

Optimization of CTAB-based RNA Extraction for *in planta Fusarium oxysporum f. sp. cubense* Gene Expression Study

(Pengoptimuman Pengekstrakan RNA Berasaskan CTAB untuk Kajian Pengekspresan Gen *Fusarium oxysporum f. sp. cubense* Secara *in planta*)

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ABSTRACT

A crucial prerequisite for an insightful gene expression study is the quality of the nucleic acid extracted. High-quality nucleic acids allow comparative downstream analyses for both organisms during a phytopathogen infection. However, RNA extraction of pathogen-infected host materials usually involves extraction methods that are optimised individually for either the pathogen or the host. Different sets of buffers or specialised commercial kits are often required. In this study, a streamlined CTAB-based extraction protocol was optimised for both the pure culture of *Fusarium oxysporum f. sp. cubense* (Foc) and infected banana roots. Foc cultures were grown on PDA overlaid by a nylon membrane and total nucleic acids were successfully extracted from mycelia with a ratio of 100 mg mycelia powder mass to 2 mL of CTAB buffer. Using the optimised protocol, LiCl-precipitated RNAs showed higher values of $A_{260/280}$ (2.064 ± 0.021) and $A_{260/230}$ (1.937 ± 0.076) compared to ethanol precipitated RNAs. Similar observation was observed for inoculated banana roots where LiCl-precipitated RNAs showed higher values of $A_{260/280}$ and $A_{260/230}$ compared to ethanol precipitated RNAs. qRT-PCR analysis using a pair of Foc specific primers, FoTEF1 α , confirmed that the LiCl-precipitated RNA was more suitable for downstream gene expression studies. This extraction protocol is applicable for Foc *in planta* gene expression study with a high potential to be extended to other filamentous fungal pathogens.

Keywords: *Fusarium oxysporum f. sp. cubense*; hexadecyltrimethylammonium bromide (CTAB); *in planta* gene expression

ABSTRAK

Prasyarat penting untuk kajian pengekspresan gen yang tepat adalah kualiti asid nukleik yang diekstrak. Asid nukleik berkualiti tinggi membolehkan analisis hiran komparatif bagi kedua-dua organisma semasa jangkitan fitopatogen. Walau bagaimanapun, pengambilan RNA perumah yang dijangkiti patogen biasanya melibatkan kaedah pengeksstrakan yang dioptimumkan secara individu untuk sama ada patogen atau perumah. Pelbagai penimbal atau kit komersial khusus sering diperlukan. Dalam kajian ini, protokol pengeksstrakan berasaskan CTAB dioptimumkan untuk kedua-dua kultur tulen *Fusarium oxysporum f. sp. cubense* (Foc) dan akar pisang yang dijangkiti. Foc dikultur pada PDA yang dilapisi oleh membran nilon dan asid nukleik berjaya diekstrak daripada miselium dengan nisbah 100 mg serbuk miselium kepada 2 mL penimbal CTAB. Dengan protokol yang dioptimumkan, RNA yang dimendak oleh LiCl menunjukkan nilai $A_{260/280}$ (2.064 ± 0.021) dan $A_{260/230}$ (1.937 ± 0.076) yang lebih tinggi berbanding RNA yang dimendak menggunakan etanol. Pemerhatian yang sama dicerap untuk akar pisang yang diinokulasi dengan RNA yang dimendak oleh LiCl menunjukkan nilai $A_{260/280}$ dan $A_{260/230}$ yang lebih tinggi berbanding RNA yang dimendak menggunakan etanol. Analisis qRT-PCR menggunakan pasangan pencetus khusus Foc, FoTEF1 α , mengesahkan bahawa RNA yang dimendak oleh LiCl lebih sesuai untuk kajian pengekspresan gen hiran. Protokol pengeksstrakan ini boleh digunakan untuk kajian pengekspresan gen Foc secara *in planta* dan berpotensi tinggi untuk diperluaskan kepada patogen kulat filamen lain.

Kata kunci: *Fusarium oxysporum f. sp. cubense*; heksadesiltrimetil ammonium bromida (CTAB); pengekspresan gen *in planta*

INTRODUCTION

Fusarium wilt of banana presents a devastating threat worldwide. This plant disease has inspired investigations of the pathosystem from various biological perspectives, including its molecular mechanisms. There are many protocols optimised for DNA extraction from *Fusarium* spp. (Brandfass and Karlovsky 2008; Gontia-Mishra et al. 2014; González-Mendoza et al. 2010; Yang et al. 2016), but only a single report is dedicated to RNA

extraction from pure culture of this species (Schumann et al. 2013). Similarly, nucleic acid extraction procedures were often optimised for banana leaf (Johari & Majumder 2015; Sharma et al. 2013) while efficient nucleic acids extraction for banana root is lacking (Mbéguié-A-Mbéguié et al. 2008). To this date, no comprehensive protocol has been demonstrated to be able to efficiently isolate total nucleic acids from both fungus and host together after infection.

