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Identification of Gram negative bacterium *Candidatus liberibacter asiaticus* (Clas) causing *Citrus* Greening Disease by Multicopy gene

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Abstract

A non-cultivable proteobacterium called *"Candidatus liberibacter asiaticus"* (Clas) is linked to citrus Huanglongbing (HLB, yellow shoot disease), which is currently endangering citrus production all over the world. There are five copies of the gene *nrd* B, which codes for the β-subunit of ribonucleotide reductase (RNR), a key enzyme in bacterial proliferation. Three multicopy *nrd*B gene copies were in long form (*nrd* BL, 1,059 bp), and the other two were in short form (*nrd*BS, 378 bp). *nrd*BS had no active sites and shared >99% of its identity with the 32 end of the long form of the *nrd*B gene (*nrd*BL). The *C*Las *nrd*B gene sequences in Eubacteria developed a unique monophyletic lineage. Using 25 HLB samples taken from Northern India, a *nrd*B-based primer pair, *nrd*BL2F1/R1, was created and tested using PCR to take advantage of the high copy number characteristic of the multicopy *nrd*B gene. Hence, the *nrd*B-based PCR offers sensitive and accurate *C*Las detection with a wide range of applications, particularly for the early identification of HLB. This aids in restricting the disease's spread to healthy plants and contributes to maintaining the citrus crop. The present study proposes a method for recognising pathogens in their initial stages and afterwards restricting their further spreading, assisting in the management of Citrus Greening Disease.

Key words : Citrus greening disease**,** *Candidatus liberibacter asiaticus* (6), primer, *Diaphorina citri* (Asian citrus psyllid), Huanglongbing (HLB), Multicopy *nrd*B gene.

Huanglongbing (HLB), widely known as citrus greening disease or yellow shoot disease, is a phloem-limited proteobacterium that is linked to *Candidatus* Liberibacter asiaticus (*C*Las), which is decimating citrus production globally[1,10]. Citrus is an evergreen

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crop with medicinal value; it was first cultivated in Southeast Asia and is now grown all over the world[2]. Citrus trees with HLB infection show yellow shoots, foliar blotchy mottle that may resemble zinc deficiency symptoms, vein corking that may imitate *Citrus tristeza virus* infection symptoms, poor blooming, and stunting. Chronically HLB-infected citrus trees exhibit scant foliage, severe twig or limb die-back, and eventually die in three to five years. Citrus with HLB infection has a productivity reduction of 30% - 100% and inferior fruit quality[6]. Due to the fatal citrus greening disease, citrus production has recently decreased globally[1,7,13]. Infected trees may eventually, and over a considerable length of time, start to show symptoms of defoliation and mineral deficiency. On fruit-laden trees, the most obvious indicators of rapid decline are generally observed. The most important commercial citrus species in India are the mandarin (*Citrus reticulata*), sweet orange (*Citrus sinensis),* and acid lime (*Citrus aurantifolia),* which share 41, 23, and 23 percent, respectively, of all citrus fruits produced in the country. In India during 2018–19, citrus was cultivated on 1034000 ha and production was 13200000 MT (NHB 2018–19), with productivity at an average of 12.76 tonnes per hectare, which is far lower than other major citrus producing countries like China, Brazil, and the USA (30–40 tonnes per hectare). Numerous biotic and abiotic factors are responsible for this gloomy scenario.

Detection of "*Ca*. Liberibacter asiaticus" is very challenging because of its low concentration and irregular distribution in its citrus host. The highly effective technique known as PCR has made it much easier to find phytopathogens. Plant material with a lower pathogen content is optimal for PCR. Because of its dependability, speed, and low relative cost, it does provide rapid detection and an alternative technique. Researchers have tried to develop PCR based assay for "*Ca*. liberibacter asiaticus" detection in citrus plants[4,8,9,11]. However, the recent discovery of multicopy copies (five) of the *nrdB* gene, encoding β-subunit of ribonucleotide reductase (RNR), a critical enzyme involving bacterial proliferation, was found. Three *nrdB* copies were in long form (*nrd*BL, 1,059 bp), and two were in short form (*nrd*BS, 378 bp). *nrdBS* shared >99% identity with the 32 end of *nrdBL*[14]. Therefore, the development of PCR primers based on the *nrd*B gene will detect the pathogen early from insect vectors as well as citrus plants, be used for the implementation of a bud wood certification programme, and subsequently stop the further dissemination of the pathogen. Hence, the design of PCR primers based on the *nrd*B gene will enable early disease detection from insect vectors as well as citrus plants. This primer will then be utilised to implement the bud wood certification programme, which will eventually stop the spread of the pathogen.

