ISOLATION OF TOTAL RNA AT YELLOW STAGE OF *JATROPHA CURCAS* SEEDS FROM THAILAND HYBRID

TERRY MADATANG

25278

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Department of Molecular Biology

Faculty of Resource Science and Technology

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List of Abbreviations

AGE	Agarose Gel Electrophoresis
cDNA	Complementary deoxyribonucleic acid
CIA	Chloroform: Isoamyl
CTAB	Cethyl trimethyl ammonium bromide
DEPC	Diethyl pyrocarbonate
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
PCR	Polymerase Chain Reaction
PVP	Polyvinylpyrrolidone
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse transcription- PCR
TAE	Tris-Acetate
tRNA	Transfer RNA

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Isolation of Total RNA at Yellow Stage of *Jatropha Curcas* Seeds from Thailand Hybrid

Terry Madatang

Resource Biotechnology
Department of Molecular Biology
Faculty of Resources Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

Jatropha curcas seeds contain a number of secondary metabolites and polysaccharides, which make the isolation of total RNA difficult to be done. The aim for this study is to isolate high quality of total RNA from yellow stage of the *J. curcas* seed. RNA extraction is done by using several RNA extraction protocol with modifications. Three extraction methods have been used in this research. Those methods were CTAB-LiCl I, CTAB-LiCl II and CTAB-LiCl III. For this research, seeds from the *J. curcas* hybrid from Thailand was taken as sample. Among these three methods, the CTAB-LiCl III was the preferred method. This method was able to yield up to 0.531 (μg/μl) with the the A_{260}/A_{280} and A_{260}/A_{230} ratio ranged from 1.704-1.795 and 0.796-0.918 respectively. By using the CTAB-LiCl III protocol, the RNA was successfully isolated.

Keywords: Jatropha curcas, yellow stage seed, total RNA, RNA extraction, CTAB-LiCl

ABSTRAK

Biji daripada buah Jatropha curcas mengandungi banyak metabolit sekunder yang telah menyebabkan proses pemencilan bagi jumlah keseluruhan RNA dalam J. curcas agak sukar untuk dijalankan. Tujuan utama kajian ini adalah untuk memencilkan jumlah RNA yang berkualiti tinggi daripada biji peringkat kuning J. curcas. Pemencilan RNA telah dilakukan dengan menggunakan beberapa kaedah pemencilan RNA yang telah diubah suai. Kaedah-kaedah tersebut ialah CTAB-LiCl I, CTAB-LiCl II dan CTAB-LiCl III. Untuk kajian ini, hanya biji daripada J. curcas hybrid dari Thailand sahaja yang dipilih. Di antara kesemua kaedah tersebut, CTAB-LiCl III adalah kaedah yang paling sesuai untuk memencilkan RNA. Kaedah ini dapat menghasilkan sebanyak 0.531 (μg/μl) dengan nisbah A_{260}/A_{280} dan A_{260}/A_{230} berkisar dari 1.704-1.795 dan 0.796-0.918 masing-masing. RNA dalam biji J. curcas telah berjaya dipencilkan dengan menggunakan kaedah CTAB-LiCl III.

Kata kunci: Jatropha curcas, biji peringkat kuning, jumlah RNA, pemencilan RNA, CTAB-LiCl

1.0 INTRODUCTION

Jatropha curcas is a tropical shrub native to Mexico and Central America, but is widely distributed in wild or semi cultivated stands in Latin America, Africa, India and South-East Asia. The tree is well adapted to arid conditions. It is suitable for sand dune stabilization and soil conservation areas (Openshaw, 2000). *J. curcas* has achieved significant economic importance due to its industrial uses (Openshaw, 2000) and as a very promising source of non-edible oil that can be used as feedstock for production of bio-diesel.

The studies of the seeds and also its oil content have showed that the *J. curcas* potential in biodiesel aspect still not widely known and used. Unlike the palms biodiesel, *J. curcas* biodiesel cannot be used in food industry. It is not possible to use the seed oil as cooking oil due to the content of toxic compounds (Martinez-Herrera, 2006). The use of *J. curcas* oil can be widen by determining the gene which expressed fatty acid inside the seeds for further use in biodiesel production. cDNA library should be constructed in order to determine the specific gene, and RNA isolation is necessary as the first step for this purpose.

