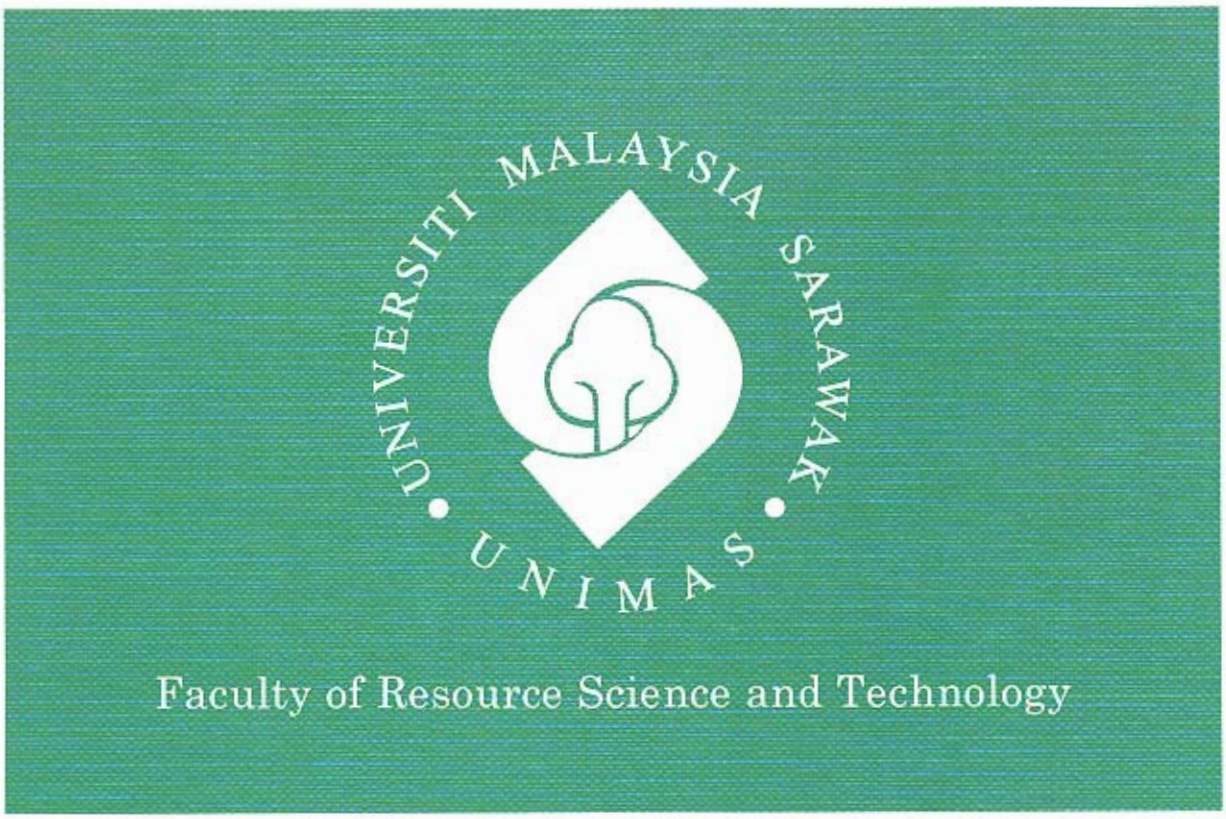

MOLECULAR CLONING AND CHARACTERIZATION OF PARTIAL cDNA ENCODING FOR DEHYDROASCORBATE REDUCTASE FROM KELAMPAYAN (*NEOLAMARCKIA CADAMBA*)

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DECLARATION

I declare that this thesis is of my original work except for quotations and citations, all of which have been duly acknowledged. I also declared that it has not been previously submitted for any other degree at UNIMAS or any other institutions.

