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Isolation of retro-transcribed RNA from *in vitro* *Mycosphaerella fijiensis*-infected banana leaves

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ABSTRACT. High polyphenol and polysaccharide levels in plant tissues such as banana fruit and leaves constitute a significant challenge to the extraction of sufficient amounts of high-quality RNA required for cDNA library synthesis and molecular analysis. To determine their comparative effectiveness at eliminating polyphenols, polysaccharides and proteins, three protocols for RNA extraction from *in vitro* banana plantlet leaves were tested: Concert™ Plant RNA isolation kit, a small-scale protocol based on Valderrama-Cháirez, and a modified version of the Valderrama-Cháirez protocol. RNA quantity and purity were evaluated by UV-spectrophotometry using DEPC-treated water and Tris-HCl, pH 7.5. Purity was greater using Tris-HCl. The Concert™ Plant protocol produced the poorest quality RNA. Reverse transcription into cDNAs from RNA isolated from *in vitro* banana plantlet leaves infected with *Mycosphaerella fijiensis* using the modified Valderrama-Cháirez protocol, followed by PCR using primers designed against γ-actin from banana and *M. fijiensis*, yielded products of the anticipated size. In addition, this protocol reduced the processing time, lowered costs, used

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less expensive equipment, and could be used for other plants that have the same problems with high polyphenol and polysaccharide levels.

Key words: Banana; *Mycosphaerella fijiensis*; Polyphenols; Polysaccharides; RNA isolation; Black leaf streak disease

INTRODUCTION

The isolation of large amounts of high-quality RNA from plant tissues rich in polysaccharides and polyphenols, including tannins, pigments and other metabolites, requires an efficient method. Several technical protocols have been described for RNA extraction from these plant tissue types (Louime et al., 2008; Montenegro et al., 2008; Suzuki et al., 2008; Wang et al., 2008; Vasanthaiah et al., 2008), and biotechnology companies have developed kits or protocols suitable for particular cells, organs or tissues. However, an efficient protocol specifically for recalcitrant plant tissues does not yet exist, meaning that successful protocols must be adapted or developed through the modification of existing methods, while considering the particular features of the target plant material (MacRae, 2007).

Banana (*Musa* spp) is cultivated in tropical and subtropical regions worldwide, and its fruit represents an important carbohydrate resource in developing countries. Like most other commercial crops, banana is affected by pathogens, the most important being the fungus *Mycosphaerella fijiensis* Morelet, responsible for black Sigatoka or black leaf streak disease, a major threat to banana crops worldwide. In molecular studies of banana-*M. fijiensis* interaction, the isolation of large amounts of high-quality RNA is crucial for cDNA library construction. Although protocols for RNA extraction from banana fruit (peel and pulp) (Manrique-Trujillo et al., 2007; Mbéguié-A-Mbéguié et al., 2007; Xu et al., 2007), root (Van den Berg et al., 2004) and greenhouse leaves (Sánchez-Rodríguez et al., 2008) have been described, there is currently no available efficient method for the isolation of large amounts of high-quality RNA from *in vitro M. fijiensis*-inoculated banana leaves that produces RNA suitable for cDNA library preparation. Our research required the isolation of high-quality RNA from *in vitro* banana plantlet leaves, which has proved to be an ongoing challenge. Initially, we compared the Trizol® reagent and CTAB or SDS-containing protocols, also we tested the method reported by Rodrigues et al. (2007), but even after repeated adjustments, these produced low yields or poor-quality RNA (data not shown). We then tested three protocols commonly used to eliminate polyphenols, polysaccharides and proteins: Concert™ Plant RNA Reagent, a commercially available total RNA extraction kit appropriate for eliminating these metabolites from plant tissues; a small-scale protocol developed to extract RNA from cactus fruit rich in polysaccharides (Valderrama-Cháirez et al., 2002), which has been successfully applied with *Tagetes erecta* (Paredes-López et al., 2007) and prickly pear (Rosas-Cárdenas et al., 2007), and a modified version of this protocol. Quality and quantity of the RNA isolated from banana were assessed by UV-spectrophotometric analysis either in DEPC-treated water or Tris-HCl buffer, pH 7.5; electrophoresis on TAE-agarose gels, reverse transcription into cDNAs and subsequent polymerase chain reaction (PCR) amplification using primers designed to specifically amplify γ-actin from cDNAs of banana and *M. fijiensis*. The modified protocol efficiently removed polyphenols, polysaccharides and proteins from *in vitro* banana plantlet leaves and yielded high-quality RNA in sufficient quantities for cDNA preparation.

