

is not permeable to ICM cells, while H333342 has the complete potency to enter all cells. Keeping this in mind, this approach is proposed as a feasible technique for assessment of blastocyst quality. For examining the quality of the embryos, around 50 stained blastocysts were observed under an epifluorescence microscope with excitation wavelength of 330–385 nm and barrier filter 400 nm. Photographs were taken and total nuclei stained were manually counted. ICM cells are shown in blue colour, while TE cells are shown in red colour (Figure 1). The results of this technique might be useful for examination effect of chemical modifiers, culture system, different physiological conditions on embryos health both for animal and human applications.

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ACKNOWLEDGEMENTS. This work was supported by the National Agriculture Innovative Project grant (1(5)/2007-NAIP-2) to S.K.S. We thank M. K. Singh for encouragement and valuable suggestions.

Received 29 January 2011; revised accepted 19 March 2012

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An RNA isolation protocol for recovery of high quality functional RNA from fungi and plants

Obtaining high quality, intact RNA is the first and often the most crucial step in performing many fundamental molecular biology experiments, including reverse transcriptase PCR (RT-PCR), Northern analysis, nuclease protection assays, RNA mapping, *in vitro* translation and cDNA library construction. However, the presence of high levels of polyphenols, polysaccharides and RNAase in the tissues of both prokaryotes and eukaryotes makes RNA extraction often difficult, challenging and requires extensive alteration in RNA isolation protocols. Thus, RNA recovery differs based on the levels of polyphenols and polysaccharides in different tissues¹.

Trichoderma (class Ascomycota, order Hypocreales, family Hypocreaceae), the free-living, beneficial fungi, commonly found in soil, with capability to produce antibiotics and lytic enzymes (cellulase, hemicellulase, xylanase, chitinase) of industrial interest, are useful for plant protection purposes in agriculture². Recent discoveries show that they are opportunistic, avirulent plant symbionts as well as parasites of other fungi. At

least some strains establish robust and long-lasting colonization of root surfaces and penetrate into the epidermis and a few cells below³. Unavailability of suitable RNA isolation protocols leads to troubles in obtaining sufficient amounts of good quality RNA from *Trichoderma*, which poses hindrance to several molecular biological studies. Cell walls or fungal structures, viz. conidia, hyphae, etc. which are resistant to lysis slow down high-quality RNA isolation, often leading to modifications of existing protocols⁴. There are several reports on total RNA isolation from filamentous fungi⁵. However, an optimized methodology that could provide large quantity of pure total RNA for gene expression studies is always lacking.

Plants are diverse, and individual species and organs or tissues of plants behave differently during RNA isolation. Hence, a range of extraction protocols has also been devised, depending on the tissue or genotype being extracted. The most common problems encountered while extracting RNA from plants include high levels of RNAases; the

presence of large quantities of polysaccharides; different kinds of phenolics, including tannins; low concentrations of nucleic acids (high water content), and tissue, such as lignin (wood), that is difficult to break up. Several CTAB, SDS and GT methods have been developed to isolate total RNA from tissue containing high levels of polyphenols and polysaccharides^{6–8}. In addition, there are several commercial kits and triazole (Sigma-Aldrich Co., USA) available for RNA isolation. However, a single protocol for isolation of high-quality RNA both from fungi and plants tissue is not available till date. Therefore, the objective of the present study was to develop a single, rapid and versatile method for the isolation of total RNA from *Trichoderma* spp. and rice (*Oryzae sativa*).

Five millimetre discs of actively growing mycelium of *Trichoderma harzianum* was taken from the margins of fungal growth and transferred to petri dishes (9 cm in diameter) containing about 20 ml PDA (200 g potato, 2% dextrose, 2.5% agar in 1 l of water). The agar percentage was increased (from 2 to 2.5)

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from normal PDA so as to scrap the mycelium with the help of a spatula without disturbing the surface of the PDA, which may otherwise hamper the isolation of high-quality RNA. Plates were incubated at $27 \pm 2^\circ\text{C}$ till the mycelial growth of the fungus completely covered the medium surface.