Isolation of RNA was more difficult compared to isolation of DNA because RNA sequences were not as stable as the DNA sequences. The main problem which arises in this research was the amount of polyphenols, polysaccharides and proteins which present in a large amount in the *J. curcas* seeds. According to Pawlowski *et al.* (1994), the high quantity content of those secondary metabolites substances in the tissues of *J. curcas* seeds can interfere in the total RNA extraction process because those substances were co-precipitated with the RNA.

The main aim of this research was to determine and generate an effective method in isolating the high quality of total RNA in the yellow stage of *J. curcas* hybrid from

Thailand. Various RNA extraction methods were used in order to find out which method able to isolate the total RNA of *J. curcas* efficiently. For this research, seed from yellow stage were used because from previous research, the seed from this stage have produced highest fatty acid content compared to other stages (Emil *et al.*, 2010). The seeds from Thailand hybrid were used rather than the Malaysia hybrid because according to Emil *et al.*, (2010), the Thailand hybrid produced higher fatty acid content compared to Malaysia hybrid.

Three methods have been applied to compare the efficiency of each method in isolating the total RNA in *J. curcas* seeds. The methods used were CTAB-LiCl method I, CTAB-LiCl method II and CTAB-LiCl method III. LiCl were used because it was able to purify and precipitate RNA extracted from DNA and other contaminants (Barlow *et al.*, 1963). The total RNA extracted was evaluated by using the RNA quantification and the success of the high quality RNA extracted was determined by less contamination of the secondary metabolites substances and by distinct bands of 28S and 18S RNA. The extracted total RNA can be used in some of the bio-molecular applications such as cDNA library construction so that the specific gene which related to the fatty acid production can be determine and amplified for further use in biodiesel aspect.

Thus, the objectives of this study are:

1. To isolate high quality of total RNA from yellow stages of *J. curcas* seeds, by using the *J. curcas* hybrid from Thailand as the sample.
2. To generate and modifiy an optimized method or protocol for total RNA isolation of *J. curcas* seeds.

2.0 LITERATURE REVIEW

2.1 *Jatropha curcas*

J. curcas was introduced in Africa centuries ago and is now naturally growing in drier areas in many countries. It is a multipurpose and drought resistant large plant or small tree. The main stem of the plant should be cut once the tree is 1 m tall. This will lead to increased branching of the tree. The more branches a plant has, the higher the production of fruits and therefore more seeds. The fruits of *J. curcas* mature 2-3 months after flowering. The fruits have three seeds and they mature when the nut changes from green to yellow after two to three months of maturity (Figure 2.1). The nut split when mature and dries to release the seeds. The seeds are black or greyish and thin shelled when mature. This plant is currently seen as the new sources of biodiesel. It has become more attractive because of it environmental benefits and renewable resources (Ma and Hanna, 1999).

The fact that *J. curcas* oil cannot be used for nutritional purposes without detoxification makes its use as energy or fuel source very attractive as biodiesel. In Madagascar, Cape Verde and Benin, *J. curcas* oil was used as mineral diesel substitute during the Second World War (Agarwal, 2007). *J. curcas* is a multipurpose small tree belonging to the family of Euphorbiaceae. It is a plant with many attributes, multiple uses and considerable potential. The plant can be used to prevent and control erosion, to reclaim land, grown as a live fence, especially to contain or exclude farm animals and be planted as a commercial crop. It is a native of tropical America, but now thrives in many parts of the tropics and sub-tropics in Africa and Asia (Martınez-Herrera *et al.*, 2006).

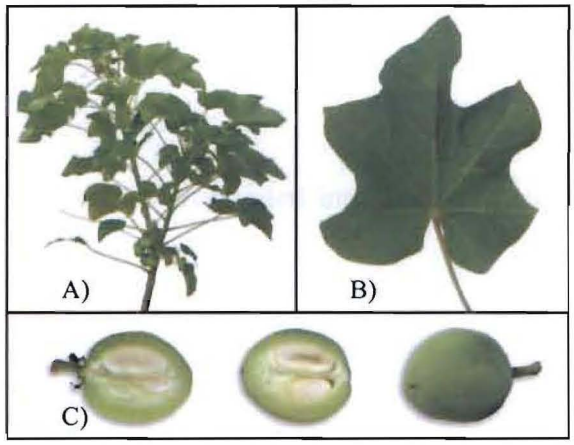

Figure 2.1: Morphology of *Jatropha curcas*. A) tree B) leaves C) fruit and seed.