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MATERIAL AND METHODS

Fungal material

Mycosphaerella fijiensis conidia, strain C1N3H2 (accession No. 389280 IMI), were harvested following an *in vitro* conidium production protocol (Peraza-Echeverría et al., 2008). Briefly, 100 mL/L V8® juice was added to 0.2 g/L $CaCO_3$ and 20 mg/L microbiological agar, autoclaved and placed on Petri dishes. Individual dishes were inoculated with 2 mL mycelium solution (1 g mycelium macerated with 5 mL sterile water) and left to grow at 20 ± 2°C under continuous, cool-white fluorescent and black light. After seven days, conidia from each Petri dish were harvested with 2.4 mL 1% gelatin (w/v), filtered through 2 pieces of fine cheesecloth and collected in a 50-mL Falcon tube. A suspension containing 200 conidia/µL was spread with a camel hair brush onto the abaxial portion of *in vitro* banana leaves.

Plant material

Banana leaves (*Musa acuminata*, subgroup Cavendish Grand-Nain) were collected from 3-month-old *in vitro* plants grown on MS solid culture medium containing 30 g/L sucrose and 2 g/L gelling agent (Gelrite, Sigma) performed at 28 ± 2°C under 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in a 16-h light/8-h dark photoperiod. The leaves were weighed, immediately submerged in liquid nitrogen and stored at -80°C until use. For RT-PCR analysis, *in vitro* banana plantlet leaves were artificially inoculated with *M. fijiensis* conidia and leaf samples taken 15 days post-inoculation.

The sterile distilled water used to prepare solutions and buffers and to dissolve RNA was treated with 0.1% (v/v) diethyl pyrocarbonate (DEPC; Sigma). All tips, tubes, and DEPC-treated water were autoclaved twice at 121.1°C for 20 min. Mortars, pestles and spatulas were washed with distilled water containing 3% (w/v) NaOCl, rinsed with sterile distilled water and autoclaved at 121.1°C for 20 min (twice).

Concert ™ Plant RNA Reagent (C protocol)

Freshly harvested *in vitro* banana leaves (0.5 g) were frozen with liquid N_2 and powdered in a mortar and a pestle. Small portions of powder were added to a vial containing 2.5 mL cold (4°C) Plant RNA Reagent, and the contents were mixed briefly with a micropipette and divided into 4 tubes (0.625 mL). Each tube was agitated briefly to completely resuspend the sample, incubated for 5 min at room temperature and centrifuged at 12,000 *g* for 2 min. The supernatants were transferred to RNAse-free tubes along with 125 µL 5 M NaCl, which were vortexed. After mixing, 375 µL chloroform was added; the suspension was completely mixed by inversion and centrifuged at 12,000 *g* for 10 min at 4°C to separate the phases. The aqueous phase was transferred to RNase-free tubes. RNA was precipitated by adding an equal volume of isopropyl alcohol and mixing, and after 10 min at room temperature, RNA was centrifuged at 12,000 *g* for 10 min at 4°C. The supernatants were decanted to recover the pellets, 1 mL 75% ethanol was added, and the suspension was centrifuged at 12,000 *g* for 1 min at room temperature. The supernatants were decanted carefully and the tubes briefly centrifuged to remove any residual liquid. The pellet (RNA) in each tube was dissolved in 10 µL DEPC-treated water. The suspen-

sions were pooled in a new tube and analyzed in a spectrophotometer. The RNA was stored at -80°C until use.

Small-scale Valderrama-Cháirez et al. protocol (VC protocol)