Rice seeds (var. Pusa Basmati-1) were sterilized in 1% (v/v) sodium hypochloride for 20 min and thoroughly rinsed in sterile distilled water. After the rinsing seeds were placed on a wet blotting paper till germination. The germinated seeds were then grown hydroponically in tap water for a week, and the seedlings were transferred to nutrient solution and grown for two weeks. Total RNA was isolated from leaves of three-week-old rice seedlings.

The tubes, bottles, pestle and mortar were treated with 0.1% DEPC solution at 37°C overnight, autoclaved twice at 121°C for 20 min, and then dried at 100°C before use. Microtips used for RNA extraction were RNAase and DNAase-free (Axygen®, USA). Triazole reagent (Invitrogen®) was used as extraction buffer. Additionally, a mixture of chloroform/phenol (1:1 v/v) was also prepared. All buffers and solutions were incubated at 37°C overnight prior to autoclaving.

For RNA isolation from *T. harzianum*, the petri dishes with full growth of the fungus were taken off the incubator and the total mycelial growth was scrapped using sterilized spatula inside laminar flow. The mycelial mat was stored in sterilized filter paper and lyophilized. For RNA isolation, 1 ml extraction buffer was added to 10 mg of lyophilized mycelium, which was ground with DEPC-treated, sterilized pestle and mortar in liquid nitrogen and shaken vigorously for 30 s. Then 0.5 ml of chloroform-phenol mixture was slowly added and incubated at 65°C for 5 min. The mixture was cooled to room temperature and centrifuged at $10,000\text{ g}$ at 4°C for 5 min. The supernatant was transferred to a new microtube, and one-fold volume of cold isopropanol or ethanol was added and mixed thoroughly for precipitating total RNA at -20°C for 20 min. The mixture was then centrifuged at $12,000\text{ g}$ for 10 min. The pellet was washed with 75% ethanol twice and centrifuged at $10,000\text{ g}$ at 4°C for 5 min. The supernatant was discarded and the pellet was resuspended in 0.1 ml RNA storage solution (Am-

bion®), and stored at -20°C for further use.

Similarly, for isolation of total RNA from rice plants, 20 mg of plant material was ground thoroughly with DEPC-treated, sterilized pestle and mortar in liquid nitrogen. Other steps were the same as mentioned earlier. Concentration and yield of RNA was checked based on absorbance at 260 and 280 nm (A_{260}/A_{280} ratios) with $5\ \mu\text{l}$ of resuspended total RNA. Then $5\ \mu\text{l}$ of total RNA solution was loaded onto 1% agarose gel and electrophoresed to separate RNA. To visualize the size and distribution of total RNA, ethidium bromide (EtBr) and UV light were used.

Two microgram of total RNA was used to prepare cDNA. For $12\ \mu\text{l}$ reac-

tion volume the following components were added to a nuclease-free $200\ \mu\text{l}$ PCR tube: RNA $2\ \mu\text{g}$, oligo dT (Qiagen, USA) $-1.25\ \mu\text{l}$, dNTP Mix (Fermantas, Canada) ($10\ \text{mM}$) $-1\ \mu\text{l}$, and sterile distilled water to make the total volume to $12\ \mu\text{l}$. Reaction mixture was heated to 65°C for 5 min using a thermocycler (Eppendorf AG 22331, Germany) followed by quick chill on ice and a brief centrifugation to collect the contents. Subsequently the following components were added: $5\times$ first strand buffer (Invitrogen, UK) $-4\ \mu\text{l}$, $0.1\ \text{M}$ DTT (Invitrogen, UK) $-2\ \mu\text{l}$ and RNAase inhibitor (Ambion, USA) ($40\ \text{units}/\mu\text{l}$) $1\ \mu\text{l}$. The contents of the tube were mixed gently and incubated at 37°C for 2 min. Then $1\ \mu\text{l}$ ($200\ \text{units}$) of MMLVRT (Invi-

