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IMPACT OF IMPROVED DNA EXTRACTION METHOD FROM CITRUS LEAVES MIDRIB AND PCR FOR THE DETECTION OF CITRUS GREENING (*CANDIDATUS LIBERIBACTER*)

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A B S T R A C T

Citrus greening/ yellow shoot disease/ HLB, caused by *Candidatus liberibacter* asiaticus, showing mosaic/ mottling pattern on leaves, stunting of plants, de-shape, pre-mature fruit drop and yellowing of reticulate venation as characteristic symptoms, is the most concerned disease prevailing in citrus groves of Punjab, Pakistan. For the detection of pathogen and downstream studies, a high-quality DNA is required. In citrus, due to the variety of species, different age groups in plants, thick waxy cuticle of leaves, high production of phenolics, polysaccharides and other compounds, it is very difficult to extract good quality DNA from leaves and especially from main midrib where the fastidious bacterium is residing. CTAB and SDS are two devised methods for the extraction of total genomic DNA from citrus leaves while the current research suggests some modified protocols for the detection of DNA from infected/ healthy samples. 100mg leaf midrib sample was crushed in liquid nitrogen, homogenized in 500µl CTAB, incubated at 60°C in water bath for 45 minutes, centrifuged at 12000rpm for 5 minutes, supernatant was transferred to new tube and 5µl RNase-A was added, incubated at 37°C for 20 minutes. Equal volume of chloroform/ isoamyl alcohol (24:1) was added, vortexed for 5 minutes, centrifuged for 2 minutes at 12000rpm for phase separation and upper phase was transferred to new tube where 500µl of chilling isopropanol was added, kept at -20°C for 15 minutes, centrifuged at 12000rpm, pallet was washed twice with 70% ethanol. CTAB method was modified by increasing the incubation time i.e., 45 minutes for cell lysis in water bath for determination the variation in quality of DNA and the amount of beta mercaptethanol was also doubled from the normal one. Modified protocols have been proved excellent for the extortion of total genomic DNA from citrus. In SDS, addition of TE buffer, RNAase, Isoamylalcohol: Chloroform (24:1) along with 20% SDS and 2M Sodium Acetate gave high quality DNA from citrus leaves. Hence, DNA was extracted by four different ways but the modified CTAB and SDS methods gave improved quality DNA as confirmed by its quantification through nanophotometer. The DNA was quantified by nanophotometer and presence was observed on gel documentation apparatus. Hence, DNA was found suitable for PCR and RFLP analysis and long-term storage on -80 ^{0}C.

Keywords: Citrus greening, CTAB and SDS Extraction Protocol, Fastidious bacteria, PCR,

INTRODUCTION

Citrus huanglongbing (HLB), also known as citrus greening disease is major devastating disease (Deng *et al.,* 2016) was detected for very first time in United States (Eveland & Brown, 2019) and have greatest biotic threat to citrus production globally. The disease has long been endemic form only in Arabian Peninsula, Subcontinent, Asia, Mascarene islands and Africa (Chung & Brlansky, 2006). This disease is associated with gram-negative bacteria which belongs to subdivision of proteobacteria (Garnier & Bové, 1983) is constantly associated with the disease but this bacteria cannot be isolated on artificial

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media, so the name of the organism has provisional ('Candidatus') status in nomenclature and Koch's postulates for this pathogen and disease can never be fulfilled. The disease is established by three forms of HLB bacteria: 'Ca. Liberi-bacter asiaticus', 'Ca. L. africanus', and 'Ca. L. americanus'. The disease can be dispersing rapidly by activity of vector psyllids *Diaphorina citri* and by the propagation of infected plant materials. The symptoms of this disease include an unparalleled mottling of the leaves, frequently the midribs of the leaves to yellow from green. Canopy of plant decline and dieback, lead to death of the whole plant.

Table 1. Various pathovars of *C. liberibacter*

Sr. No.	Type	Insect Vector	Tolerance of heat
1.	Asian	*Diaphorina citri* (psyllid)*	Tolerant (Yes)
2.	African	*Trioza erytreaae* (psyllid)*	Sensitive (No)
3.	American	*Diaphorina citri* (psyllid)	Tolerant (Yes)

Table 2. Various names of the (Huanglongbing) HLB called upon throughout the world.

Sr. No.	Continent	Country	Local Name	Reference
1.	Asia	China	Haunglongbing/Yellow shoot/ branch/ dragon disease	(Gottwald *et al.,* 1989)
2.	Asia (South East)	Philippines	Mottle leaf	(Garnier & Bové, 1993)
3.	Asia	India	Die-back	(Fraser & Singh, 1968)
4.	East Asia	Taiwan	Likubin	(Garnier & Bové, 1993)
5.	Asia (South East)	Indonesia	Vein phloem degeneration	(Bové *et al.,* 2000)
6.	Africa	South Africa	Greening	(Planet *et al.,* 1995)
7.	Asia	Pakistan	Greening	(Yaqub *et al.,* 2017)
8.	North America	USA (Florida)	HLB	(Bové, 2006)

*Out of the cited facts HLB has become the official name of this disease because it was first used in the literature and in the history.

