THESIS APPROVAL

GRADUATE SCHOOL, KASETSART UNIVERSITY

Master of Science (Biochemistry)

DEGREE

Biochemistry	Biochemistry
FIELD	**DEPARTMENT**

TITLE: Effect of Light Duration, Sucrose Deprivation and Potassium Cyanide on Gene Expression of Alternative Oxidase in Eucalypt (*Eucalyptus camaldulensis*)

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THESIS

EFFECT OF LIGHT DURATION, SUCROSE DEPRIVATION AND POTASSIUM CYANIDE ON GENE EXPRESSION OF ALTERNATIVE OXIDASE IN EUCALYPT (*Eucalyptus camaldulensis*)

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A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Master of Science (Biochemistry)
Graduate School, Kasetsart University
2009

Boon Kittisarawanno 2009: Effect of Light Duration, Sucrose Deprivation and Potassium Cyanide on Gene Expression of Alternative Oxidase in Eucalypt (*Eucalyptus camaldulensis)*. Master of Science (Biochemistry), Major Field: Biochemistry, Department of Biochemistry. Thesis Advisor: Associate Professor Poontariga Harinasut, Dr.Agr.Sci. 120 pages.

Alternative oxidase pathway in plants, fungi and some protists are the alternative pathway, either it supports the flow of electron through the main pathway or when the obstruction of electron transport pathway happens. This research was attempted to investigate the expression of *alternative oxidase (AOX)* gene at the transcriptional level under various factors such as light duration, sucrose and potassium cyanide (KCN) content by semi-quantitative RT-PCR. *Eucalyptus camaldulensis* clone T5 was selected to investigate the expression of *AOX* gene within 15 days. By observing the morphology, it was found that *E. camaldulensis* clone T5 could endure in the state of 24 hour-light a day or in the dark. It could grow in the absence of sucrose, or insufficient sucrose, 15 mM sucrose. However, it could not stand in 5 mM and 10 mM KCN. Duration of light affected the alteration of the expression of *AOX* gene. When the *E. camaldulensis* were in the longest duration of light, 24 hour-light a day, the expression of *AOX* gene reduced. Contrastingly, when they were in the dark, the expression of *AOX* gene increased. Reduction of sucrose supplementation affected the expression of *AOX* gene. When reduced concentration of sucrose in the same duration of light receiving of 24, 16, 8, 0 hours a day, the expression decreased. 5 mM and 10 mM KCN would activate the expression of the *AOX* gene in the first period of treatment within 5 days but after that the expression of the *AOX* gene decreased. Upon the relation among duration of light, reduction of sucrose and KCN supplementation, it was found that cyanide may be the most effective factor to the expression of *AOX* gene, the second one may be quantity of sucrose and duration of light, in order.

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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to my advisor, Associate Professor Dr. Poontariga Harinasut, for her kindness, support, valuable guidance, warm encouragement and helpful suggestions.

I would like to express my special sincere gratitude and appreciation to my committee advisor Assistant Professor Dr. Somchai Pornbunlualap for his kindness, valuable guidance, attentive interest, understanding and support throughout the course of this research which enable me to complete this dissertation. And this thesis could not have been completed without the advice and the supply of eucalypt *E. camaldulensis* clone T5 that was used as the plant model on my research, from Associate Professor Dr. Somsak Apisitwanich.

My thank is extended to the Department of Biochemistry and the Department of Genetics, Faculty of Science, Kasetsart University for providing laboratories for the experiments.

My appreciation is also expressed to staffs, undergraduate and graduate students in the Department of Biochemistry, Faculty of Science, Kasetsart University, especially all member in 7214 and 7216 laboratories for their great helps and wonderful friendship.

Boon Kittisarawanno

August, 2008

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LIST OF ABBREVIATIONS

AOX	=	alternative oxidase
bp	=	base pair
ºC	=	degree celsius
Da	=	Dalton
dATP	=	deoxyadenosine-5′-triphosphate
dCTP	=	deoxycytosine-5′-triphosphate
dGTP	=	deoxyguanosine-5′-triphosphate
dTTP	=	deoxythymidine-5′-triphosphate
dNTP	=	deoxynucleotide-5′-triphosphate
DNA	=	deoxyribonucleic acid
E. camaldulensis	=	*Eucalpytus camaldulensis*
E. coli	=	*Escherichia coli*
EDTA	=	ethylene diamine tetraacetic acid
EtBr	=	ethidium bromide
g	=	gram
IPTG	=	isopropyl thiogalactoside
kb	=	kilobase pair
KCN	=	potassium cyanide
LB	=	Lulia-Bertani medium
µl	=	microlitre
µg	=	microgram
µM	=	micromolar
mg	=	milligram
min	=	minute
ml	=	millilitre
mM	=	millimolar
MW	=	molecular weight
OD	=	optical density

LIST OF ABBREVIATIONS (Continued)

rpm	=	revolution per minute
RT-PCR	=	reverse transcription polymerase chain reaction
SDS-PAGE	=	Sodium dodecyl sulfate polyacrylamide electrophoresis
TBE	=	Tris borate EDTA electrophoresis buffer
TE	=	Tris EDTA
Tm	=	melting temperature
Tris	=	Tris (hydroxymethyl) aminomethane
U	=	unit
UV	=	ultraviolet
X-gal	=	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

EFFECT OF LIGHT DURATION, SUCROSE DEPRIVATION AND POTASSIUM CYANIDE ON GENE EXPRESSION OF ALTERNATIVE OXIDASE IN EUCALYPT *(Eucalyptus camaldulensis)*

INTRODUCTION

The higher green plants posses a machinery in the chloroplast to capture light energy and convert it to ATP under photochemical reaction of the process, namely photosynthesis. By biochemical reaction coincidently, carbon dioxide is fixed in the expense of ribulose bisphosphate oxygenase carboxylase, using the ATP and reducing power, NADPH. Sucrose, the photosyntate carbohydrate is transported to various living cells through the whole plant. Under respiration process in an aerobic living cell, carbohydrate is combused completely to CO_2, ATP. The respiratory process undergoes in the common metabolic pathway such as glycolysis, Kreb's cycle and electron transport pathway. The electron transport pathway takes place in the inner envelope membrane of the mitochondria. Unlike animal mitochondria, the unique component in electron transport chain of plant mitochondria is alternative oxidase. Therefore, it is interesting of how importance and regulation of the enzyme.

The architecture of electron transport chain components in the inner envelope membrane facilitate the released electron from NADH and succinate flow through complex I and complex II , respectively, to ubiquinone. The reduced ubiquinone donates electron to cytochrome of complex III and complex IV. Proton is also released into intermembrane space while electron transport to complex I, complex III and complex IV. Proton motive force drives ATP synthesis via fo- fi ATP synthetase. However, in plant the alternative pathway that electron is donated from ubiquinone to quinol-oxidising alternative oxidase, exist. Therefore, uncoupling oxidative phosphorylation occurs via the alternative pathway.

The aim of this research is to study the effect of three factors; duration of light, sucrose deprivation and potassium cyanide (KCN) on the expression of *AOX* gene. *Eucalyptus camaldulensis* were used for the special interest because of its importance in Thai's industry.

OBJECTIVES

1. To investigate the regulation of *AOX* gene expression at transcriptional level under three factors; duration of light, sucrose deprivation and potassium cyanide (KCN).

2. To determine the intergral effect of the three factos; duration of light, sucrose deprivation and potassium cyanide (KCN) on the *AOX* gene expression.

LITERATURE REVIEW

1. Background information of Alternative oxidase (AOX)

The alternative oxidase (AOX), which catalyses cyanide insensitive respiration, has been studied in a wide variety of plant species over the last 40 years. In voodoo lily *(Sauromatum guttatum*), *AOX* is expressed at high levels during flowering and was used as a model to initially clone *AOX* (Rhoads and McIntosh, 1991). In recent years, Arabidopsis has become the pre-minent plant model with the availability of the complete genome sequence allowing experimental approaches which are only possible with extensive, high quality sequence data (Bevan and Walsh, 2005).

The alternative pathway of mitochondrial respiration branches from the cytochrome pathway in the inner mitochondrial membrane at the ubiquinone pool and passes electrons to a single terminal oxidase, the alternative oxidase (Figure 1). The alternative oxidase apparently reduces molecular oxygen to water in a single four electron transfer step (Day *et al*., 1995). The alternative pathway is nonphosphorylating. The oxidase is resistant to cyanide and inhibited by substituted hydroxamic acids such as salicylhydroxamic acid (SHAM) and n–propyl gallate (Siedow and Bickett, 1981). All angiosperms, many algae, and some fungi contain the genetic capacity to express this pathway (McIntosh, 1994). The significance of this pathway may be that its electron transport from ubiquinone to oxygen does not contribute to a transmembrane potential and thus wastes two of the three energy coupling sites that are part of the cytochrome pathway. The phosphorylating potential from site I (NADH dehydrogenase) is retained, thus allowing some energy production (Figure 1). In plants and fungi the alternative pathway of mitochondrial respiration requires a single nuclear gene (existing as a small gene family in some plants) *AOX1*, encoding the alternative oxidase. All plant species tested possess the genetic capacity to express the alternative pathway under any number of developmental and environmental conditions (McIntosh, 1994).