Fresh *in vitro* banana leaves (0.5 g) were frozen with liquid N_2 and powdered for 5 min using a mortar and a pestle previously chilled to -20°C with liquid N_2 added continuously. Small portions of powdered sample were added to a vial containing 3 mL extraction buffer (150 mM Tris base (hydroxymethyl-hydrochloride), 2% (w/v) SDS, 100 mM EDTA, adjusted to pH 7.5 with saturated boric acid), and 30 µL β-mercaptoethanol (1%, v/v) added just before use. This suspension was quickly mixed using a cut tip to avoid RNA damage, transferred to Eppendorf tubes (750 µL per tube), precipitated with 66 µL 5 mM potassium acetate, and 150 µL absolute ethanol, and vortexed for 1 min. Next, 850 µL chloroform-isoamyl alcohol (49:1, v/v) was added, and the suspension vortexed for10 s and centrifuged at 16,000 *g* for 20 min at room temperature. The supernatants were recovered and transferred to new tubes, 850 µL phenol-chloroform-isoamyl alcohol (25:24:1, v/v/v) was added per tube, and the tubes were vortexed for 10 s and centrifuged at 16,000 *g* for 15 min at room temperature. The supernatants were recovered and transferred to new tubes, along with 850 µL chloroform-isoamyl alcohol. The tubes were vortexed for 10 s and centrifuged at 16,000 *g* for 15 min at 4°C. The supernatants were recovered, 12 M LiCl was added to a final concentration of 3 M, and the tubes were gently mixed by inversion and left to stand overnight at -20°C. The samples were then centrifuged at 16,000 *g* for 20 min at 4°C, and the pellets were washed twice with 70% ethanol (500 µL each) and centrifuged again at 16,000 *g* for 10 min at 4°C. The pellets were dried at room temperature and resuspended in 10 µL DEPC-treated sterile distilled water. The suspensions were pooled in a new tube, and RNA quantified in a UV-light spectrophotometer. RNA was stored at -80°C until use.

Modified small-scale Valderrama-Cháirez et al. protocol (MVC protocol)

This was carried out as described above for the VC protocol, but with some modifications: 4% (w/v) SDS and 2% (v/v) β-mercaptoethanol, and the addition of 3% (w/v) PVP-40 in the extraction buffer (3 mL), where the last two reagents were added just before use. An additional step was added, the incubation of the homogenate (material powdered with modified extraction buffer) at 65°C for 10 min. After allowing the samples to return to room temperature, potassium acetate and absolute ethanol were then added.

Total RNA analysis

RNA quantity and quality (purity and integrity) were determined using spectrophotometry at 260 nm, and $A_{260/210}$, $A_{260/230}$ and $A_{260/280}$ absorbance ratios, as well as by visual analysis on gels. Given that the $A_{260/280}$ ratio is considerably influenced by the pH of the solution in which it is diluted for measurement (Wilfinger, 1997; Okamoto and Okabe, 2000), the samples were diluted (1:100) in 10 mM Tris-HCl, pH 7.5, or in DEPC-treated sterile distilled water before comparison of their absorbance values. For analysis by gel electrophoresis, 2 µg RNA samples were mixed with 17 µL denaturing solution [641 µL 2.77 M formamide (Sigma-

Aldrich); 128.2 µL 3-[N-morpholine] propanesulfonic acid (Sigma-Aldrich) (MOPS) 10X buffer, pH 7, and 230.8 µL 12.3 M formaldehyde (Sigma-Aldrich); final volume = 1 mL]. The samples were then incubated for 5 min at 65°C, immediately chilled on ice for 5 min, and 2 µL loading buffer [50% glycerol, 1 mM EDTA, 10 µL ethidium bromide (10 mg/mL) and 0.025% bromophenol blue; final volume = 1 mL] was added. Samples (25 µL) were loaded onto 1.2% agarose gels in 1X TAE buffer following Masek et al. (2005) and run at 70 V for 40 min.

Three replicates were done for all experiments. All statistical analyses were done with the STATGRAPHICS Plus version 4.1 software package (Manugistics, Rockville, USA).

Retro-transcribed RNA analysis

RNA performance in generating cDNA was tested by first treating 1 µg RNA sample with 1 unit DNase following manufacturer instructions (Invitrogen). RNA (0.5 µg) was used to synthesize the first cDNA strand by applying Superscript III reverse transcriptase following manufacturer instructions (Invitrogen). The cDNA was analyzed by amplification of γ-actin genes from the host and the pathogen. In PCR, 2 µL cDNA was used as a template, and *M. fijiensis* and *M. acuminata* genomic DNA (1 ng) samples were used as positive controls. Reaction primers were specifically designed for cDNAs from *M. fijiensis*: γ-actin forward primer (5'-CTTGACTCCGGTGACGGTGTCACTC-3') and γ-actin reverse primer (5'-CGTCAGG AAGCTCGTAGGACTTCTC-3'), and *Musa acuminata*: γ-actin forward primer (5'-CTGGT GATGGTGTGAGCCACACTGTTC-3') and γ-actin reverse primer (5'-CACTGAGAACGA TGTTGCCATACAGGTC-3'). Conditions for PCR were: 95ºC for 5 min; 36 cycles of 94°C for 30 s, 65°C for 30 s, 72°C for 1 min, and 72°C for 7 min. Denaturing gel electrophoresis was not used, only the products amplified were denatured, avoiding the use of highly toxic formaldehyde in 1.2% (w/v) TAE-agarose gels. Analysis performance was unchanged from that obtained with denatured gel (data not shown).

