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COMPARISON OF METHODS FOR ISOLATING HIGH QUALITY GENOMIC DNA AND TOTAL RNA FROM THE MYCELIA OF AN OIL PALM PATHOGEN, *Ganoderma boninense*

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ABSTRACT

Isolation of high quality and intact nucleic acids are important steps for a number of molecular techniques. The problems that are normally associated with nucleic acid isolation from filamentous fungi are the presence of high polysaccharide contaminants and tough cell walls. In this study, we compared several published protocols for efficient DNA and RNA isolation from the mycelia of an oil palm pathogen, *Ganoderma boninense*. Three protocols were analysed for the isolation of genomic DNA, with the best protocol being the hexacetyltrimethylammonium bromide (CTAB) method. This method yielded 336.5± 56.9 µg/ml genomic DNA from 0.1 gm of mycelia, with minimal carbohydrate contamination. This method also produced DNA of sufficient quality for PCR amplification of *G. boninense* DNA. A total of four protocols were analysed for RNA extraction. Among the four protocols, LiCl, SDS and phenol yielded the highest amount of total RNA, wherein a total of 359.83 ± 67.8 µg/ml total RNA from 0.1 gm of mycelia was obtained. Spectrophotometric assessment of the RNA indicated relatively high purity and an absence of carbohydrate contamination. This method produced RNA of sufficient quality that was suitable for RT-PCR of *G. boninense* genes.

Key words: Isolation, protocol, genomic DNA, total RNA, *Ganoderma boninense*

INTRODUCTION

Ganoderma boninense is one of the most threatening pathogens in the oil palm industry and causes basal stem rot (BSR) (Flood *et al*., 2002). It has the capacity to destroy large amounts of living stand with a small amount of *G. boninense* inoculums. Although control methods are available to suppress the proliferation of *G. boninense*, these methods are time consuming and not effective in the field (Hushiarian *et al.*, 2013). Thus, more accurate methods are needed to combat *G. boninense* infection. Therefore, understanding its pathogenicity at the molecular level is important for the development of strategies that can control *G. boninense* effectively. However, due to limitations in molecular techniques, attempts to dissect the molecular events that lead to the *G. boninense* pathogenicity are still in their infancy.

Isolation of high purity and intact nucleic acids is a critical step for a number of molecular techniques. The isolation of pure genomic DNA is crucial for studies involving gene isolation by Polymerase Chain Reaction (PCR), Southern blotting and the construction of DNA libraries, while high quality RNA is important in analysing gene expression profiles, which use techniques such as reverse transcriptase-polymerase chain reaction (RT-PCR), northern blot, real-time PCR and microarray analysis. However, the problems that are usually associated with isolating high quality nucleic acid from filamentous fungi include the presence of high polysaccharide contamination and tough cell walls of the mycelia (Karim *et al.*, 2007). Therefore, a number of protocols have been developed for the isolation of fungal DNA and RNA (Sokolovsky *et al.*, 1990; Viogt *et al*., 1999; Kamaruddin *et al*., 2007; Molsem *et al*., 2010). Many of these protocols seem to be suitable for certain species of fungi and may not be versatile and efficient for other fungal species (Viogt *et al.*, 1999). Therefore, it is important to consider and optimise several protocols to obtain a good quantity and quality of nucleic acid when working with new

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fungal species. Thus, this study will identify the best nucleic acid extraction protocol for the further molecular genetic studies in this fungus.

MATERIALS AND METHODS

Fungal strain and cultivation

G. boninense strain PER71 was obtained from the Malaysian Palm Oil Board (MPOB). Single spores were isolated from the fruiting body and maintained on Potato Dextrose Agar (Oxoid, USA) until further manipulation. Mycelia that were grown on PDA were cut, transferred into 150 ml of Potato Dextrose Broth (Difco, France) and incubated on an orbital shaker (150 rpm) at 28°C for 1 week. The mycelia were collected, washed with distilled water and frozen in liquid nitrogen to facilitate the genomic DNA and RNA extraction process.

Genomic DNA isolation and validation

A total of four protocols were analysed for genomic DNA isolation. The first protocol used was the extraction method using hexacetyltrimethyl-ammonium bromide (CTAB) as described by Voigt *et al*. (1999) (hereafter referred to as the Voigt method). The second protocol was the extraction protocol using CTAB, polyvinylpyrrolidone (PVP) and β-mercaptoethanol as described by Aras *et al.* (2007) (hereafter referred to as the Aras method). The third protocol used organic acids as described by Molsem *et al*. (2010) (hereafter referred to as the Molsem method). The concentration and quality of the extracted genomic DNA was analysed using a spectrophotometer (Eppendorf, Germany). To determine the quality of the DNA for enzymatic activity, DNA was used as a template in a PCR reaction to amplify the internal transcribe spacer (ITS) region of *G. boninense* as described by Latiffah *et al*. (2002).