Ethics statement :

The research complied with protocols for plant protection and was conducted in controlled conditions so that HLB disease should not infect a normal citrus plantation.

Sample sources and DNA preparation :

From 2020 to 2022, 120 citrus leaf samples (42 showed blotchy leaves and 29 showed yellow shoots) were collected from

25 different citrus cultivars in Delhi/NCR that covered almost all citrus fields, including the Indian Agricultural Research Institute (IARI, New Delhi) at 28.6377° N, 77.1571° E.

All samples were handpicked and stored at -4ºC (Fig. 1A). Leaves from the infected plants (Fig. 1 B) were washed with running tap water and blotted dry with paper towels. The midribs of leaves were extracted through cutting with surgical knives (Fig. 1 C). Then, 100 mg of each sample were grinded in liquid nitrogen (Fig. 1 D) and polyvinylpyrolidone, and DNA was extracted using the CTAB method. The extracted DNA was dissolved in 50 µl of SDW. The quality and concentration of DNA were checked by a NanoDrop One (ND-ONE-W Thermo Scientific).

Primers design :

One set of primers was designed for the PCR to amplify the three larger and two smaller subunits of the multicopy *nrd*B gene (Accession No.: CP010804.1). The primer sets were designed manually and verified through primer BLAST (NCBI primer designing tool) with the following criteria: Tm = 55 ±2ºC and primer length = 18–22 BP. To ensure amplification efficiency, designed primers were checked through the OligoAnalyzer program, and the primer sets were selected that had the least possibility of forming a hairpin, self/cross dimer structures. One set of PCR primers was validated (*nrd*B L2F1/R1).

Validation of the specificity of designed PCR primers :

To validate the PCR primer specificity, we first BLASTed the primer against *Candidatus* Liberibacter asiaticus genome sequences. To ensure that the designed primers were specific for Las, citrus greening disease positive sample were identified and separated from collected samples of citrus. Moreover, one DNA of *C*Las and one omp gene primer were used to identify a CGB positive sample from collected DNA samples. Objective genes were confirmed by electrophoresis in 1.6% agarose gels, sequencing PCR products and BLASTing them online.

In this study, CGB positive samples were tested using the PCR primer *nrd*B L2F1/R1. PCR products were separated by electrophoresis in 1.6% agarose gels and detected after staining with ethidium bromide.

Validation of the sensitivity of PCR :

Usually, the determination of primer sensitivity was based on different templates or different concentrations of the same template. Firstly, five suspected samples were amplified by PCR primer pairs (*nrd*B L2F1/R1) and conventional PCR primer (oi2c). Then we detected the concentration of the conventional PCR positive product with a NanoDrop one, and adjusted the dilution to 100 ng/µl. The PCR mixture (25 µl) was prepared using (12.5 µl) DreamTaq™ Hot Start Green PCR Master Mix (Thermo Scientific™), and amplification was proceeded using the following parameters: 94ºC for 4 min, followed by 35 cycles at 94ºC for 45 sec, 52ºC for 45 sec ,72ºC for 60 sec , 72ºC for 10 min and 4ºC for ∞ for the PCR. All PCR mixtures (25 µl) included 1 µl of primer pair. CGB negative DNA samples were amplified as negative

Fig. 1. **(A)** Samples were handpicked from citrus fields of IARI, New Delhi, **(B)** Infected leaves were collected and used for the study, **(C)** The midribs of leaves were extracted through cutting with surgical knives, **(D)** 100 mg of each sample were grinded in liquid nitrogen and polyvinylpyrolidone for DNA extraction.

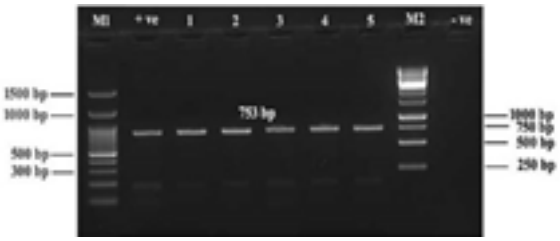

Fig. 2. The 1.6% agarose gel electrophoresis result of products of PCR of the *Candidatus* Liberibacter asiaticus (*C*Las) gene through *nrd*B gene primer.