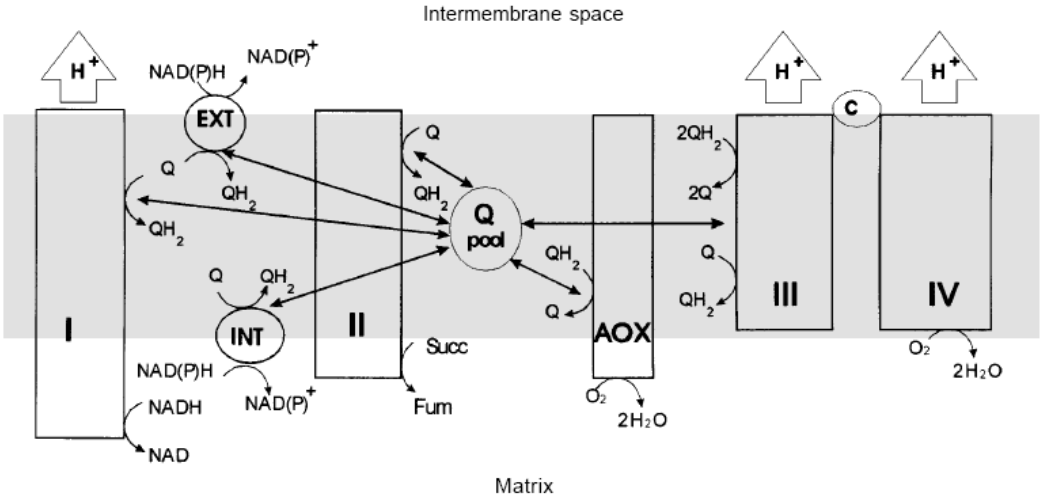


Figure 1 Organization of the branched respiratory chain of plant mitochondria. Complex I,NADH dehydrogenase; complexII, succinate dehydrogenase;complex III, cytochrome c; complex IV, cytochrome oxidase; AOX, alternative oxidase;c, cytochrome c; Q, ubiquinone;QH2, ubiquinol; INT, internalNAD(P)H dehydrogenase; EXT,external NAD(P)H dehydrogenase.

Source: Sluse and Jarmuszkiewicz (1998)

2. Alternative oxidase gene

The alternative oxidase (AOX) is encoded in two discrete gene subfamilies in higher plants. *AOX1* is most widely known for its induction by stress stimuli in many tissues and is present in both monocot and eudicot plant species. *AOX2*, on the other hand, is usually constitutive or developmentally expressed in eudicot species but is absent from the genomes of all monocot species examined to date. This molecular distinction suggests a divergence of *AOX* across plant families and may even have implications for the role of this enzyme in different plant species.

AOX genes are classified into two families, *AOX1* and *AOX2*. Comparison of *AOX1* members indicates that they display higher sequence similarity to each other between species than they do to *AOX2* members within a species (Considine *et al*.,

2002). However it is clear that by the process of duplication and subsequent divergence that the number of members of specific gene types have expressed in some species. Thus, while *AOX1* type genes have expressed in Arabidopsis, *AOX2* type genes have expressed in legumes such as soybean and cowpea (Costa *et al*., 2004). In tobacco, another widely used model to study *AOX* (Donald *et al*., 2002), it appears that there is one of each type of gene, but two allelic copies of each exist due to the amphidiploid nature of tobacco. In monocots only *AOX1* type genes have been characterised to date (Karpova *et al*., 2002). Given this diversity in the number and type of *AOX* genes in any species, knowing the sequence of all genes, as in Arabidopsis, is aconsiderable advantage for expression analysis if the role and regulation of *AOX* is to be comprehensively understood.

3. Characteristics of AOX protein

Primary structure of AOX

The amino acid sequence of a protein can be derived from the cDNA sequences. By analysis of the primary sequence of a protein, an insights into its structure can be deduced. AOX primary sequences obtained in such away contain a mitochondrial transit peptide at the N-terminus. The mature AOX protein from *S. guttatum*, as an example, has a calculated molecular mass of 32.2 kDa and contains 283 amino acids (Rhoads and McIntosh, 1991). Hydropathy plot analysis of several AOX amino acid sequences (Moore and Siedow, 1991) has indicated important conserved features existed in the protein: a) two hydrophobic regions with a strong α-helical character and a highly conserved sequence that have been proposed to be membrane-spanning helices, b) these two regions are separated by about 40 amino acids including an amphipathic helix probably exposed to the intermembrane surface, and c) two hydrophilic regions of about 100 amino acids on both terminal sides with highly conserved short regions at the C-terminus. A topologic model for the AOX protein has been proposed (Day *et al*., 1995), with the amphipathic helix exposed on the cytosolic side and the hydrophilic domains extending into the matrix. Both cytosolic and matricial parts are linked by the two membrane-spanning helices.

Alternative oxidase structure

Two structural models of the AOX currently exist. The first model proposed by Siedow and Umbach (1995), was based on relatively few AOX sequences and classified the AOX as a member of the di-iron family of proteins that also includes the R2 subunit of ribonucleotide reductase and the hydroxylase component of methane monooxygenase. Based on hydropathy analysis, the AOX was predicted to contain two transmembrane helices that are connected by a helix located in the intermembrane space (Moore and Siedow, 1991), (Fig. 2A). Since this model was proposed, more AOX sequences had been sequenced and identified. Anderson and Nordland (1999) proposed an alternative structural model (Fig. 2B). Although the second model also classifies the AOX as a di-ironprotein, it differs from the frist model by the precise ligation sphere of the di-ironcenter (Andersson and Nordland, 1999) For instance, one of the C-terminal Glu-*X*-*X*-His motifs identified by Siedow and Umbach (1995), containing Glu-270, appeared not to be fully conserved in the newly identified sequences and consequently seemed unlikely to play a role in ligating iron. Instead, Andersson and Nordlund used the second Glu-*X*-*X*-His motif (that contains Glu-217, which is located in the intermembrane space according to the Siedow and Umbach (1995)) model to coordinate the iron atoms. Since such a choice implies that the transmembrane helices can no longer be retained, Andersson and Nordlund (1999) proposed that the AOX is an interfacial rather than a transmembrane protein.

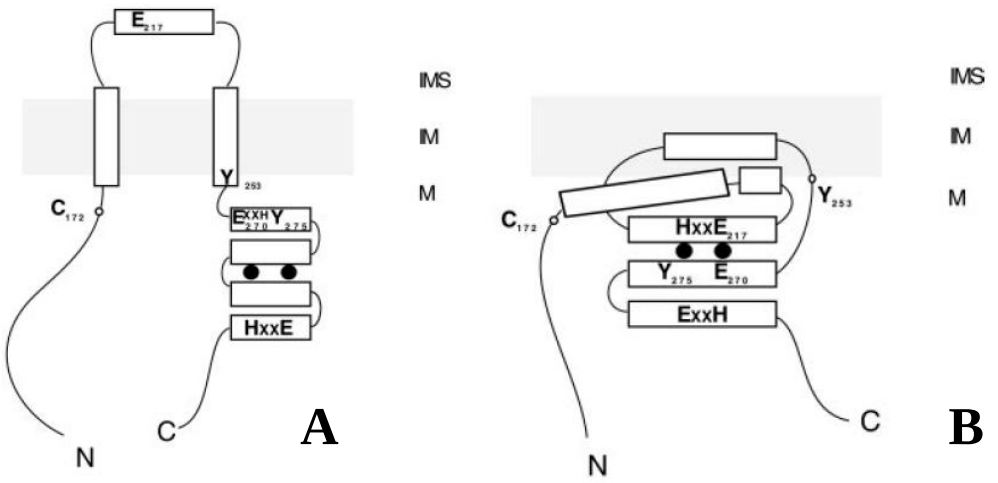


Figure 2 Two proposed structural models of the AOX**.** ***A***, the transmembrane model proposed by Siedow and Umbach (1995) ***B***, the interfacial model proposed by Andersson and Nordlund (1999). The residue numbering refers to the *S. guttatum* deduced amino acid sequence. The structures are presented in a two-dimensional format, with filled spheresre presenting the iron atoms, and show the respective iron-binding motifs (E*XX*H). *IMS*,intermembrane space; *IM*, inner membrane; *M*, matrix.

Source: Albury *et al.* (2002)

Active sites of AOX

The ability of AOX to reduce oxygen to water involves a four-electron reduction reaction (Moore and Siedow, 1991) to avoid production of damaging partially reduced oxygen species. Therefore, an active site containing transition metals has been predicted (Moore and Siedow, 1991). Evidence that makes iron the leading metal candidate for the metal associated with the AOX active site comes from experiments with culture of the yeast *Hansenula anomala* in which the absence of iron leads to an inactive 36-kDa AOX protein (Minagawa *et al*., 1990). Its activity is recovered by the addition of iron to the culture medium. All of the plant AOX amino acid sequences (in the C-terminal domain) reveal the presence of two copies of the conserved iron-binding motif (E-X-X-H), providing the additional argument that AOX

contains iron. By comparison with coupled binuclear iron proteins, an iron-binding four helical bundle model of the AOX active site has been proposed analogous to that of methane monooxygenase (Siedow and Umbach, 1995).The reducing substrate (i.e. ubiquinol (QH2)) binding site has been postulated to be situated on the matrix side of the mitochondrial membrane in a hydrophobic pocket formed by the two membrane-spanning helices (Siedow and Umbach, 1995).The three fully conserved residues (T, E, Y) in the pocket are potential ligands for ubiquinone. Then, in the proposed structural model of AOX, the binuclear iron center where oxygen is reduced to water should be close to the postulated binding site of reducing QH2 which itself is in the vicinity of the binding site for the allosteric effector of the enzyme, pyruvate
(Figure 3).

Dimeric structure of AOX

The structural property of AOX has been revealed by SDS-PAGE and immunoblotting using chemical cross-linkers and oxidizing and reducing agents (Umbach and Siedow, 1993).These experiments have demonstrated that AOX exists as a dimer of 65 kDa in the inner mitochondrial membrane and that two distinctstates of the dimer can be identified: an oxidized state in which the dimer is covalently cross-linked by a disulfide bridge(-S-S-) and a reduced state (-SH HS-) which is maintained through non-covalent interactions. The intermolecular disulfide bridge is formed at the level of a conserved cysteine residue (in the plant AOX) situated in the N-terminal domain exposed to the matrix of each monomer (Figure 3). A second conserved cysteine residue (in the plant AOX) implicated in pyruvate binding is situated in the same N-terminal domain close to the membrane surface of each subunit (Umbach and Siedow, 1996).