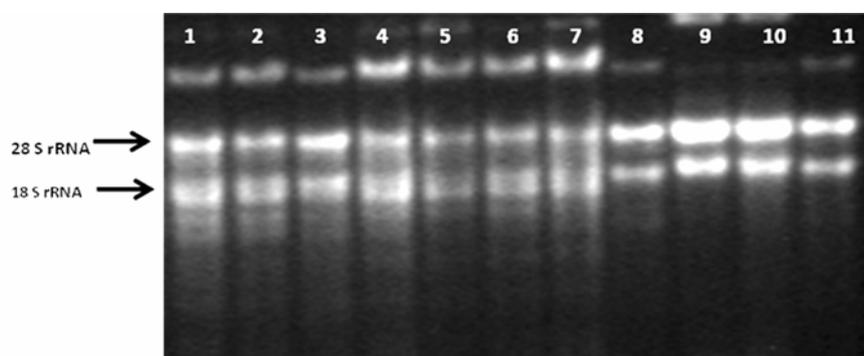


Figure 1. Total RNA isolated from different *Trichoderma harzianum* (lanes 1–7) and rice (lanes 8–11) samples.

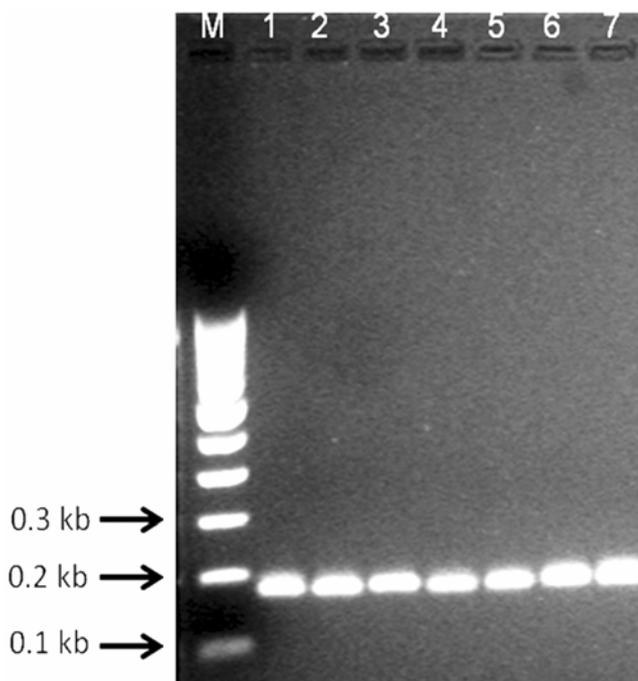


Figure 2. Electrophoresis of RT-PCR products of *actin* transcript from *T. harzianum* on 1.2% (w/v) agarose gel. M, marker; lanes 1–7, actin gene from different samples.

trogen, UK) was added and mixed by pipetting gently up and down. The mixture was incubated for 50 min at 37°C. Reaction was inactivated by heating at 70°C for 15 min and the cDNA prepared was stored at -20°C till further use. *T. harzianum* actin gene-specific primers, actin forward (5'-ATG TGC AAG GCC GGT TTC GC-3') and actin reverse (5'-TAC GAG TCC TTC TGG CCC AT-3') were designed using Primer 3 software from reference sequences. For rice, tubulin-specific primers, i.e. tubulin-forward (5'-CCC CCA TGC TAT CCC TCG TCT C-3') and tubulin reverse (5'-CTC GGC CGT TGT GGT GAA TGA-3') were used for expression study⁹.