The tree produces yellow shoots when it is completely infected. In Chinese, HLB meant "yellow shoot", so it is characteristic symptom of HLB disease. Infected fruits become lopsided, off flavored, and often contain aborted seed. Capacity of plant to produce fruit is reduced, fruit remain small and usually drop prematurely. The tree dies and declines within 2-3 years.

The bacteria penetrate through phloem vessels and attack the vascular bundles, cause the blockage of veins, and radically inhibit the nutrients and water transportation. During 1919 in China, this disease was already described. HLB was affecting and causing serious damage to citrus yield in many citrus groves in India, South Africa and South East Asia in early 20th century. It was reported from Brazil in 2004 (Coletta-Filho *et al.,* 2004, do Carmo Teixeira *et al.,* 2005) and it was appeared in different localities in Florida in 2005 (Spreen *et al.,* 2014). The HLB bacteria detected from Florida was *L. asiaticus* and from Brazil *L. americanus* was found associated with HLB. The occurrence of greening disease in Florida is supposed to have extremely affected and reduced the citrus yield. After realizing the serious damaging effect of greening disease, the citrus growers of Florida had sold their citrus groves to real estate developers. The per acre yield was declined and growing area of citrus was reduced. Citrus greening caused great economic loss of billions of dollars in Florida.

Spreen *et al*., (2014) predicted that the presence of HLB would have a detrimental effect on planting new citrus trees in the future. In summary, there is a strong reason to describe HLB as the most significant calamity facing Florida's citrus industry today. Three species of Liberibacter are differentiated in their temperature response. *L. Asiaticus* tolerate heat and can live at temperatures above 30°C, whereas *L. africanus* is thermo-labile and likes temperatures between 22 and 25°C. It is difficult to detect pathogen in the contaminated plant or vector (Manjunath *et al.,* 2008).

As like Asiatic strain, African strain of HLB does not need high temperature for symptoms expression. On artificial culture media, HLB cannot be cultured. Two psyllid insects are responsible for spread of this disease and it can also be spread in field by vegetative propagation (Porebski *et al.,* 1997). Asiatic strain is mostly vectored

by *Diaphorina citri* while African strain in transmitted by *Trioza erytreae,* but both vectors are able to spread either strain of HLB. Eradicating the diseased plants after disease is detected, use of healthy planting material, biological control and use of chemical insecticides to control vector are the best ways to prevent the disease. In molecular research, isolating high-quality DNA is important for detection of certain pathogens (Fang *et al.,* 1992).

Polysaccharide contamination is a major problem when extracting DNA from plant tissues, and melicera colloidal hyalosome infect DNA samples that are insoluble in Tris-EDTA buffer or DEPC treated water, resulting in a reduction in enzyme activity (Schlink and Reski, 2002). Several DNA extraction methods have been published for the removal of polysaccharides (Möller *et al.,* 1992, De La Cruz *et al.,* 1997, Porebski et al., 1997, Schlink and Reski, 2002). On the other hand, in certain woody fruit crops with high levels of polysaccharide, such as Citrus spp., protocols could only be applied to robust tissue, and the DNA isolated was not of adequate consistency to be used in PCR and RFLP tests (Porebski *et al.,* 1997). This method is ideal for separating genomic DNA from fruit crops with high polysaccharide content, such as citrus. The aim of the current study was to compare different methods of DNA extraction in order to obtain high-quality DNA for PCR amplification and long-term storage. In light of the above, the current study aimed to compare various DNA extraction protocols for high-quality genomic DNA from the midribs of citrus leaves.

MATERIALS AND METHODS

Sampling of different citrus cultivars: The plant materials were collected from different citrus cultivars viz., including orange, mandarin, tangerine, grapefruit, pummelo, kumquat, trifoliate orange, showing the characteristic symptoms of the citrus haunglongbing and tagged in the course of surveys. Plant samples of leaves from above ground parts roots and fruit were also collected in plastic bags, kept on ice and brought to the Laboratory for the further processing.