RESULTS AND DISCUSSION

Isolated RNA yields with the MVC and C protocols were not significantly different, but the A_{260}/A_{230} ratio indicated a significant difference in RNA purity (Table 1). The MVC protocol produced RNA free of polyphenols and carbohydrate contaminants (A_{260}/A_{230} ratios >2), while RNA using the C protocol contained substantially higher contamination (A_{260}/A_{230} ratios = 0.55-0.63). Compared to these two protocols, the VC protocol produced less RNA but of significantly higher purity (A_{260}/A_{230} ratios = 1.73-1.84) (Table 1). Although the A_{260}/A_{230} ratio results suggest that the MVC protocol was the most effective at removing phenols and carbohydrates from the RNA, this protocol's A_{260}/A_{210} ratio (0.8) suggests the weak presence of other contaminants. However, the C protocol produced RNA with a lower A_{260}/A_{210} ratio (0.29), indicating a higher contaminant level, which is also supported by the A_{260}/A_{230} ratio. The A_{260}/A_{280} ratio is affected by acidic pH (Wilfinger, 1997; Okamoto and Okabe, 2000), and many manufacturer instructions for RNA extraction (Qiagen, Trizol, Omega Bio-tek, Stratagene) recommend the use of a buffered solution to dilute RNAs. Taking this into account, we compared the A_{260}/A_{280} ratios of samples diluted with DEPC-treated water, pH 4.23, or Tris-HCl, pH 7.5. Those diluted with DEPC-treated water apparently exhibited substantial protein contamination with the VC and MVC protocols, leading to RNA purity underestimation. This

agrees with Okamoto and Okabe (2000), who reported that DEPC-treated water does not affect absorbance at 260 nm but does increase it at 280 nm. This affects the A_{260}/A_{280} ratio, producing underestimation of RNA quality.

Table 1. Comparison of means of absorbance ratios and total yields of RNA obtained from *in vitro* banana plantlet leaves using three extraction protocols.

Method	$A_{260/210}$	$A_{260/230}$	$A_{260/280}$	RNA concentration (µg/g fresh weight)
VC[1]	0.745[c]	1.73833[b]	1.96167[bc]	127.038[b]
MVC[1]	0.882667[a]	2.31[a]	2.06167[ab]	202.293[a]
C[1]	0.291667[d]	0.63[c]	1.91333[c]	175.182[ab]
VC[2]	0.712167[c]	1.84833[b]	1.52167[d]	129.193[b]
MVC[2]	0.815167[b]	2.37667[a]	1.58[d]	199.848[a]
C[2]	0.296667[d]	0.553333[c]	2.15833[a]	203.897[a]

VC = small-scale Valderrama-Cháirez et al. protocol; MVC = modified small-scale Valderrama-Cháirez et al. protocol; C = Concert™ Plant protocol. [1]RNA diluted with Tris-HCl, pH 7.4. [2]RNA diluted with DEPC-treated sterile distilled water. The same superscript letters within the same column indicate no significant difference (P = 0.05) as determined by the Fisher least significant difference procedure.

On gels, the RNA samples exhibited two bright bands corresponding to 28S and 18S rRNA (Figure 1), with a ratio of intensities of 2:1, indicating a good-quality RNA with the VC and MVC protocols.

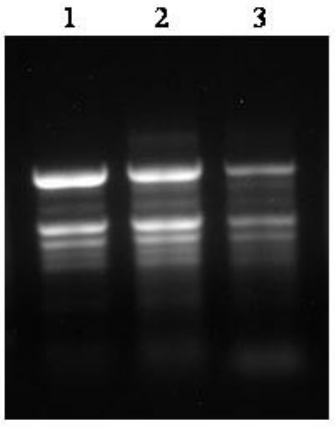

Figure 1. Total RNA extracted from *in vitro* banana plantlet leaves using three protocols: *Lane 1* = Small-scale Valderrama-Cháirez et al. protocol; *lane 2* = modified small-scale Valderrama-Cháirez et al. protocol; *lane 3* = Concert™ Plant RNA Reagent.

