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A STEP WISE MOLECULAR TECHNIQUES FOR DETECTION OF BACTERIAL PATHOGEN (*Spiroplasma citri*) IN CITRUS PLANT: A MINI REPORT

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INTRODUCTION

Spiroplasma citri is a bacterium without cell wall that cause disease in citrus plants. It was first discovered in 1915 in Washington navel orange trees in California (Fawcett, 1944). It is a stubborn disease widely distributed in the South-western United States, North Africa and Mediterranean. Citrus is not the only host of *S. citri,* however many non-citrus plants species throughout the world have also been reported to naturally and experimentally infected with *S. citri*, for instance, *Catharanthus roseus* (L) commonly called Periwinkles was the first non-rutacea plants to be reported infected with *S citri* in California and Arizona (Allen, 1975) and recently its presence in Periwinkle plant was reported in Malaysia (Nejat, Vadamalai, Sijam, & Dickinson, 2014) nevertheless no report of the pathogen in Malaysian citrus. This research was carried out to determine wether *S. citri* are found in Malaysian citrus plants or not and to establish a molecular method for detection of the pathogen.

DNA extraction from citrus plant using CTAB DNA extraction protocols are as follows:

Procedure

Step 1: Citris plant sample was obtained from Ladang 16 at Faculty Pertanian (Agriculture), Universiti Putra Malaysia. 2g of leaf tissue (4 mid-ribs) were surface sterilized, cut into small sizes and grinded in the presence of liquid nitrogen into fine power using mortar and pestle then transferred into 1.5 ml microcentrifuge tube.

Step 2: Eight hundred (800) µL of CTAB buffer was added immediately followed by 20 µL of β–mercaptoethanol and incubated at 60^{o}C for 25 min in water bath. To enhance proper mixing, the tubes were occasionally inverted intermittently 2 to 3 times during incubation.

Step 3: After incubation, 600µL of chloroform-isoamyl alcohol (24:1) was added and the mixture was vortexed for 30 seconds followed by centrifugation at 13,000 rpm for 8 minutes. The resultant supernatant was transferred into clean 1.5 ml microcentrifuge tube. This step was repeated once.

Step 4: DNA was precipitated with an equal volume of isopropanol added to the volume of supernatant and incubated in -20^{o}C for 2 hours then centrifuged at 13,000 rmp for 10 min. The resultant supernatant was discarded.

Step 5: Pellet obtained were rinsed three times with 70% ethanol and air-dried at room temperature. The pellets were then suspended in 50 µL of double distilled water.

Step 6: A colourless, clean pure DNA pellets were obtained and preserved at -20^{o}C for further analysis.

POLYMERASE CHAIN REACTION (PCR) TECHNIQUES

The Internal transcribed spacer (ITS) region is universally used for identification and elucidating species relatedness hence Nested PCR technique was adopted to amplify the ITS region from the citrus plant DNA extracted above.

PCR components 1x (µL)

1.	PCR master Mix	12.5
2.	Forward primer	1.0
3.	Reverse primer	1.0
4.	Water	5.5
5.	DNA samples	5.0
	Total	25µL

Conventional PCR conditions

Conditions	Temperature (^{oC})	Time (mins)	Cycles
Early denaturation	94	2	1
Denaturation	94	1	35
Annealing	61	2	35
Extension	72	3	35
Final extension	72	10	1

Method: A nested PCR was conducted for four (4) hours 22 minutes for 35 cycles; specific primers for the PCR (ScR16F1/ScR16R1); Expected size 1800 bp. Same conditions was used in conventional PCR but 60^{o}C was used for the annealing temperature. Primers used for nested PCR are ScR16F1A/ScR16R2 and the Expected band size is 1500bp.

AGAROSE GEL ELECTROPHORESIS

Gel electrophoresis was employed to visualize, quantify and measure the length of DNA fragments in the sample as well as PCR product amplified. A reaction mixture was prepared as follows:

- Agarose gel
- 1XTBE buffer
- DNA samples (PCR product)
- Silva nitrite
- 5% acetic acid
- Loading dye (20µL)
- Molecular marker/ladder
- Ethidium bromide
- 10% ethanol

Procedure

One and half percent (1.5%) of agarose gel was prepared. 3g of agarose was mixed with 200ml of 1X TBE buffer and heated for 2.5 minutes in a microwave until dissolved and formed a gel. The gel was poured into a gel-cast and allowed to cool (solidify) for 15 minute before loading the sample. About 20 – 40 µL of 1X TBE buffer was used to rinse the well just before loading. Twenty (20 µL) of samples (PCR product) was carefully loaded into six (6) well using micro pipette. The gel was run for about 30 minutes at 200 volt and then fixed twice. Firstly the gel was washed with solution containing 5% acetic acid and 10% ethanol and shaked on Belly dancer shaker for 5 minutes and secondly, using 10% acetic acid/5% ethanol for 7 minutes. Acetic acid helps in fixing DNA onto the gel while ethanol opens the gel pores to enable acetic acid access to the DNA.

The gel was then washed with 3% silver nitrite (aids in fixing & visualization of the gel) under dark condition (to avoid light reflection) then rinsed with water and finally the gel was visualized using Gel Documentation and photographed.

RESULTS AND DISCUSSION

The result obtained from the gel electrograph (figure 1) showed no visible band correspond to the ladder in all the six replicates hence indicated poor or unsuccessful PCR performance. Several inter-laboratory studies have been performed to assess the factors affecting the reproducibility and comparability of PCR as a molecular detection technique (Kim, Yang, Bae, & Park, 2008). Inhibition of DNA polymerase cause poor PCR performance and could be minimized by increasing the polymerase concentration, dNTP concentration and PCR elongation time thereby allowing for the rebust amplification of larger amplicons (Dietrich *et al.*, 2013).

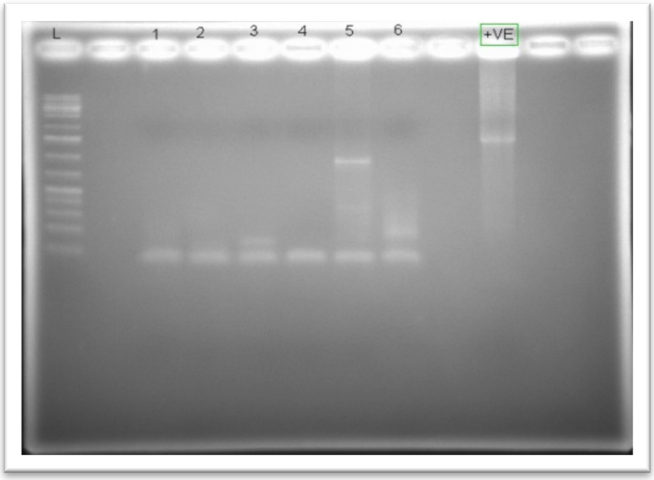

Figure 1: Gel electrograph of DNA extracted from citrus plant.

A strange band appears in 3, 5 and 6 wells which do not correspond to the molecular marker and positive control band.

CONCLUSION

The result indicated that the citrus plant sample is free from *S citri* pathogen, Non-existence of band length in the sample indicated that the sample DNA might have been denatured due to prolong time during extraction process.

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