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SHORT COMMUNICATION

A low-cost disruption of rhizospheric microorganisms for the extraction of total RNA using modified RNeasy Mini Kit protocols

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Abstract A low-cost disruption of jute (*Corchorus olitorius*) rhizospheric microorganisms (*Aspergillus niger* and *Arthrobacter* sp.) with naturally occurring sand for extracting total RNA using modified QIAGEN RNeasy Mini Kit protocols is described. The method yielded distinct 18S and 28S, and 16S and 23S subunits of the fungal and bacterial ribosomal RNAs, respectively, on formaldehyde agarose gels. Total RNA extracted using this method was suitable for reverse transcription PCR to study the differential expression of mRNAs under the stress of herbicides. Primers OPM 1 and OPP 15 were effective in identifying upregulated transcripts in glyphosate and paraquat stressed *A. niger*. Similarly, primers OPP 13 and OPR 7 were effective in identifying upregulated transcripts in glyphosate-stressed *Arthrobacter* sp. This is the first report to show that natural sand can be combined with QIAGEN RNeasy Mini Kits for the extraction of total RNA from rhizospheric microorganisms. Novelty of the present method lies in the grinding of samples without the need of liquid nitrogen and then direct purification of RNA using modified QIAGEN RNeasy Mini Kit protocols, without chloroform extraction.

Keywords *Aspergillus niger* · *Arthrobacter* sp. · Disruption · Rhizosphere · RNA extraction · RNeasy

Disruption of cells for the extraction of ribonucleic acids (RNA) from biological samples is the first and foremost step in any protocol. Thorough and fast disruption not only increases the yield, but also protects the RNA from degradation, which is very important for downstream analysis. Chemical, enzymatic and mechanical or physical methods are available for disruption of cells obtained from different biological samples (Burden 2012). It is suggested that the cells that are not efficiently disrupted by chemicals and enzymes, such as Gram-positive mycobacteria, should also be disrupted by mechanical means (Mangan et al. 1997). Mechanical disruption coupled with chemical or enzymatic treatments may speed up the process, and gives a high yield of good quality RNA. However, in some cases where chemical or enzymatic disruption do not give good results, mechanical disruption of cells is the only option (Burden 2012). Mechanical disruption of cells includes grinding in liquid nitrogen with mortar and pestle, shearing in dounce glass homogenizers, beating using glass beads and shocking using sonicators.

Mechanical disruption of cells with glass beads or liquid nitrogen has been performed for the extraction of RNA using QIAGEN RNeasy Mini Kits (Zhang et al. 2006; Lim et al. 2008; García-Nogales et al. 2010; Leite et al. 2012; Atkinson et al. 2013). High yields of good quality total RNA are reported to have been extracted by mechanical disruption of filamentous fungi and Gram-positive bacteria using glass beads (Oh and So 2003; Leite et al. 2012). However, mechanical disruption using glass beads is time consuming and needs special expensive pieces of equipment that are not always available to every laboratory. Similarly, liquid nitrogen may not be available at all times and its handling can be hazardous. Therefore, a low-cost, easily available, suitable and safe material that can efficiently disrupt both prokaryotic and eukaryotic cells for the extraction of high yield, good quality RNA without the need of enzymes, hazardous chemicals such as liquid nitrogen, or special equipment is essential.

Some microorganisms, such as the filamentous fungus *Aspergillus niger* and the Gram-positive *Arthrobacter* sp., may not be efficiently disrupted either chemically or

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enzymatically. This is due to their rigid and thick cell walls, which release certain enzymes that help them degrade various substances. *Aspergillus niger* can release highly active endogenous RNases during disruption; whereas *Arthrobacter* sp. can degrade a variety of substances, including chemicals, to use as a carbon and energy source (Singh and Walker 2006; Sallau et al. 2013). With endogenous RNases released by *A. niger* and degradation of chemicals by *Arthrobacter* sp., these microorganisms should be disrupted rapidly to protect those RNAs with short half-lives from degradation. However, this is not possible either with enzymes or chemicals, because the disruption process involving these substances is very slow. Consequently, chemical and enzymatic disruption of these microorganisms may result in a very low yield and poor quality RNA. In this paper, we introduce naturally occurring sand as an easily available, safe and very cheap material for mechanical disruption of jute (*Corchorus olitorius*) rhizospheric *A. niger* and *Arthrobacter* sp.