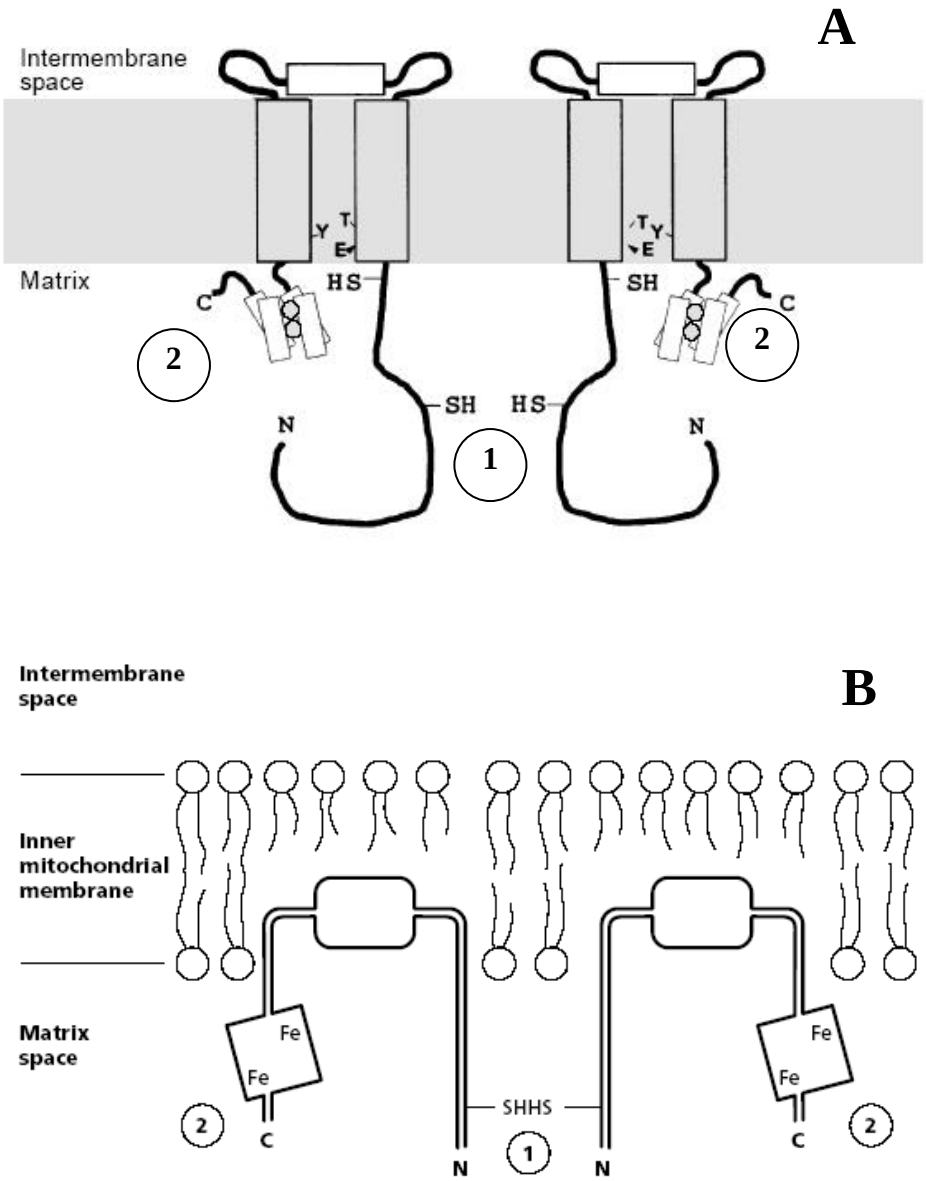


Figure 3 Schematic representation of the alternative oxidase dimer (AOX) and its proposed conformation in the inner mitochondrial membrane. ***A***, the transmembrane model ***B***, the interfacial model. The AOX is embedded in the inner mitochondrial membrane via an inter-membrane helix region. 1, Sulfydryls that are involved in redox regulation and pyruvate activation; 2, proposed iron-active site of AOX.

Source: Sluse and Jarmuszkiewicz. (1998)

4. Background information of Eucalypt

The genus *Eucalyptus* is extraordinarily prevalent in much of the woody vegetation of Australia (Groves, 1994) and has become a symbol of the Australian plant. The genus includes about 700 species, a number of which are unnamed. Species of the genus *Eucalyptus* are commonly known as “eucalypts” throughout the world, although in Australia they are often called “gum trees” because of the gum (kino) that exudes from the trunk of older trees. The possession of opercula (single or double) covering the flora buds, and the lack of petals distinguishes the eucalypts within the Myrtaceae (Boland *et al*., 1984). Eucalypts belong to the family Myrtaceae, subfamily Leptospermoideae, which includes dry-fruited forms such as *Angophora, Syncarpia, Tristania, Melaleuca* and *Leptospermum,* so in the broad sense have been classified traditionally the eucalypts as two genera: *Angophora* Cav. And *Eucalyptus* L’Her (Eucalyptus alliance of Briggs and Johnson, 1988). *Angophora* is a small genus with only 11-13 taxa confined to eastern Austrlia 11 species and sub-species in Chippendale (1988) and 13 species in Hills (1972). It has flower with free, green sepals, and free, creamy white petals. *Eucalyptus* includes approximately 800 species, some undescribed, and has flowers that lack showy sepals and petals. In 1995 new evidence, largely genetic, indicated that some prominent eucalypt species were actually more closely related to *Angophora* than to the other eucalypts; they were split off into the new genus *Corymbia*. Although separate, the three groups are allied and it remains acceptable to refer to the members of all three genera *Angophora*, *Corymbia* and *Eucalyptus* as "eucalypts".

Most eucalypts are found naturally only in Australia, with a few species occurring to the others. Ten species are common to southern New Guinea (Irian Jaya and Papua New Guinea) and northern Australia. Four tropical species do not occur in Australia: *Eucalyptus deglupta* is endemic to the Indonesian islands of Sulawesi and Ceram, Mindanao in the southern Philippines, and northern New Guinea, *E. urophylla, E. wetarensis* and *E. orophila* occur in Timor and adjoining islands. There are no extant species in New Zealand or New Caledonia, although New Caledonia is home to

Arillastrum gummiferum, which, together with *Eucalyptopsis, Allosyncarpia* and unnamed genus, are the closest relatives of the eucalypts (Johnson and Briggs, 1984).

The most readily recognisable characteristics of *Eucalyptus* species are the distinctive flower and fruits. The name *Eucalyptus* means "well-covered"; it describes the bud cap (technically called an operculum). This cap forms from modified petals and falls off as the flower opens. Thus flowers have no petals, decorating themselves instead with many showy stamens.The woody fruits are roughly cone-shaped and have valves at the end which open to release the seeds. Most eucalypt are “forest trees” or “woodland trees” or “mallees” (Specht, 1970). “Forest trees” are generally between 30 and 50 metres in height with the single stem. In natural forests, the crown forms a minor proportion of trees height, with the combined crowns covering at least 30% of the ground area. Some forest species such as *E. regnans, E. diversicolor, E. grandis and E. deglupta* reach more than 70 metres in height, with specimens of *E. regnans* reaching over 100 metres. The tallest *E. regnans* surveyed last century was 110 metres (Handy, 1935). “Woodland trees” are around 10-25 metres in height; although single-stemmed, they may branch a short distance above ground level. Their crown generally occupies a major proportion of the tree height, with the combined crowns covering less than 30% of the ground. Two further tree forms, only found in Western Australia, are considered notable. One of these is the “mallet”, which is a smooth-barked, small to medium-sized tree, usually of steep-branching habit, sometimes fluted at the base of the trunk, and often with a conspicuously dense, terminal crown. “Mallees” are multistemmed from ground level, usually less than 10 metres in height, often with the crown predominantly at the ends of the branchets. Individual plants may combine to form either an open or a closed formation (Brooker and Kleinig, 1990).

Nearly all eucalypts are evergreen but some tropical species lose their leaves at the end of the dry season. As in other members of the Myrtle family, eucalypt leaves are covered with oil glands. The copious oils produced are an important feature of the genus. Eucalypts also exhibit leaf dimorphism. When young, their leaves are opposite and often roundish and occasionally without petiole. When several years old, the leaves become alternate, quite slender and with long petioles. Plants do not flower

until adult foliage starts to appear, except in *E. cinerea*.

The bark dies annually and species can be roughly grouped based on its appearance. In smooth-barked trees most of the bark is shed, leaving a smooth surface that is often colourfully mottled. With rough-barked trees the dead bark persists on the tree and dries out. Many trees, however, have smooth bark at the top but rough bark on the trunk or its bottom. The types of rough bark is often used to broadly label a group of eucalypts but are not always well correlated with taxonomic groupings. They are:

Stringybark consists of long-fibres and can be pulled off in long pieces. It is usually thick with a spongy texture.

Ironbark is hard, rough and deeply furrowed. It is soaked with dried kino (a sap exuded by the tree) which gives a dark red or even black colour.

Tessellated, bark is broken up into many distinct flakes. They are corkish and can flake off.

Box has short fibres. Some also show tessellation.

Ribbon, this has the bark coming off in long thin pieces but still loosely attached in some places. They can be long ribbons, firmer strips or twisted curls.

The appearance of eucalypt bark varies with the age of the plants, the manner of bark-shed, the length of the fibres, the degree of the furrowing, the thickness, hardness and colour of the bark. Additionally, some species that grow over a wide range of environments have different bark forms in different areas. For example, both *E. viminalis* in southeastern Australia and *E. loxophleba* in southwestern Australia have subspecies with different bark characteristics. The ecological basis for the wide variation in bark type is still uncertain, although the tolerance of eucalypt in southern Australia to fire has been discussed in relation to bark type (Gill and Ashton, 1968).

Eucalypt dominate the forests and woodlands of the coastal regions of Australia and vast areas of the drier country, particularly in the south, are covered by eucalypt mallee shrublands. Only in the arid interior are eucalypt lacking in dominance. In addition to a broad geographic range, eucalypt inhabit a range of temperature and rainfall regimes from the warmer, summerrainfall rigions of the tropics and subtropics to the temperate, cooler regions of southern Australia where rainfall is either uniform or falls predominantly in winter. As might be expected in a genus so widespread and comprising hundreds of species, a wide range in form is exhibited by members growing under different environments; a mosaic of habitats differing in climate, topography and soil type will usually result in a corresponding mosaic of eucalypt species. Other species are distributed over wide geographic and environmental gradients and show a relatively wide tolerance to environmental conditions such as soil type. For example, *E. pauciflora* can be found from the sub-alpine tree-line to sea-level on mainland Australia (Williams and Ladiges, 1985) and occurs on a number of soil types across this wide elevational range.

