One economical extraction method to detect all graft transmissible pathogens of citrus

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ABSTRACT

A simple and applicable extraction method for the isolation of high quality nucleic acid (DNA and RNA) to detect all graft transmissible pathogens of citrus including prokaryotic pathogens, DNA and RNA viruses and viroid provides a new approach to virus and virus like detection and identification. The method provides a pure preparation of un-degraded RNA and DNA in high yield and can be completed within 2 h. It is particularly useful for processing large number of samples and for isolation of RNA and DNA from small quantities of tissues. The method does not require expensive and environmentally hazardous reagents and equipment. It can be performed even in low technology laboratories. The amount of tissue required by this method is ~50–100 mg. The quantity and the quality of the nucleic acid extracted by this method is high enough to perform hundreds of PCR-based reactions and also to be used in other DNA manipulation techniques such as restriction digestion, Southern blotting and cloning.

INTRODUCTION

The extraction of biomolecules, DNA and RNA is most crucial method used in molecular biology Wink (2006). Plant species often produce secondary metabolites, i.e. alkaloids, flavonoids, phenolic compounds and gummy polysaccharides, which interfere with successful nucleic acid isolation and follow-up reactions such as DNA digestion, amplification and cloning. Isolation and purification of nucleic acid from plant species are faced with problems that include degradation of nucleic acid due to endo-nucleases, co-isolation of highly viscous polysaccharides and, co-isolation of inhibitor compounds like polyphenols and other secondary metabolites which directly or indirectly interfere with enzymatic reactions Khanuja *et al* (1999) and Loomis (1974). Generally,

successful nucleic acid purification required four important steps: effective disruption of cells or tissue; denaturation of nucleoprotein complexes; inactivation of nucleases, for example, RNase for RNA extraction and DNase for DNA extraction; away from contamination Doyle (1996).Citrus facing the destruction of 10 to 12 diseases and may be more caused by variable agents like virus, viroid, bacteria and Prokaryotic …act. Definition the infection agents is the first important step to resist diseases Roistacher and Moreno (1991). Molecular biology tools one of that knowledge's which developed so fast during the last few years, and become dependable to detect and diagnose the diseases. Egypt and most of developing countries facing some difficult in using this tools for two reasons, knowledge and cost, which still a big problem especially with the economic difficulties. One of our goals was how can reduce the cost, and this was the basic strategy in this work depending on two axes, the first - developing a protocol using cheaper chemical than the commercial kits. The second – the applied protocol should be usable with most of the graft-transmissible diseases agents.

MATERIALS AND METHODS

Source of samples: Samples were kindly provided and tested in national clonal germplasm repository for citrus and dates – ARS-USDA. To validate the method and PCR protocol with Egyptian isolates, another set of Egyptian samples provided and tested in diagnosis and monitoring for citrus disease project – PpathRI–ARC-EG. Macrophylla was used as healthy samples, Navel orange; Madame Venues and Citron were used as infected samples.

Nucleic acid extraction:

About 0.1 gm mid rib scrapings tissue placed in 2 ml screw cap microfuge tube. One steel sling shot pellet was added and mixed with 1.25 ml of extraction buffer (100 mM tris base; 50 mM EDTA, pH8.0; 500mM NaCl; 0.7µl/ml β-mercaptoethanol immediately prior to use), Homogenized with Bead-Beater for 2.5 min twice (ground in liquid nitrogen could be used). 100 µl of 20 % SDS was added and incubated for 30 min at 65ºC. The solution was then centrifuged at 10 000 xg for 1 min. The aqueous phase was transferred (about 850µl) to fresh tube(from this step all the treatment done under cooling), and 500 µl of 5M potassium acetate was added with vigorously shaking and incubated on ice for 20 min,

then centrifuged at 15 000 xg, 4ºC for 20 min. The aqueous phase was then transferred (about 900µl) to new tube and the nucleic acid was precipitated with 2/3 volume (about 600µl) of ice cold isopropanol and kept at -20ºC for 20min. The nucleic acid was then centrifuged for 30 min at 4ºC, then the supernatant were removed and tubs were kept on ice, the pellet washed twice with 1 ml cold 70% ethanol and centrifuged for 5 min each at 4ºC. The pellet was air dried for 15 min and dissolved in 100µl Tris-EDTA buffer or nuclease free water.

Viral cDNA synthesis and PCR amplification for RNA samples:

Two-step PCR begins with the reverse transcription of total RNA. First –strand cDNA synthesis was performed using recombinant Moloney murine leukemia virus (MMLV) reverse transcriptase (SuperScript III; Invitrogen Corporation) and total RNA prepared from mid rib tissue. In a 0.2ml thin walled PCR tube, 5 µl of RNA extract was mixed with an equal volume of RT master mix, composed of 250 mM Tris-HCL, pH 8.3, 375 mM KCL, 15 mM MgCl2, 0.20 mg of BSA per ml, 20 mM dithiothreitol, 1 mM deoxy nucleoside tri-phosphates (dNTPs), 1µM complementary primer (see Table 1.), and 2u/µl of SuperScript III RT, and incubate for 30 min at 50ºC. Following RT, 50 µl of PCR master mix containing 2µl of cDNA, 5µl of 10x PCR buffer (200 mM Tris-HCL, pH 8.4, 500mM KCL),1.5µl of 50 mM MgCl2, 1 µl of 10 mM (dNTPs), 1 µl of 10µM of each primer (cachexia viroid primer kindly designed and provided by Manjunath Keremane ARS-USDA) and 2u/µl of Taq DNA polymerase (Promega, Corporation). Thermo-cycling was carried out as follows: 94ºC for 2 min, then 35 cycles of 94ºC for 1 min, 58ºC for cachexia (viroid) and 63ºC for CTV (Citrus tristeza Virus) for 30 sec and 72ºC 30 sec. A final incubation 72ºC for 7 min. The samples were then chilled to 4ºC.

DNA amplification:

PCR reactions were conducted with 3.0 µl of DNA templatein 25 µl of reaction mixture. Which Included 1xGoTaq buffer, 0.5µl of each 10mM dNTPs (Promega), 10µM of each primer pair (XDT) the primer tested and expected size listed in (Table 1), and 0.2µl of GoTaq (Promega). Reaction were performed on PTC-200 Thermo cycler (MJ Research) as follow: 3 min of denaturation at 95ºC; followed by 40 cycles of 95ºC for 30 s, 56ºC for 30 s, and 72ºC for 90 s; followed by 7 min of elongation at 72ºC.

Table1. Primers used to amplify nucleic acid extracted from citrus plant tissue.

Primer	Nucleic Acid	Sequences	bp	Authors
Spiralin f	DNA	GTCGGAACAACATCAGTGGT	657	Foissac*et al.*(1996)
Spiralin r		TGCTTTTGGTGGTGCTAATG		
P89f	DNA	ATTGACTCAACAAACGGGATAA	707	Berho*et al.* (2006)
P89r		CGGCGTTTGTTAATTTTTGGTA		
P58-6f	DNA	GCGGACAAATTAAGTAATAAAAGAGC	450	Comer *et al.*(2007)
P58-4r		GCACAGCATTTGCCAACTACA		
MDHf	DNA	GCTCCTGTGGAAGAGACCC	995	Ramadugu *et al.* (2013)
MDHr		GCTCCAGAGATGACCAAAC		
CTV-65f	RNA	ATGACGACGCCACGGGTATAACGTACACTC	353	Lair *et al.* (1994)
CTV-64r		TGACATTAGTAACTACGACATCATCAGCCC		
Cachexia-f	RNA	CTTGAGCCCCTCCTGGGGAATTC	300	Personal communications
Cachexia-r		CTCTGCCGCGGATCCTCTCTTG		

Electrophoretic analysis of PCR products: Aliquots (7 µl) of PCR amplified DNA were analyzed by electrophoresis through 1% agarose gels for about 1 h in 1x TPE buffer(90 mMTris-phosphate, pH 8.2, 2mM EDTA) at 120 V. Separated fragments were visualized using Gel Documentation System following staining with ethedium bromide (1µg/ml). The size of amplified DNA products were determined using DNA Trackit™ 100bp ladder; Life Technologies.

RESULTS

A simple protocol for the extraction of nucleic acid from different organisms is of high scientific value for a wide range of applications. In the present study, we developed a universal method suited to nucleic acid extraction. We used this protocol to extract DNA from citrus plants to detect stubborn disease caused by *Spiro Plasma Citri* using different pair of primers (Figure 1),and to identify the malate dehydrogenase gene in citrus genome (Figure 2). We use also the same method to extract RNA from citrus plant tissue to detect citrus tristeza virus which cause heavy losses in citrus cultivars (Figure 3), in addition to use this method to detect Cachexia viriod (Figure 4).

Table 2.Price comparison between using modified extraction method and commercial kits.

Comparability	Modified method	Commercial kits
Nucleic acid	Both DNA and RNA together	DNA or RNA
Amount tissue	~100mg	~ 100 mg
Sample price	1EGP	15-20 EGP for DNA 30-50 EGP for RNA

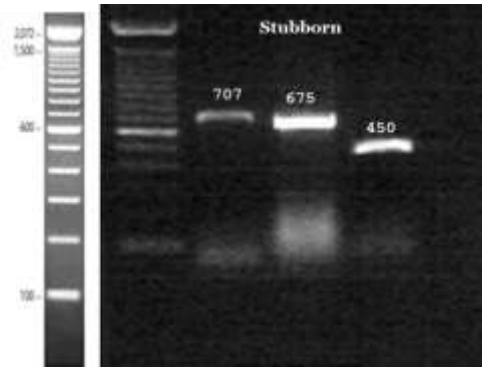

Figure 1.Agarose gel electrophoresis analysis of PCR product for genomic DNA extraction from infected citrus trees using the modified extraction method, Lane 1, Trackit™ 100bp ladder; lane 2,3 and 4 PCR product from infected citrus trees using different pair of primers for stubborn disease.