The microorganisms *A. niger* (GenBank Accession No. HM136829) and *Arthrobacter* sp. (sequence not submitted to GenBank) were isolated from jute (*C. olitorius*) rhizosphere, as described previously (Kharbikar et al. 2009). Internal transcribed spacer (ITS) regions of the ribosomal RNAs (rRNAs) were sequenced to identify the microorganisms using BLAST search. For disruption of cells, acid-washed fine sand (particle size<2 mm) was used instead the of liquid nitrogen and glass beads that are recommended for the disruption of fungi and bacteria, respectively. The sand was submerged in 37 % hydrochloric acid (Merck, India) in a glass vial and vigorously shaken for few minutes. Acid was decanted and the sand was thoroughly washed twice by submerging in RNase-free water before autoclaving.

For the total RNA extraction from *A. niger*, the original RNeasy Plant Mini Kit (QIAGEN, India) protocol was modified at the second and third steps. Briefly, 100 mg of mycelia was harvested from culture grown in potato dextrose broth by centrifugation at 10,000 x *g* for 10 min at 4 °C. The supernatant was decanted and any remaining media was carefully removed by aspiration. Since centrifugation was used in later steps, pellets were heated to 20 to 25 °C and washed twice with RNase-free water. For washing, 500 μl of RNase-free water was added to the pellets, vortexed for 10 to 15 s and centrifuged at 5,000 x *g* for 5 min at 4 °C. The supernatant was decanted and the washing was repeated. Four hundred and fifty microliters of Buffer RLC was added to the pellets and vortexed vigorously for 15 s. The resulting suspension was transferred into 1.5 ml Safe-Lock tubes containing the acid-washed fine sand, and cells were disrupted with full force using a small plastic pestle for 5 min. Disruption was performed on ice. The supernatant was incubated for 3 min at 56 °C and centrifuged for 20 s at 10,000 x g. All the other steps in the protocol were performed at room temperature according to the manufacturer's instructions.

For the extraction of total RNA from *Arthrobacter* sp., the original RNeasy Mini Kit (QIAGEN, India) protocol for total RNA extraction from bacteria was modified at the first, second, third and fourth steps. An additional step (washing) was incorporated after the second step. In the first step, 50 mg acid-washed fine sand was used instead of glass beads. In the second step, 1 ml of log phase culture (*ca*. 1×10^9 bacteria), grown overnight, was pipetted out in 2 ml microcentrifuge tubes and centrifuged at 5,000 x *g* for 5 min at 4 °C. The supernatant was decanted and the tubes were again filled with 1 ml of the same culture (*ca*. 1×10^9 bacteria). Centrifugation was repeated to get the final pellets. Pellets obtained in the second step were washed twice with RNase-free water (ca. 500 μl), as described for *A. niger*. In the third step, 525 μl instead of 350 μl of Buffer RLT was added and vortexed vigorously for 5 to 10 s. In the fourth step, cells were disrupted with full force in 1.5 ml Safe-Lock tubes containing acid-washed fine sand for 5 min using a small plastic pestle. This step was performed on ice. All the other steps in the protocol were performed at room temperature according to manufacturer's instructions.

Total RNAs extracted from *A. niger* and *Arthrobacter* sp. were quantified by a spectrophotometer (Aquarius 7000 Series, Cecil Instruments, India). The samples with an acceptable purity (260/230 ratio≥2.0) were electrophoresed on a 1 % denaturing formaldehyde (HCHO) agarose gel to check the quality. Suitability of RNA for downstream analysis was confirmed by differential display reverse transcription PCR (DDRT-PCR) of glyphosate and paraquat (25 ml l^{-1} and 10 ml l^{-1}, respectively)-stressed *A. niger* and glyphosate (25 ml l^{-1})-stressed *Arthrobacter* sp. First strand cDNA was prepared using M-MuL V reverse transcriptase (Fermentas, India) and amplified by PCR, using random primers as anchored and arbitrary primers. Differentially displayed mRNAs were detected by resolving 10 μl of each PCR product mixed with 2 μl of 6X gel loading dye on 1.6 % agarose gels.

Electrophoretograms of total RNA preparations from *Arthrobacter* sp. (Lanes 1, 2 and 3) and *A. niger* (Lanes 6 and 7) on an ethidium-bromide–stained denaturing formaldehyde gel are shown in Fig. 1. The method yielded distinct 16S and 23S subunits of the bacterial and 18S and 28S subunits of the fungal rRNAs, respectively. Total RNA extracted using this method was suitable for RT-PCR to study the differential expression of *A. niger* and *Arthrobacter* sp. mRNAs under the stress of herbicides. DDRT-PCR of glyphosate and paraquat-stressed *A. niger* using random primers yielded differentially displayed bands in the gel (Fig. 2a). Primers OPM 1 and OPP 15 were effective in identifying upregulated transcripts. Similarly, DDRT-PCR of glyphosate-stressed *Arthrobacter* sp. using random primers also yielded differentially displayed bands in the gel (Fig. 2b). Primers OPP 13 and OPR 7 were effective in identifying upregulated transcripts.