Genomic DNA extraction of citrus through modified CTAB method : 100 mg of leaf midrib was crushed in liquid nitrogen and homogenized in 500 µl of CTAB buffer, incubated at 60°C in water bath for 45 minutes. After incubation, the mixture was centrifuged at 12000 rpm for 5 minutes. The upper supernatant layer was transferred to new tube and 5 µl RNase A was added, incubated at 37°C for 20 minutes. An equal volume of chloroform/ isoamyl alcohol (24:1) was added, vortexed for 5 minutes. Centrifuged for 2 minutes at 12000 rpm for separation of phases. The upper phase was transferred to new tube and 500 µl of chilling isopropanol was added, kept at -20°C for 15 minutes. Centrifuged at 12000 rpm. Supernatant was discarded without disturbing the DNA pallet. Pallet was washed twice with 70% ethanol for removal of salts and debris. Discard ethanol and air dried overnight. 50 µl of DEPC treated water was added after 12 hours. CTAB method was modified by increasing the incubation time i.e., 45 minutes for cell lysis in water bath for determination the variation in quality of DNA. Similarly, the amount of beta mercaptethanol was also doubled from the normal one.

Genomic DNA extraction of citrus through SDS method: Leaves showing citrus greening disease were crushed in liquid nitrogen and grinded in pestle and mortar, transferred to Eppendorf tubes. Samples were centrifuged at 10,000 g for 5 minutes. Supernatant was discarded and 450µL TE buffer, 4 µL RNase and 30 µL of 20% SDS were added in each tube. The tubes were subjected to vortex and incubated at 65°C for 30 minutes. In each tube, 500 µL Isoamylalcohol: Chloroform (24:1) was added and mixed gently. Centrifuged and upper layer was taken into new tubes and then 50 µL of 2M Potassium Acetate was added. Centrifuged again and upper aqueous layer was drained. 500 µL of chilled 2-Propanol was added and again supernatant was discarded after centrifuge at 12000 rpm. Tubes were washed twice without disturbing the DNA pallet. Dried overnight and 100 µL of DEPC treated water was added in each tube and subjected to Nanophotometer for determination the quality and purity of extracted DNA.

Genomic DNA extraction of citrus through modified SDS method: Mid rub tissues of citrus leaves were cut and grind by liquid nitrogen. Grinded tissue was transferred in Eppendorf tube. 800 µl of extraction buffer (Tris HCL, EDTA and NaCl) containing 2% v/v beta mercaptethanol and 2% w/v PVP, was added in each tube and mixed gently. The tubes were incubated at 65°C in water bath for 20 minutes and were gently blending for every 5 ~ 10 min. Tubes were treated with 2 µl of RNAse A and Protease K. After cooling 225 µl

Potassium acetate was added and kept on ice for 1 hour. Centrifuged at room temperature with 12000 RPM for 10 min, supernatant was saved. Chloroform and isoamyl alcohol (24:1) was added to the supernatant and mixed. Centrifuged at 12000 RPM for 10 min and supernatant was saved again Resuspended the nuclei pellet with equal volume of frozen isopropanol. Tubes were washed twice with cold 75% ethanol. Air dried overnight and 100 µl TE buffer was added in each tube.

Molequle-on kit method for the extraction of genomic DNA: Fresh leaf samples were crushed and grind in pestle and mortar, 200µl of TE buffer was added. 400 µl of digestion solution was added and mixed well, 3 µl of Proteinase K solution was added in each tube and incubated on water bath at *55*°C for 5 minutes. 260 *µl* of pure ethanol (100%) was added and mixed gently. The homogenous mixture was shifted to molequle-on spin column placed in 2 ml collection tube and centrifuged at 8000 rpm for 2 minutes. The upper layer was drained and 500 µl of wash solution was added, again centrifuged and this step was repeated. Again, discard the supernatant and spin at 8000 rpm to remove the debris of wash solution. The molequle-on column tube was placed on clean Eppendorf tube and *30* µl of elution buffer was added to middle part of membrane in column. Tubes were incubated at room temperature 2-3 minutes to obtain high quality DNA. To Elute genomic DNA from column, tubes were subjected to centrifuge at 8000 rpm for 5 minutes. Purified DNA was kept at -20.

PCR detection of HLB: The PCR amplification was done by My Cycler (Bio Rad) by using four set of primers i.e. A2 F& J5 R, Oi1 F & Oi2 R, Laas & Lass, S3 & S4. PCR amplification was performed by using DNA which was extracted by four methods. Amplification was carried out in 25 µl reaction by using conventional method and Master Mix Method.

Table 3. List of primers used for the detection of *C. Liberibacter* after extraction of DNA through various methods to optimize the best protocol for extraction.