In a 50 µl PCR reaction mixture the following components were added: PCR buffer (Invitrogen, UK) (10×) 5 µl, MgCl₂ (Invitrogen, UK) (50 mM) 1.5 µl, dNTP Mix (Fermantas, Canada) (10 mM) 1 µl, forward primer (10 µM) 1 µl, reverse primer (10 µM) 1 µl, *Taq* DNA polymerase (Invitrogen, UK) (5 U/µl) 0.4 µl, cDNA (from first-strand reaction) 2 µl and nuclease free water (Promega, USA), 38.1 µl. The temperature profiles used for the PCR were 94°C for 5 min (initial denaturation) followed by 35 cycles of 94°C for 30 s (denaturation), 55°C for 30 s (annealing) and 72°C for 1 min (extension) and final extension for 10 min. After completion of the PCR cycles, 8 µl of the PCR product was analysed on 1.2% agarose gel by electrophoresis.

Special care and precautions are required for RNA isolation as it is susceptible to degradation¹⁰. RNA is especially unstable due to the ubiquitous presence of RNAases, which are the enzymes present in blood, all tissues, as well as most bacteria and fungi in the environment¹¹. RNA extraction relies on good laboratory and RNAase-free techniques. RNAases are heat-stable and refold following heat denaturation. They are difficult to inactivate as they do not require cofactors. The two most common RNA isolation methods¹⁰ which have been in use since decades include utilization of 4 M guanidinium thiocyanate and of phenol and SDS. Many standard protocols and commercial kits available in the market do not work well when it comes to high-quality RNA isolation, indicating that sample preparation for RNA isolation may contain such high levels of secondary metabolites that the RNA is readily lost together with these complexes during subsequent rounds of extraction or precipitation. Due to the biochemical diversity and structural divergence of organisms, a common RNA extraction protocol for different groups of organisms does not exist. Here we have made an attempt to resolve this problem to a certain extent. A common protocol has been devised for the isolation of total RNA from *T. harzianum* mycelium and rice leaves.

In the current protocol higher-strength SDS or β -mercaptoethanol was not

used¹²; rather Triazole reagent was used as lysis buffer. Phenol/chloroform was used¹² to denature RNAases and remove proteins much more effectively than chloroform and β -mercaptoethanol¹³. The 100% cold isopropanol or ethanol allowed better RNA precipitation, rendering it more stable¹².

Before treatment of the fungus or plant tissues with triazole reagent, grinding with pestle and mortar in liquid nitrogen produced better results compared to the absence of this step. Gonzalez-Mendoza *et al.*¹⁴ also reported better yield of RNA when tissues were ground before chemical lysis.

RNA was proficiently extracted with no degradation, but some DNA contamination was observed in the fungus as well as plant RNA. In all RNA samples, two distinct bands corresponding to 28S and 18S rRNA could be seen (Figure 1), with clear distinction and poor degradation. To evaluate quantity, quality and integrity of isolated RNA, the absorbance A_{260/280} was determined. The A_{260/280} ratio was in the range of 1.65–1.8, suggesting better purity of RNA fraction in the mixture and possibility of its further use in other studies like expression profiling, Northern blotting, nuclease protection assays, RNA mapping, *in vitro* translation and cDNA library construction.

Concentration of RNA was 123.25 and 117 µg/mg fresh weight of *T. harzianum* mycelium and rice leaves respectively, which was almost similar to the concentration (126 µg RNA/mg fresh wt) obtained by Li *et al.*¹⁵ in *Lentinula edodes* mycelium using SDS-phenol method of RNA isolation. Keeping sensitivity of reverse transcription to impurities in view, purity of isolated RNA or its suitability for RT-PCR was further tested by RT-PCR analysis of the actin gene of *T. harzianum* and tubulin gene of rice. In both the cases gene-specific primer efficiently amplified the actin gene of *T. harzianum* (Figure 2) and tubulin gene of rice (Figure 3), suggesting high purity of mRNA in the sample. Thus the protocol proposed here is proficient (about 2 h) and less expensive for the isolation of good-quality RNA from fungi as well as from plants.