There are many compounds in eucalypts. Some compounds can be found in only one or a few species. Within species the quantity of essential oil and the specific compounds in the essential oil and extracts of dry and fresh leaves, buds, mature fruit, and bark vary with the origin of the tree and the age of the leaves. The following discussion and list of eucalypts and the compounds in them is not comprehensive. The quantity of the compounds in the oils and plant parts are estimates based on several sources. *E. globulus*is one of the more important species of eucalypt. The essential oil in the leaves is commonly used for medicinal purposes such as anesthetic, antibronchitic, anticatarrh, antilaryngitic, antipharyngitic, antiseptic, antitussive, cns-stimulant, choleretic, counterirritant, dentifrice, expectorant, fungicide, hepatotonic, herbicide, hypotensive, pesticide, and sedative.

The other production from eucalypts is carried out in Australia and overseas. Many different species are used both from natural forests and from plantations. Eucalypt plantations can be found in more than 90 countries with the largest overseas

plantations being in Brazil which has over 1 million hectares. Some of the uses for eucalypts are:

Building (for termite resistance) such as *E. camaldulensis, E. marginata*

Furniture such as *E. maculata, E. globulus*

Woodchips such as *E. camaldulensis, E. globulus, E. viminalis*

Paper such as *E. botryoides, E. camaldulensis, E. grandis*

Fuel such as *E. camaldulensis, E. globulus, E. saligna*

5. *Eucalyptus camaldulensis*

The most widely distributed of all the eucalypts, *E. camaldulensis* (river red gum) is also one of the most variable. In some less arid areas *E. camaldulensis* occur away from its typical riverine habitat on plains and hill slopes. It can be found on the margins of salt lakes and on a variety of soil types. Soil are typically alluvial silts and sands. Except for a few populations in South Australia and Western Australia that occur on shallow soils over limestone, the species is not adapted to calcareous soils. *E. camaldulensis* grows in temperate and tropical climates with rainfall from 200 mm to more than 1100 mm annually, with summer rains in the north and winter rains in the south of the continent. The most extensive natural stands are found along the banks and wide flood plains of the Murray-Darling river system in the inland of south-eastern Australia.

There have been several physiological investigations which might help explain why one provenance is faster growing and more drought hardy than another. Awe *et al*. (1976) study root growth of *E. camaldulensis* by grew seedling in tubes one metre long. They found that roots of the Lake Albacutya provenance reached the bottom of the tube more quickly than Kantherine and other provenances. The capacity to produce a massive root system very rapidly would help the Lake Albacutya seedling to reach water at greater depths in the soil, avoid drought, and continue to grow rapidly when

shallower rooted plants were under severe moisture stress. Stomatal conductance and CO_2 uptake for the Lake Albacutya provenance were shown by Moreshet, 1981 to be greater than for the slower growing Kantherine provenance in trees of four-year-old trial in Israel, but only when water was available. In the hot dry summer both provenances increased growing and their dormancy was indicated by very low evapotranspiration.

E. camaldulensis has been highly regarded for some time as a tree with useful tolerance of salinity in the soil. Some populations were found to have greater tolerance than the others (Karschon and Zohar, 1975), but only in recent years have well controlled experiments been made to test for genetic variation in tolerance of salinity. In a study of seedlings of 52 species of eucalypts in saline solution culture Blake (1981) found that *E. camaldulensis* was the second most resistant species. (The most resistance species in this experiment was *E. woodwardii*.) Sands (1981) examined the salt tolerance of three southern provenances and found that at a salt concentration of 400 mM the seedlings from Lake Albacutya were all healthy, though their growth was reduced, and the seedlings from Shepparton were nearly all dead; seedlings from Port Lincoln were intermediate.

Eucalyptus spp. was first planted at Thailand in 1950, which brought 15 species from Australia to be planted in 4 stations in different regions of Thailand: Chaing Mai, Kanchanaburi, Sisaket and Surat Thani.The experiment showed that the best species was *E. camaldulensis* , in term of surviral and growth rate. It is adaptable to saline soils in north eastern of Thailand and tolerates a variety of climate condition (Terra, 2004).

The boom of Eucalyptus spp. in Thailand happened with the government's support. Its purpose was to provide enough raw material for the pulp and paper industry and the purpose of selling seedling. Eighty-six percent of the pulp and paper production of today depends on Eucalyptus spp. tree for its raw material as the others such as rice straw, bagasse, kenaf and bamboo, had problems of consistency and transport. Using Eucalyptus spp. tree as raw material helped expand the investment in

the pulp and the paper industry. The Thai pulp and paper industry aimed its production not only as an import substitution but also at exports to the lucrative European and Japanese paper markets. At present, Thailand's production capacity of short-fiber pulp is ranked second among the Association of Southeast Asian (ASEAN) countries and is fifth overall in Asia after China, Japan, Indonesia and India. Thailand's annual production capacity of one million tones comes from five main pulp producers.

6. Amplification of Specific Alternative Oxidase cDNA Fragment by Reverse Transcription Polymerase Chain Reaction (RT- PCR)

RT-PCR is a highly sensitive and specific method used in gene expression studies. RNA cannot serve as a template for PCR, so it is reverse transcribed into cDNA using specific primers for synthesis of the first-stranded cDNAs. Avian myeloblastosis virus (AMV) and Malony murine leukemia virus (MoMuLV) reverse transcriptases are commonly used for RT reaction. Complementary DNA of interest was amplified using first-stranded cDNA as a template. This powerful tool has utilized in numerous research applications, including library construction, the study of developmental gene expression and differential display.

7. Photosynthesis

Photosynthesis is a metabolic pathway that converts light energy into chemical energy. Its initial substrates are carbon dioxide and water; the energy source is sunlight (electromagnetic radiation); and the end-products are oxygen and (energy-containing) carbohydrates, such as sucrose, glucose or starch. This process is one of the most important biochemical pathways, since nearly all life on earth either directly or indirectly depends on it as a source of energy. It is a complex process occurring in plants, algae, as well as bacteria such as cyanobacteria. Photosynthetic organisms are also referred to as photoautotrophs (Bryant *et al*., 2006).

Photosynthesis uses light energy and carbon dioxide to make triose phosphates (G3P). G3P is generally considered the first end-product of photosynthesis. It can be

used as a source of metabolic energy, or combined and rearranged to form monosaccharide or disaccharide sugars, such as glucose or sucrose, respectively, which can be transported to other cells, stored as insoluble polysaccharides such as starch, or converted to structural carbohydrates, such as cellulose or glucans.
A commonly used slightly simplified equation for photosynthesis is:

$$6\ CO_{2(g)} + 12\ H_2O_{(l)} + \text{photons} \rightarrow C_6H_{12}O_{6(aq)} + 6\ O_{2(g)} + 6\ H_2O_{(l)}$$

carbon dioxide + water + light energy → glucose + oxygen + water

The equation is often presented in introductory chemistry texts in an even more simplified form as:

$$6\ CO_{2(g)} + 6\ H_2O_{(l)} + \text{photons} \rightarrow C_6H_{12}O_{6(aq)} + 6\ O_{2(g)}$$

Photosynthesis occurs in two stages. In the first stage, light-dependent reactions or photosynthetic reactions (also called the light reactions) capture the energy of light and use it to make high-energy molecules. During the second stage, the light-independent reactions (also called the calvin-benson cycle, and formerly known as the dark reactions) use the high-energy molecules to capture and chemically reduce carbon dioxide (CO_2) (also called carbon fixation) to make the precursors of carbohydrates.

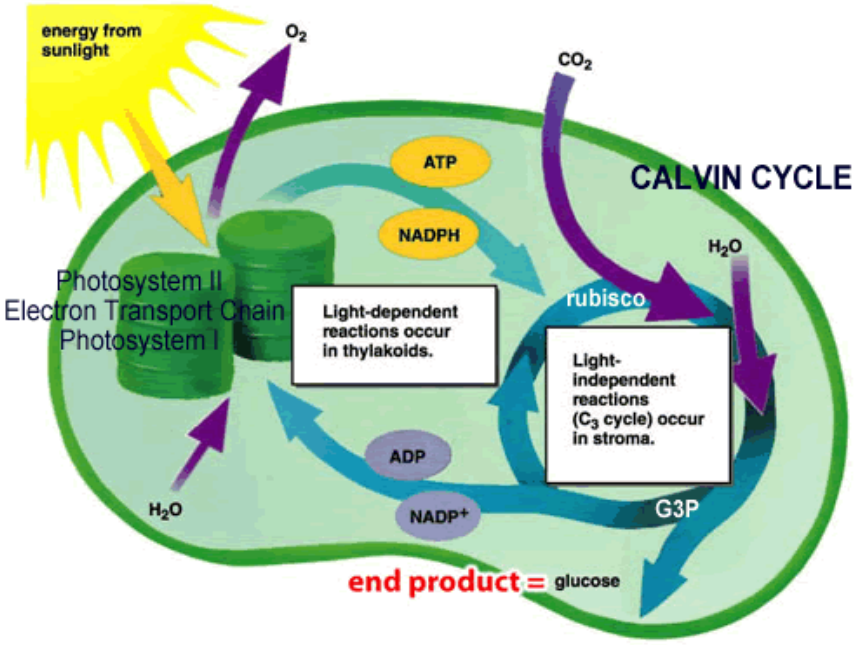


Figure 4 Photosynthesis occurs in two stages. In the first stage, light dependent reactions. The second stage, the light-independent reactions.

Source: Bryant *et al*. (2006)

In the light reactions, one molecule of the pigment chlorophyll absorbs one photon and loses one electron. This electron is passed to a modified form of chlorophyll called pheophytin, which passes the electron to a quinone molecule, allowing the start of a flow of electrons down an electron transport chain that leads to the ultimate reduction of NADP to NADPH. In addition, this creates a proton gradient across the chloroplast membrane; its dissipation is used by ATP Synthase for the concomitant synthesis of ATP. The chlorophyll molecule regains the lost electron from a water molecule through a process called photolysis, which releases a dioxygen (O_2) molecule.

In the Light-independent or dark reactions the enzyme RuBisCO captures CO_2 from the atmosphere and in a process that requires the newly formed NADPH, called the Calvin-Benson Cycle, releases three-carbon sugars, which are later combined to form sucrose and starch.

Photosynthesis may simply be defined as the conversion of light energy into chemical energy by living organisms. It is affected by its surroundings, and the rate of photosynthesis is affected by the concentration of carbon dioxide in the air, the light intensity, and the temperature.