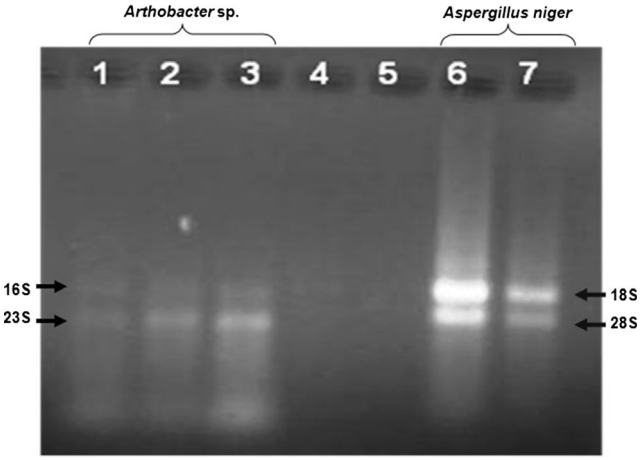

Fig. 1 Ethidium-bromide–stained denaturing formaldehyde gel electrophoresis of RNA preparations from rhizospheric microbes obtained after cell disruption using natural sand. Individual extractions were repeated thrice for *Arthrobacter* sp. and twice for *A. niger*. Lanes 1, 2, and 3: *Arthrobacter* sp. 16S and 23S rRNA; lanes 6 and 7: *Aspergillus niger* 18S and 28S rRNA

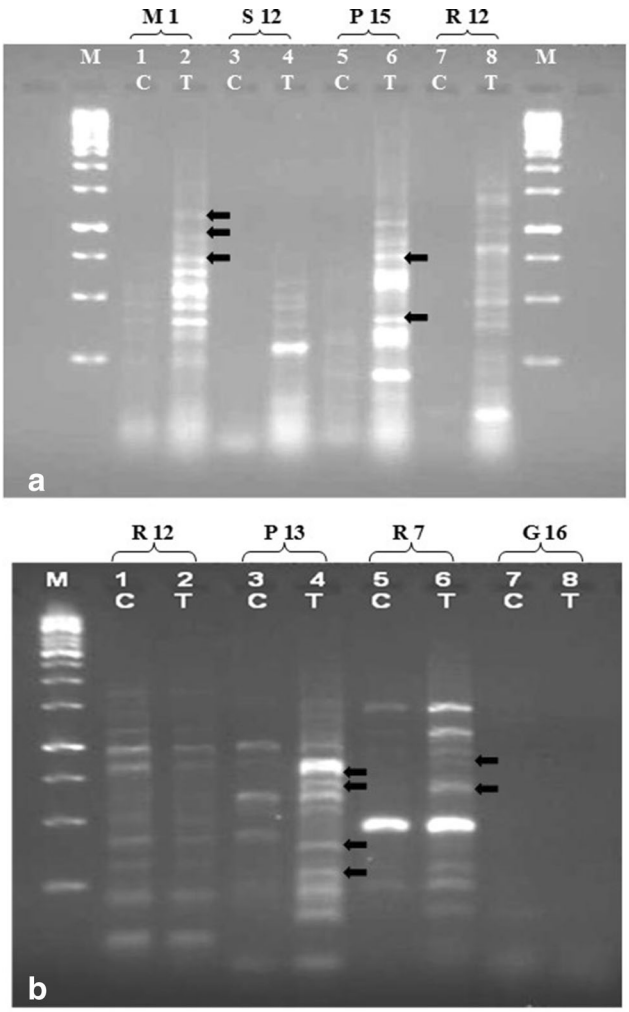

Fig. 2 Differential display reverse transcription PCR (DDRT-PCR) profiles of glyphosate+paraquat (25 ml l^{-1} and 10 ml l^{-1} respectively)-stressed *Aspergillus niger* (**a**) and glyphosate (25 ml l^{-1})-stressed *Arthrobacter* sp. (**b**). Differentially displayed bands in DDRT-PCR of RNA extracted from control (C) and herbicide-stressed (T) microbes using different random primer combinations have been marked with *black arrows*. Primers OPM-1 (Lanes 1 and 2) and OPP-15 (Lanes 5 and 6) in *A. niger* and primers OPP-13 (Lanes 3 and 4) and OPR-7 (Lanes 5 and 6) in *Arthrobacter* sp. were effective to identify upregulated transcripts. M: 1 kb DNA ladder

This is the first report to show that natural sand can be combined with QIAGEN RNeasy Mini Kits for the extraction of total RNA from rhizospheric microorganisms. Sand has already been applied in previous studies for the extraction of RNA from environmental samples (Hurt et al. 2001; Rittmann and Holubar 2014) and a filamentous fungus (Shu et al. 2014). In those studies, sand was combined with liquid nitrogen to grind the samples and the RNAs were extracted using chloroform, before purification with either commercial kits or indigenous protocols. The novelty of the present method lies in the grinding of samples without the need of liquid nitrogen, and then direct purification of RNA using modified QIAGEN RNeasy Mini Kit protocols, without chloroform extraction.