Nucleic acid extraction from an infected plant root is time consuming and technically challenging. RNA extraction of pathogen-infected host material usually involves extraction methods that are optimised individually for either the pathogen or the host with different sets of buffers (Sánchez-Rodríguez et al. 2008). The challenge is contamination of the roots retrieved from soil with inhibitory compounds, such as humic acid and elevated amounts of phenolic compounds, which often compromise enzymatic reactions. Banana plants have been reported to show high contents of polyphenols and polysaccharides (Bryant 1996) and roots are also well known to produce high amounts of secondary metabolites (Khan et al. 2007). Plant and fungi possess rigid cell walls. Therefore, a critical step is the physical disruption of these cell walls in order to release the cellular contents, such as nucleic acids from the cells. Bead-beating is a common method in extracting nucleic acids from filamentous fungi (Gontia-Mishra et al. 2014; Leite et al. 2012), but requires specialised equipment. Freezing the mycelia in liquid nitrogen followed by a mortar and pestle pulverisation is another method and has been proven to be superior in RNA yield and quality (Bernáldez et al. 2017), while requiring no specialised equipment.

Subsequently, lysis buffer, generally consisting of salts and chaotropic agents is used to denature the proteins. In general, hexadecyltrimethylammonium bromide (CTAB) is a surfactant widely used in plant nucleic acid extraction (Clarke 2009). CTAB was first introduced as a method for plant DNA extraction (Doyle & Doyle 1987), but was later adapted to be used in RNA extraction (Gambino et al. 2008). For filamentous fungi, attempts to isolate RNA using CTAB have reportedly failed (Islas-Flores et al. 2006; Sánchez-Rodríguez et al. 2008), thus TRIzol or guanidium thiocyanate-based reagents have been routinely applied for such procedures instead (Schumann et al. 2013; Tan & Yiap 2009). Typically, β -mercaptoethanol and polyvinylpyrrolidone (PVP) are used for this process since binding to the polyphenols facilitate the elimination of impurities from nucleic acids (Martínez-Fuentes et al. 2015).

Organic solvents, such as phenol and chloroform, are used in the subsequent phase extraction step to separate the nucleic acids from impurities. Finally, in high-salt conditions, the precipitation agent will induce the nucleic acids to form aggregates, which are then collected through high-speed centrifugation (Rubio-Piña & Zapata-Pérez 2011). Widely adopted nucleic acid precipitation agents, such as ethanol and isopropanol, are often supplemented with monovalent cations, such as sodium and ammonium salts. Lithium chloride is another commonly used precipitant, particularly for RNA precipitation, usually used on its own or added in low amounts to enhance ethanol precipitation. For further downstream applications, RNase A can be added to the DNA fraction to obtain RNA-free genomic DNA whereas DNase I can be used in RNA fractions to obtain DNA-free RNA.

Fusarium oxysporum f. sp. *ubense* (*Foc*) is a filamentous fungus of the ascomycota division. It is an important pathogen for a wide range of plantains and banana cultivars. *Foc* invades the root of the banana host and colonises the vascular system to complete its life cycle while remaining dormant as chlamydospores in the soil (De Cal et al. 1997). The fungus is routinely isolated from infected plant materials (such as xylem vessels and saps) and cultured on potato dextrose agar (PDA). In order to study *in planta* gene expression responses, banana roots are inoculated with *Foc* prior to RNA extraction. At the onset of an infection, *Foc* conidia will germinate and penetrate the roots, with hyphae and mycelia colonising the roots and then becoming inseparable from the infected plant samples. Our interest in studying the *Foc in planta* expression profile has prompted us to develop a simple and cost-effective way to isolate nucleic acids from *Foc* and its infecting form in the banana host in a single extraction method. Moreover, co-extraction of DNA and RNA in a single reaction helps to reduce technical bias caused by the different extraction attempts.

High-quality DNA-free RNA is a crucial prerequisite for a successful quantitative gene expression analysis. The present study introduces a modified CTAB-based nucleic acids extraction protocol applicable to both *Foc* and banana, suitable for either individual or infected samples. The same set of buffers can be used in the nucleic acid extraction procedure of these sample types. Moreover, we demonstrated that both the DNA and RNA extracted are highly compatible with conventional PCR and qRT-PCR applications. This streamlined extraction protocol provides a convenient and efficient way to study molecular plant-pathogen interactions and *in planta* gene expressions of *Foc* and banana.