Photosynthesis uses only 1% of the entire electromagnetic spectrum, and 2% of the visible spectrum.

In plants : Most plants are photoautotrophs, which means that they are able to synthesize food directly from inorganic compounds using light energy - for example from the sun, instead of eating other organisms or relying on nutrients derived from them. This is distinct from chemoautotrophs that do not depend on light energy, but use energy from inorganic compounds.

$$6\ CO_2 + 12\ H_2O \rightarrow C_6H_{12}O_6 + 6\ O_2 + 6\ H_2O$$

The energy for photosynthesis ultimately comes from absorbed photons and involves a reducing agent, which is water in the case of plants, releasing oxygen as product. The light energy is converted to chemical energy (known as light-dependent reactions), in the form of ATP and NADPH, which are used for synthetic reactions in photoautotrophs. The overall equation for the light-dependent reactions under the conditions of non-cyclic electron flow in green plants is (Raven *et al*., 2005):

$$2\ H_2O + 2\ NADP^+ + 2\ ADP + 2\ P_i + \text{light} \rightarrow 2\ NADPH + 2\ H^+ + 2\ ATP + O_2$$

Most notably, plants use the chemical energy to fix carbon dioxide into carbohydrates and other organic compounds through light-independent reactions. The overall equation for carbon fixation (sometimes referred to as carbon reduction) in green plants is:

$$3\ CO_2 + 9\ ATP + 6\ NADPH + 6\ H^+ \rightarrow$$
$$C_3H_6O_3\text{-phosphate} + 9\ ADP + 8\ P_i + 6\ NADP^+ + 3\ H_2O$$

To be more specific, carbon fixation produces an intermediate product, which is then converted to the final carbohydrate products. The carbon skeletons produced by photosynthesis are then variously used to form other organic compounds, such as the building material cellulose, as precursors for lipid and amino acid biosynthesis, or as a fuel in cellular respiration. The latter occurs not only in plants but also in animals when the energy from plants gets passed through a food chain. Organisms dependent on photosynthetic and chemosynthetic organisms are called heterotrophs. In general outline, cellular respiration is the opposite of photosynthesis: Glucose and other compounds are oxidized to produce carbon dioxide, water, and chemical energy. However, the two processes take place through a different sequence of chemical reactions and in different cellular compartments.

Plants absorb light primarily using the pigment chlorophyll, which is the reason that most plants have a green color. The function of chlorophyll is often supported by other accessory pigments such as carotenes and xanthophylls. Both chlorophyll and accessory pigments are contained in organelles (compartments within the cell) called chloroplasts. Although all cells in the green parts of a plant have chloroplasts, most of the energy is captured in the leaves. The cells in the interior tissues of a leaf, called the mesophyll, can contain between 450,000 and 800,000 chloroplasts for every square millimeter of leaf. The surface of the leaf is uniformly coated with a water-resistant waxy cuticle that protects the leaf from excessive evaporation of water and decreases the absorption of ultraviolet or blue light to reduce heating. The transparent epidermis layer allows light to pass through to the palisade mesophyll cells where most of the photosynthesis takes place.

Plants convert light into chemical energy with a maximum photosynthetic efficiency of approximately 6%.(Photosynthesis Outline) By comparison solar panels convert light into electric energy at a photosynthetic efficiency of approximately 10-20%. Actual plant's photosynthetic efficiency varies with the frequency of the light being converted, light intensity, temperature and proportion of CO_2 in atmosphere.

8. Respiration

In biochemistry, respiration may refer to:

- Cellular respiration, the process in which the chemical bonds of energy rich molecules such as glucose are converted into energy usable for life processes
- Aerobic respiration, a process that allows respiration with use of oxygen
- Anaerobic respiration, a process that allows respiration without use of oxygen
- Plant respiration, the enzymatic oxidation of substrates in plants, leading to the release of carbon dioxide respiration.

Plant respiration

Plant respiration is the oxidation of certain substrates by enzymes, leading to a release of carbon dioxide. It can be loosely thought of as the opposite of photosynthesis, though the net release of carbon dioxide in respiration is less than carbon uptake in photosynthesis. Sources of glucose for respiration for plants are starch or directly from photosynthesis.

Plant respiration convert biochemical energy from nutrients into adenosine triphosphate (ATP), and then release waste products. The reactions involved in respiration are catabolic reactions that involve the oxidation of one molecule and the reduction of another.

Nutrients commonly used by animal and plant cells in respiration include glucose, amino acids and fatty acids, and a common oxidizing agent (electron acceptor) is molecular oxygen (O_2). Bacteria and archaea can also be lithotrophs and these organisms may respire using a broad range of inorganic molecules as electron donors and acceptors, such as sulfur, metal ions, methane or hydrogen. Organisms that use oxygen as a final electron acceptor in respiration are described as aerobic, while those that do not are referred to as anaerobic.

The energy released in respiration is used to synthesize ATP to store this energy. The energy stored in ATP can then be used to drive processes requiring energy, including biosynthesis, locomotion or transportation of molecules across cell membranes. Because of its ubiquity in nature, ATP is also known as the "universal energy currency".

Respiration is an essential life process in plants. It is necessary for the synthesis of essential metabolites including carbohydrates, amino acids and fatty acids, as well as for the transport of minerals and other solutes between cells. It consumes between 25 and 75% of all the carbohydrates produced in photosynthesis at ordinary growth rates. (Lambers *et al*., 2005)

Aerobic respiration

Aerobic respiration requires oxygen in order to generate energy (ATP). It is the preferred method of pyruvate breakdown from glycolysis and requires that pyruvate enter the mitochondrion to be fully oxidized by the Krebs cycle. The product of this process is energy in the form of ATP (adenosine triphosphate), by substrate-level phosphorylation, NADH and $FADH_2$.

Simplified reaction: $C_6H_{12}O_{6\,(aq)} + 6O_{2\,(g)} \rightarrow 6CO_{2\,(g)} + 6H_2O_{\,(l)}\ \Delta H_c$ -2880 kJ

The reducing potential of NADH and $FADH_2$ is converted to more ATP through an electron transport chain with oxygen as the "terminal electron acceptor". Most of the ATP produced by aerobic cellular respiration is made by oxidative phosphorylation. This works by the energy released in the consumption of pyruvate being used to create a chemiosmotic potential by pumping protons across a membrane. This potential is then used to drive ATP synthase and produce ATP from ADP. Biology textbooks often state that between 36-38 ATP molecules can be made per oxidised glucose molecule during cellular respiration (2 from glycolysis, 2 from the Krebs cycle, and about 32-34 from the electron transport system). Generally, 38 ATP molecules are formed from aerobic respiration. However, this maximum yield is never

quite reached due to losses (leaky membranes) as well as the cost of moving pyruvate and ADP into the mitochondrial matrix.

Aerobic metabolism is 19 times more efficient than anaerobic metabolism (which yields 2 mol ATP per 1 mol glucose). They share the initial pathway of glycolysis but aerobic metabolism continues with the Krebs cycle and oxidative phosphorylation. The post glycolytic reactions take place in the mitochondria in eukaryotic cells, and in the cytoplasm in prokaryotic cells.

Glycolysis

Glycolysis is a metabolic pathway that is found in the cytoplasm of cells in all living organisms and is anaerobic, or doesn't require oxygen. The process converts one molecule of glucose into two molecules of pyruvate, and makes energy in the form of two net molecules of ATP. Four molecules of ATP per glucose are actually produced; however, two are consumed for the preparatory phase. The initial phosphorylation of glucose is required to destabilize the molecule for cleavage into two triose sugars. During the pay-off phase of glycolysis, four phosphate groups are transferred to ADP by substrate-level phosphorylation to make four ATP, and two NADH are produced when the triose sugars are oxidized. The overall reaction can be expressed this way:

$$\text{Glucose} + 2\ NAD^+ + 2\ P_i + 2\ ADP \rightarrow 2\ \text{pyruvate} + 2\ NADH + 2\ ATP + 2H^+ + 2\ H_2O$$

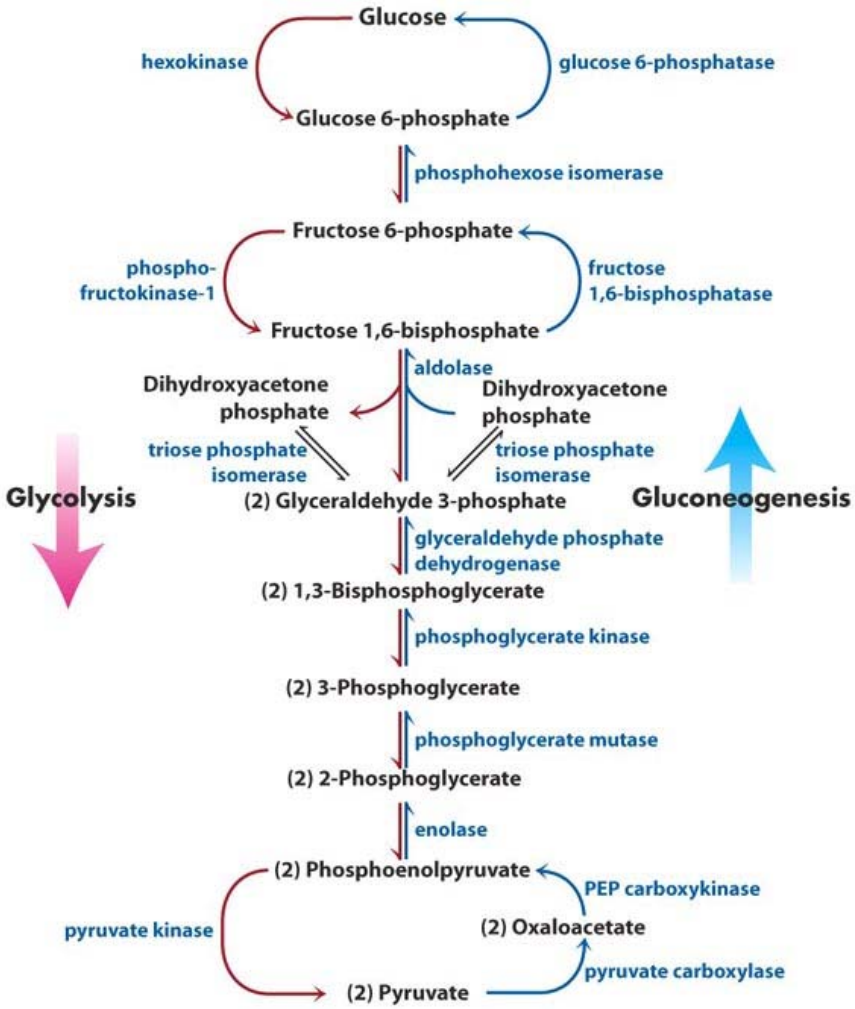


Figure 5 Glycolysis and Gluconeogenesis pathway.