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List of Abbreviations

A	Ampere
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
cDNA	Complementary DNA
CTAB	Cetyltrimethylammonium Bromide
ddH_2O	Double-distilled water
DHAR	Dehydroascorbate reductase
DEPC	Diethylpyrocarbonate
dH_2O	Distilled water
dNTP	Deoxyribonuclotide triphosphate
EDTA	Ethylenediamine tetraacetic acid
LB	Luria Broth
min	Minute
mm	Millimeter
mRNA	Meseenger RNA
NCBI	National Centre for Biotechnology Information
PCR	Polymerase chain rection

RNA	Ribonucleic acid
rpm	Revolution per minute
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
TAE	Tris-Acetate EDTA
T_m	Melting temperature
UV	Ultra-violet

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ABSTRACT

Neolamarckia cadamba or locally known as Kelampayan is an evergreen and tropical forest tree. It has high commercial value for timber production and possesses several medical values, such as anthelmintic and astringent production. The intention of this study was to isolate and characterize the partial complementary DNA (cDNA) of Dehydroascorbate reductase (DHAR) gene by using RT-PCR in Kelampayan. Dehydroascorbate reductase (DHAR) is an important reducing enzyme in ascorbate-glutathione cycle. It maintains the level of ascorbate in the plants. Ascorbate (AsA) protects the plants from environmental stress. It also involved in controlling the appropriate signaling in guard cell. In this study, ribonucleic acid (RNA) of *N. cadamba* was extracted from fresh young leaves of Kelampayan and the purified polymerase chain reaction (PCR) product was sent for direct sequencing. Results showed that the partial cDNA of *N.cadamba* was successfully isolated. The sequence had high similarity when compared with DHAR in other species using BLASTn and BLASTp. The partial cDNA of DHAR gene isolated was the middle region when compared to the complete cDNA of DHAR in *Populus tementosa*.

Keywords: *Neolamarckia cadamba*, complementary DNA (cDNA), Dehydroascorbate reductase, RT-PCR, direct sequencing

ABSTRAK

Neolamarckia cadamba atau dikenali sebagai Kelampayan merupakan species pokok tropika malar hijau. Kelampayan mempunyai nilai komersial yang tinggi untuk kegunaan industry kayu dan mempunyai nilai perubatan, seperti penghasilan anthelmintic dan astringen. Tujuan kajian ini adalah untuk mengeluarkan dan mencirikan DNA pelengkap separa (cDNA) daripada gen *Dehydroascorbate reductase* (*DHAR*) pada Kelampayan menggunakan teknik RT-PCR. *Dehydroascorbate reductase* (*DHAR*) adalah enzim yang penting dalam kitaran askorbat-glutation yang mengekalkan tahap askorbat dalam tumbuhan. Askorbat (AsA) boleh melindungi tumbuh-tumbuhan daripada tekanan alam sekitar. AsA juga terlibat dalam mengawal isyarat yang sesuai dalam sel pengawal. Dalam kajian ini, asid ribonucleic (RNA) *N. cadamba* telah diekstrak daripada daun segar Kelampayan dan produk polymerase chain reaction (PCR) yang telah dibersihkan akan dihantar untuk penjujukan langsung. Keputusan menunjukkan bahawa cDNA separa N.cadamba telah berjaya diekstrak. Urutan ini mempunyai persamaan yang tinggi jika dibandingkan dengan *DHAR* spesies lain-lain menggunakan BLASTn dan BLASTp. cDNA *DHAR* terletak di bahagian tengah berbanding dengan mRNA lengkap DHAR di *Populus tementosa*.

Kata kunci: *Neolamarckia cadamba*, DNA pelengkap separa (cDNA), *Dehydroascorbate reductase* (DHAR), RT-PCR, penjujukan langsung

CHAPTER I

INTRODUCTION

Deforestation declines area of the natural forest which leads to the decrease in natural timber sources. According to Appanah (2001), demands for timber of all categories increased sharply, locally and worldwide after the Second World War (1945-50). Based on PERKASA (2009), Sarawak carned RM 4.4 million from the export of timber and timber products to China in the year 2008. Fast-growing exotic timber plantations were established to avoid timber shortage. For example Kelampayan, one of Lesser-known Commercial Known Timber (LKCT) is used timber stock and also in wood-based industry (Tchin *et al.*, 2012). According to Plantation Industries and Commodities Minister, Datuk Peter Chin Fah Kui, the loans are given to the companies to plant eight highly prized timber species to establish total of 375,000 ha of forest plantations by 2020 and one of these species is the *Neolamarckia cadamba* (Malaysian Timber Council, 2008).

