necessary to have quick and precise methods for identifying *Lxx*-infected plants in nurseries and production fields to ensure sugarcane seeds do not exhibit RSD symptoms. Laboratory-based techniques are needed to diagnose RSD effectively so that sugarcane seeds are free from the disease. It is a very specific and more sensitive method of DNA-based diagnosis known as the Polymerase Chain Reaction (PCR) test. The PCR method is known to be applied for plant pathogen detection because it is very simple and easily practised. This DNA-based method is applied by directly taking infected plant fluids or tissues so that they are more sensitive and accurate [8]. In Indonesia, sugarcane varieties used as planting material are very diverse, including AAS Agribun and AMS Agribun varieties. The varieties were released by the Ministry of Agriculture in 2018. A high-yielding variety that has the advantages of hablur, productivity and high yield. There is no information in sugarcane nurseries about the two varieties associated with RSD disease. The spread of RSD in nurseries and in production fields in Indonesia is still not intensive. Therefore, there is a need for early detection activities to prevent disease transmission from seed fields to production fields. This study aimed to detect sugarcane seeds infected with RSD by the Polymerase Chain Reaction (PCR) method.

2 Materials and methods

Sample collection of sugarcane plants from the Indonesian Institute for Sweetener and Fiber Crops Standard Instrument Testing Nursery. The sugarcane seeds used were G0 seedlings of AAS Agribun, AMS Agribun and BQ (RSD positive control) aged 3 and 7 months. For the sample, we did use sugarcane midribs and stalks. The DNA extraction method was according to Geneaid DNA extraction KIT protocol. PCR analysis was conducted at the laboratory of KKI Purwodadi, Pasuruan. We used specific Lxx's primers [8]. The total reaction volume was 20 µL, including 1 µL DNA template, 1 µL F primary (Cxx1), 1 µL R primary (Cxx2), 7.5 µL dreamtaq 2X, 9.5 µL H_2O. The amplification was achieved by the following protocol: 95°C one cycle for 2 minutes, 95°C 35 times 30 minutes (denaturation), 55°C 30 minutes (annealing), 72°C 1 minute (elongation) (Each step is repeated 35 times), final elongation at 72°C and 10°C for 10 minutes. The electrophoresis was conducted by taking 3 µL PCR product and loaded onto 1% agarose. The electrophoresis was carried out for 90 minutes with 60 Volts in TBE buffer.

3 Results and discussion

Although RSD does not have an externally recognised symptom, it has a characteristic symptom within its nodes. The symptom is usually identified as a red-orange spot located on the xylem tissue (Fig. 1) that can only be seen when the node is split. The only visible symptom of RSD is abnormal growth of sugarcane. Nevertheless, this symptom is quite similar to nutrient deficiency. Therefore, it is important to apply an early detection method for the RSD, one of which is using the PCR method.

Fig. 1. Symptoms of sugarcane plants infected with RSD

Our result revealed that the PCR method could detect the existence of *Lxx* bacteria within the sugarcane stem and midrib of the BQ variety at the age of seedlings 3 and 7 months with a band size was about 439 bp (Fig. 2). The results also showed that the primary sequence specifications of CXX1 and CXX2 were able to recognise *Lxx* bacteria in the total DNA genome in young seedlings (3 months). In contrast, in both AAS Agribun and AMS Agribun varieties aged 3 and 7 months, there were no DNA bands seen in stem or midrib samples. This might indicate that neither variety was infected by *Lxx*. Gao et al. stated that the results of a modified PCR test that does not require organic ethanol solvents are identical to those of PCR tests carried out according to the CTAB protocol [9]. All PCR amplicons are 439 bp with the same nucleotide sequence. According to Grisham et al., PCR detection can be applied on sugarcane leaves inoculated with *Lxx* bacteria in greenhouses at 3 and 4 months [5]. Taylor et al. stated that two 10-mer oligonucleotide primers and PCR-based polymorphic DNA amplification were utilised in the detection process on the fibrovascular fluid of sugarcane plants [10]. The *Lxx*-specific markers produced by OPC-02 and OPC-11 primers are roughly 800 bp and 1000 bp in length.

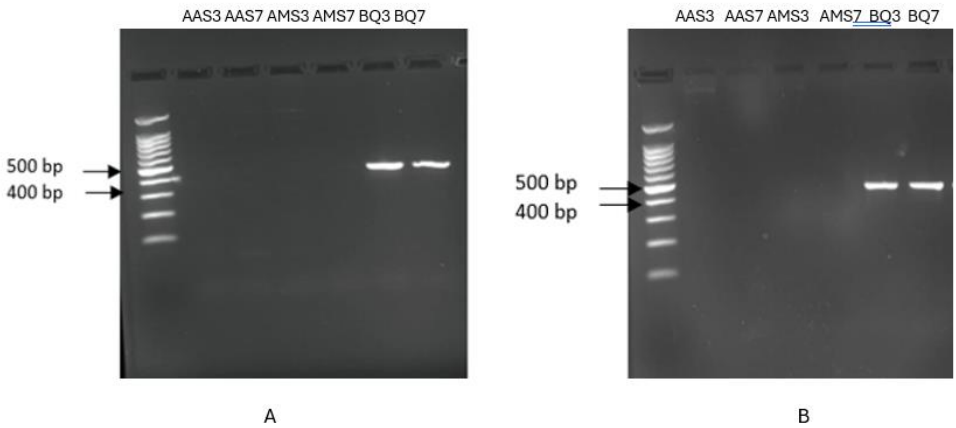

Fig. 2. Results of *Lxx* DNA amplification by PCR using Cxx1 and Cxx2 primers.
A: sample from midrib, B: sample from stem

Plant disease detection activities using molecular is one rapid and accurate identification method. Molecular characteristics will easily detect plant pathogens because we do not need to culture the pathogen on artificial media. In addition, this detection can be directly conducted by taking parts of plants that have disease symptoms. The PCR method is expected

to be used for efficient, accurate, and easy detection, and it can also be cheaper because pathogen culture costs are absent [11]. Mills et al. stated that PCR-based detection is a rapid, highly sensitive and inexpensive method that it can both detect and differentiate Lxx from *L. cynodontys* and *L. xyli* [12]. This PCR-based detection activity is expected to help identify problem areas where control measures are taken. It needs to be increased to prevent further spread of the disease.

4 Conclusion

The PCR method can be used to detect the existence of *Lxx* bacteria within symptomatic sugarcane samples at the early growth stage.

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