Source: Lambers *et al*. (2005)

Oxidative decarboxylation of pyruvate

The pyruvate is oxidized to acetyl-CoA and CO_2 by the Pyruvate dehydrogenase complex, a cluster of enzymes-multiple copies of each of three enzymes-located in the mitochondria of eukaryotic cells and in the cytosol of prokaryotes. In the process one molecule of NADH is formed per pyruvate oxidized, and 3 moles of ATP are formed for each mole of pyruvate. This step is also known as the link reaction, as it links glycolysis and the Krebs cycle.

Citric acid cycle

This is also called the Krebs cycle or the tricarboxylic acid cycle. When oxygen is present, acetyl-CoA is produced from the pyruvate molecules created from glycolysis. Once Acetyl CoA is formed, two processes can occur, aerobic or anaerobic respiration. When oxygen is present, the mitochondria will undergo aerobic respiration which leads to the Krebs cycle. However, if oxygen is not present, fermentation of the pyruvate molecule will occur. In the presence of oxygen, when acetyl-CoA is produced, the molecule then enters the citric acid cycle (Krebs cycle) inside the mitochondrial matrix, and gets oxidized to CO_2 while at the same time reducing NAD to NADH. NADH can be used by the electron transport chain to create further ATP as part of oxidative phosphorylation. To fully oxidize the equivalent of one glucose molecule, two acetyl-CoA must be metabolized by the Krebs cycle. Two waste products, H_2O and CO_2, are created during this cycle.

The citric acid cycle is an 8-step process involving 8 different enzymes. Throughout the entire cycle, Acetyl CoA changes into Citrate, Isocitrate, α-ketoglutarate, succinyl-CoA, succinate, fumarate, malate, and finally, oxaloacetate. The net energy gain from one cycle is 3 NADH, 1 FADH, and 1 GTP. Thus, the total amount of energy yield from one whole glucose molecule (2 pyruvate molecules) is 6 NADH, 2 FADH, and 2 GTP.

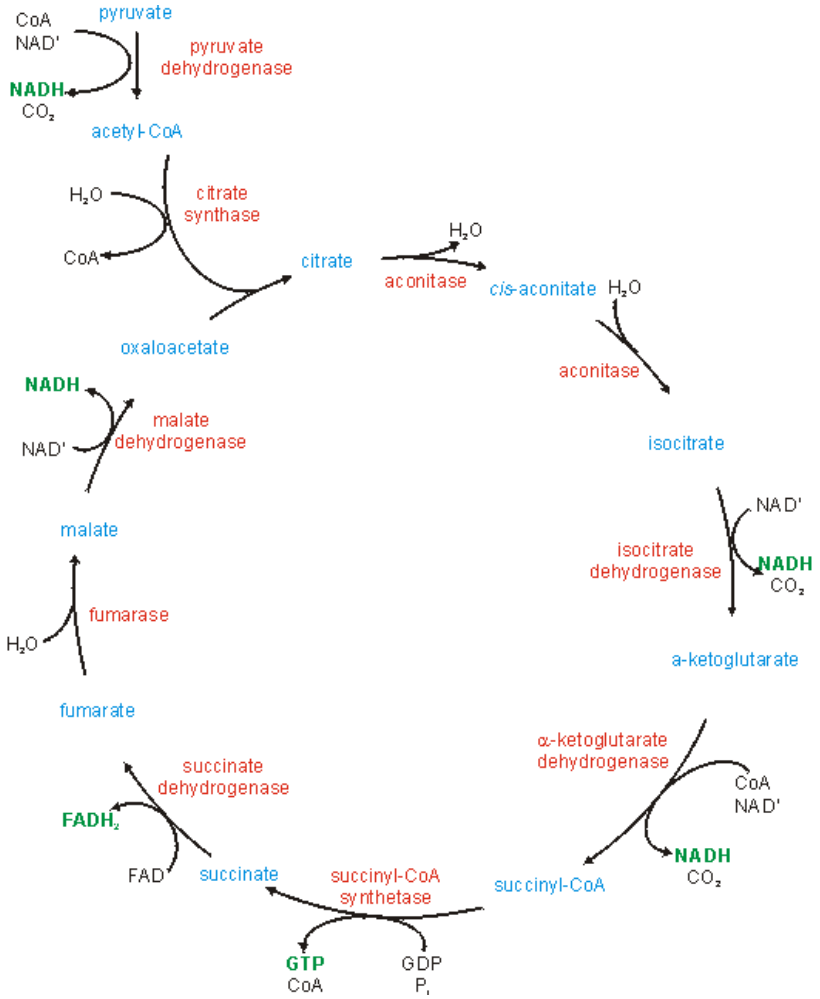


Figure 6 Citric acid cycle.

Source: Berg *et al.* (2002)

Oxidative phosphorylation, Electron transport chain, Electrochemical gradient, and ATP synthase

In eukaryotes, oxidative phosphorylation occurs in the mitochondrial cristae. It comprises the electron transport chain that establishes a proton gradient (chemiosmotic potential) across the inner membrane by oxidizing the NADH produced from the Krebs cycle. ATP is synthesised by the ATP synthase enzyme when the chemiosmotic gradient is used to drive the phosphorylation of ADP.

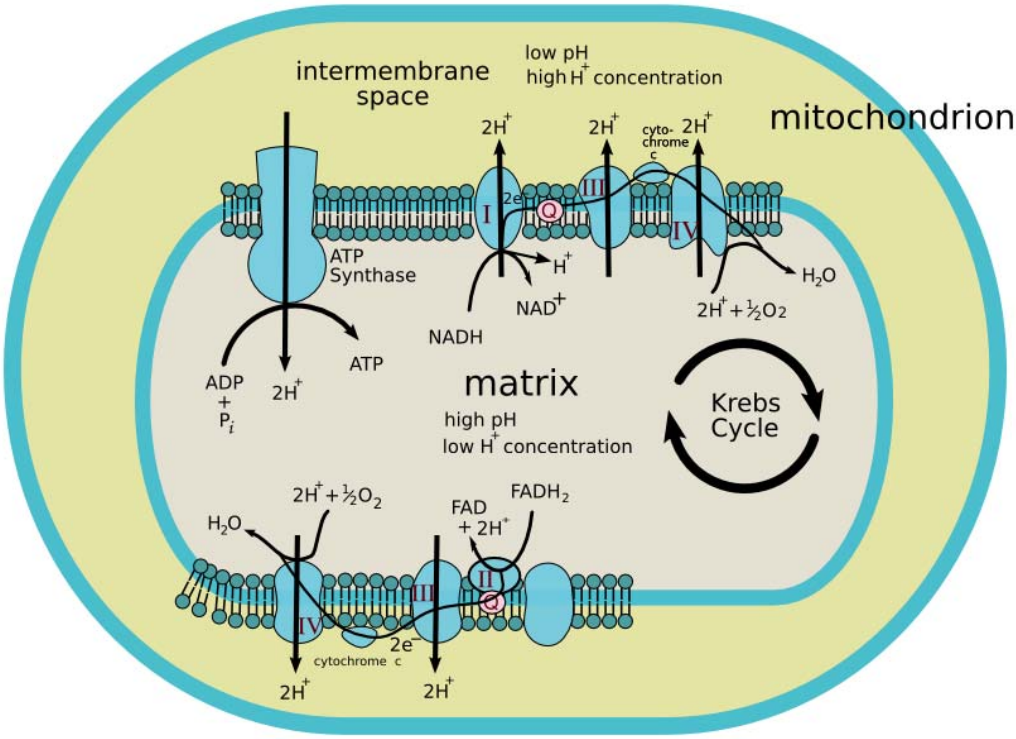


Figure 7 Electron transport chain.

Source: Krauss *et al.* (2005)

Theoretical yields

The yields in the table below are for one glucose molecule being fully oxidized into carbon dioxide. It is assumed that all the reduced coenzymes are oxidized by the electron transport chain and used for oxidative phosphorylation.

Table 1 Total ATP yield of one glucose molecule oxidized into CO_2

Step	coenzyme yield	ATP yield	Source of ATP
Glycolysis preparatory phase		-2	Phosphorylation of glucose and fructose 6-phosphate uses two ATP from the cytoplasm.
Glycolysis pay-off phase		4	Substrate-level phosphorylation
	2 NADH	4 (6)	Oxidative phosphorylation. Only 2 ATP per NADH since the coenzyme must feed into the electron transport chain from the cytoplasm rather than the mitochondrial matrix. If the malate shuttle is used to move NADH into the mitochondria this might count as 3 ATP per NADH.
Oxidative decarboxylation of pyruvate	2 NADH	6	Oxidative phosphorylation
Krebs cycle		2	Substrate-level phosphorylation
	6 NADH	18	Oxidative phosphorylation
	2 $FADH_2$	4	Oxidative phosphorylation
Total yield		36 (38) ATP	From the complete oxidation of one glucose molecule to carbon dioxide and oxidation of all the reduced coenzymes.

Source: Bryant and Frigaard. (2006)

Although there is a theoretical yield of 36-38 ATP molecules per glucose during cellular respiration, such conditions are generally not realized due to losses such as the cost of moving pyruvate (from glycolysis), phosphate, and ADP (substrates for ATP synthesis) into the mitochondria. All are actively transported using carriers that utilise the stored energy in the proton electrochemical gradient.

9. Relationship between photosynthesis and respiration

In the term of common metabolite between photosynthesis and respiration, two molecules of glyceraldehyde-3-phosphate (G3P) are the initial products formed when glucose is split in glycolysis pathway for the direction of the ultimate energy (ATP) gain by aerobic respiration in mitichondria. But they also are the molecules that react to from glucose in the eventual process of photosynthesis.