MATERIALS AND METHODS

PLANT GROWTH CONDITION

Rooted and *in vitro* propagated one-month old plantlets of *Musa acuminata* var Berangan (AAA) were purchased from Granatech Sdn. Bhd. Plantlets were individually planted in a plastic pot containing sterile sand and acclimatised in a constant temperature of 25°C and 12/12-h day/night cycle for a week. After the acclimatisation period, surviving plants were transferred to a plastic pot containing sterile soil medium (a mixture of clay:coarse vermiculite = 1:1, v/v) for 2 months. Healthy plants were used for *Foc* inoculation and nucleic acid extraction.

FUNGAL STRAIN, GROWTH AND INOCULATION METHOD

Foc strain C1HIR_9889 (VCG type 01213/16), an isolate from Kuala Terengganu, Terengganu, Malaysia was used in this study. Agar plugs with pure culture of *Foc* mycelia were placed on Potato Dextrose Agar (PDA) supplied with 50 μ g/mL streptomycin with and without a nylon membrane placed on top of the agar. The *Foc* culture

was allowed to grow on the PDA for 7 days. Mycelia were harvested from the agar or nylon membrane using a sterile spatula and used for fungal inoculation and total nucleic acids extraction.

For *Foc* inoculation, the double tray method described by Mohamed et al. (1999) was used. Twenty healthy *M. acuminata* var Berangan (AAA) (10 to 15 cm in height) were used for this study. Spore suspension in Potato Dextrose Broth (PDB) with a concentration of 10^6 spores/mL was used for artificial inoculation. Roots of 15 plants were immersed in the spore suspension and allowed to incubate for 2 h. The remaining five plants were immersed in sterile distilled water, serving as control (mock inoculation). After 2 h, all plants were retrieved. Five plantlets retrieved from the spore suspension were randomly selected to represent the 0 hpi (hours post inoculation) group. The remaining plantlets were replanted into sterile soil until the designated harvesting time points (48 hpi and 96 hpi). Harvested root samples were rinsed and then frozen in liquid nitrogen. All root samples were stored at -80°C before total nucleic acid extraction was performed.

TOTAL NUCLEIC ACID EXTRACTION

Total nucleic acids were extracted from samples using a CTAB method modified from Doyle and Doyle (1987). The modified lysis buffer consisted of 100 mM Tris-HCl (pH8.0), 25 mM EDTA (pH8.0), 2% (w/v) CTAB, 2 M NaCl, 2% (w/v) PVP-10. Two percent (v/v) of β -mercaptoethanol was added to the lysis buffer prior to the extraction. Mycelia were ground to fine powder (abbreviated as 'mycelia powder' hereafter) in liquid nitrogen with a sterile mortar and pestle and transferred to sterile microcentrifuge tubes. Prewarmed CTAB lysis buffer was added to microcentrifuge tubes to resuspend the fine powder. Samples were vortexed vigorously until all powder dissolved in the buffer and then incubated at 65°C for 10 min. The remaining steps were carried out under cool conditions ($\leq 4^\circ\text{C}$). Chloroform: isoamyl alcohol (24:1) extraction was carried out twice followed by pure chloroform extraction. All centrifugation steps were done at $16000 \times g$ for 15 min. Aqueous phases collected from the last step were added with either 2.5 volumes of absolute ethanol or 2.5 M lithium chloride (LiCl), then kept at -20°C overnight. Samples were centrifuged at $16000 \times g$ for 10 min to recover pellets, supernatants were discarded. Pellets were washed with 70% ethanol twice, air-dried and resuspended in nuclease-free water. The same extraction protocol was used for mock-inoculated and *Foc*-infected banana samples. RNeasy plant mini kit (QIAGEN, Germany) was used to extract nucleic acids from 100 mg of mycelia powder according to the manufacturer's instructions.

TOTAL NUCLEIC ACID MEASUREMENT AND QUALITY CONTROL

Integrity and concentration of the total nucleic acids were tested with agarose gel electrophoresis and Nanophotometer

(Implen GmbH, Germany). In general, an $A_{260/280}$ ratio of ~ 1.8 or ~ 2.0 is considered high quality for DNA and RNA, respectively. DNA and RNA with an $A_{260/230}$ ratio of 2.0 and above are considered to be free of contaminants (Lim et al. 2016). In this study, total nucleic acids extracted from 100 mg of mycelia powder (PDA with nylon membrane) were used for further nuclease treatment. Total nucleic acids from infected roots were treated according to the schematic diagrams (Figure 1) to produce DNA and RNA used for further PCR and qRT-PCR assessments. All samples were stored at -80°C until nuclease treatments.