N. cadamba is one of the eight highly prized timber species because of its characteristics which are easy to grow and manage and has high dense fibre which result in better wood quality (Panshin & De Zeeuw, 1980). It is also one of the plantation tree species that is selected by Sarawak State Government for planted forest establishment due to its fast growth rate (PERKASA, 2009). It can self-prune and grows well in exploited and denuded areas (Ismail *et al.*, 1995).

N. cadamba with the local name Kelampayan is from the family of Rubiaceae (Joker, 2000). It is distributed naturally from India, Nepal, through Thailand and Indo-China and eastward in the Malaysian Archipelago to Papua New Guinea (Joker, 2000). It can grow up to 45m tall with diameter up to 100cm. Other than one of the best raw materials for chips, sawn timber, composites, veneer and pulp (Monsalud & Lopez, 1967),

Kelampayan can also be used in medical purpose, for example, its bark and leave has anti-hepatotoxic effect (Kapil *et al.*, 1995) and its aerial part has anti-inflammatory effect (Chandrashekar *et al.*, 2010).

Dehydroascorbate reductase (DHAR) enzyme has been reported as the enzyme that catalyses the two electron reduction of dehydroascorbate to ascorbate (Chen & Gallie, 2006). DHAR has been proven to play a significant role in establishing the cellular ascorbate redox state in leaves (Chen & Gallie, 2006) which help in proper signalling for opening and closure of stomata in guard cells. To date, DHAR in higher plants has been purified and characterized from spinach leaves, spinach chloroplasts, rice and wheat (Amako *et al.*, 2005).

In this regard, this project aimed to isolate the high quality of total RNA from young leaves of Kelampayan, amplify the cDNA encoding sequence of dehydroascorbate reductasewith designed primer. After sequencing, the partial cDNA of dehydroascorbate reductase in Kelampayan was then characterized after comparing the homologous sequence of Dehydroascorbate reductase with other plant species in the GenBank.

CHAPTER II

LITERATURE REVIEW

2.1. *Neolamarckia cadamba*

Neolamarckia cadamba (Roxb.) Bosser, also known as Kelampayan, belongs to the Rubiaceae family. It is an evergreen and fast growing tropical tree species. *N. cadamba* are well distributed from India, Nepal, through Thailand and Indo-China and eastward in the Malaysian Archipelago to Papua New Guinea (Joker, 2000). It can self-prune and grows well in exploited and denuded areas (Ismail *et al.*, 1995). The mature tree can grow up to 30 to 45 m and about 2.4 m in girth with a straight cylindrical bole (Acharyya *et al.*, 2010). The leaf length is 13 to 32 cm long with simple and elliptic-oblong in shape but its flower is small, yellow or orange in colour and has the shape of hairy ball (Ismail *et al.*, 1995).

N. cadamba is one of the eight plantation tree species that is selected by Sarawak State Government for planted forest establishment because of its fast growth rate (PERKASA, 2009). This aims to meet the increasing demand in timber industry from both domestic and international markets (PERKASA, 2009). It is one of the best raw materials for chips, sawn timber, composites, veneer and pulp (Monsalud & Lopez, 1967). Kelampayan is also important in medical purpose. It can be used as antimalarial, antidiuretic and antidiarrhoeal drugs (Kitagawa *et al.*, 1996), dysentery, skin diseases, improvement in semen quality (Acharyya *et al.*, 2010), fever treatment and lowering blood sugar in diabetes mellitus patients (Bussa & Pinnapareddy, 2010). Figure 2.1 shows the spreading branches, flowers and leaves of *N. cadamba*.

(a) **(b)**

Figure **2.1(a)** Spreading branches of *N. cadamba* (Adapted from http://www.flickr.com/photos/dinesh_valke/2078207818/); **(b)** Flowers and leaves of *N. cadamba*. (Adapted from http://davesgarden.com/guides/pf/showimage/129495/#b).