(Source:http://carboncapture.us/img/jatropha_curcas.JPG&imgrefurl=http://carboncaptue.us/docs/Jatropha_Curcas_080424).

2.2 *Jatropha curcas* seeds

J. curcas seeds are recalcitrant, that is, they are sensitive to desiccation. Therefore, they should be moderately dried to enable the fruits release the seeds. The temperatures should not exceed 45 °C because at that temperature the fruits will open up releasing the seeds respectively, facilitating the separation from the fruits. During drying the temperatures should not be above 45 °c because the proteins (oil) within the seeds will get de-natured. Therefore, temperature control must be well managed. While under direct sunlight the seeds must be well spread in thin layers with a good aeration and turned over frequently. The seeds should not be stored or put in polythene bags which can cause suffocation or create a "green house" over the seeds, because this will destroy the oil and germination of the seeds. The seeds should be stored at low temperatures or at least at de-ionized water to prevent bacteria and fungus growth. Oxygen is needed for these seeds. Since they continue to respire they should be stored in open or perforated gunny bags or the seeds should be processed into oil or sown out immediately after collection and extraction. The fatty acid derived from the seeds of the *J. curcas* can be used to produce biodiesel. According to US

Department, they estimated that 50% of the diesel fuel can be replaced by biodiesel (Vivek and Gupta, 2004).

The seeds of *J. curcas*, which divided into 5 stages; according to its fruits color, (Figure 2.2), contain viscous oil, which can be used for manufacture of candles and soap, in cosmetics industry, as a diesel or paraffin substitute or extender. This latter use has important implications for meeting the demand for rural energy services and also exploring practical substitutes for fossil fuels to counter greenhouse gas accumulation in the atmosphere. These characteristics along with its versatility make it of vital importance to developing countries (Kumar and Sharma, 2008). In view of these, the present research was designed to study the psycho-chemical properties including the fatty acids and TAGs composition of *J. curcas* oil seed from Malaysia.

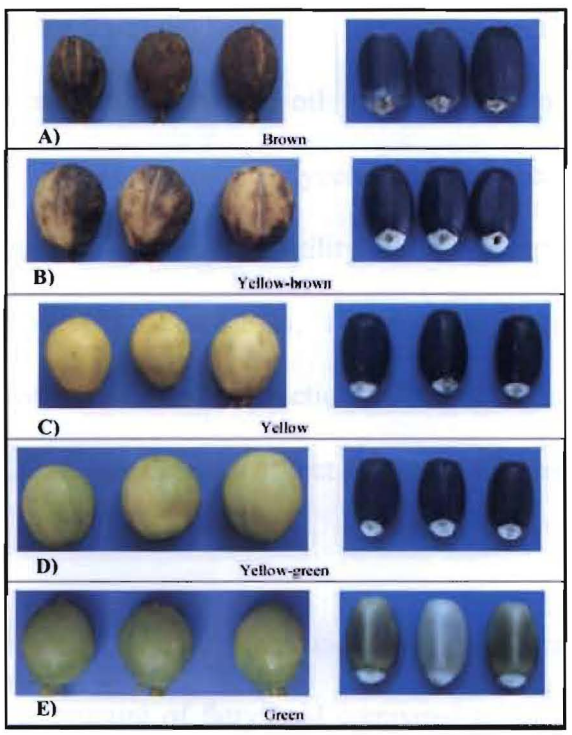

Figure 2.2: The five stages of *J. curcas*. A) Stage V (dark, mature and ripen fruits), B) Stage IV (yellow-dark fruit), C) Stage III (yellow fruit), D) Stage II (yellow-green fruit) and E) Stage I (immature fruits).

(Sources:http://www.scielo.br/scielo.php?pid=S141370542012000100005&script=sci_arttext).

2.3 *Jatropha curcas* hybrid from Thailand

In Thailand, *J. curcas* is called "Saboo Dam" which is often found near the rice fields as the farmers planted them and using them as herb. It is a large shrub with thick branches and numerous large leaves attaining a height of 3–4 m. in 3 years. It can be successfully cultivated both in irrigated and rain fed conditions. The plants grow quickly forming a thick bushy fence in a short period of time of 6–9 months, and growing to heights of 4 m. with thick branches in 2–3 years. Seeds resemble castor seeds in shape, but are smaller and brown. *J. curcas* leaves also yield a dye and latex which has many medicinal uses that could support potential pharmaceutical industry. (Augustus et *al.*, 2002).