Commercially available natural sand is over 60 times cheaper than the glass beads recommended for the disruption of bacteria. The liquid nitrogen, which is recommended for the disruption of filamentous fungi, is very cheap, but its cryogenic container is extremely expensive. The container for liquid nitrogen storage may not be considered as a full contribution towards the cost of RNA extraction protocol in big laboratories. However, the container is a big deal in small laboratories such as in developing and underdeveloped countries, and adds to the total cost of cell disruption. Hence, disruption of rhizospheric microorganisms using natural sand for RNA extraction is highly cost-effective and suitable for small laboratories having limited resources.

One may argue that commercial RNA extraction kits are usually very expensive (Shu et al. 2014). However, when both qualitative and quantitative recovery of the RNA is particularly important, an anion-exchange–based commercial kit (QIAGEN, Santa Clarita, California) is better than the traditional Trizol-reagent–based method (Life Technologies) of RNA extraction (Hurt et al. 2001). To minimize the cost of RNA extraction, we initially tried the trizol-reagent–based method, but failed to extract the total RNA both from *A. niger* and *Arthrobacter* sp. (data not shown). Shu et al. (2014) also failed to extract the total RNA from the sclerotia of a filamentous fungus, *Rhizoctonia solani*, using a modified Trizol method. However, they could amplify the specific gene fragments in RT-PCR following the RNA extraction using a commercial fungal RNA kit (E.Z.N.A.™, Omega, GA, USA). Considering this, we used QIAGEN RNeasy Mini Kits, even

if they are bit expensive, for the guaranteed RNA extraction in the present study.

In previous studies, QIAGEN RNeasy Mini Kits have been used for the extraction of RNA from various biological samples, including plants (Atkinson et al. 2013), microorganisms (Lim et al. 2008; García-Nogales et al. 2010; Leite et al. 2012), viruses (Zhang et al. 2006) and animals (Gelsthorpe and Sokol 1997). The authors followed the kit manufacturer's instructions where glass beads or liquid nitrogen were used for the disruption of cells. However, non-availability of these materials during the period of our study prompted us to search for an easily available, suitable material that could efficiently disrupt both prokaryotic and eukaryotic cells for the extraction of high quality RNA. Enzymes or chemicals could have been used for the disruption of cells. However, enzymes take more time for the disruption of cell walls (De et al. 2010). This might be due to the presence of chitin, (1–3)-β-D-glucan, (1–6)-β-glucan, lipids and peptides in the cell walls of filamentous fungi and a thick peptidoglycan layer composed of two sugar derivatives (N-acetylglucosamin and N-acetylmuramic acid), a small group of amino acids (L-alanine, D-alanine, D-glutamic acid, and lysine) and pentaglycine interbridge in cell walls of Gram-positive bacteria (Francesconi et al. 2008; Vollmer et al. 2008). Similarly, a variety of chemicals, including those involved in cell disruption, can be degraded by Gram-positive bacteria. (Singh and Walker 2006). Hence, to obtain a high yield of good quality RNA from a filamentous fungus or a Gram-positive bacterium such as *A. niger* and *Arthrobacter* sp., the cells should be disrupted rapidly. In addition to this, the chemicals involved in the disruption process should not be degraded by these microorganisms. Since the disruption process involving enzymes is very slow and the chemicals may be degraded by such microorganisms, enzymes and chemicals are not good options for the disruption of such cells.

Mechanical disruption of filamentous fungi and Gram-positive bacteria using glass beads has been reported to give high yields of good quality total RNA (Oh and So 2003; Leite et al. 2012). Leite et al. (2012) used equipment such as a homogenizer and a tissue lyser for bead beating, and extracted the RNA using the RNeasy® Plant Mini Kit (Qiagen, Germany). Whereas, Oh and So (2003) used a vortex machine for bead beating and extracted the RNA using a hot phenol method. Equipment is not always available to every laboratory and phenol is hazardous. Therefore, we decided to use a material similar to glass beads but without enzymes, hazardous chemicals such as liquid nitrogen and phenol, or special equipment.