Primer	Target gene	Sequence	Amplicon Size	Reference
Las606 & Lss	Las 16S rDNA	5′-GGA GAG GTG AGT GGA ATT CCG A-3′ 5′-ACC CAA CAT CTA GGT AAA AAC C-3′	500 bp	(Fujikawa & Iwanami, 2012)
Oi1 & Oi2	16S rDNA	5′-GCGCGTATGCAAGAGCGGCA-3′ 5′-GCCTCGCGACTTCGCAACCCAT-3′	1160 bp	(Jagoueix *et al.,* 1994)
A2 & J5	16S rDNA	5′-TATAAAGGTTGACCTTTCGAGTTT-3′ 5′-ACAAAAGCAGAAATAGCA CGAACAA-3′	703 bp	(Jagoueix *et al.,* 1996)
S3 & S4	16S rDNA	GTAAACGATGAGTGCTAGCTGT-3′	359 bp	(Hong *et al.,* 2019)

For conventional method 10X PCR buffer was taken in 5 µl, DNTPs 0.5 µl, $MgCl_2$ 1.7 µl, Taq DNA polymerase 0.3 µl, Primer F and Primer R were taken in 2 µl, dH_2O 14.8 and DNA template was 1 µl. For Master mix reaction, master mix was taken in 12.5 µl, dH_2O 9.5 µl, Primer F and Primer R were used in 2 µl and DNA was used in same quantity as taken in conventional method. All Samples were amplified for 30 cycles using My cycler. Denaturation took 30 seconds at 94°C, annealing took 30 seconds at 55-60°C, extension took 60 seconds at 72°C, and final extension took 10 minutes at 72°C. Amplifications were observed on a 1% agarose gel containing ethidium bromide and observed under ultraviolet UV lights.

RESULTS

Genomic DNA extraction from citrus leaves: Various factors were considered in each of the methods for extracting DNA from citrus leaf midrib tissues in order to assess the consistency of DNA on an agarose gel. We looked at DNA concentration (ng L-1), performance (ng mg-1 DNA from plant tissues), purity (260/280 nm absorbance ratio), the presence or absence of protein contaminants, and the integrity of the relationship to total DNA non-degradation. Following the evaluation of parameters, it was discovered that the DNA obtained from the SDS and modified CTAB methods, where the change was rendered by raising the incubation time, i.e., 45 minutes for cell lysis in a water bath for determining the variance in DNA content, was of higher quality. The quality of DNA was greatly improved as a

result of this small improvement in the process. (Table 4, Fig: 1, 2, 3, 4).

Table 4. Quantification of DNA with various extraction methods through nanophotometer

Varieties	Methods	Quantity	Quality		Detection
		*Conc.	A260/280	A260/230	
Kinnow	Modified CTAB	61	2.31	2.36	
	SDS	42	1.95	1.98	+
	Modified SDS	54	2.19	2.24	
	KIT	49	2.69	3.25	+
Lemon	Modified CTAB	49	1.93	2.5	
	SDS	39	1.9	2.01	+
	Modified SDS	46	2.23	2.48	
	KIT	74	2.04	2.77	+
Grapefruit	Modified CTAB	67	1.97	2.65	_
	SDS	41	1.52	1.97	_
	Modified SDS	56	1.99	2.36	_
	KIT	77	2.33	3.75	_
Sweet Orange	Modified CTAB	59	1.99	2.3	+
	SDS	34	1.53	1.88	+
	Modified SDS	44	1.96	2.26	_
	KIT	81	2.73	3.55	+
Lime	Modified CTAB	50	2.32	2.75	_
	SDS	38	1.84	1.98	+
	Modified SDS	46	2.19	3.13	+
	KIT	69	1.99	2.39	+

All the values and readings were taken at the factor 10 of nano photometer, * Conc. = Concentration

