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Comparison of various RNA extraction methods, cDNA preparation and isolation of calmodulin gene from a highly melanized isolate of apple leaf blotch fungus *Marssonina coronaria*

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ABSTRACT

Marssonina coronaria causes apple blotch disease resulting in severe premature defoliation, and is distributed in many leading apple-growing areas in the world. Effective, reliable and high-quality RNA extraction is an indispensable procedure in any molecular biology study. No method currently exists for RNA extraction from *M. coronaria* that produces a high quantity of melanin-free RNA. Therefore, we evaluated eight RNA extraction methods including manual and commercial kits, to yield a sufficient quantity of high-quality and melanin-free RNA. Manual methods used here resulted in low quality and black colored RNA pellets showing the presence of melanin, despite all the modifications employed to original procedures. However, these methods when coupled with clean up resulted in melanin-free RNA. On the other hand, all commercial kits used were able to yield high-quality melanin-free RNA having variable yields. TRIzol™ Reagent + RNA Clean & Concentrator™-5 and Ambion-PureLink® RNA Mini Kit were found to be the best methods as the RNA extracted with these methods from 15 day old fungal culture grown on solid medium were free of melanin with good yield. RNA extracted by this improved methodology was applied for RT-PCR, subsequent PCR amplification, and isolation of calmodulin gene sequences from *M. coronaria* and infected apple leaf pieces. These methods are more time effective than traditional methods and take only an hour to complete. To our knowledge, this is the first report on the method of isolation of high-quality RNA for cDNA synthesis as well as isolation of the calmodulin gene sequence from this fungus.

1. Introduction

Marssonina coronaria (Ellis and J.J. Davis) [teleomorph known as *Diplocarpon mali* (Y. Harada & Sawamura)] is a causative agent of Marssonina blotch causing premature leaf fall problem of apple. It affects apple production in India as it also attacks the fruit by causing circular dark brown spots on all the commercial cultivars, thereby downgrading the quality of the marketable produce (Sharma et al., 2004; Zhao et al., 2010). *In vitro* raised colonies of *M coronaria* are slow growing, show typical characteristics of having dark brown to black colouration without aerial mycelia and forming a wrinkled surface (Sharma et al., 2004; Lee et al., 2011). Localized melanization of appressoria is a crucial factor for the pathogenicity of *Marssonina sp.* (Emma et al., 2010).

Many fungal species produce melanin, often providing protection from ultraviolet radiation, oxidizing agents and ionizing radiation. It also contributes to the virulence of pathogens and plays a role in fungal pathogenesis (Eisenman and Casadevall, 2012). Melanin contained in pigment cells in a variety of tissues gets co-purified with nucleic acids (RNA or DNA) in standard extraction procedures and inhibits the activity of thermostable DNA polymerase in PCR as well as reverse transcription polymerase chain reactions (RT-PCR) (Yoshii et al., 1993; Giambernardi et al., 1998; Eckhart et al., 2000). Islas-Flores et al. (2006) while working with *Mycosphaerella fijiensis* have already reported the precipitation of melanin and faced a significant challenge during RNA extraction in terms of quantity and quality of RNA for preparation of cDNA libraries.

Our laboratory has been working on the genetic transformation of apple in a view to develop transgenic apple resistant to fungal pathogens (Sharma et al., 2012). In this context, the work has been initiated on host RNA interference (RNAi) mediated silencing of vital genes of the target phytopathogenic fungus, *M. coronaria* for fungal resistance.

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We are particularly interested in the isolation of partial gene sequences from the genome of *M. coronaria* and use for the development of RNAi construct in later stage. We focused on isolation of the calmodulin gene sequence because of its role in fungal differentiation and in the development of specialized structures (appressoria) among other fungi (De Carvalho et al., 2003; Warwar and Dickman, 1996; Magalhães et al., 1991).

For this, extraction of a sufficient quantity of high quality RNA is the first essential step for cDNA synthesis, subsequent PCR amplification, and isolation of gene sequences. Different RNA extraction methods vary in yield, quality and integrity of the RNA, which affect the downstream applications (Kałużna et al., 2016). In our initial attempts to isolate RNA, we took 30 d old fungal cultures grown in Peptone supplemented Potato Dextrose Agar (PPDA) and faced problems of low yield, poor quality and melanin co precipitation during the process. Therefore, keeping in view the above points, an extensive evaluation of different RNA extraction methods was carried out in order to obtain a good quantity of pure, melanin-free RNA from *M. coronaria* suitable for RT-PCR and isolation of calmodulin gene sequence.

2. Materials and methods

2.1. Collection of fungal isolate and inoculum preparation

Infected apple leaves were collected from an apple orchard in Himachal Pradesh during the month of July 2015 and the single spore isolation of the pure culture was performed as described by Lee et al. (2011) with slight modifications in the final medium composition and temperature. In the final step, after incubation at 25 °C for another week, isolated single colonies were transferred to final medium PPDA [PDB, HiMedia, India (200 g potato, 20 g dextrose), 10 g peptone, 15 g agar in 1 L of distilled water and pH 7.0] and were allowed to grow for 30 d at the same temperature (25 °C). The colony obtained was directly used as an inoculum to extract RNA from solid medium (PPDA) grown fungus. Simultaneously, the resulting colonies were detached from the PPDA with the help of an autoclaved spatula and ground with an autoclaved mortar and pestle in 1 mL of sterile water and then diluted in 50 mL of sterile water to make an inoculum for fresh fungus culture to be grown in Peptone supplemented Potato Dextrose Broth (PPDB) and used for RNA extraction.