DNASE I AND RNASE A TREATMENT

Total nucleic acids were treated with DNase I (New England Biolabs, UK) to obtain DNA-free RNA. Ten microgrammes of total nucleic acids were treated with either 2 units of DNase I for LiCl-precipitation or 4 units of DNase I for ethanol-precipitated samples. Samples were incubated at 37°C for 1 h. One volume of phenol:chloroform (1:1, pH 4.5) was added in order to clean-up the residual DNase I by phase separation. The mixtures were centrifuged for 15 min and supernatant was collected. The treated-RNAs were precipitated from the supernatant by incubated overnight at -20°C with 2.5 M of LiCl.

For RNase A treatment, total nucleic acids were treated with 50 $\mu\text{g}/\text{mL}$ of RNase A (Vivantis Technologies Sdn. Bhd., Malaysia) and incubated for an hour at 37°C . One volume of phenol:chloroform:isoamyl alcohol (125:24:1, pH6.7) was used to purify the RNase-treated DNAs and centrifuged for 15 min. The DNAs were precipitated overnight at -20°C using 0.1 volume of 3 M sodium acetate (pH5.2) and 2 volumes of absolute ethanol.

RNA and DNA pellets were air dried and resuspended in nuclease-free water. Integrity and concentration were determined with agarose gel electrophoresis and Nanophotometer (Implen GmbH, Germany). All centrifugations were done at $16000 \times g$ at 4°C .

cDNA SYNTHESIS, RT-PCR AND QUANTITATIVE RT-PCR ANALYSIS

cDNA was synthesised from 2 μg of DNA-free total RNA using the SuperScript IV™ First Strand Synthesis System (Thermo Fisher Scientific, USA) according to the manufacturer's instructions.

A standard curve for the primer FoTEF1 α (Table 1) was determined with five serial dilutions (100 ng to 0.01 ng) of *Foc* DNA on the Applied Biosystems™ QuantStudio™ 12K Flex Real-time PCR System. The reaction specificity of the PCR reaction was monitored by melting curves. The qRT-PCR reaction was set up with 2 μL of *Foc* DNA, 1X SensiFAST Lo-ROX mix (Bioline Reagent Ltd., UK), 0.1 μM of forward and reverse primers (Table 1), and nuclease free water in a 10 μL reaction volume. The standard curve was plotted using C_q and \log_{10} of DNA concentrations. Primer FoTEF1 α was then used to quantify the expression of *Foc* translation elongation factor 1 α in cDNA of infected banana roots.

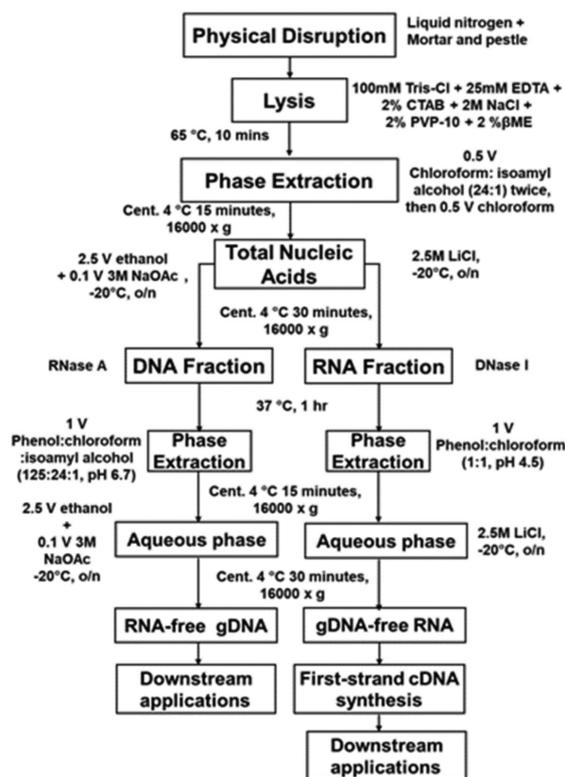


FIGURE 1. Schematic diagram of streamlined CTAB-based method. V, volume; cent., centrifuge; o/n, overnight; mins, minutes; hr, hour

TABLE 1. List of primers

Primer	Sequence	Melting temperature	Amplicon size
FoTEF1 α -F	5'-TCGGCTACAACCCCAAGGCTG-3'	62.0°C	120 bp
FoTEF1 α -R	5'-CGGACTGTGATCTCACGCTCCCA-3'	61.0°C	
FocSIX1-F	5'-GGGAGTGTCCCAGATAACAGTG-3'	57.0°C	92 bp
FocSIX1-R	5'-CGTCTCGGTCTGAACACTATCG-3'	56.9°C	

RESULTS AND DISCUSSION

NUCLEIC ACID EXTRACTION

Symptoms of *Fusarium* wilt were observed on the *M. acuminata* var Berangan infected with *F. oxysporum* f. sp. *cubense* Tropical Race 4 (*Foc*TR4) isolate C1HIR_9889. The leaves started to show the wilting symptoms from the outer older leaves and progressively to the inner younger leaves from week 2 to week 5 after inoculation (Figure 2).