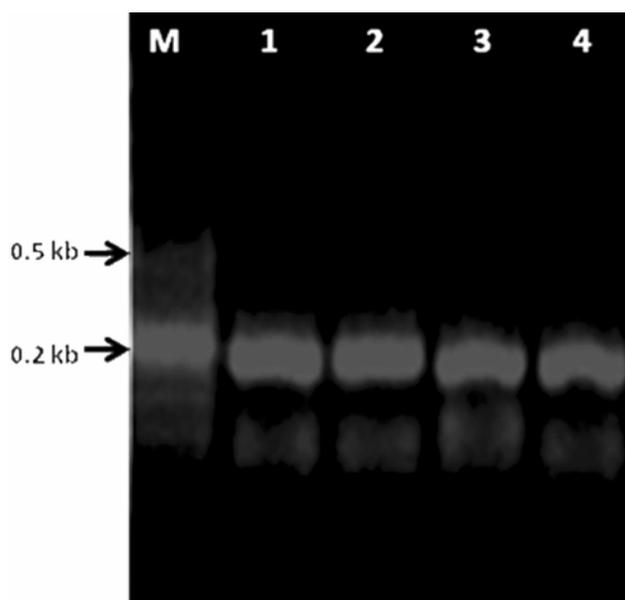


Figure 3. Electrophoresis of RT-PCR products of *tubulin* transcript from rice on 1.2% (w/v) agarose gel. M, Marker; lanes 1–4, tubulin gene from different samples.

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Received 15 July 2011; accepted 26 March 2012

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A lion figurine with non-Acheulian Lower Palaeolithic implements

The Lower Palaeolithic culture in India has yielded some of the earliest evidences of palaeoart and these are consistent with the rest of the world¹. Evidences of palaeoart are crucial in considering the cognitive and intellectual status of the early hominins. Earliest palaeoart forms include various types of perforated objects, figurines, petroglyphs, petrographs, manuports, etc.

Among the perforated objects, beads were identified from some of the Acheulian sites in France and England² and their characteristics were confirmed with the discovery of disc beads made of ostrich egg shells dated by Th/U to 2 lacks from a Late Acheulian site in Libia, El Greifa³. The discovery of a cupule from *Olduvai Gorge*⁴, and a grinding stone from South Africa is attributed to the Lower Palaeolithic.

Lower Palaeolithic sites at Bilzingsleben have yielded engraved bone fragments, ivory and engraving on a quartzite slab along with thousands of non-Acheulian implements⁵ roughly of 300 ka (refs 6 and 7). The engraved rectangular pattern on Blombos Cave hematite slab of 77 ka and several engraved bones found with Micoquian industry and Middle Pleistocene fauna from the gravel pit at Thuringia, Germany⁸, a fragment of banded ironstone bearing a set of seven curved, sub-parallel lines incised with stone tools from a late fauresmith context⁹ dated between 420 ka and 260 ka

are some of the earliest known palaeoart in those remote times, which suggest that long-lived conventions definable as ‘traditions’ already existed¹⁰.

The existence of figurines in the Lower Palaeolithic has only recently been seriously considered and till now only two specimens have been designated as proto-figurines. Figurines should resemble another object and must have the indication of modification by human hand in order to emphasize their iconicity. One such find from Berekhat Ram,

Israel¹¹, has been considered older than 230 ka. It has been further studied and its artefact nature confirmed by Marshack¹², and his findings were corroborated by d’Errico and Nowell¹³. The second figurine from Tan-Tan, Morocco, is considered 400 ka old on the basis of the Lithic typology¹⁴. Here lies the importance of a line figurine discovered from Abhayagiri, Kerala.

The Lower Palaeolithic rock art in India includes the petroglyphs in the Auditorium Cave at Bhimbetka, Madhya



Figure 1. Lion figurine from Abhayagiri.