

FINDING THE ROOT CAUSE OF FREQUENTLY PREVALENT DISEASE IN SUGARCANE FROM SOME DISEASED FIELDS OF SINDH, PAKISTAN

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خلاصہ

گنا (*Saccharum officinarum*, L.) پاکستان کی تیسری بڑی فصل ہے۔ یہ صرف ایک اہم چینی فصل نہیں بلکہ پاکستان میں مختلف زراعت کی بنیاد پر صنعتوں کے لیے خام مال کا ذریعہ بھی ہے۔ کچھ بیماریوں کی طرف سے ایشاء کے بہت سے حصوں میں شدید اضافہ ہوا ہے جس میں سفید پتی اور گھاس گولی مار فیلوٹولوزما کی بیماریاں شامل ہیں۔ موجودہ تحقیق کا مقصد گنا کی فصل میں بیماری کا سبب بننے والے ذمہ دار عوامل کی نشاندہی کرنا تھا۔ سندھ کے متاثرہ علاقوں سے نمونے حاصل کیے گئے تھے۔ پاکستان میں فائیکوپلازما کے وہابی امراض قریبی علامت سے جڑے ہوئے ہیں۔ فائیکوپلازما کا پتہ لگانے کے لیے، جینڈ-پی سی آر کا مقصد انجام دینے کا مقصد تھا۔ فائیکوپلازما کا پتہ لگانے کے لیے قابل اعتماد سالماتی ہتھیار جینڈ-پی سی آر استعمال کرتے ہوئے P1/P7 کا ہونا پایا گیا تھا۔ پلانٹ کے مختلف حصوں سے کل DNA مثال کے طور پر جڑوں، شاخ، پتیوں اور سفید کھیسوں سے متاثر ہونے والی پتیوں کو C-TAB اور فینول/کلوروفارم نکالنے کے طریقوں سے نکال دیا گیا تھا۔ فائیکوپلازما میں SrRNA16 جین کا ایک حصہ بڑھانے کے لیے یونیورسل پرائمر جوڑی P1/P7 استعمال کیا جاتا تھا۔ منفی نتائج حاصل کیے جاتے تھے جب اثر مکمل پلانٹ میزبان پی سی آر کے نتائج تھے اور پھر جیل الیکٹروفورسز کیے گئے تھے۔ موجودہ مطالعہ کے نتائج سے پتہ چلتا ہے کہ فائیکوپلازما کی بیماری کی علامات جیسے کلوروس اور ناکام ترقی کی علامات فیلڈ مٹی میں معدنی/غذائی اجزاء (لوہا اور دیگر) کی کم سطح کا بھی نتیجہ ہو سکتا ہے۔ زمین میں یا متاثرہ فصل کے حصے میں فیرس سلفیٹ کی درخواست علامات کا علاج اور گنے کی پیداوار میں اضافہ کر سکتا ہے۔

Abstract

Sugarcane (*Saccharum officinarum*, L.) is the third major crop of Pakistan. It is not only an important sugar crop but also a source of raw material for various agro-based industries in Pakistan. Sugarcane yields have been severely reduced in many parts of Asia by some diseases which include white leaf and grassy shoot phytoplasma diseases. The current research was intended to identify the causative agent responsible for the disease in sugarcane crop. The samples were collected from the diseased ridden fields of Sindh, Pakistan with the symptoms closely related to phytoplasma infection. For the detection of phytoplasma, nested-PCR was aimed to perform. The reliable molecular tool for the detection of phytoplasma was found to be Nested PCR using P1/P7. Total DNA from different parts of plant such as; roots, stem, leaves and the leaves infected with white fly, were extracted by CTAB and phenol/ chloroform extraction methods. Universal primer pair P1/P7 were used to amplify a portion of the 16S rRNA gene in phytoplasma. Negative results were obtained when symptom full plant host were subjected to PCR and then gel electrophoresis were carried out. Results of the current study indicated that the indication of phytoplasma disease symptoms such as chlorosis and stunted growth, can also be the result of low levels of minerals/ nutrients (iron and others) in the field soil. Application of ferrous sulphate in soil or in parts of the affected crop might cure the symptoms and increase the yield of sugarcane.