For RNA extraction from filamentous fungi, TRIzol is routinely used, since guanidium salt is an excellent RNase denaturant (Chomczynski & Sacchi 2006; Cox 1968). However, TRIzol and other related products such as TRI Reagent consist of 30 to 60% phenol and guanidium thiocyanate, which are hazardous. Hexadecyltrimethylammonium bromide (CTAB) is a relatively safer alternative to TRIzol (Abu Almakarem et al. 2012). The chloride concentration in CTAB lysis buffer is increased to more than 2 M and can therefore prevent polysaccharide precipitation (Fang et al. 1992).

While previous attempts to utilise CTAB-based buffer for RNA extraction in filamentous fungi failed (Islas-Flores et al. 2006; Sánchez-Rodríguez et al. 2008), our study showed that CTAB lysis buffer was suitable for isolating DNA and RNA from *Foc* mycelia grown on solid media.

We found that the ratio of mycelia powder mass to CTAB lysis buffer was critical for the reproducibility of *Foc* nucleic acids extraction. Filamentous fungi nucleic acids extractions are often compromised by its rich content of carbohydrates (i.e. polysaccharides) (Sánchez-Rodríguez et al. 2008). The resulting lysate tends to be extremely viscous, which traps the buffer and hinders the following phase separation. In this study, 100 mg of mycelia powder resuspended in 2 mL of CTAB lysis buffer resulted in a clean separation of cell debris from the aqueous phase. Mycelia powder (250 mg) collected from PDA resuspended in 2 mL CTAB buffer failed to separate after centrifugation (Figure 3(a)). Hence 100 mg of mycelia powder to 2 mL lysis buffer was the optimal ratio for *Foc* nucleic acid extraction.

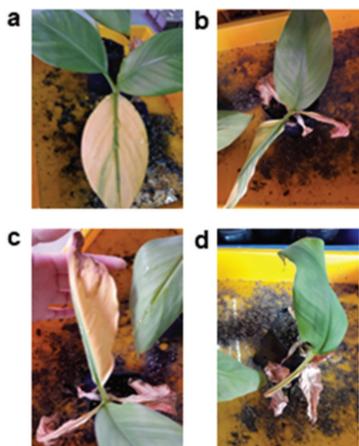


FIGURE 2. Banana plant shows symptoms at 2 weeks to 5 weeks (a to d) post-inoculation

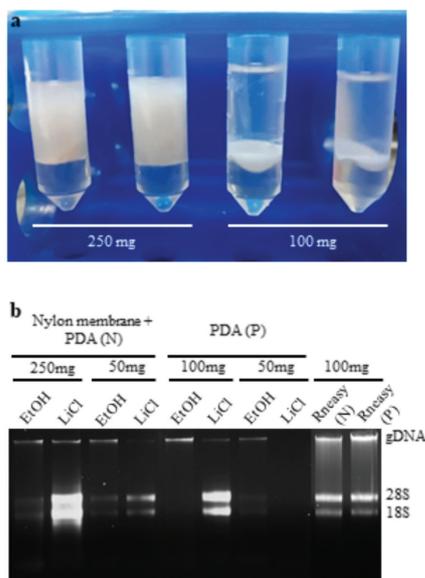


FIGURE 3. (a) Chloroform isoamyl alcohol extraction step after lysis (b) Total nucleic acids extracted with different ratios of mycelia powder to 2 mL CTAB buffer

Increasing the ratio of mycelia powder to buffer resulted in higher DNA but lower RNA yields, when the nucleic acids were precipitated using ethanol (Figure 3(b)). On the other hand, high quality RNA was obtained by reducing the ratio of mycelia powder to buffer (Table 2). To investigate the effect of the precipitation agent on the quality of RNA, lithium chloride (LiCl) was tested as an alternative precipitant. Using LiCl allowed for a higher ratio of mycelia powder to buffer (Figure 3(b)). This suggested that high amount of genomic DNA might compete with the RNA precipitation when ethanol was used as precipitation agent. The phosphodiester bonds of nucleic acids are able to form ionic bonds with cations and consequently the higher molecular weight results in a higher precipitation rate when using ethanol of more than 64% (Shackelford 2018). Therefore, in order to efficiently precipitate RNA, higher volumes of ethanol are needed (Williams et al. 2007).

