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Rapid Detection of Citrus Greening (Haunlongbing) by Iodine Kit Method and its Validation using Polymerase Chain Reaction (PCR)

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A B S T R A C T

Citrus greening (HLB) is deadly diseases caused due to gram negative bacterium called α-probacteria. The transmission of disease occurs by a vector psyllid (*Diaphori citri*). It cannot be controlled fully by chemical means only the production of diseases free panting material and removal of infected part are the measures to overcome this disease. The early detection of this disease helps to keep the orchards free of diseases and losses. On the basis of visual symptoms the diagnosis of HLB under field condition is very difficult. The symptoms occurred due to HLB are like blotchy mottle pattern of leaves and yellowing of leaves which can also be the reason of nutrient deficiency like zinc, manganese and iron. So, to avoid this confusion present study was designed to produce rapid diagnostic test for the disease. The Iodine kit method was standardized for the detection of disease in field with accuracy and rapidity. The HLB infected leaf produce high starch compare to healthy leaf because of that there is production of brown colour on reaction with iodine while yellow colour was observed in healthy leaves this shows the absence of HLB infection. Later the polymerase chain reaction (PCR) was used to confirm and validate the result obtain by iodine kit method using specific primer to 16S rDNA region of Indian '*Ca*. L. asiaticus' i.e. OI1/OI2c species. The amplification was observed in HLB infected leaf at 60^0c with amplicon size of 1160 bp, which was absent in healthy leaf sample. Thus, the iodine kit method has great potential to provide an improved, cost effective, rapid, user friendly and in situ method for diagnosis of *Ca. L. asiaticus'* for the farmer, nurseryman, mobile plant pathology laboratories, bud wood certification programme and quarantine programme and in offices. The present methods of disease diagnosis helps in the reduction of disease outbreak and keep the orchard free from diseases by helping us in the screening of disease free planning material.

Introduction

Citrus is one of the most valuable and widely grown fruits in the world which provides nutrition to the local people and is used as an indispensible cash crop. But now a days Asian citrus plantation have brought serious yield loss and deterioration of fruit quality due to diseases associated with it (Whiteside, 1988). Citrus greening (Haunlongbing) is one of the devasting diseases which cause severe loss in citrus yield. There is 30-100% loss occur in citrus yield due to citrus greening infection globally (Ghosh and Das, 2012) while vidharbha region of maharashtra 8-43% of sweet orange and 6% Nagpur mandarin were infected by greening. It is characterized by chlorosis of leaves on one or more limbs (Fig 1 a), followed by twig dieback, sparse foliage, distinct yellow shoots, and fruits which do not fully colour at stylar end and remain green hence the name greening (fig 1b). Later the trees may show an open, spare foliage, severe fruit drop and many small yellow shoots in many cases result into severe decline and death (Roistacher, 1991).

The greening is caused by a gram negative bacterium called α-probacteria. It is widely distributed and serious disease in India transmitted by psyllid vector shown in fig 1 c (*Diaphoria citri*) and vegatative propagation (Ghosh, 2012). They are classified based on geographic location and sensitivity to temperature into three species: '*Ca L. asiaticus*' '*Ca. L. africanus*' and '*Ca.L. americans'* (Bove 2006; Jagoueix *et al.*,1997). On the basis of visual symptoms the diagnosis of HLB under field condition is very difficult.

The symptoms like blothchy mottle pattern of leaves and yellowing of leaves can also be the reason of nutrient deficiency like zinc, manganese and iron. So, to avoid this confusion a rapid diagnostic test (Iodine kit method) used for diagnosis of disease. It works on the principle of starch production.

The infected leaves produce six times more starch than healthy leaves. Recently number of researcher used this technique for diagnosis of HLB. It is not hundred percent sure techniques for diagnosis of citrus greening so to make it more accurate the results were validated using improved technology i.e. polymerase chain reaction (PCR).

The polymerase chain reaction (PCR) based on molecular techniques are powerful methods which has greatly facilitated detection of plant pathogen that otherwise would have been difficult or time consuming to detect using conventional technique (Dilip Ghosh and Das, 2012). Citrus being a predominantly vegetatively propagated crop, presence of pathogen in mother plants in nursery.