In aerobic respiration, glucose is oxidized, and its electrons are passed to the electron transport chain in the inner mitochondrial membrane. In photosynthesis, chlorophyll a is oxidized by light (photo-oxidized), and its electrons are passed to the electron transport chain in the thylakoid membrane.

In both cases, the energy released while the electrons as they pass through the electron transport chain is used to actively transport (pump) hydrogen ions across a membrane-across the inner mitochondrial membrane into the intermembrane space in mitochondria, and across the thylakoid membrane into the thylakoid lumen in chloroplasts.

Both the inner mitochondrial membrane and the thylakoid membrane are impermeable to hydrogen ions. Diffusion occurs only via specific transport proteins. This diffusion is called chemiosmosis. In both aerobic respiration and photosynthesis, the transport protein is an enzyme called ATP synthase. As the hydrogen ions (protons) diffuse through ATP synthase, they cause the central structure of the enzyme to rotate. This rotation somehow enables the synthesis of ATP from ADP and inorganic phosphate.

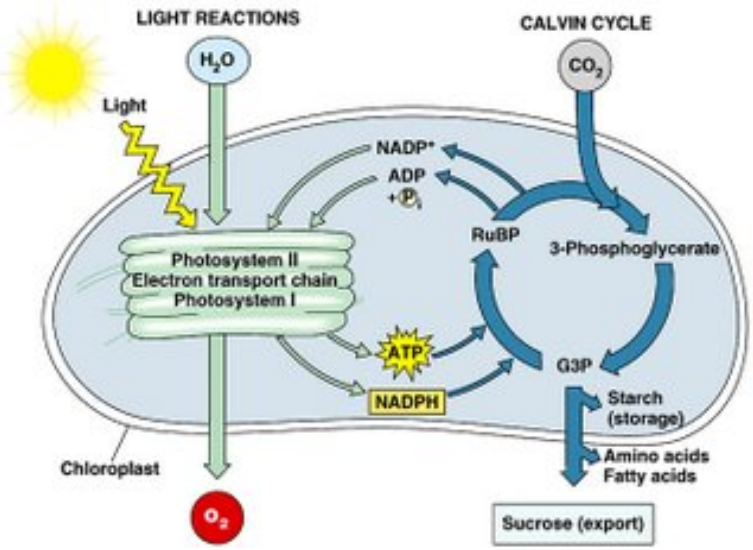


Figure 8 Relationship between photosynthesis and respiration.

Source: Bryant *et al*. (2006)

Probably the most striking similarity between the processes of cellular respiration and photosynthesis is that their overall reactions are basically the reverse of each other. The reactants in aerobic respiration are the products in photosynthesis, and vice versa.

Both processes produce ATP by chemiosmosis, and both have similar electron transfer compounds: NAD^+ in aerobic respiration and $NADP^+$ in photosynthesis. Both processes are associated with cell organelles surrounded by a double membrane—the mitochondrion in aerobic respiration and the chloroplast in photosynthesis.

The effects of photosynthesis on respiration

Both photosynthesis and respiration are vital parts of plant metabolism; however, there is still debate about the magnitude of mitochondrial respiration during photosynthesis.

In recently it was believed that an increase in the ATP/ADP ratios during photosynthesis would prevent operation of mitochondrial electron transport chain.

Several reports have provided evidence that ATP/ADP ratios, as influenced by photosynthesis, are probably not an important regulatory mechanism for mitochondrial electron transport chain activity. First, cellular ATP/ADP ratios are relatively constant, and not greatly affected by light/dark transitions. Second, *in vitro* evidence has shown that extremely high ATP/ADP is necessary to inhibit mitochondrial electron transport chain activity and such conditions are unlikely during steady-state photosynthesis. Indeed, *in vivo* electron transport chain activity is probably much more sensitive to the absolute ADP concentration than to the ATP/ADP ratio. Therefore, any biosynthetic process which consumes ATP and produces ADP could conceivably stimulate electron transport chain activity. Finally, the existence of alternative pathway respiration would minimize ATP/ADP effects on electron transport chain activity, especially if electron flow through the alternative pathway is completely uncoupled from ATP production through utilization of the rotenone resistant bypass (Turpin *et al.,* 1997).

MATERIALS AND METHODS

Materials

1. Plant Samples

In vitro cultures of eucalypt (*Eucalyptus camaldulensis*) clone T5 were obtained from Department of Genetics, Faculty of Science, Kasetsart University.

2. Chemicals and Reagents

All chemicals and reagents used in this study were molecular biological and analytical grade.

2.1 General Chemicals and Reagents

Absolute ethanol (Merck, Germany)
Glacial acetic acid (J.T. Baker, USA)
Magnesium chloride (Merck, Germany)
β-mercaptoethanol (Sigma, USA)
Methanol (Merck, Germany)
N,N,N′,N′-tetramethyl ethylenediamine (Sigma, USA)
Phosphoric acid (Merck, Germany)
Potassium chloride (Sigma, USA)
Sodium acetate (Merck, Germany)
Sodium chloride (Merck, Germany)
Sodium dodecyl sulfate (Pharmacia,USA)
Sodium dihydrogenphosphate (Merck, Germany)
Sodium hydroxide (BDH, UK)
Trichloroacetic acid (Merck, Germany)

2.2 Chemical for Tissue Culture

Ammonium nitrate (Merck, Germany)
Ammonium molybdate (Riedel-dehaen, USA)
Boric acid (Merck, Germany)
6-Benzylaminopurine (Sigma, USA)
Biotin (Sigma, USA)
Calcium chloride (Sigma, USA)
Cobalt chloride (Sigma, USA)
Copper sulfate (Sigma, USA)
D-Cal Panthothenic acid (Sigma, USA)
Ethylene diamine tetraacetic acid, (Merck Germany)
Ferrous sulfate (Sigma, USA)
6-Furfurylaminopurine (Fluka, Australia)
Magnesium sulfate (Carlo erba, Italy)
Manganese sulfate (Sigma, USA)
Potassium dihydrogen phosphate (Carlo erba, Italy)
Potassium iodide (Carlo erba, Italy)
Potassium nitrate (Carlo erba, Italy)
Zinc sulfate (Sigma, USA)

2.3 Chemicals for Bacterial Culture

Bacto agar (Difco, USA)
Bacto tryptone (Difco, USA)
Tryptone (Difco, USA)
Yeast extract (Difco, USA)

2.4 Chemicals and Reagent for Molecular Cloning

Absolute ethanol (Merck, Germany)
Agarose (Sekem)

Ampicillin (Sigma, USA)

5-bromo-4-chloro-3-indoyl-®-D-galactopyranoside (Sigma, USA)

Chloroform (Merck, Germany)

Diethyl pyrocarbohydrate (DEPC)

dATP, dCTP, dGTP, and dTTP (Promega)

DNA marker, and 100 bp ladder (Gibco,USA)

Ethidium bromide (EtBr) (Sigma, USA)

Formaldehyde (BDH, UK)

Isopropyl-®-D-thiogalactoside (IPTG) (Sigma, USA)

Phenol (Sigma, USA)

Tris-(hydroxy methyl)-aminomethane (Sigma, USA)

2.5 Reagent Kit for Molecular for Molecular Analysis

NeucleoSpin Plasmid Extraction Kit (M.N Germany)

pGEM-T vector Systems (Promega, USA)

ImProm-II^{TM} Reverse transcription System Kit (Promega, USA)

MasterAmp High Fidelity RT-PCT Kit (Epicentre)

2.6 Enzyme and Restriction enzymes

Proteinase K (Gibco, USA)

Restriction enzymes, *Eco*R I (Promega, USA)

Taq DNA polymerase (Finnzyme, Finland)

3. Equipments for analysis

Autoclave: Model HA-300MAutopipette: Pipetteman, Gilson, France

A -20 °C Freezer

A -70°C Freezer

Balance: Satorious

Centrifuge, refrigerated centrifuge: Model Sorvall Biofuge stratos

Centrifuge, microcentrifuge: Model Spectrafuge 16M

Electrophoresis unit: Submerged Agarose Gel Electrophoresis System

Gel documentation Transilluminator: ImageMaster®VDS

Heating block

Hot air oven: Model 838F (Fisher Scientific)

Hot plate

Incubator water bath: Model INNOVA 3100

Magnetic stirrer

Microwave oven

Nanodrop spectrophotometer: Model ND-1000

Protein electrophoresis: mini protean 3 electrophoresis (Bio-rad, USA)

pH meter: Fisher Scientific

Thermal cycle: Model 9700 (GeneAmp PCR system)

UV/ Visible spectrophotometer: Model UV-1601

Vortex mixer

Methods

1. Plant Materials and Stress Treatment

An *in vitro* culture of eucalypt (*Eucalyptus camaldulensis*) cultivars was propagated for multiple shoots on MS6 medium. Plantlets were maintained under 25°C with light period of 16 hours per day and 8 hours per day in dark period.

In vitro cultures of *E. camaldulensis* cloned T5 were propagated for multiple shoots on the modified Murashige and Skoog (Murashige and Skoog, 1962) semi-solid medium supplemented with 20 mg/L kinetin, 50 mg/L 6-benzylaminopurine, 10 mg/L biotin and 10 mg/L panthothenic acid. Plantlets were maintained under 25°C condition with 16 hours daylight of 2000 lux. For 45-day-old plantlets, duration of light and sucose or potassium cyanide content were varied to study their effect on alternative oxidase gene expression as in Table 2. The control was defined as multiple shoots with light period 16 hours per day without anything supplementation.

Experimental condition were performed by three factors; duration of light, sucrose deprivation and potassium cyanide (KCN) in Table 2.