Introduction

Saccharum officinarum L. (Sugarcane) crop is prone to diseases caused by phytoplasma. These plant pathogens are responsible for causing excessive damage to agricultural crops worldwide. Due to white leaf and sugarcane grassy shoot disease; the sugarcane industry in Asia is suffering substantial and considerable crop loss. Two closely related species of phytoplasma are actually responsible for these types of losses which specifically infect sugarcane (Marcone, 2002). The symptoms of infection associated with phytoplasma can be clearly observed phenotypically. Leaves of the infected plant are relatively smaller in size and because of the rapid loss of chlorophyll they turned into yellowish or pale white coloration. Excessive tillers results in characteristic bushy appearance and dwarfism. The transmission of phytoplasma can occur during the vegetative propagation and by insects, particularly *via* leaf hoppers (Nasare *et al.*, 2007). Furthermore, other utmost important factors include; chlorosis (loss of chlorophyll) which can also be associated with iron deficiency in plant. It also occurs due to the deficiency of iron contents in the soil. Addition of ferrous

sulphate in the field soil and application on leaves in the form of spray has found to improve the sugarcane yield (Rakkiyappan *et al.*, 2002).

Phytoplasma belong to class of Mollicutes. They are obligate intracellular bacteria that lack cell wall and require some complex medium in order to culture in the laboratory (Martini *et al.*, 2014). Generally, they are present in the phloem of infected plants and also in those insects that feed on the phloem of these plants. (Hogenhout *et al.*, 2008). All these problems led to the hindrance for the detection of phytoplasma and create a huge barrier for their classification and characterization. The recommended and widely used methods for the detection and identification includes some molecular techniques such as those based on Polymerase chain reaction (PCR), Loop-mediated isothermal amplification (LAMP), and Restriction fragments length polymorphism (RFLP) analysis are recommended and widely used. These methods are sensitive, rapid and provide efficient way for the diagnosis and classification of almost all types of phytoplasmas. (Srivastava, 2016).

According to the report of integrated management of sugar cane diseases published in Jan 07, 2008: In Pakistan, sugarcane production endures a significant economic value. Sugarcane industry contributes enormously in total Gross domestic production (GDP). It is used as cash crop and its by-products proved as a source of cheap raw material for the production of various goods. Some of the most important and widely used by-products include bagasse which is used in paper, plastic, and paints industry; molasses for the production of chemicals such as ethyl alcohol, citric acid, acetic acid, etc., and the agro-waste as animal feed, and as fertilizer. In Pakistan the data of sugarcane crop protection revealed that the sugarcane crop in country is usually affected by Red rot, Whips smut, mosaic virus, and Ratoon stunting diseases. Detailed studies on phytoplasma affecting sugarcane in Pakistan has not undertaken yet. Furthermore, it has also been reported to cause diseases in mungbean, chickpea, , potatoes and citrus fruits in Pakistan (Akhtar *et al.*, 2010; Akhtar *et al.*, 2008; Gosnell and Long, 1969 ; Mannan *et al.*, 2009). The current study aims to detect the presence of phytoplasma in sample collected from disease ridden fields of Sindh, Pakistan. The infected samples, with symptoms similar to phytoplasma infection, analysed *via* nested-polymerase chain reaction.

Materials and Method

Chemicals and Media

Primers for Phytoplasma P1/P7 (Penicon), Nutrient Agar (Oxoid), Nutrient Broth (Oxoid), Phosphate Buffered saline (Oxoid), Amphotericin B (Sigma), CTAB (Sigma), Mercaptoethanol (Diaging), Tris-HCl (Anal R), NaCl (Anal R), EDTA (Scharlau), Chloroform (Merck), Isoamyl alcohol (Murmapur), Agarose, Sodium Acetate (Sigma), Ethanol (GPR-BDH laboratory), Master Mix (Thermo-scientific) , Ladder (Thermo-scientific).

Sample processing / Plant sample processing

Leaves, eggs, roots and stems were collected from the diseased fields of Sindh (Fig.1). All samples were washed with autoclaved distilled water and stored at 4°C and lower temperatures, or dried with anhydrous calcium chloride, until processed for DNA extraction.

DNA Extraction

Method 1: CTAB method

CTAB extraction method for the extraction of total genomic content from various parts of sugarcane. Samples of leaves, roots, stem, eggs and white fly infected leaves were kept at -80°C and pulverized. A stock solution of 50ml CTAB extraction buffer was prepared as follows: (1g CTAB was added in a buffer containing 20mM EDTA, 100mM Tris-HCl, 1.4M NaCl, and 2% 2-Mercaptoethanol) EDTA and Tris-HCl were set to pH 8. The solution was placed in water bath at 65°C for approximately 45 minutes. Each sample was added in CTAB extraction buffer. The suspension was incubated in a water bath at 65°C for 60 minutes. Prior to centrifugation, solution of Chloroform: isoamyl alcohol in a ratio of 24: 1 was added in equal proportion into each tube. All samples were centrifuged at 13000rpm for 5 minutes. The supernatant was transferred to another tube and equal volume of isopropanol was added in each sample. After vortexing the samples were centrifuged at 13000rpm for 5 minutes and supernatant was recovered. For precipitation of DNA 1\10 volume of 3M sodium acetate (pH 5.2) solution and 2x volume of cold ethyl alcohol (absolute) was added in supernatant and placed at -20°C for overnight. The precipitates were pelleted out through centrifugation at 13000rpm for 15 minutes. The supernatant was discarded and pellet was washed serially with 70% and 95% ethanol. The pellet was air dried and the resulting DNA precipitates were re-suspended in 0.5-1mL TE buffer. (Parmessur *et al.*, 2002)