2.1. Dehydroascorbate Reductase (DHAR)

Environmental stress, for example salinity, drought, low or high temperature (Hasegawa *et al.*, 2000), excess light, soil water deficiency, UV-B radiation and water logging (Zou *et al.*, 2006) or metabolic process such as photosynthesis enhances production of reactive oxygen species (ROS). Examples of ROS are super oxidant, ozone and hydrogen peroxide in plants (Asada & Takahashi, 1987). These ROS can reduce the photosynthesis capacity of plants and even causes extensive damage in plant (Kabir & Wang, 2010; Pell *et al.*, 1997) to the plants (Hasegawa *et al.*, 2000). This in turn affects plant growth and productivity (Gao *et al.*, 2008). Thus, plants have different protective materials, such as antioxidants and ROS-scavenging materials in response to scavenge free radicals and ROS (Yoshida *et al.*, 2006).

Ascorbate (AsA), present in most cellular compartment, synthesized mainly from D-glucose (Wheeler *et al.*, 1998) is the major antioxidant molecule that involved in the detoxication of ROS in plants (Asada & Takahashi, 1987). Smirnoff (2000) revealed that there is high concentration (1-300mM) of ascorbate in plants (Amako *et al.*, 2005).AsA is one of the necessary nutrients for maintain growth (Hou *et al.*, 2010) and helps to enhance the ability of plants against environmental stress. AsA is involved in the regeneration of α-tocopherol (Vitamin E) from the tocopheroxy radical (Asada, 1994), acts as the cofactor for enzymes such as 2-oxoacid-dependent dioxygenases and prolyl and lysyl forming enzymes (Morell *et al.*, 1997). Furthermore, Morell *et al.* (1997) also revealed that AsA is involved in the cell elongation and progression. Thus, accumulation and recycling of AsA in plants are important to maintain the activity of the active oxygen-scavenging system and the xanthophylls system (Shimaoka *et al.*, 2003) for the normal growth in plants (Hou *et al.*, 2010).AsA can be synthesized by plants, and regenerated from dehyroascorbate (DHA), the oxidized form of ascorbic acid (Yoshida *et al.*, 2006).

Ascorbate-glutathione cycle which involves a series of redox reactions (Yamasaki *et al.*, 1999) have been reported as one of the primary ROS-scavenging systems in higher plants. In study presented by Hou *et al.*, (2010), dehydroascorbate reductase (DHAR) is an important reducing enzyme (Shin *et al.*, 2008) in regenerating ascorbic acid (AsA). Once used, AsA is oxidized to the monodehyroascorbate (MDHA) radical (Asada, 1994). Then, this MDHA radical can be reduced by MDHA reductase to AsA in the chloroplast or cytosol in an NADPH-dependent reaction (Morell *et al.*, 1997). MDHA that is produced by violaxanthin deepoxidase in the thylakoid lumen is rapidly disproportionate to AsA and dehyroascorbate (DHA) when the pH of the lumen is low (Morell *et al.*, 1997). Then, DHA is reduced to AsA by DHAR with the presence of glutathione (GSH) as the electron donor.By these findings, it has been revealed that DHAR is very important for plant growth as it is the key enzyme in regenerating AsA (Hou *et al.*, 2010). It has been proved that the lack of DHAR can cause the immediate loss of AsA in plant, which in turn affects plant growth and development (Ye *et al.*, 2000).

Chen and Gallie (2006) revealed that increasing in DHAR expression in tobacco and maize elevates the AsA content. Thus, Yoshida *et al.* (2006) suggests that DHAR is important in determine the pool size of AsA. Besides that, Chen and Gallie (2006) showed that increasing in DHAR expression which cause the elevation of AsA can also enhance the ability of plant in ozone tolerance, whereas plants suppressed to DHAR were ozone susceptible. Yoshida *et al.* (2006) stated that DHAR expression may determine the sensitivity of plant to ozone. Other than that, DHAR also enhance the resistant ability of plant to heat, cold and saline environment (Hou *et al.*, 2010). Furthermore, Morell *et al.* (1997) reported that level of DHAR expression is important for appropriate signaling in guard cell. This is proved by the study done by Chen and Gallie (2006) which showed that guard cells of plants expressing DHAR exhibit a higher AsA redox state, lower level of

H_2O_2 and ABA signaling, results in greater stomatal opening; whereas suppression of DHAR expression results in higher level of H_2O_2 in guard cell and enhance stomatal closure.