Interestingly, we found *Foc* RNA to be more unstable than RNA extracted from banana roots. A significantly higher RNA yield was observed when using our optimised CTAB method. With a commercial kit tested, 100 mg of *Foc* mycelia powder gave a yield of 5.0 to 6.0 μg of total nucleic acids compared to 20.0 to 30.4 μg when the optimised CTAB-based method with LiCl-precipitation was used (Table 2). Similar finding was reported by Yaffe et al. (2012) where the authors described a novel method of RNA extraction, called LogSpin, using combination of guanidine hydrochloride buffer and plasmid DNA spin column. The authors showed that the RNA quantity extracted using LogSpin method was higher than commercial kit with similar purity values. Prominent amount of genomic DNA was also found in the kit-extracted *Foc* samples (Figure 3(b)) and low $A_{260/230}$ ratio especially for samples collected directly from PDA (Table 2). Commercial kits restricted method optimisation of all sample types needed for an *in planta* pathogenic gene expression study, whereas the method described here allowed modifications to suit the purpose. Moreover, the cost for total nucleic acid extraction could be as low as USD1.90 per reaction with

TABLE 2. Spectrometric readings of *Foc* RNA extracted with the optimized CTAB-based method

Culture media	Precipitation reagent	Mass (mg)	Yield ($\mu\text{g}/100 \text{ mg}$)	$A_{260/280}$	$A_{260/230}$
PDA ^a	ND	250	ND	ND	ND
PDA	Absolute ethanol	100	10.6	1.912	1.207
PDA	2.5 M LiCl	100	12.5	2.088	1.975
PDA	Absolute ethanol	50	2.5	1.986	1.521
PDA ^b	2.5 M LiCl	50	ND	ND	ND
PDA	RNeasy plant mini kit	100	1.6	2.022	0.659
Nylon membrane + PDA	Absolute ethanol	250	9.6	1.937	1.469
Nylon membrane + PDA	2.5 M LiCl	250	12.6	2.055	1.986
Nylon membrane + PDA	Absolute ethanol	100	28.2	1.965	1.529
Nylon membrane + PDA	2.5 M LiCl	100	23.4	2.050	1.850
Nylon membrane + PDA	Absolute ethanol	50	3.2	2.009	1.733
Nylon membrane + PDA ^b	2.5 M LiCl	50	ND	ND	ND
Nylon membrane + PDA	RNeasy plant mini kit	100	1.6	1.966	1.683

^aFailed to separate at first chloroform isoamyl alcohol phase separation, ^bResulting pellets after precipitation were unrecoverable, ND Not determined

this optimised method compared to commercial kits, such as USD7.60 for RNeasy plant mini kit (USD379.00 for 50 preps; QIAGEN, Germany). This cost-effectiveness is especially advantageous for high-throughput total nucleic acid preparation.

NUCLEIC ACIDS QUALITY ASSESSMENTS

Ethanol-precipitated nucleic acids contained both genomic DNA and RNAs (Figure 4(a)) while LiCl efficiently precipitated high quality RNA with reduced amount of genomic DNA and small RNA was not visible on the gel (Figure 4(a) and 4(b)). In seven of our samples, a nylon membrane was placed on top of PDA to facilitate the collection of mycelia as described in Schumann et al. (2013). Using CTAB buffer, we were able to isolate RNA from mycelia grown directly on the agar as well as on the membrane. Precipitation with 2.5 M lithium chloride yielded RNA with values of $A_{260/280}$ (2.064 ± 0.021) and $A_{260/230}$ (1.937 ± 0.076) (Table 2), indicating very low levels of contamination with proteins, polysaccharides and phenolics (Martínez-Fuentes et al. 2015).

The advantage of using LiCl as precipitant is that it reduces co-precipitation of DNA, proteins, polyphenolics and carbohydrates (Barlow et al. 1963; Rubio-Piña & Zapata-Pérez 2011). Ethanol, on the other hand, was more efficient in precipitating RNA of smaller species, while the threshold for efficient precipitation with LiCl was for RNA around 300 bases long. Although ethanol effectively precipitated nucleic acids, the mode of action was nonselective as it also precipitated afore-mentioned impurities as reflected by low $A_{260/230}$ readings (Table 3). In the case of infected root samples, $A_{260/230}$ readings of ethanol-precipitated nucleic acids were always below 1.0 despite multiple repeats of chloroform isoamyl alcohol or phenol-chloroform extractions. The use of 2.5 M LiCl, however, drastically improved the $A_{260/230}$ value to above 2.0 (Table 3) indicating a reduced level of impurities.