RNA isolation and validation

A total of four methods were analysed for RNA isolation from *G. boninense*. The first RNA extraction protocol used CTAB and PVP in the lysis buffer as described by Yang *et al*. (2008) (hereafter referred to as the Yang method). The second protocol used LiCl and phenol in the lysis solution as described by Rosas-Cardenas *et al*. (2011) (hereafter referred to as the Rosas-Cardenas method). The third protocol followed the method described by Kansal *et al.* (2008) (hereafter referred to as the Kansal method) wherein Urea and LiCl were added to the lysis buffer. The final method followed the protocol described by Sokolovsky *et al*. (1990) (hereafter referred to as the Sokolovsky method) wherein organic acids were used during the extraction. The concentration and quality of the total RNA extracted were analysed using a spectrophotometer (Eppendorf, Germany). To determine the quality of the RNA in an enzymatic reaction, RT-PCR was performed. For the RT-PCR analysis, 50 ng of RNA was reverse transcribed using the Access RT-PCR kit (Promega, USA) with the following parameters: 48°C for 45 minutes (reverse transcription step) and 94°C for 2 min (reverse transcriptase inactivation), which was then followed by the PCR amplification steps: 95°C for 5 min (1 cycle); 95°C for 20 sec, 65°C for 30 sec, and 72°C for 1 min (39 cycles); finally an extension at 72°C for 20 min (1 cycle) was performed. The forward primer 5' CCTTAAGGTATGCGAATTCGG 3' and reverse primer 5' GTGTACTGCTTGAACGT GAGCA 3' were designed to amplify the mitogen-activated protein kinase (MAPK) with an expected amplicon of 200 bp.

RESULTS AND DISCUSSION

Although various protocols have been developed to extract nucleic acid from filamentous fungi, these methods are usually efficient for certain groups of fungi or for different fungal morphological forms. Hence, it is important to establish the best protocol for nucleic acid isolation from a particular fungus. This will help facilitate the subsequent steps in molecular genetic studies, especially during experiments that involve enzymatic reactions. As *G. boninense* is a major fungal pathogen of the oil palm, the establishment of suitable DNA and RNA isolation protocols that can produce high yields of genetic materials with minimal contaminants is an important starting point for subsequent molecular genetic studies.

The three protocols that were assessed for DNA extraction in this work were reported to produce good quality genomic DNA from fungi and lichen. Table 1 shows the yield and quality of *G. boninense* DNA that was isolated using the three protocols while Fig. 1A shows the integrity of the extracted DNA on agarose gel. The results from three independent DNA extractions show that the protocol using organic acids from the Molsem method produced the highest yield of DNA. In this method, a total of 378.3 ± 20.0 µg of DNA from 0.1 g of mycelia was obtained. However, the quality of the DNA isolated using this method is lower than the other two protocols. Protein and carbohydrate contaminations were present because the absorbance ratios of $A_{260/280}$ and $A_{260/230}$ were lower than 1.8. For a high purity preparation of nucleic acid, the $A_{260/280}$ ratio, which represents protein contamination, should be between 1.8 to 2.0, while

Table 1. Comparison of the yield and quality from various genomic DNA isolation methods. All of the values are the means of a triplicate analysis

Methods	DNA yield (µg/gm mycelia)	$A_{260/280}$	$A_{260/230}$
Aras method	290.7±35.7	1.91±0.23	1.67±0.31
Molsem method	378.3±20.0	1.70±0.71	1.67±0.18
Viogt method	336.5±56.9	1.81±0.36	1.75±0.43