The one obvious thing is its oil can be used in place of kerosene and diesel. It has been well promoted to make rural areas self-sufficient in fuels for cooking, lighting and motive power or converted into its methyl ester by the trans-esterification process. This involves making the triglycerides of *J. curcas* oil to react with methyl alcohol in the presence of a catalyst (NaOH/KOH) to produce glycerol and fatty acid ester which known as bio-diesel. Considering its wide spectrum of utility and the prospects of it growing in wastelands without competing with other crops, Thailand have selected *J. curcas* for investigation of its feasibility in the mass production as bio-diesel in the theme of Life Cycle Assessment and the Life Cycle Costing aspect was used to measure the economical value (Emil *et al.*, 2010).

According to Emil *et al.* (2010), the fatty acid content of *J. curcas* from Thailand hybrid were producing higher amount of fatty acid compared to Malaysia hybrid (Table 2.1). The amount of saturated and unsaturated fatty acid comparison between the hybrids from two countries can be clearly seen and observed (Table 2.1).

Table 2.1: Fatty acid composition of the *J. curcas* oil extracted from the seeds of Malaysia and Thailand.

(Source: Emil *et al.*, 2010).

Fatty acids	**Relative Composition in (%)**	
	Malaysia	Thailand
Unsaturated		
Oleic acid (18:1)	44.7	48.8
Linoleic acid (18:2)	32.8	28.8
Palmitoleic acid (16:1)	0.7	0.6
Linolenic acid (18:3)	0.2	0.1
Saturated		
Palmitic acid (16:0)	14.2	13.2
Stearic acid (18:0)	7.0	7.7
Arachidic acid (20:0)	0.2	0.3
Margaric acid (17:0)	0.1	0.1
Myristic acid (14:0)	0.1	0.1

2.4 RNA

RNA is a powerful biological material. It is an active molecule that is directly involved in performing or controlling many biological functions. Unlocking the role of the various types of RNA present in biological cellular processes is critical to developing new methods of diagnosis and understanding and treating disease (Ambros *et al.*, 2003).

The backbone of RNA contains ribose rather than 2' deoxyribose. The ribose has a hydroxyl group at the 2' position. RNA also contains uracil in place of thymine in DNA. Uracil has the same single-ringed structure as thymine, except that it lacks the 5' methyl group. Thymine is in effect 5' methyl-uracil. RNA is usually found as a single

polynucleotide chain. Except for the case of certain viruses, RNA is not the genetic material and does not need to be capable of serving as a template for its own replication. RNA functions as the intermediate, the mRNA, between the gene and the protein-synthesizing machinery (Jennifer, 2000).

RNA also functions as an adaptor, the tRNA, between the codons in the mRNA and amino acids. RNA can also play a structural role as in the case of the RNA components of the ribosome. RNA can also be a regulatory molecule, which through sequence complementarity binds to and interferes with the translation of certain mRNAs (Martin *et al.*, 2001). In all of these cases, the RNA is copied as a single strand off only one of the two strands of the DNA template, and its complementary strand does not exist. RNA is capable of forming long double helices, but these are unusual in nature.

According to Martin *et al.* (2001), since RNA only contains the exon, which are the functional coding region, analysis of total RNA will provide the information about the gene expression, regulation and function. Many applications have been using the extracted total RNA nowadays, such as in the RT-PCR, cDNA library construction, gene expression study and in vitro translation.

2.5 RNA extraction

RNA extraction is the purification of RNA from biological samples. This procedure is complicated by the ubiquitous presence of ribonuclease enzymes in cells and tissues, which can rapidly degrade RNA. There are many different types of RNA, each of which carries out diverse and important functions in the cell. The need to extract and understand the roles of these RNAs is important not only for an understanding of biology but also for

developing therapeutics. RNAs have been shown to function in transcription (pri - RNA, mRNA), splicing (snRNA), translation (tRNA, tmRNA), and post - transcription regulation (siRNA, miRNA). Unlike DNA, which is quite rugged, RNA is transient. RNA is also fragile, elusive, and difficult to recover. Proteins are expressed from DNA using RNA, with the cell carefully controlling which proteins are active, largely through overseeing the synthesis and degradation of RNA. The expression of DNA through RNA to produce new proteins could not be accomplished if the "old" RNA were still present from previous expressions of proteins; consequently, the cells routinely remove the "old" RNA by degrading it with RNase enzymes (Gjerde, 2009).