Method 2: DNA Extraction by Phenol/ Chloroform

Extraction method was used as an indirect method to detect Phytoplasma in the samples. The strategy involved the growth of respective organism in broth medium and the extraction of total genomic content was done by Phenol-chloroform method. The crude extracts from both methods (CTAB / Phenol-chloroform) were run on agarose gel to confirm the presence of DNA in the samples to assure the method's validity (Green *et al.*, 2017).

Polymerase Chain reaction

Conventional PCR was performed for the detection of phytoplasma DNA in extracted DNA of sugarcane samples. The universal primer pair P1/P7 Forward Primer: 5' AAG AGT TTG ATC CTG GCT CAG GAT T 3', Reverse Primer: 5' CGT CCT TCA TCG GCT CTT 3' were used to amplify a portion of the 16S rRNA gene in phytoplasma. P1/P7 which amplifies a 1784 bp DNA fragments (Schneider *et al.*, 1997). The reaction was performed in 25µL volume. The reaction mixture was consisting of 12.5µL PCR Master Mix (thermo-fisher scientific), 0.5µL of each primer, 1µg/µL DNA sample and deionized water to make up the volume up to 25µL. The PCR cycle initial denaturation at 94°C for 5 minutes. 30 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute and 1 cycle of final extension at 72°C for 10 minutes. The PCR products were detected by Gel electrophoresis into 1% agarose gel, stained with ethidium bromide and photographed on an UV trans-illuminator

Results and Discussion

Sugarcane disease has a great impact on country's economy. It is needed to find out the root cause of disease. For this purpose nested PCR approach is usually used to detect the phytoplasma (Schneider and Gibb, 1997) even when it is present in low levels or it is disseminated in their plants and insect host (Goodwin *et al.*, 1994; Andersen *et al.*, 1998). Suspected samples of phytoplasma were assayed via Polymerase chain reaction and amplicons were analyzed by agarose gel electrophoresis (Fig. 2). Same samples were further processed with the culture medium by phenol chloroform method to find out the possibility of phytoplasma and the results were further confirmed by PCR followed by electrophoresis. It was observed that in direct PCR assay there was no amplification of phytoplasma DNA when extracted from sugarcane by CTAB method, using the primer pair P1/P7. Also no PCR product was formed in any of the DNA sample (Extracted by Phenol-Chloroform method) from various parts of the plants (Fig.3).

The sugarcane disease was first diagnosed almost 50 years ago and it has been found in all the sugarcane cultivating countries resulting in marked loss of country's economy. Some diagnostic methods have been adopted that detect the phytoplasma in sugarcane include serology and DNA based tests (Sdoodee, 2001 ; Srivastava *et al.*, 2003). Among all of these diagnostic techniques PCR was considered to be the most sensitive method. In order to verify the identity, phylogenecity and genetic relatedness of phytoplasma, DNA sequencing would be helpful. Another main reason for such samples of the diseased plants is the deficiency of various minerals/ nutrients such as iron, zinc etc. Nutrition in sugarcane are responsible for the improving the cane yield of sugarcane (Kumar, 2013).

The results of the current study indicated that the phytoplasma was not the causative agent for the disease in the plant samples used for the detection, the pathological conditions might have been due to the deficiency of any nutrient for instance zinc and iron etc., as the symptoms showed by the infected plant can also appear due to lack of availability of a particular nutrient or low concentration of a particular nutrient (Kumar, 2013). Deficiency of nitrogen, sulphur, iron, calcium, and boron results in yellowish appearance of leaves (Gosnell and Long 1969 & Radhamani *et al.*, 2013) However, according to a report, leaves chlorosis is only a measure of iron deficiency and could be cured by 0.25% of ferrous sulphate solution. (Radhamani *et al.*, 2013) Decreased concentration of phosphorous, nitrogen, zinc, iron and boron may also lead to aberrant plant growth (Kumar, 2013)

Conclusion

Current study was focused with the identification of the causative agent for sugarcane disease in the disease ridden field of Sindh. The likely reason behind such studies could be the deficiency of nutrients in the soil of sugarcane field and indicated that the phytoplasma was not the causative agent for the disease in plant samples provided.