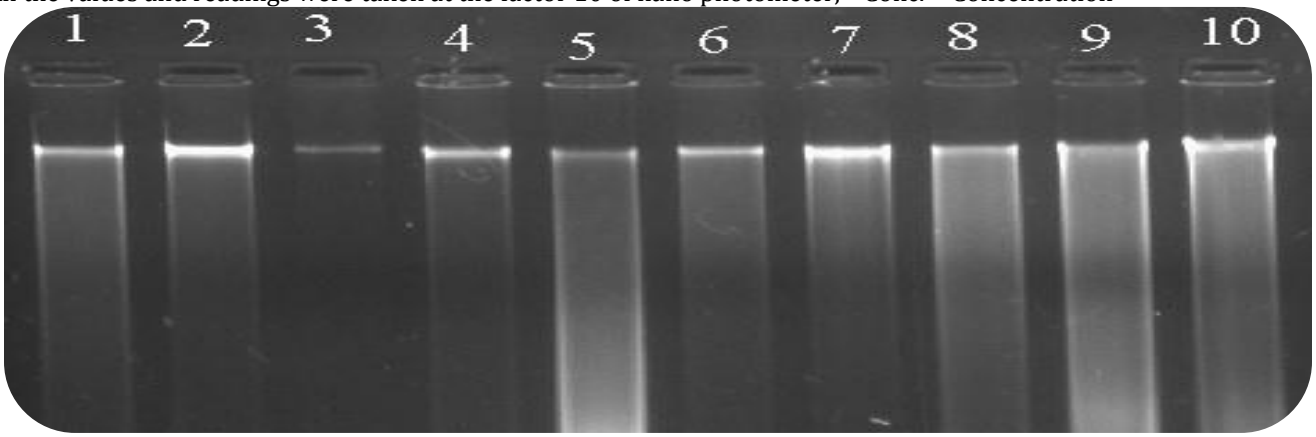

Figure 1. Extraction of DNA by CTAB method

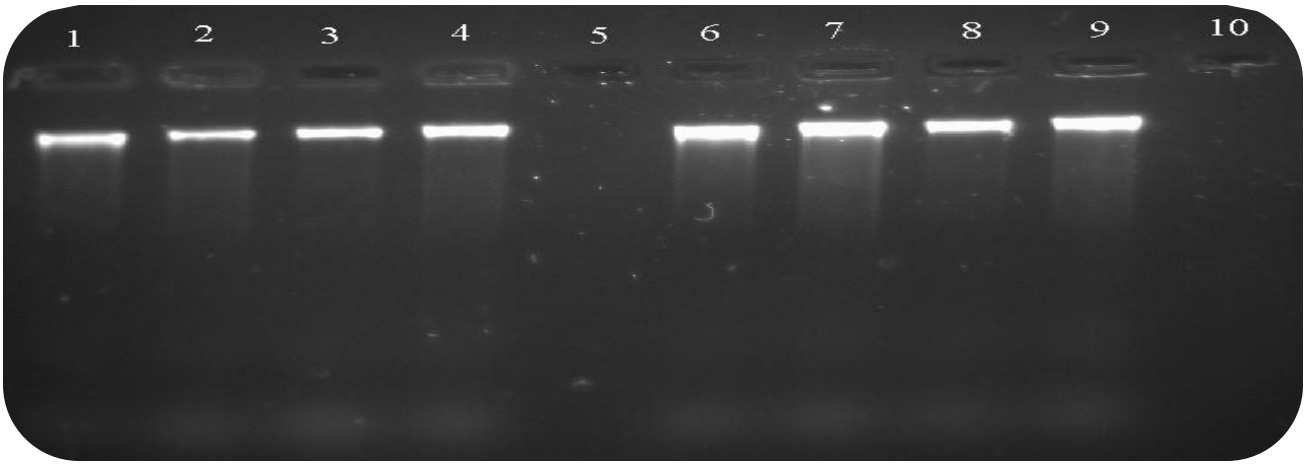

Figure 2. Extraction of DNA by Modified CTAB method

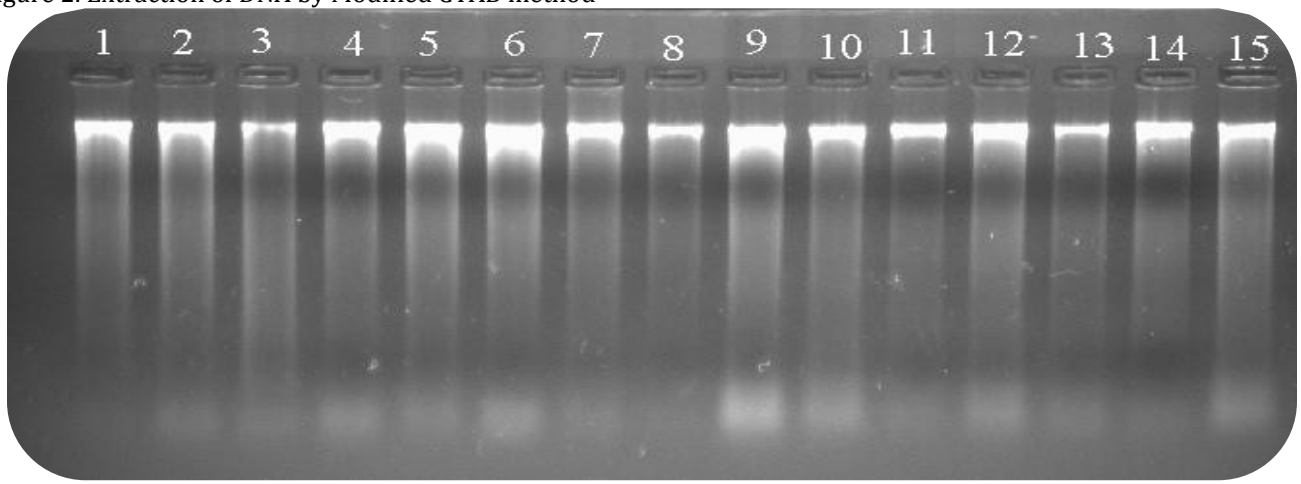

Figure 3. Extraction of DNA by SDS method

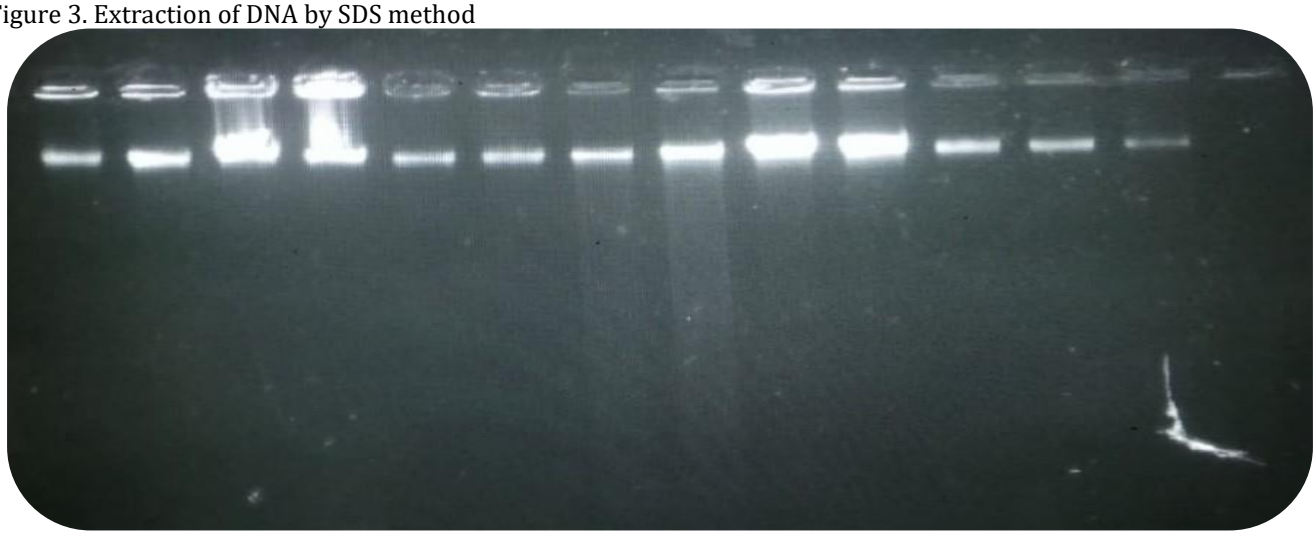

Figure 4. Extraction of DNA by Kit Method

Detection of genomic region of *Candidatus liberibacter* with different primers: PCR was used to detect 'Ca. L. asiaticus' from extracted DNA samples, and the sequences of