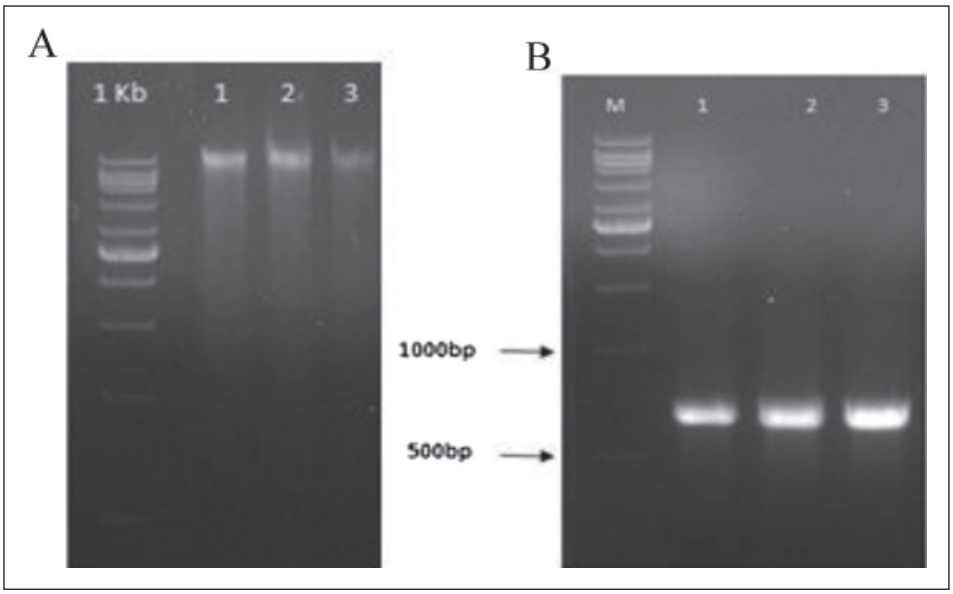

Fig. 1. A) Genomic DNA extracted using three different protocols electrophoresed on agarose gel. B) PCR product of ITS region amplified via various protocols. Lane 1: Genomic DNA extracted using the Aras method, Lane 2: Genomic DNA extracted using the Molsem method and Lane 3: genomic DNA extracted using the Viogt method, M: Marker 1 kb (Invitrogen, USA)

the $A_{260/230}$ ratio, which represents carbohydrate contamination, should be more than 2.0 (Sambrook and Russel, 2001). In the Molsem method, a relatively simple DNA extraction buffer was used, and the quantity of DNA obtained was higher than the other methods. With the aid of sodium dodecyl sulphate (SDS), the disruption of the mycelia cell wall and cell membrane were much more efficient. However, higher carbohydrate contamination, as detected by spectrophotometry, indicated that this protocol failed to remove some of the polysaccharides contaminants during the extraction protocols. Meanwhile, the protocols that use CTAB (for both the Voigt and the Aras methods) yielded a better a quality of DNA, but at a lower quantity. CTAB, coupled with certain concentration of sodium chloride, has the ability to remove polysaccharides by increasing their solubility in the isopropanol and preventing their co-precipitation with the DNA (Puchooa, 2004). The addition of PVP to the extraction buffer removed phenolic compounds and prevented the browning of DNA, which is caused by polyphenol oxidation. However, the addition of β-mercaptoethanol in the Aras method resulted in a lower yield of DNA being extracted. The addition of β-mercaptoethanol breaks the disulphide bonds in protein molecules, thereby reducing the presence of protein contaminant in the final product. However, β-mercaptoethanol has the ability to bind to phenolic compounds, which later forms a cross-link to a high molecular weight DNA, resulting in a loss of DNA due to co-precipitation (Nguyen *et al.*, 2009). To test the integrity and the suitability of the extracted DNA for enzymatic reactions, PCR was carried out using primers designed to amplify a 650-bp amplicon of the *G. boninense* ITS region. Fig. 1B shows that all of the samples could be used to amplify the targeted DNA fragment. This suggests that all three protocols were able to produce a relatively good quality DNA that is suitable for PCR reaction.

A total of four methods were analysed for *G. boninense* total RNA extraction using CTAB lysis buffer from the Yang method, lithium chloride coupled with phenol lysis buffer from the Rosas-Cardenas method, urea and lithium chloride from the Kansal method and organic solvent from the Sokolovsky method. Table 2 shows the yield and quality of *G. boninense* total RNA that was isolated from different methods. The data show that the Rosas-Cardenas method produced the highest yield of total RNA, wherein 359.83 ± 67.8 µg of RNA was obtained from 0.1 g of mycelia. However, a very minimal amount of protein contamination was detected from this protocol. Fig. 2A shows the results of the gel electrophoresis of the total RNA extracted from the mycelia of *G. boninense*. All of the protocols led to the isolation of RNA from *G. boninense*, which resulted in two distinct bands of 28S and 18S ribosomal RNA. In addition, no traces of genomic DNA were observed when samples were electrophoresed on ethidium bromide-stained agarose gel, thus proving that all four protocols resulted in an efficient removal of genomic DNA from the RNA. Lithium chloride has been used in all of the protocols except in the Yang method. It offers major advantages over other RNA precipitations in which, with the aid of lithium chloride, the efficient precipitation of DNA, protein and carbohydrate was observed for easy removal. It also removes inhibitors of translation from RNA preparations (Cathala *et al.*, 1983). However, in each method described above (except the Yang method), the concentration of lithium chloride varies. In addition to lithium chloride, surfactant agents, such