Since no chemical method is effective against this graft transmissible pathogen establishment of pathogen free nursery system is the only way to control this disease. This increases the necessity of conducting various experiments to detect the pathogen in citrus sample. So, the present study reports an effective Iodine kit method and its validation by PCR for faster and reliable detection of greening pathogen (*CLa*) in citrus cultivar and its usefulness to implement citrus bud wood certification programme in India.

Materials and Methods

The HLB infected leaves and healthy leaves of sweet orange (*Citrus sinesis*) were collected (Fig 2) from College of Horticulture, Dr. Punjabrao Deshmukh Krishi Vidyapeeth, Akola. The mature citrus leaves showing symptoms were collected in a polythene bag. The upper surface of the infected leaf was scratched about 40 times with a piece of sandpaper.

The sand-paper piece harbouring tissue debris was then placed in a small polythene bag with a sealing mouth and 1 ml of pure water added in it. The sand-paper piece was rubbed in water thoroughly for washing of the tissue debris into the water. After that, a drop of iodine solution was added into the suspension in the bag and the solution was mixed by shaking. At last, the solution was observed for colour-change.

Extraction of DNA from infected leaf sample

DNA isolation method for detection of HLB was optimized as per standard protocol given by Dellaporta *et al.,* (1983). The quality of DNA obtained after extraction was confirmed by running it on 0.8% agarose gel (containing ethidium bromide @0.5 µg/ml) in a horizontal gel electrophoresis system.

Primers and polymerase chain reaction (PCR) for citrus greening

The PCR was carried out using citrus greening specific primers (OI1/OI2c) given in Table 1. The primers were designed on the basis of the sequence information reported and which is conserved among Asian strains of the greening bacterium (Jagoueix *et al.,* 1994). The PCR was carried out by using following parameters: one cycle at 94^0c for 3 min, 35 cycles at 94^0c for 1 min, 55-60^0c for 1 min and 72^0c for 45 sec followed by one cycle at 72^0c for 10 min. The PCR products were analysed by electrophoresis using 1% agarose gel and visualized in gel documentation unit.

Results and Discussion

Detection of citrus greening (HLB) using iodine kit

The test was carried out to distinguish between confusing nutrient deficiency symptoms and leaves that may be HLB positive and also helpful to select the best sample for PCR analysis thus helping to reduce the number of negative samples.

The iodine kit detection was carried out by collecting healthy and citrus greening (HLB) infected leaf samples of sweet orange. The piece of sandpaper was scratched on both HLB infected and healthy leaf sample and this scratched sandpaper was put into zip lock polyethylene bag so that after adding a drop of iodine solution into the zip lock bag, it can be rubbed properly to loose the leaf tissue debris which reacts with iodine solution. After adding iodine solution it was observed that there was a development of black colour in HLB infected leaf sample (fig 3)

This was due to the reaction of accumulated starch in the HLB infected leaves while the development of yellow colour in the healthy leaf sample showed no starch accumulation ultimately showed the absence of HLB infection to the healthy leaf plant shown in plate 1. A similar test was carried out by Hong and Truc (2003) on citrus species and they got similar results. This showed that the test was carried out successfully.

Validation of iodine kit method using polymerase chain reaction (PCR)

The results of iodine kit method were validated using polymerase chain reaction (PCR). The DNA isolated by CTAB method was confirmed on 0.8 percent agarose gel and quantified using a spectrophotometer. The DNA was treated with ribonuclease-A for RNA to cut off. Then pure quality DNA was used for PCR amplification with 50 ng/µl concentration. PCR was carried out using OI1 /OI2c primers specific to 16S r DNA region of α-probacteria and the amplified products were run on 1% agarose gel. The OI1 /OI2c amplify amplicon size 1160 bp in the infected leaf sample and no amplification was observed in healthy leaf as well as negative control sample shown in Fig 4.

Ghosh and Das (2012) while working on HLB detection in different citrus cultivars used similar OI1/OI2c primers and got similar band size *i.e.* 1160 bp for greening infected mosambi, Nagpur mandarin and acid lime plant while no band in a healthy plant.