Table-1. General information about primer sets for the multicopy *nrd*B gene of "*Candidatus liberibacter asiaticus*"

Name	Type	Primer sequence 5'→3'	Nucle-otide (bp)	Annealing tempe-rature	Gene
*nrd*B L2F1	Forward	GCATTTAACCCCACGA ACCC	753bp	59°C	*nrd*B, β-subunit of ribonucleotide reductase
*nrd*B L2R1	Reverse	ATACGGATGGGCATGG GAAA			

controls, and one CGB positive sample already identified was amplified as a positive control. PCR products were separated by electrophoresis in 1.6% agarose gels and visualized after staining with ethidium bromide.

Identification of multiple-copy regions in the A4 genome :

PCR primer *nrd*BL2F1/R1 produced the target product without primer dimers. The amplification product 753 bp is shown in Fig. 2.

Specificity of the nrdBL2F1/R1 primer set:

Primer set *nrd*BL2F1/R1 was designed based on the 753 bp of the *nrd*B gene in the *C*Las genome. A BLASTn search using primer sequences in NCBI that contained *C*Las genome sequences returned hits strictly to the *nrd*B gene of *C*Las. PCR of DNA samples extracted from five CGB-positive citrus leaves shows amplification around 753 bp in gel electrophoresis against a 1Kb and 100 bp DNA ladder as a marker, and one *C*Las-free plant leaf reared in our laboratory showed no amplification with primer set *nrd*BL2F1/R1 by PCR (Table-1).

The insight provided herein relates to primers for molecular diagnosis of the pathogenetic agents that cause citrus plant HLB disease. The method used in the present research to identify the specific *nrd*B gene in *C*Las is based on PCR. Also, the application offers primer sequences for use in quick and accurate PCR detection.

Despite extensive management efforts made over the years all around the world, the major citrus crop is still under threat from the complicated, challenging, and devastating disease known as citrus greening[5,13]. Citrus trees with HLB infection show sparse foliage, severe twig or limb die-back, and final mortality after three to five years. When citrus is infected with HLB, productivity is reduced by 30% to 100%, and the fruit quality is decreased[6,13]. During the past ten years, scientists, producers, and businesspeople have worked together to study citrus greening disease.

Multiple genomic loci have been used as HLB detection molecular markers. For

instance, the outer membrane protein (OMP) gene, the bacteriophage-type DNA polymerase area, and the 16S rRNA gene are frequently used in the molecular detection of HLB[15]. It was reported that molecular detection techniques were sensitive to false negative results due to the HLB bacteria's uneven distribution among citrus and even among cells within distinct tissues, resulting in samples that were both positive and negative from the same citrus tree. Moreover, the same HLB bacterium species were significantly different depending on the geographic region but did not differ significantly based on the citrus cultivar[3]. Subsequently, it is essential to choose a suitable assay technique and genetic locus to detect the suspicious HLB samples coming from a particular geographical region.

Recently, a group of researchers developed *C*Las detection assay using simple alkaline heat DNA lysis and loop-mediated isothermal amplification coupled hydroxy-naphthol blue (AL-LAMP-HNB) method[12]. Pathogen detection techniques should ideally be sensitive, precise, timely, and cost-effective, as well as sensitive and focused. Disease surveys, epidemiological studies, plant quarantine, seed certification, and breeding programmes may all quickly and precisely detect citrus pathogens when diagnostic techniques are accessible. The identification of phytopathogens has become significantly simpler thanks to the incredibly effective PCR method. PCR works best with plant material that has fewer pathogens. It does offer faster detection and an alternative technique because of its dependability, speed, and relatively low cost.

Therefore, the creation of PCR primers based on the multicopy gene *nrd*B facilitates the early and even low-concentration detection of "Ca. L. asiaticus." The primers created for the current study are a very helpful addition to the methods already used for *C*Las detection. This will aid in limiting the disease's spread to unaffected plants and help to preserve the citrus crop. The current study offers ways to identify pathogens in the early stages and then prevent their further spread, aiding in the treatment of Citrus Greening Disease.

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