Table 2. Comparison of the yield and quality of RNA isolation via different protocols. All of the values are the means of triplicate analysis

Methods	RNA yield (µg/gm mycelia)	$A_{260/280}$	$A_{260/230}$
Yang method	182.13±34.1	2.09±0.04	1.85±0.08
Sokolovsky method	224.21±67.0	1.88±0.12	1.70±0.15
Kansal method	207.33±10.0	1.86±0.07	1.99±0.04
Rosas-Cardenas method	359.83±67.8	1.75±0.11	1.94±0.16

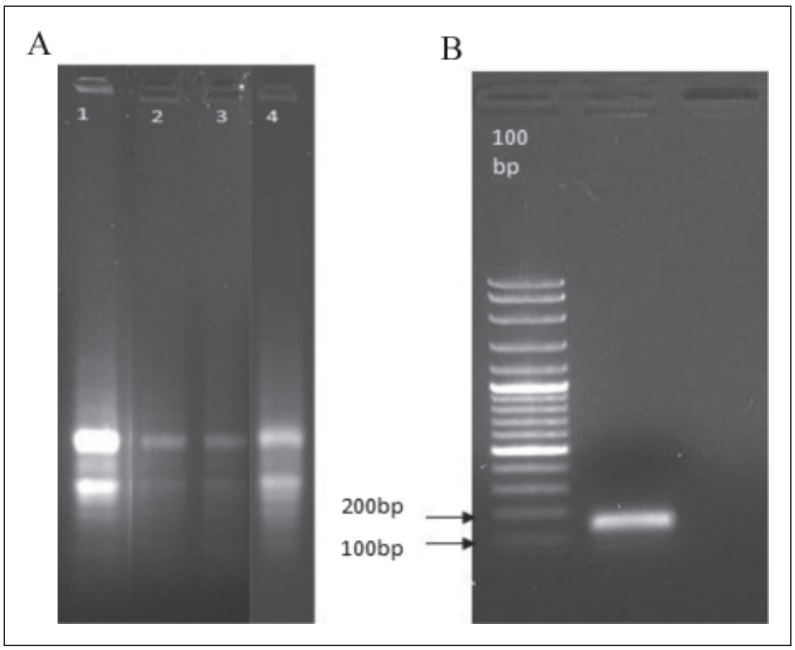

Fig. 2. A) Total RNA extracted using different protocols electrophoresed on agarose gel. Lane 1: the Rosas-Cardenas method, lane 2: the Yang method, lane 3: the Sokolovsky method, lane 4: the Kansal method. B) RT-PCR amplicon of a MAP kinase gene using RNA extracted from Rosas-Cardenas method

as CTAB or SDS, were used in these protocols. However, methods that included SDS, with the aid of phenol, gave better yields of total RNA compared to CTAB. In addition to this, potassium acetate was also used in the Yang, Rosas-Cardenas and Kansal methods to aid in the removal of polysaccharides from the solution mixture. Efficient removal was observed in the Rosas-Cardenas method, in which the $A_{260/280}$ ratio as 1.94±0.16. This indicates that the sample was clean from polysaccharide contamination and should be suitable for downstream processes. Because the Rosas-Cardenas method produced the highest yield of RNA of high purity, we tested the suitability of RNA extracted using this protocol for a RT-PCR reaction. Fig. 2B shows the 150-bp RT-PCR product of a MAP kinase gene electrophoresed on an agarose gel. The presence of a single intact band indicates that the RNA extracted via the Rosas-Cardenas method was essentially free of contaminants that could inhibit enzymatic reactions and suitable for RNA profiling techniques.

In conclusion, our results show that the Voigt method is the most suitable method for DNA extraction from the mycelium of *G. boninense*. This method produced the most considerable amount of genomic DNA, with very minimal carbohydrate contamination. Similarly, the Rosas-Cardenas method is the most effective method to extract a high yield and good quality of RNA from *G. boninense* mycelium.

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