Foc nucleic acids extracted from 100 mg mycelia powder (Figure 4(a)) were further digested with DNase I to yield DNA-free RNA. Noticeably, RNA precipitated with LiCl maintained its integrity better than ethanol-precipitated RNA (Figure 4(b)). The activity of cellular RNases severely affected the quality of extracted RNA. Lithium chloride precipitation followed by phenol-chloroform (1:1, pH4.5) extraction after DNase I treatment effectively slowed down the RNA degradation process. Lithium chloride does not

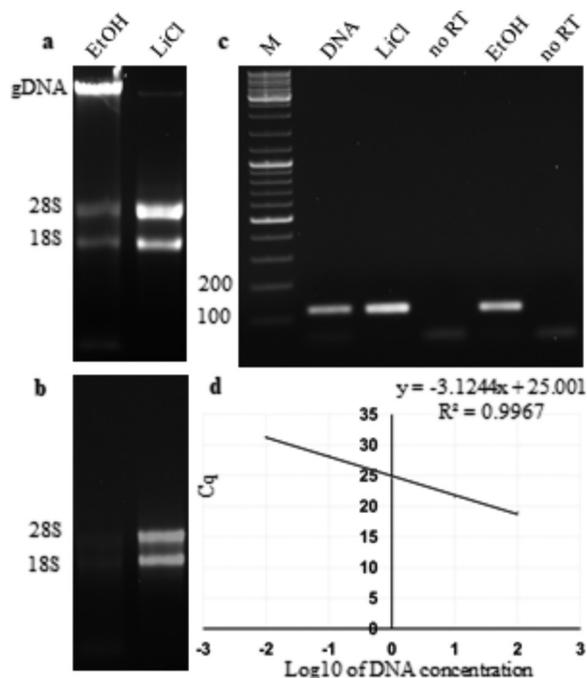


FIGURE 4. *Foc* DNA and RNA extracted with CTAB-based method and their quality assessments. (a) Nucleic acids extracted from 100 mg of mycelia powder (nylon membrane + PDA) in 2 mL of CTAB buffer (b) *Foc* RNA after DNase I treatment (c) End-point PCR analysis of FoTEF1 α gene using *Foc* DNA and cDNA. M - O' Gene ruler DNA ladder mix (Thermo Fisher Scientific, USA) and (d) FoTEF1 α primer efficiency analysis using qRT-PCR on *Foc* DNA

effectively precipitate DNA and proteins (Barlow et al. 1963; Sambrook & Russel 2001) and hence any potential degradation caused by *Foc* endogenous RNases can be minimised. Moreover, phenol acts through the disruption of protein structures and hence helps in denaturing RNases (Abu Almakarem et al. 2012).

Environmental samples, infected roots retrieved from soil in our case, are prone to be contaminated with a myriad of inhibitory compounds. DNase I activities in removing genomic DNA from RNA samples with high level of inhibitors was proved incomplete (Lim et al. 2016). Here, we showed that DNase I treatments were inefficient for ethanol-precipitated samples as amplification signals were detected on RNA controls containing no reverse transcriptase (no-RT) (Figure 5). According to Lim et al.

TABLE 3. Spectrometric readings of nucleic acids extracted from infected banana roots precipitated with absolute ethanol and 2.5 M lithium chloride

Root samples	Ethanol-precipitated		LiCl-precipitated	
	$A_{260/280}$	$A_{260/230}$	$A_{260/280}$	$A_{260/230}$
Mock	2.327 ± 0.217	0.705 ± 0.090	1.986 ± 0.041	2.369 ± 0.067
0HPI	2.359 ± 0.079	0.577 ± 0.015	1.997 ± 0.012	2.412 ± 0.019
48HPI	2.268 ± 0.160	0.590 ± 0.024	2.021 ± 0.019	2.380 ± 0.016
96HPI	2.191 ± 0.104	0.645 ± 0.036	2.082 ± 0.069	2.097 ± 0.422

(2016), DNase I treatment is considered incomplete when amplification in qRT-PCR is detected earlier than a threshold of 35 cycles. It is critical to completely eliminate residual gDNA in RNA samples when conducting gene expression analyses. Small amounts as low as 0.002% of gDNA contributed to as high as 60000 copies of false transcripts in qRT-PCR assays (Lim et al. 2016). In the present study, total nucleic acids extracted from infected roots subjected to DNase I digestion showed no noticeable RNA degradation

and 2 Units of DNase I were sufficient to remove gDNA from the LiCl-precipitated nucleic acids (Figure 6(a) and 6(b)).