established 16s rDNA regions were compared. The results of PCR detection of 'Ca. L. asiaticus' revealed that LAS primers amplified the 16s rDNA region with an estimated product size of 500 bp, while OI1/OI2 primers amplified a 1160 bp band, and HLB specific primers A2 and J5 produced 703 bp bands. (Fig. 5, 6, 7, 8).

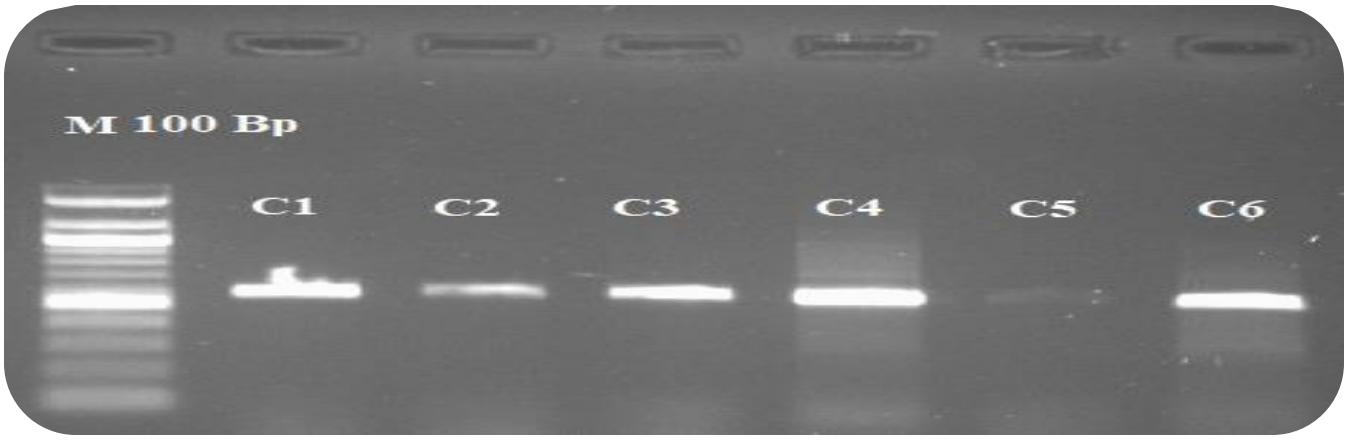

Figure 5. (1.5%) TBE agarose gel shows band of 500bp using specific primer of HLB i.e., Lass from symptomatic leaves (Lane 1-6) Lane M: 100bp Ladder (Invitrogen)