Fig 1. Sugarcane plant sample from the disease ridden fields of Sindh (a) White fly eggs (b) Leaves (c) Stem (d) Roots

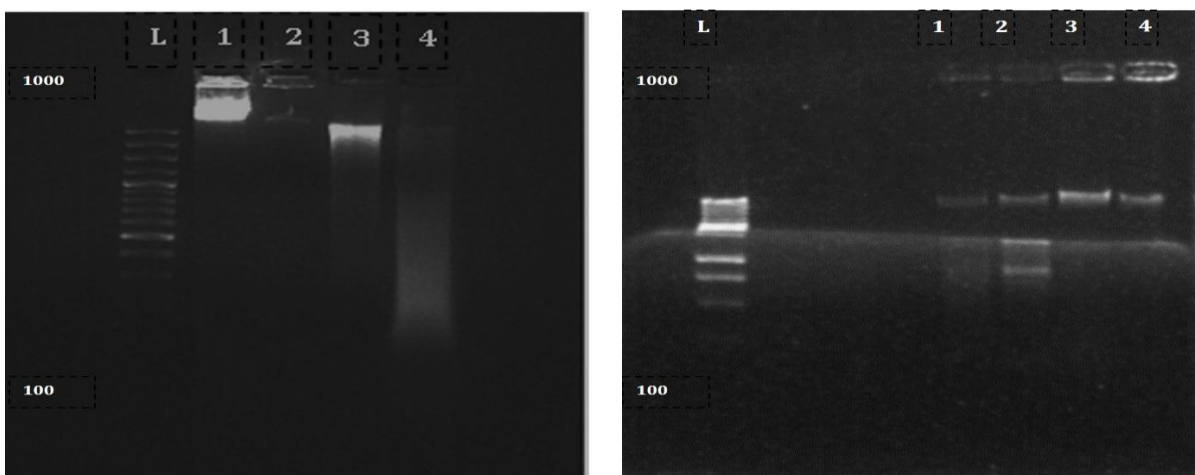


Fig 2. Results of DNA extraction from different parts of sugarcane plant by CTAB extraction method (a) and extraction of microbial DNA by Phenol/chloroform extraction method (b). Crude DNA samples were run on agarose gel prior to PCR whereas; L=DNA ladder, 1=Roots, 2=White fly infected leaves, 3=Stem, and 4=Leaves

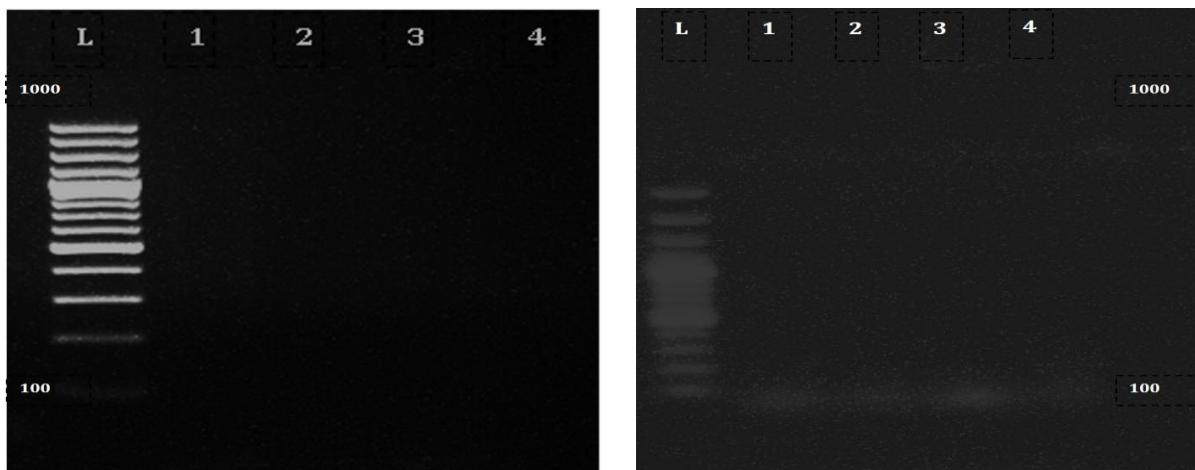


Fig 3. Results of PCR Products. No bands were observed in the PCR products; L=DNA ladder, 1=Roots, 2=White fly infected leaves, 3=Stem, and 4=Leaves

Acknowledgements:

We are highly grateful to the Gene Manipulation and Molecular Pathology Labs of Dow research institute of biotechnology and Biomedical sciences, Dow University of Health Sciences, Ojha Campus.

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