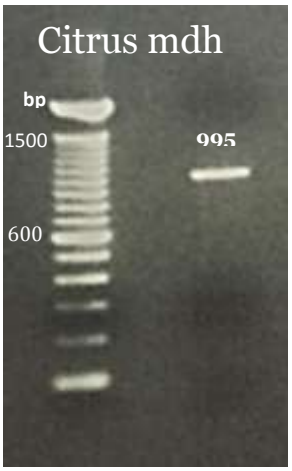

Figure 2.Agarose gel electrophoresis analysis of PCR product for genomic DNA extraction from citrus trees using the modified extraction method, Lane 1, Trackit™ 100bp ladder; lane 3 PCR product for Malate dehydrogenase gene (MDH) isolated from citrus plant genome.

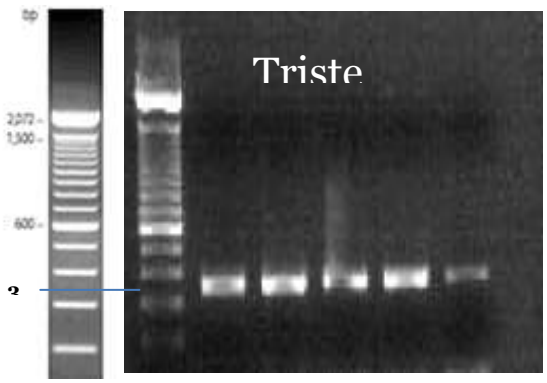

Figure 3.Agarose gel electrophoresis analysis of PCR product for RNA extraction from infected citrus trees using the modified extraction method, Lane 1, Trackit™ 100bp ladder; lane 2,3,4,5 and 6 PCR product for *Citrus Tristeza Virus* Disease (CTV).

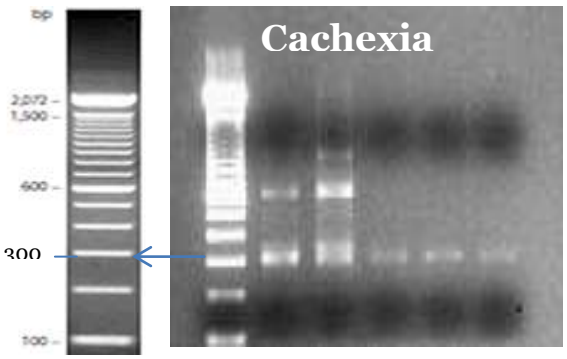

Figure 4.Agarose gel electrophoresis analysis of PCR product for RNA extraction from infected citrus trees using the modified extraction method, Lane 1, Trackit™ 100bp ladder; lane 2,3,4,5 and 6 PCR product for *Citrus Cachexia Viroid*.

DISCUSSION

The extraction of high quality nucleic acid procedures are crucial step when hundreds of samples need to be analyzed rapidly, for purposes such as a disease screening, mutant screening and marker assisted programs and other PCR-based techniques Khanuja *et al*. (1999). However, a low cost and time efficient extraction protocol is often hindered by two major challenges now. The first challenge is the reduction of secondary chemical reactions in the initial crude tissue extract, which otherwise could lead to loss of nucleic acid yield; another is the lack of universal extraction protocol suitable for different organisms because of the differences in compounds between

them Kotchoni and Gachomo (2009).

The traditional DNA and RNA extraction methods usually requires from 0.5 to several grams of plant tissue, making it impractical to analyze individual plants during early seedling stage. Also, the methods are time consuming and laborious due to their multistep procedures. Furthermore, large amount of hazardous chemical solvents are required Jinfa and James (2000). Many commercial kit are available from different biotech companies, but the main problem with these kits is their high cost per sample when hundreds or thousands of samples are extracted for DNA or RNA Kang and Yang (2004).

In the present study, modifications have been made to be compatible with different plant species. This includes using SDS as a detergent to disrupts the membranes, β-mercaptoethanol as a reducing agent which helps in denaturing proteins by breaking the disulfide bonds between the cysteine residues and for removing the tannins and polyphenols present in the crude extract, EDTA as chelating agent which chelates the magnesium ions required for DNase activity, buffer which is Tris pH8 and sodium chloride as a salt which aids in precipitation by neutralizing the charges on nucleic acid so that the molecules can come together. This method requires small amount of plant tissue to reduce inhibitor agents. This method also, simple, universal, inexpensive, and safe for extracting nucleic acid from different organisms, which can subsequently be used for PCR amplification. The method needs only a few reagents, and it could reduce risk of contamination and even be suitable for other purposes following PCR amplification, enzyme digestion and cloning. It is, therefore, suitable not only for large scale sample extraction but also for high quality extraction in laboratories with relatively limited equipment or funds.

Conclusion

By using this simple and rapid protocol, it was possible to isolate nucleic acid and perform PCR for a large number of samples in a single working day. The efficiency and the speed of this method together with the use of inexpensive facilities and the absence of toxic chemicals make the present method an attractive alternative for the extraction of plant nucleic acid. These results show that the nucleic acids produced by this simple, low coast, fast and safe protocols can be used in PCR-based techniques on a wide range of organisms, and in laboratories leaking state-of –the-art equipment's and technology .

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