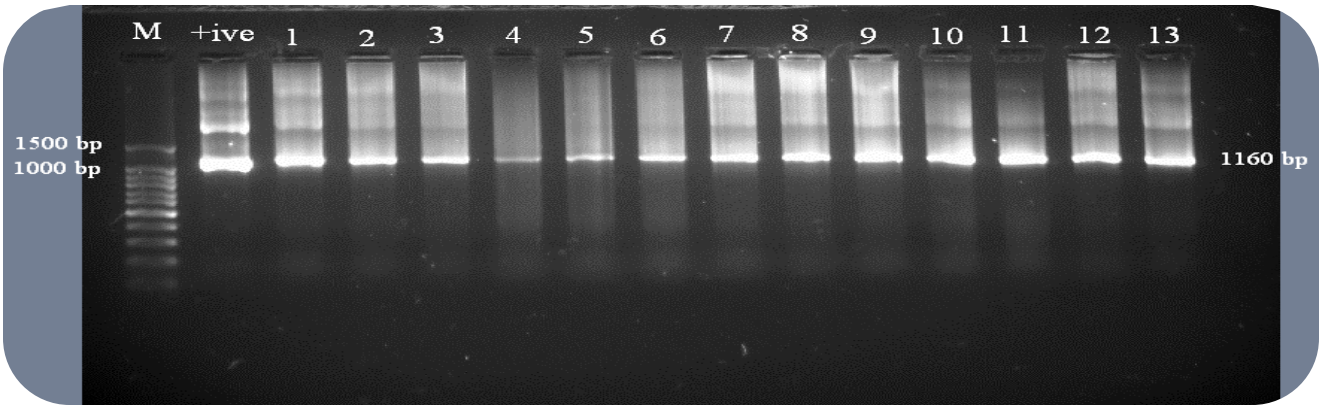

Figure 6. (1.5%) TBE agarose gel shows band of 1160bp using specific primer of HLB i.e. OI1 & OI2 from symptomatic leaves (Lane 1-13) Lane M: 100bp Ladder (Invitrogen) for molecular weight

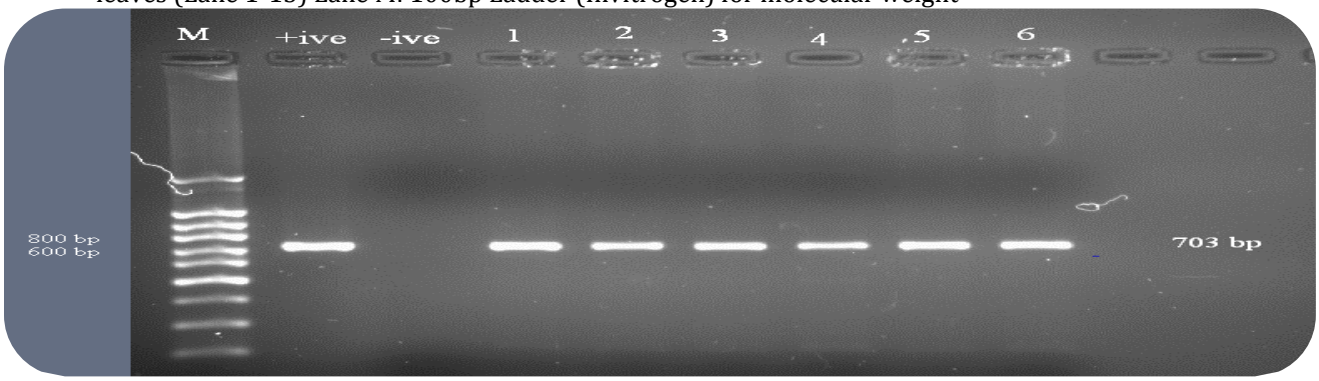

Figure 7. (1.5%) TBE agarose gel shows band of 703bp using specific primer of HLB i.e. A2 & J5 from symptomatic leaves (Lane 1-6) Lane M: 100bp Ladder (Invitrogen)