2.2. Harvest of fungal material for RNA extraction

For solid medium cultures, the fungal colonies growing on PPDA in petriplates (Fig. 1a) were harvested after 10^{th}, 15^{th}, 20^{th}, 25^{th} and 30^{th} d of culturing by lifting the colony from the surface using the flat side of clean spatula and 100 mg of the fresh biomass was directly processed for RNA extraction. In case of liquid cultures, 1 mL of the previously obtained inoculum was added to each 150-mL culture flask containing 50 mL of PPDB and incubated at 25 °C with constant shaking at 150 rpm for 15 d. The fungal biomass from liquid culture was collected on the 15th d of culturing by centrifuging at 13,000 × *g* for 5 min followed by snap chilling in liquid nitrogen and then stored at -80 °C until further use.

2.3. DNA extraction and PCR amplification of internal Transcribed Spacer (ITS) and 28S rRNA from M. coronaria

Genomic DNA was extracted from 100 mg of 25 d old fungal biomass as described by Liu et al. (2000) followed with a final clean up step using Dneasy® Kit (Qiagen, Germany) as per the manufacturer's instructions. The ITS and partial 28S rRNA were amplified using universal primer pairs, ITS1: 5′-TCC GTA GGT GAA CCT GCG-3′/ITS4: 5′-TCC TCC GCT TAT TGA TAT GC-3′ (White et al., 1990) and NL1: 5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′/NL4: 5′-GGT CCG TGT TTC AAG ACG G-3′ (O'Donnell, 1993). Amplification reactions were performed in a total volume of 20 μL containing 10 μL of EmeraldAmp® GT PCR Master Mix (Takara, Japan), 0.25 μM of each primer, 200 ng of genomic DNA of *M. coronaria* and final volume with H_2O (Takara, Japan). PCR was performed using a GeneAmp® PCR System 9700 (Applied Biosystems, US) with the following program: an initial denaturation stage of 5 min at 94 °C, followed by 25 cycles of denaturation for 1 min at 94 °C, annealing for 30 s at 50 °C (ITS1/ITS4) or 55 °C (NL1/NL4), extension for 1 min at 72 °C, and a final 7 min extension at 72 °C. Amplified products were analyzed by gel electrophoreses on 1% agarose gel with 1 kb DNA ladder (GeneRuler ™, Thermo Scientific, US) as a marker.

2.4. M. coronaria inoculation on apple leaves

Plants of apple cultivar 'Starking Delicious' were obtained from the fruit science nursery of Dr. Y. S. Parmar University of Horticulture & forestry and maintained in the glass house of the Department of Biotechnology. *In vitro* inoculation of *M. coronaria* on healthy apple leaves was performed according to the procedure outlined by Li et al. (2014) with some modifications. Inoculated leaves were incubated in the dark for 2 d at 25 °C with a relative humidity of 95–100% and then shifted to normal photoperiod (16 h light/8 h dark). After 20 d post-inoculation, dark green patches and black lesions having fungal growth on infected leaves (Fig. 1d) were cut with a sharp sterile blade and leaf pieces were immediately frozen in liquid nitrogen and stored at −80 °C until further use. A few infected leaf pieces were cultured on PPDA and resulting colonies were morphologically characterized to complete the Koch's postulates.

2.5. RNA extraction methods

100 mg harvested fungal biomass and the same quantity of infected leaf pieces were ground separately in liquid nitrogen to a fine powder using a pre-chilled mortar and pestle. In order to avoid thawing of the homogenized tissue, the material was transferred to a 2-mL Eppendorf tube, prefilled with the different extraction buffers as per the method used followed by quick vortexing (10 s). Water used to prepare the solutions and buffers was treated with 0.1% diethyl pyrocarbonate (DEPC; Sigma-Aldrich, US). The tips, tubes and bottles were treated with 0.1% DEPC solution at 37 °C overnight, autoclaved twice at 121 °C for 20 min, and baked at 60 °C for 6 h before use. Glass containers, mortars, pestles, and spatulas were washed with distilled water, autoclaved twice at 121.1 °C for 15 min and then baked overnight at 200 °C.

2.5.1. TRIzol™ Reagent

For TRIzol alone, a method was modified from the manufacturer's (Invitrogen, US) instructions. Briefly, after the sample was agitated vigorously for 15 s in 1.5 mL TRIzol™ Reagent, the aqueous phase containing RNA was separated from the interphase and the organic phase by the addition of an extra chloroform step followed by an RNA precipitation step at −80 °C for 4 h, using 0.5 mL isopropanol (IPA). The RNA pellet was then washed in 75% ethanol. Finally, the pellet was air-dried for 10 min, redissolved in 40 μL of nuclease-free water, and stored at −80 °C.

2.5.2. TRIzol™ Reagent + RNA Clean & Concentrator™-5

Carefully transferred the upper aqueous phase obtained in the previous method after double chloroform treatment into an RNase-free tube and followed the manufacturer's instruction as per the RNA Clean & Concentrator™-5 kit (Zymo Research, US) for each volume of the aqueous phase. Finally, the RNA was eluted in 40 μL nuclease-free water and stored at −80 °C.

2.5.3. CTAB-PVP

The CTAB extraction method of Wang and Stegemann (2010) with slight modification was used. For this, each sample powder was