Ruangwong and Akarapisan (2006) reported that a polymerase chain reaction (PCR) with specific primers OI1/OI2c used for detection of HLB bacterium produced specific band of 1160 bp for diseased leaves whereas no product from healthy citrus plants.

Table.1 Primer used for detection of citrus greening (HLB)

Primer	Nucleotide sequence	Amplicon size
FP (OI1/OI2C)	GCGCGTATGCAATACGAGCGGCA	1160 BP
RP (OI1/OI2C)	GCCTCGCGACTTCGCAACCCAT	

Fig.1 a) Chlorosis of leaves symptoms due to citrus greening infection, b) Greening of fruits symptoms induced by citrus greening c) Citrus Psyllid, *Diaphori citri* feeding on citrus plant

a b c

Fig.2 a, b) HLB infected leaf sample of sweet orange (*Citrus sinesis*), c) Healthy leaf sample of sweet orange (*Citrus sinesis*)

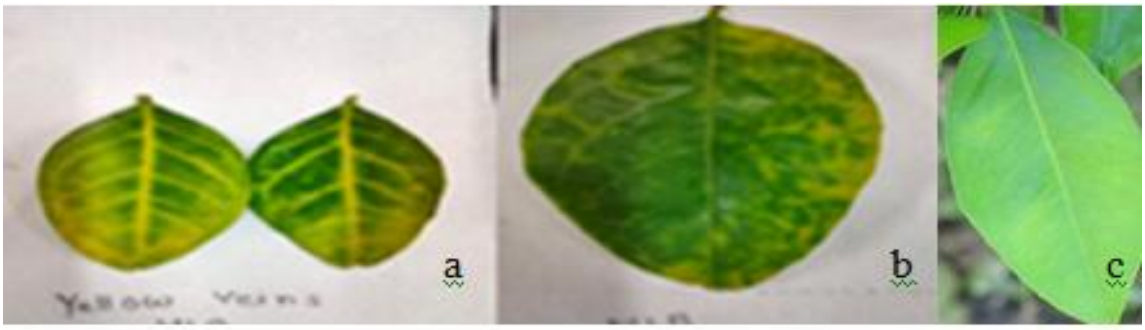
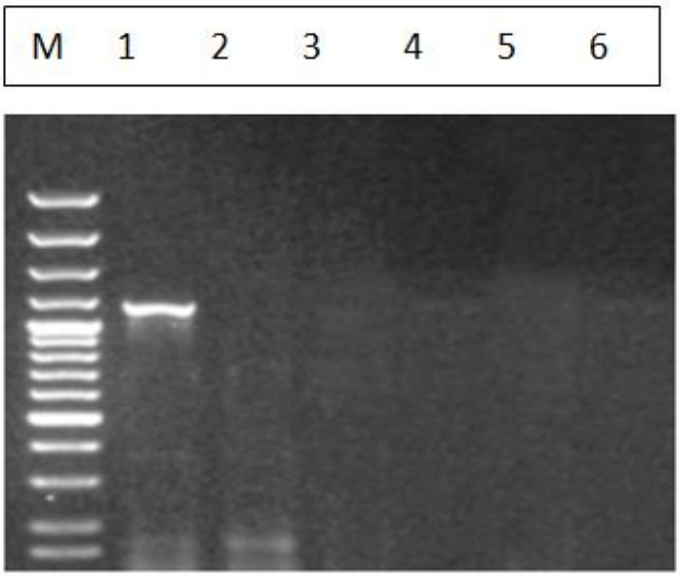


Fig.3 Iodine kit method for detection of citrus greening
a) Healthy leaf sample of sweet orange, b) Citrus greening infected leaf sample, c) sand paper, d) Iodine solution, e) yellow colour development in healthy leaf sample, f) Black colour development in citrus greening infected sample

Fig.4 Detection of citrus greening (HLB) infection in sweet orange (*Citrus sinesis*)



The developed protocol will be helpful for rapid, early, cost effective detection of citrus greening (HLB) infection among citrus species. The development of molecular detection technology resulting in more convenient, effective and specific assays has opened the door to greater use of these tests for detecting plant pathogens.

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