Other than that, Morell *et al.* (1997) revealed that plants suppressed in DHAR expression has slower rate of leaf expansion and shoot growth, delayed flowering time and lower foliar dry weight. They also suggest these phenotypes happen due to loss of chlorophyll a and lower rate of CO_2 assimilation in mature leaves when the DHAR expression is suppressed. By this finding, DHAR expression on leaf aging has been proved to be inversely correlated with the level of lipid peroxidation, which indicate that the efficiency of Asc recycling was important in regulating ROS-mediated damage and DHAR is contributed significant role in this regard (Morell *et al.*, 1997).

To date, DHAR in higher plants has been purified and characterized from spinach leaves, spinach chloroplasts, rice and wheat (Amako *et al.*, 2006). Kabir and Wang (2010) stated that "ascorbic acid recycling by DHAR and the protection of plants from abiotic and oxidative stress are attractive areas of research". By having better understanding of the structure and functions of DHAR gene, it makes cloning of this gene possible which in turn increases the content of AsA.

2.3 Characterization of Partial cDNA

In this project, total RNA from young leaf tissues of Kelampayan (*Neolamarckia cadamba*) is isolated. Then, reverse-transcription polymerase chain reaction (RT-PCR) is used to amplify defined sequences of RNA which is then reverse transcribed enzymatically into cDNA with suitable primers (Rappolee *et al.*, 1988) .cDNA that is generated can be used as template for replication and allows for the analysis of minute or limited amount of RNA samples.

Generally, RT-PCR reaction vessel contains a mixture of nucleotides, buffers, primers, thermostable polymerase and RNA from the specimen of interest. The process involves denaturation of the RNA, annealing of primers and primer elongation (Schochetman *et al.*, 1988). Denaturation of cDNA is done by heating the cDNA to 95-100 °C to separate the duplex held together. In the annealing process, the specific primers, which is a single stranded sequence of oligonucleotide which are each complementary to one of the original DNA strands, to either the 5' or 3' sides of the sequence of the gene of interest is used to anneal to the cDNA. The primers have to be in high excess concentration so single-stranded cDNA can anneal to primers rather than self-annealing between cDNA. The forward and reverse primers are designed based on the conserved regions of the gene of interest (Dehydroascorbate reductase) when compared to other species in the GenBank. After annealing process, the number amplification of gene of interest is amplified by DNA polymerase. DNA polymerase is an enzyme used to synthesize new strand of DNA which is complementary to the unpaired DNA strand after it is annealed with specific primers. According to Schochetman *et al.* (1998), amplication of DNA by PCR can increase the number of DNA up to 1 million copies. This allows for the analysis of minute or limited

amount of samples. After performed agarose gel electrophoresis, the stained gel was visualized under UV transilluminator.

CHAPTER III

MATERIALS AND METHODS

3.1 Collection of Plant Materials

Fresh young leaves of Kelampayan (*Neolamarckia cadamba*) were used for RNA extraction. After the 2nd and 3rd fresh branching leaves were collected, they are immediately put in the plastic bag in the container containing liquid nitrogen.

3.2 Reagent Preparation

Stock solution:

1. 1M Tris-HCl (pH8.0)

   60.57g of Tris base was dissolved in 300ml distilled deionised water and stirred vigorously. The volume was top up to 500ml after adjusting the pH. Then, it was autoclaved.

2. 0.5M EDTA (pH 8.0)

   93.06g of EDTA disodium salt dehydrate was dissolved in 300ml distilled deionised water and stirred vigorously. The volume was top up to 500ml after adjusting the pH. Then, it was autoclaved.