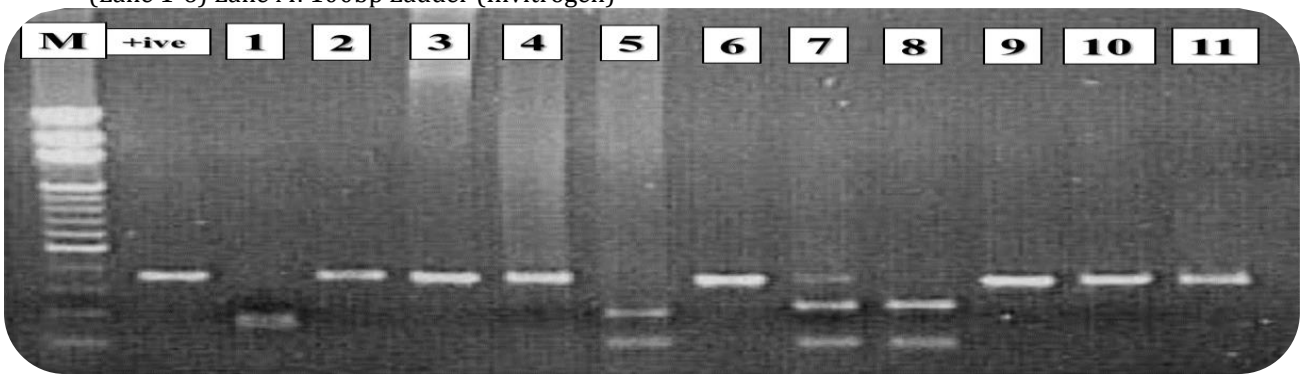

Figure 8. (1.5%) TBE agarose gel shows band of 359bp using specific primer of HLB i.e., S3 and S4 from symptomatic leaves (Lane 1-11) Lane M: 100bp Ladder (Invitrogen)

DISCUSSION

Citrus greening diseases is of high importance for citrus industry all over the world and especially for Pakistan where this fruit ranked 2nd after banana due to its dietary value and taste. The fastidious bacteria are responsible for the damages in this disease causing the destruction of vascular bundles in citrus plants. In the current research various DNA extraction protocols were compared to determine the best one in terms of obtaining the quality DNA. Because it is almost very difficult to get a highly stabile DNA by using the simple CTAB or SDS method. The subtropical or tropical and citrus spp. are perennial woody plants and therefore even their young tissues contain more Polysaccharide content than other field crops. The old DNA extraction procedures i.e. CTAB (Saghai-Maroof *et al.,* 1984) and SDS (Dellaporta *et al.,* 1983), were found inefficient to remove polysaccharides. Rather a minor change or amendment in the fundamental protocol gave a high-quality genomic DNA from the citrus leaves' midribs. In our research modified SDS and CTAB method gave high quality product for the amplification of the bacterial genome as it reduces the phenols and

polysaccharides or completely remove the polysaccharides in the product. Although the changes made were not so drastic but simple variation in the protocol leads us to the quality DNA product (Fang *et al.,* 1992, Möller *et al.,* 1992, Porebski *et al.,* 1997). CTAB method was modified by increasing the incubation time i.e., 45 minutes for cell lysis in water bath and by increasing the double amount of beta mercaptethanol for determination the variation in quality of DNA. With this treatment, polysaccharides concentrated in the interphase while the DNA still dissolved in the bottom aqueous phase. By discarding gel like interphase, we can get rid of polysaccharides.

We were able to amplify 16s rDNA region of *Candidatus liberobacter* by utilizing 4 set of primers. The modifications adopted in CTAB method found a quick and efficient method for DNA extraction from citrus midrib tissues. So, this method can perform well for future molecular study involving large numbers of different plant samples. As a result of these modifications, we obtained pure and high-quality DNA suitable for further molecular analysis. Moreover, modified method reflects the competence of the protocol and proves its suitability for further analysis like PCR amplifications. In the current study, we optimized a modified SDS-based DNA extraction method and results was then compared to those extracted by old SDS method and DNA extraction kit. In this regard, we revealed that the Modified SDS method gave higher DNA yield and cell lysis more effectively, lower DNA shearing, and higher diversity scores than other two methods. The modified CTAB method was compared with extraction kit such as *molequle on* kit for genomic DNA from plants for PCR-based detection of HLB. Our comparative tests revealed that DNA which were extracted by modified methods contain low quantity of phenolic compounds. The modified extraction methods make DNA able for long term storage.

CONCLUSION

A good quality DNA can be obtained from citrus leaf midribs by making some amendments in normal protocols. Increase in the lysis time in water bath and by doubling the amount of beta mercaptethanol removes the high density of polysaccharides in the samples.

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Muhammad Shahid	:	Analyzed data
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Muhammad Umair	:	Helped in manuscript writeup