Because plant material represents the bulk of biological biomass in the pathogen-host interaction compared with *M. fijiensis* biomass, we evaluated the above RNA isolation protocols to determine which produced the highest quality and quantity of RNA in *in vitro* banana plantlet leaves. The MVC protocol produced the highest purity and yield of RNA and was used to extract total RNA from banana leaves artificially infected with *M. fijiensis* conidia harvested at 15 days post-inoculation. As expected, the protocol produced large amounts of high-quality total RNA (Figure 2).

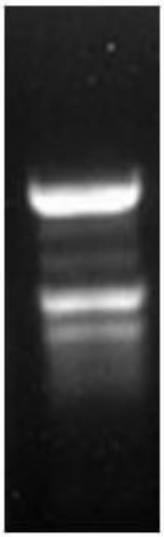

Figure 2. Total RNA extracted from *in vitro Mycosphaerella fijiensi*-infected banana plantlet leaves using the modified small-scale Valderrama-Cháirez et al. protocol.

The amplification of PCR products, 450 bp for *M. acuminata* and 277 bp for *M. fijiensis*, demonstrated that total RNA isolated with the MVC protocol was efficiently retro-transcribed into cDNA (Figure 3).

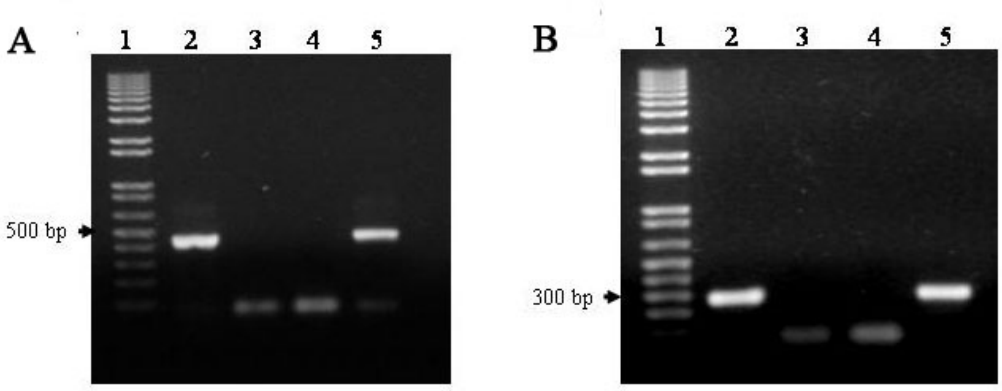

Figure 3. RT-PCR-amplified products using cDNA synthesized from total RNA extracted from *in vitro* banana plantlet leaves infected with conidia of *Mycosphaerella fijiensis*. Total RNA was extracted using the modified small-scale Valderrama-Cháirez et al. protocol. *Lane 1* = 1-kb plus DNA ladder (Invitrogen) (A, B); **A.** *Lane 2* = γ-actin fragment from banana; *lane 3* = negative control of lane 2 (sample without RT); *lane 4* = PCR control (water as template); *lane 5* = positive control, γ-actin fragment from *M. acuminata* gDNA. **B.** *Lane 2* = γ-actin fragment from *M. fijiensis*; *lane 3* = negative control of lane 2 (sample without RT); *lane 4* = PCR control (water as template); *lane 5* = positive control, γ-actin fragment from *M. fijiensis* gDNA.

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Sanchez-Rodríguez et al. (2008) reported an efficient method for isolating RNA, 48.67 ± 3.10 mg/g, from *M. fijiensis*-infected banana leaves. Nevertheless, the modified Valderrama-Cháirez et al. protocol, which produced the highest yield (202.293 mg/g) and purity of RNA from *in vitro* banana leaves, improved substantially this result with a 4.1-fold increase in total RNA yield. Additionally, the MVC protocol reduced the processing time, lowered costs (minipreparation), and used less expensive equipment (bench-top centrifuge) compared to the method reported by Sanchez-Rodríguez et al. (2008). On the other hand, the Concert™ Plant protocol produced apparently good RNA yield (overestimated), and the RNA quality (purity and integrity) was the poorest for the three tested protocols, restricting its use for cDNA libraries and ESTs. In addition, measurement of RNA purity using a buffered solution such as Tris-HCl, pH 7.4, produced a more reliable A_{260}/A_{280} ratio than with DEPC-treated water, pH 4.23. In conclusion, the highest yield and quality of RNA isolated from *in vitro* banana leaves were obtained with the modified Valderrama-Cháirez et al. protocol. Furthermore, this protocol reduced the processing time, lowered costs, used less expensive equipment, and could be used for other plants that have the same problems with high polyphenol and polysaccharide levels.

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