DOWNSTREAM APPLICATION OF THE EXTRACTED NUCLEIC ACIDS

Fusarium-specific endogenous control, translation elongation factor 1 α (FoTEF1 α) was successfully quantified in cDNA synthesised from RNA for both LiCl-

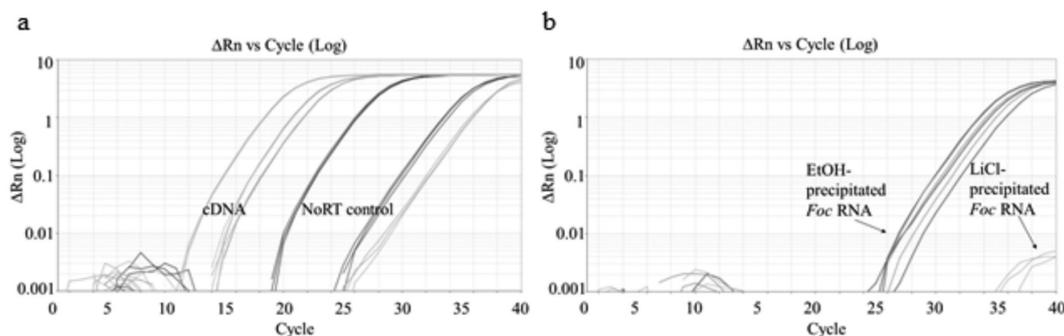


FIGURE 5. Ethanol-precipitated RNA controls containing no reverse transcriptase (noRT control) show amplification signals in qRT-PCR assays with FoTEF1 α primers (a) Infected root cDNA (b) *Foc* cDNA

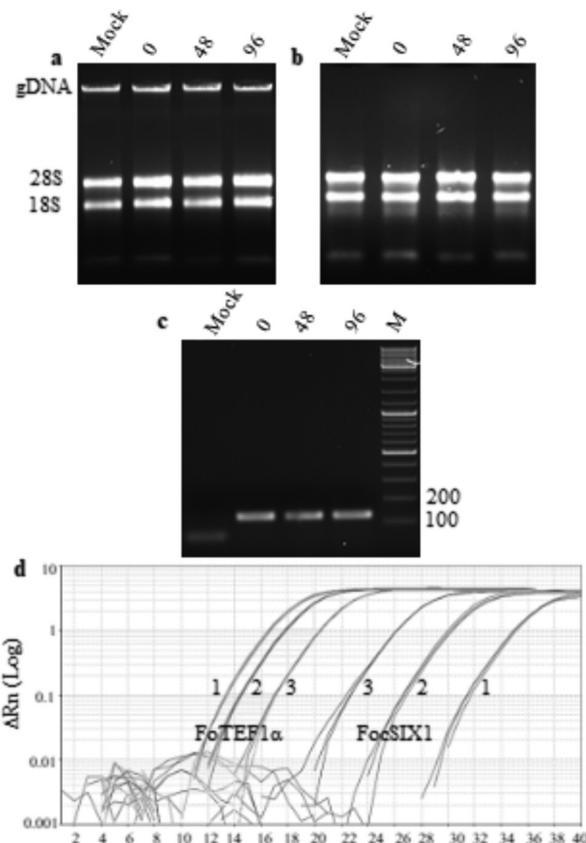


FIGURE 6. Quality assessment of RNA extracted from the infected banana roots for *Foc in planta* gene expression (a) Total nucleic acids extracted from 500 mg of infected banana root powder in 2 mL of CTAB lysis buffer, (b) Infected root RNA after DNase I treatment, (c) End-point PCR analysis of FoTEF1 α gene using infected root cDNA. M - O' Gene ruler DNA ladder mix (Thermo Fisher Scientific, USA), and (d) qRT-PCR quantification of *Foc* cDNAs using FoTEF1 α and *Foc*SIX1 primers, 1 - 2HPI, 2 - 48HPI, 3 - 96HPI

precipitated *Foc* and infected root samples (Figure 6(d)). Using our method, PCR amplification of FoTEF1 α showed high integrity (Figures 4(c) and 6(c)), despite the number of *Foc* cells attached to the banana roots being very low during the early stages of infection (Anderson et al. 2016). Amplification of another *Foc*-specific effector gene, *FocSIX1*, from sampled infected roots proved the prospect of this optimised method to be used for *in planta* pathogenic gene expression studies. This confirmed that RNAs extracted with CTAB buffer combined with LiCl precipitation, were compatible with downstream applications (Figures 4(d) and 6(d)).

CONCLUSION

We present an optimised CTAB-based nucleic acid extraction protocol applicable to both DNA and RNA extractions from pure culture *Foc* mycelia and *Foc*-infected banana roots. For *Foc* mycelia, the optimum ratio was 100 mg mycelia powder to 2 mL of CTAB lysis buffer, whereas for banana root samples it was 500 mg to 2 mL of buffer. Precipitation of RNAs using LiCl has proven to be more compatible with downstream gene expression studies, as the RNA was of high quality and free from genomic DNA contamination. This optimised method will be useful for *in planta* *Foc* pathogenic genes expression studies and has high potential to be applied on other filamentous fungal pathogens.

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