3. 0.1% DEPC buffer

   2ml of DEPC was added into 2L distilled deionised water and mixed the solution by shaker and made sure the DEPC was completely dissolved in the solution.

4. CTAB Extraction Buffer (500ml)

   50ml Tris-HCl (from 1M Tris-HCl stock solution), 20ml EDTA (from 0.5M EDTA stock solution) and 40.91g NaCl were mixed with 300ml of distilled

deionised water. The volume was adjusted to 500ml with distilled deionised water, autoclaved, and stored at room temperature.

5. CIA : Chloroform-isoamyl alcohol (24:1)

   480ml of chloroform was mixed with 20ml of Isoamyl alcohol. The solution was not autoclaved and made sure it is wrapped with aluminium foil as this solution is light-sensitive.

6. Wash Buffer (Store at 4°C)

   380ml of EtOH (absolute ethanol without additive), 0.39g ammonium acetate was mixed with sterile distilled deionised water and was top up to 500ml. The solution was not autoclaved.

7. TE buffer

   10ml Tris-HCl (from 1M Tris-HCl stock solution) and 2ml EDTA (from 0.5M EDTA stock) were mixed with distilled deionised water and top up to 100ml, then autoclaved.

8. 70% Ethanol (for disinfection purpose)

   350ml of ethanol (industrial grade) was mixed with 150ml of deionised water. The solution was not autoclaved.

3.3 Apparatus Treatment

For RNA extraction, apparatus such as pipette tips, 15 ml Falcon tubes, 1.5 ml microcentrifuge tubes, glassware, mortar and pestle were treated with 0.1% DEPC buffer before autoclaved. The PCR tubes, pipette tips and microcentrifuge tubes used for PRC work were autoclaved.

3.4 Data Mining and Primer Design

The nucleotide sequence of dehydroascorbate reductase (DHAR) gene of other plant species were obtained from the NCBI Genbank database. Then, DHAR sequence of four types of plant species, *Arabidopsis thaliana, Theobroma cacao, Pinus bungeana, Populus tomentosa* were selected to perform multiple alignment using Clustal W2 programme (http://www.ebi.ac.uk/Tools/msa/clustalw2). The forward and reverse primers were designed by the software, Primer Premier 6.0 (PREMIER Biosoft International, USA). *Populus tomentosa* was used asthe template based on the conserved regions of the dehydroascorbate reductase (DHAR) genes of other species after the alignment result.

3.5 Total RNA Extraction

3.5.1 Chemicals and Reagents

The chemicals and reagents that were used include liquid nitrogen, RLT buffer, plant RNA purification reagent (Invitrogen, Promega), β-mercaptoethanol, ethanol, DNase, 10 x DNase buffer, DEPC-treated ddH_2O

3.5.2 Total RNA Isolation Protocol

The 2nd or 3rd of fresh branching leaves was chosen from Kelampayan tree. Then, it was wiped with tissue using sterilized distilled water to remove the dirt and dust on the leaves surface. Then, the leaves were cut into smaller size with approximately 0.5g.

Then, this 0.5 g of plant leaves was grinded in the liquid nitrogen into fine powder by using the pre-chilled DEPC-treated mortar and pestle. Liquid nitrogen was used to avoid thaw when necessary. All the RNA work apparatus has to be DEPC-treated to reduce the risk of RNA degradation by RNases. The powdery sample was then transferred to a clean 15 ml Falcon tube containing a mixture of 50 µl β-mercaptoethanol and 5 ml of RLT buffer or plant RNA purification reagent. β-mercaptoethanol is a reducing agent that denatures RNases by reducing disulphide bond and destroys native conformation required in enzyme function.

The mixture was left to stand for 15 minutes. After that, it was centrifuged with the speed of 3,000 rpm for 5 minutes at 25°C. The clear lysate was transferred to a new clean 15 ml DEPC-treated autoclaved Falcon tube. Subsequently, before applying the samples to the 15ml tube of RNeasy Midi Kit Column, 0.5 volume of RNase-free 100% ethanol was added to the supernatant and mixed well immediately. The sample was applied to an