Sand is a naturally occurring granular material that can also be used for applying a force for disruption of cells by mechanical action. It can act as a physical chaotropic agent and help release the nucleic acids by disrupting the cells (Chan et al. 2004). Therefore, in the present study, cells of *A. niger* and *Arthrobacter* sp. were disrupted by applying a force of naturally occurring sand with the mechanical action of small pestles in microcentrifuge tubes. Although grinding with small pestles is time consuming, combining this method with RNeasy Mini Kits compensated for the total time required for RNA extraction. The total time required for RNA extraction using this method was comparable with other studies (Zhang et al. 2006; Lim et al. 2008; García-Nogales et al. 2010; Leite et al. 2012; Atkinson et al. 2013). In those studies, disruption of cells by various means followed by RNA extraction using RNeasy Mini Kits took approximately 30 to 60 min. Using the present method, RNA extraction from *A. niger* and *Arthrobacter* sp. took approximately 30 and 45 min, respectively.

Cell disruption using sand may increase the temperature during the grinding process, which could potentially also lead to RNA degradation. To avoid this, grinding for the disruption of cells was performed on ice. This maintained a lower temperature in the tubes and protected the RNA from degradation due to the increasing temperature. Leite et al. (2012) also cooled the samples by placing them in ice or briefly in liquid nitrogen, or even by stopping the bead beating and resuming it after a few seconds. Although their technique was also effective at maintaining a lower final temperature in the tubes and gave a higher quality RNA, it appears to be inconvenient due to its haphazard manner. Holding the tubes continuously on ice, while grinding for 5 min in our study, was more convenient as it was not haphazard.

We optimized the present RNA extraction method as a whole, including the quantity of cells and buffers and the time of incubation. The present method as a whole did work, but the Trizol method for RNA extraction from *A. niger* and *Arthrobacter* sp. did not (data not shown). The quantity of cells and buffers and the time of incubation, as mentioned in the original RNeasy Mini Kit protocol, did not give results, but the modified protocol, as described in the present study, did. However, the statistical analysis of results could not be performed, since we did not test these parameters at various levels. Other parameters, such as different cell disruption methods (e.g., using liquid nitrogen), different quantities of sand, changes in the particle size of sand, etc., could also have been optimized. However, we have not optimized these, as the RNA extracted using the present method was sufficient and suitable for downstream analysis. The yield of total RNA extracted by the present method was low, as seen in denaturing gel electrophoresis; however, it was suitable for DDRT-PCR. Since, DDRT-PCR particularly requires a small quantity of high-quality total RNA, this method of downstream analysis is useful when only small numbers of cells are available (Sturtevant 2000). The method is robust and can compare organisms under different environmental conditions. In the present study, although the yield of RNA was low, randomly primed DDRT-PCR produced distinct profiles of mRNA

expression in glyphosate and paraquat-stressed *A. niger* and glyphosate-stressed *Arthrobacter* sp., suggesting that the RNA might be of good-quality. These results suggest that the low quantity of RNA extracted using the present method may also be used in specific and comparative gene expression studies where qualitative recovery of RNA is important. A low quantity of RNA (less than 5 ng of RNA, 10^4 stem cells) has also been used to display the differential expression of genes in hematopoietic stem cells (Rosok et al. 1996).

RNA quantification using state-of-the-art analysis techniques such as Nanodrop and Agilent bioanalyzer requires expensive equipment that is not always available to every laboratory. Quantification by a simple spectrophotometer and a quality and integrity check on a denaturing formaldehyde (HCHO) agarose gel are sufficient to understand whether the RNA is suitable for further downstream analysis. The present method yielded RNA of acceptable purity (260/230 ratio≥2,.0) with distinct bands corresponding to small and large ribosomal subunits on a denaturing agarose gel. The RNA was suitable to use in DDRT-PCR for further downstream analysis. García-Nogales et al. (2010) also used a spectrophotometer having a quartz cuvette measurement system for quantification of RNA. Similarly, Shu et al. (2014) used a 1 % agarose gel to assess the quality and integrity of total RNA, which could be used for RT-PCR analysis in their study.

In the present study, the use of naturally occurring sand for disruption of microbial cells gave good quality total RNA. Sand has also been potentially incorporated for the grinding of a secondary metabolite-rich seaweed, *Gracilaria changii* (Chan et al. 2004). Grinding of *Gracilaria changii* using sand significantly increased the yield of RNA that was suitable for cDNA synthesis. The present method of cell disruption using natural sand for RNA extraction from rhizospheric microorganisms has potential for use in comparative gene expression studies, where the quality of RNA as well as its integrity are imperative.

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