Extracting and purifying total RNA from plant tissues has traditionally been a very demanding task, requiring rigorous care and significant labor and time investment. There are four processes involved in the extraction of total RNA from plant tissues. These processes were, obtaining an appropriate tissue sample from the specific part of plant; freezing and storing the tissue sample at the correct temperature; pulverizing, macerating, or homogenizing the tissue sample, either alone or in a extraction buffer; and separating the total RNA from genomic DNA, protein, and other cellular debris in the homogenized lysate (Azevedo, 2003).

There are many established methods or protocols which can be used to isolate total RNA from various plant tissues. The methods which are commonly used in RNA extraction experiments were the guanidine thiocynate method, TRIZOL reagent and CTAB-LiCl (Azevedo *et al.*, 2003). These methods have been used for *J. curcas* and sago samples and high quality of RNA have been obtained by referring to the previous researches. Experiments involving the use of guanidine thiocynate usually give positive results, but the previous finding on bean seed shows its homogenate formed sticky glue-like gel in guanidine thiocynate extraction buffer that could not be separated during

centrifugation. This will makes the pellet cannot be dissolved completely in distilled water (Ling *et al.*, 2007). CTAB-LiCl method was usually used for plant samples which rich in secondary metabolites, such as polysaccharides and polyphenols.

2.6 RNA quantification

Spectrophotometry is one of the most useful tools available to the biochemist and biotechnologist. It offers a high degree of precision, sensitivity, and accuracy. In addition, it is inexpensive and applicable to the measurement of a variety of substances. It can measure the fraction of the incident light transmitted through a solution. In other words, it is used to measure the amount of light that passes through a sample material and, by comparison to the initial intensity of light reaching the sample, they indirectly measure the amount of light absorbed by that sample (Rendina, 1976). This light that has not been absorbed by the solution in the cuvette, will strike the phototube. The photons of light that strike the phototube will be converted into electrical energy. This current that is produced is very small and must be amplified before it can be efficiently detected. The signal is proportional to the amount of light which originally struck the phototube and is thus an accurate measurement of the amount of light which has passed through or been transmitted by the sample (Schwedt, 1997).

The concentration of DNA and RNA yield can be obtained and the protein contamination degree also can be determined by the mean of quantification (Gasic *et al.*, 2004). The quantity and quality of RNA can be quantified spectrophotometrically at 280 nm and 260 nm, since the RNA had the absorption maximum approximate 260 nm. The common type of spectrophotometer which usually used is the spectrophotometer (Ultrospec 1100 pro, Amersham Pharmacia) with the ratio of (A_{260}/A_{280} and A_{260}/A_{230})

(Azevedo, 2003). The Ultrospec™ 1100 *pro* UV/visible spectrophotometer is an easy to use instrument that is optimized for the biological science teaching laboratory and for industrial, utility QC, and environmental testing situations. In addition to the basic modes of operation, the instrument has enhanced software and method storage functionality while menu options can be password-protected if required. The instrument will output alphanumeric text to a standard parallel printer and connect to a PC using a custom, serial interface lead. It can be used in conjunction with SWIFT 1000 software, or made to download directly to a spreadsheet. The UV lamp on Ultrospec 1100 *pro* can be switched off when the required measurements are in the visible region only.

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Seed samples

J. curcas samples from Thailand hybrid was used for the total RNA isolation. The samples were obtained from UiTM Samarahan, Sarawak. Seeds from the fruits of *J. curcas* in yellow stage were used as the sample for this research. The stage of *J. curcas* can be determined basically by looking at the color of the fruits skin.

3.1.2 Reagents and Equipments

The main reagents used for this experiment were the extraction buffer containing CTAB, PVP, Tris-HCL (pH8), EDTA, NaCL and β-mercaptoethanol. Spermidine was added in the extraction buffer used in CTAB-LiCl method I and CTAB-LiCl method II. The other reagents which also been used were chloroform isoamyl-alcohol (CIA), LiCL, DEPC-treated distilled water, 70 % ethanol and liquid nitrogen. The apparatus and equipment used for this experiment are; falcon tubes, round bottom flask, centrifuge machine, water bath, oven, vortex machine, microcentrifuge tubes, pipette, pipette tips and spectrophotometer for RNA quantification.

3.2 Methodology

3.2.1 Plants sample preparation

The flesh fruits of Thailand hybrid *J. curcas* of the yellow stage were cut in order to harvest the seeds. This is done to obtain and store the seeds of *J. curcas*. After that, the seeds were sliced into small pieces and kept inside the -20 °C refrigerator. The sliced *J. curcas* seeds were being weigh into approximately 1 to 4 g. The samples were washed and soaked for a while inside the DEPC-treated distilled water (Appendix A) to remove contaminants from the samples.

3.2.2 Total RNA isolation from the seeds

For this extraction process, three methods were used to extract the RNA. These methods were CTAB-LiCl method I, CTAB-LiCl method II and CTAB-LiCl method III.

3.2.2.1 CTAB-LiCl method I

This RNA extraction method was performed according to the method proposed by Azevedo *et. al.* (2003) with some modifications. First, the *J. curcas* seeds were surface sterilized by immersing them with 70% ethanol in sterile tube and rinsed by using the distilled water for two times. After that, the sample were weighed approximately in 0.8-1.0 g which then was ground in the liquid nitrogen before transferred to a pre-chilled Falcon tube.

The sample was added into 10 mL pre-heated (42 °C) total RNA extraction buffer containing 2% CTAB, 2% PVP, 100 mM Tris-HCL (pH 8.0), 25 mM EDTA, 2.0 M NaCl,

0.05% spermidine, and 2% β-mercaptoethanol. Sample was later incubated at 42 °C in a water bath for 90 minutes. Meanwhile, this sample was taken from the water bath and vortex every 5 minutes to disrupt the tissue and help fasten up RNA extraction in the buffer. After incubation and vortex, nucleic acid was extracted by adding one volume of CIA (24:1) to the sample in the fume hood. Again the sample was placed on vortex to mix. Subsequently, sample was centrifuged at 8000 rpm, for 30 minutes at 4 °C. After that, the top aqueous phase was carefully transferred into a microcentrifuge tube. The extraction was repeated with another equal volume of CIA (24:1). The sample in the microcentrifuge tube was then first mixed by vortex and later centrifuged at 13000 rpm for 20 minutes at 4 °C to separate the phase.

After centrifugation, the top aqueous phase was recovered. A ¼ volume of 9 M LiCl was added and left overnight in 4 °C. On the following day, the sample was then centrifuged at 13000 rpm for 15 minutes at 4 °C. Supernatant was discarded and remaining white pellet was washed with 2 M LiCl and centrifuged at 13000 rpm for 15 minutes at 4 °C. Again, the supernantant was collected and discarded, and the pellet was left air-dried for 5-10 minutes. After that, the pellet was dissolved in DEPC-treated distilled water and kept at -20 °C.

3.2.2.2 CTAB-LiCl method II

This RNA extraction method was performed according to the method proposed by Zeng and Yang (2002) with some modifications. This method was specific for plant samples with high amount of polyphenols and polysaccharides. 15 mL of extraction buffer which containing 2% CTAB, 2% PVP, 100 mM Tris-HCL (pH 8.0), 25 mM EDTA, 2.0 M NaCl, 0.05% spermidine, and 2% β-mercaptoethanol was pre-warmed to 65 °C in a water bath.

After that, 0.8-1.0 g sample of *J. curcas* seeds was ground in a mortar using liquid nitrogen. After the grinding process was done, the frozen powder was quickly transferred to the pre-warmed extraction buffer and mixed completely by inverting the tube.

After that, the mixture was incubated at 65 °C for 10 minutes with vigorous shaking several times. An equal volume of CIA (24:1) was added and the mixture was shaken vigorously. After that, the mixture was subjected to the centrifugation at 10,000 *g* for 10 minutes at 4 °C. After the centrifugation finish, the very viscous supernatant was transferred into a new tube and the sample was re-extracted with an equal volume of CIA (24:1). The mixture then subjected to the centrifugation again at 10,000 *g* for 10 minutes at 4 °C. Then the supernatant was collected very slowly and carefully. During this process, the chloroform and flocculent material through filaments which formed between the interphase and the tip were not be taken and avoided very carefully. The supernatant then was centrifuged at 30,000 *g* for 20 minutes at 4 °C to pellet and discard the insoluble material. The supernatant was transferred into a new tube without disturbing the transparent drop of chloroform at the bottom of the tube. After that 0.25 mL of 10 M LiCl were added to the supernatant. The mixture was mixed well and then stored at 4 °C for overnight.

On the next day, the RNA was recovered by means of centrifugation at 30,000 *g* for 30 min at 4 °C. The viscous supernatant was completely discarded, and the pellet was washed with 75% ethanol 3 times to remove the remaining mucilage, and the sample was air dried for 10 minutes. Finally, the RNA was dissolved in DEPC-treated water. The RNA samples were kept at -80 °C until use.

3.2.2.3 CTAB-LiCl method III

This RNA extraction method was performed according to the method proposed by Gasic *et. al.* (2004) with some modifications. The original method required 3 days for extraction, but the modified method just required 2 days. In this method, sodium acetate (NaOAc) was used. Extraction buffer was used to lyse the tissue samples. By the present of this reagent, the tissue samples were physically disrupted. The extraction buffer used for this research contained 2% CTAB, 2% PVP, 100 mM Tris-HCL (pH 8.0), 25 mM EDTA, 2.0 M NaCl, and 2% β-mercaptoethanol.

Initially, before the grinding process was performed, the extraction buffer was pre-warmed at 60 °C inside the water bath. 10 mL volume of the extraction buffer was added per sample in a Falcon tube. 2 g of Thailand hybrid *J. curcas* seeds at yellow stage was ground using mortar and pestle in liquid nitrogen to fine the powder during grinding. The fine powdered sample was then transferred into a Falcon tube with the pre-warmed extraction buffer which had been prepared earlier. Then the mixture was vortex briefly and followed by incubation in water bath for 15 minutes at 60 °C. This vortexing and incubating process was repeated for 3 times. After that, the Falcon tube containing the sample was taken out from the water bath and an equal volume of chloroform-isoamyl-alcohol (CIA) was added into the mixture and vortex for 2-3 minutes. After finish with the vortexing, the sample mixture was centrifuged at 7000 g for 15 minutes at 4 °C. After 15 minutes, the Falcon tube containing the sample mixture was taken out and all the supernatant was transferred into a new Falcon tube. After that, an equal volume of CIA was added again into the Falcon tube and followed by centrifuging at 7000 g for 15 minutes at 4 °C. The supernatant was then transferred into new sterile 1.5 microcentrifuge tubes. Then the volume of 1/3 of 8.0 M LiCl was carefully added into each of the new tubes. The tubes was mixed by inversion and stored for overnight incubation at -20 °C.

After overnight incubation, the frozen sample was allowed to slowly thaw on ice. Then the tubes were centrifuged at 13000 rpm for 30 minutes at 4 °C. After centrifugation, the supernatant was discarded and the pellet was washed with 500 µl of 70% ethanol. The tubes were subjected to centrifugation again with 13000 rpm for 15 minutes at 4 °C. After the centrifugation, the supernatant was discarded again and the pellets were air dried for 5 minutes. After drying, 200 µl of DEPC-treated water was added into each tube to dissolve the pellet. After that, 20 µl of NaOAc pH 5.2 and 500 µl of 70% EtOH were added to precipitate the RNA. Then the samples were stored at -20 °C for overnight.

After the overnight incubation for the second times, the samples were centrifuged at 13000 rpm for 30 minutes at 4 °C after thawing process before the centrifugation. The supernatant were discarded and the pellet were washed with 500 µl 70% EtOH, The samples were subjected to centrifugation again at 13000 rpm for 30 minutes at 4 °C. The supernatant were discarded after the centrifugation finish. Then the pellet were air dried for 5 minutes. Finally, add 40-50 µl of DEPC-treated water into the tubes containing pellet. The pooled RNA was then stored in -80 °C.

3.2.3 Agarose Gel Electrophoresis

The size and integrity of the extracted RNA were verified by running the samples on 1% agarose gel electrophoresis. The gel was prepared by weighing 0.3 g of agarose powder and was then dissolved in 30 ml of 1 x tris-acetate (TAE) buffer. The solution was then heated inside the microwave for 2 minutes. After that, the solution was cooled down before poured into the gel tray with suitable comb. Small amount of ethidium bromide was injected into the cooled solution before the solution was poured inside the tray. The gel was left for about 30 to 40 minutes to solidify. After the gel was solidified completely, the gel was placed inside the gel electrophoresis machine and followed by pouring TAE buffer