Table 2 The experimental condition

Condition I	Condition II	Condition III
Dark	Dark – no Sucrose	Dark – no Sucrose – no KCN
		Dark – no Sucrose – 5 mM KCN
		Dark – no Sucrose – 10 mM KCN
	Dark – 15 mM Sucrose	Dark – 15 mM Sucrose - no KCN
		Dark – 15 mM Sucrose - 5 mM KCN
		Dark – 15 mM Sucrose - 10 mM KCN
	Dark – 30 mM Sucrose	Dark – 30 mM Sucrose - no KCN
		Dark – 30 mM Sucrose - 5 mM KCN
		Dark – 30 mM Sucrose - 10 mM KCN
	Dark – 65 mM Sucrose	Dark – 65 mM Sucrose - no KCN
		Dark – 65 mM Sucrose - 5 mM KCN
		Dark – 65 mM Sucrose - 10 mM KCN
Light 8 h	Light 8 h – no Sucrose	Light 8 h – no Sucrose - no KCN
		Light 8 h – no Sucrose - 5 mM KCN
		Light 8 h – no Sucrose - 10 mM KCN
	Light 8 h – 15 mM Sucrose	Light 8 h – 15 mM Sucrose - no KCN
		Light 8 h – 15 mM Sucrose - 5 mM KCN
		Light 8 h – 15 mM Sucrose - 10 mM KCN
	Light 8 h – 30 mM Sucrose	Light 8 h – 30 mM Sucrose - no KCN
		Light 8 h – 30 mM Sucrose - 5 mM KCN
		Light 8 h – 30 mM Sucrose - 10 mM KCN
	Light 8 h – 65 mM Sucose	Light 8 h – 65 mM Sucrose - no KCN
		Light 8 h – 65 mM Sucrose - 5 mM KCN
		Light 8 h – 65 mM Sucrose - 10 mM KCN
Light 16 h	Light 16 h – no Surose	Light 16 h – no Sucrose - no KCN
		Light 16 h – no Sucrose - 5 mM KCN
		Light 16 h – no Sucrose - 10 mM KCN
	Light 16 h – 15 mM Sucrose	Light 16 h – 15 mM Sucrose – no KCN
		Light 16 h – 15 mM Sucrose – 5 mM KCN
		Light 16 h – 15 mM Sucrose – 10 mM KCN
	Light 16 h – 30 mM Sucrose	Light 16 h – 30 mM Sucrose – no KCN
		Light 16 h – 30 mM Sucrose – 5 mM KCN
		Light 16h – 30 mM Sucrose - 10 mM KCN
	Light 16 h – 65mM Sucrose	Light 16 h – 65 mM Sucrose – no KCN
		Light 16 h – 65 mM Sucrose - 5 mM KCN
		Light 16h – 65 mM Sucrose - 10 mM KCN
Light 24 h	Light 24h – no Sucrose	Light 24h – no Sucrose - no KCN
		Light 24h – no Sucrose - 5 mM KCN
		Light 24h – no Sucrose - 10 mM KCN
	Light 24 h – 15 mM Sucrose	Light 24 h – 15 mM Sucrose - no KCN
		Light 24 h – 15 mM Sucrose - 5 mM KCN
		Light 24 h – 15 mM Sucrose - 10 mM KCN
	Light 24 h – 30 mM Sucrose	Light 24 h – 30 mM Sucrose - no KCN
		Light 24 h – 30 mM Sucrose - 5 mM KCN
		Light 24 h – 30 mM Sucrose - 10 mM KCN
	Light 24 h – 65 mM Sucrose	Light 24 h – 65 mM Sucrose - no KCN
		Light 24 h – 65 mM Sucrose - 5 mM KCN
		Light 24 h – 65 mM Sucrose - 10 mM KCN

2. Isolation of Partial Alternative Oxidase Genes from *E. camaldulensis*

2.1 RNA Extraction

Because RNA is sensitive to degradation, all pertinent glasswares were soaked in diethylpyrocarbonate-treated water and autoclaved. Total RNA was extracted from 100 mg of multiple shoots of *E. camaldulensis* grown under three conditions; duration of light, sucrose deprivation and varius concentration of potassium cyanide in Table 2. RNA samples were isolated using Plant Total RNA Mini Kit (Real Biotech Corporation) and stored at –80 °C until analysis.

The concentration of total RNA was determined by nanodrop spectrophotometer

Quality of the purified total RNA was determined by calculating the A_{260} /A_{280} ratio. The ratio between 1.8 and 2.0 indicated good quality of the RNA. The RNA was stored at -20 °C until use.

2.2 Reverse Transcription Reaction

First strand cDNA had been done by reverse transcription from total RNA using ImProm-II™ Reverse transcription System Kit (Promega). Twenty microlitres reaction was performed at 42 °C for 1 hour in a 1.5 ml microcentrifuge tube containing 1 μg total RNA, 15 U/μg ImProm-II™ Reverse transcriptase, 0.5 μg oligo$(dT)_{15}$ primer, 5 mM $MgCl_2$, 1X Reverse Transcription buffer, 1 mM each dNTP, and 1 U/μl Recombinant RNasin® ribonuclease inhibitor. After incubation, the reaction mixture was heated at 70 °C for 15 min to denature the RNA/cDNA hybrid and inactivated the ImProm-II™ Reverse transcriptase then incubated on ice for 5 min. The first strand cDNA synthesis reaction was stored at –20 °C use as a template in PCR reaction.

2.3 Primers for PCR Amplification of Alternative Oxidase cDNA Fragments

Primers used to amplify *alternative oxidase* and 26S rRNA gene in cDNA were designed based on the alignment of the alternaive oxidase amino acid sequence from various amino acid sequence plants (Figure 2). Genbank member includes *Nicotiana tabacum* (Genbank accession no. AB281425), *Triticum aestivum* (Genbank accession no. AB078883), *Zea mays* (Genbank accession no. AY059646), *Oryza sativa* (japonica cultivar-group) (Genbank accession no. AB004864), *Oryza sativa* (Genbank accession no. AB007452), *Arabidopsis thaliana* (Genbank accession no. AJ131392), *Gossypium hirsutum* (Genbank accession no. DQ250028), *Vigna unguiculata* (Genbank accession nos. DQ100441), *Saccharum officinarum* (Genbank accession no. AY644465) revealed highly conserved boxes for primers design. The forward degenerate primers (AOX-F) and the reverse degenerate primer (AOX-R) were designed for *alternative oxidase* gene.

```
Triticum aestivum           ----MMTRGATRMTRVVMGHMGPRYFSTTVLRNDPGTGVVGGAAAGLLHG 46
Zea mays                    ----MMTRGATRMTRTVLGHMGPRYFSTAIFRNDAGTGVMSGAAV-FMHG 45
Oryza sativa(japonica)      ---MMMMMSRSGANRVAN---------TAMFVAKGLSGEVGGLRA--LYG 36
Oryza sativa                ----MSSRVAGSVLLRHLGPRVFGPTTPAAQRPLLAGGEGGAVAV----A 42
Arabidopsis thaliana        ----MSSRMAGSAILRHVG----------GVRLFTASATSPAAAA----A 32
Nicotiana tabacum           ----MSYRSIYRTLRPVLS-------SSVQSSGLGIGGFRGHLIS----- 34
Gossypium hirsutum          -------------MFRNHASRITAAAAPWVLRTACRQ-KSDAKTP----- 31
Vigna unguiculata           -------------MFRNHASRITAAAAPWVLRTACRQ-KSDAKTP----- 31
Saccharum officinarum       -------------MFRNHASRITAAAAPWVLRTACRQ-KSDAKTP----- 31

Triticum aestivum           FPANPSEKVAVTWVRHFSAMGSRSASTAALNDKQQEKESSDKKVENTATA 96
Zea mays                    VPANPSEKAVVTWVRHFPVMGSRSAMSMALNDKQHDKKAENG--SAAATG 93
Oryza sativa(japonica)      G-------------------GVRSESTLALSEKEKIEK------KVGLSS 61
Oryza sativa                MWARPLSTSAAEAAREEATASKDNVASTAAATAEAMQAA-----KAGAVQ 87
Arabidopsis thaliana        AAARPFLAGGEAVP---GVWGLRLMSTSSVASTEAAAKA-----EAKKAD 74
Nicotiana tabacum           -----------------HLPNVRLLSSDTSSPVSGNNQP------ENPIR 61
Gossypium hirsutum          ------------------VWGHTQLNRLSFLETVPVVPLR----VSDESS 59
Vigna unguiculata           ------------------VWGHTQLNRLSFLETVPVVPLR----VSDESS 59
Saccharum officinarum       ------------------VWGHTQLNRLSFLETVPVVPLR----VSDESS 59
                                                .

Triticum aestivum           AANGGAGKSVVSYWGVPPSK-ATKPDGTEWKWNCFRPWETYEADMSIDLT 145
Zea mays                    GGDGGDEKSVVSYWGVQPSK-VTKEDGTEWKWNCFRPWETYKADLSIDLT 142
Oryza sativa(japonica)      AGGNKEEKVIVSYWGIQPSK-ITKKDGTEWKWNCFSPWGTYKADLSIDLE 110
Oryza sativa                AAKEGKSPAASSYWGIVPAK-LVNKDGAEWKWSCFRPWEAYTSDTTIDLS 136
Arabidopsis thaliana        AEKE---VVVNSYWGIEQSKKLVREDGTEWKWSCFRPWETYTADTSIDLT 121
Nicotiana tabacum           TADG---KVISTYWGIPPTK-ITKPDGSAWKWNCFQPWDSYKPDVSIDVT 107
Gossypium hirsutum          EDRPTWSLPDIENVAITHKKPNGLVDTLAYRSVRTCRWL-------FDTF 102
Vigna unguiculata           EDRPTWSLPDIENVAITHKKPNGLVDTLAYRSVRTCRWL-------FDTF 102
Saccharum officinarum       EDRPTWSLPDIENVAITHKKPNGLVDTLAYRSVRTCRWL-------FDTF 102
                                      *     *           *         *
```

Figure 9 Amino acid sequences alignment of *alternative oxidase* gene from the other plants.

```
Triticum aestivum           KHHAPVTFLDKFAYWTVKVLRFPTDVFFQRRYGCRAMMLETVAAVPGMVG 195
Zea mays                    KHHAPTTFLDKFAYWTVKSLRYPTDIFFQRRYGCRAMMLETVAAVPGMVG 192
Oryza sativa(japonica)      KHMPPTTFLDKMAFWTVKVLRYPTDVFFQRRYGCRAMMLETVAAVPGMVA 160
Oryza sativa                KHHKPKVLLDKIAYWTVKSLRVPTDIFFQRRYGCRAMMLETVAAVPGMVD 186
Arabidopsis thaliana        KHHVPKTLLDKIAYWTVKSLRFPTDIFFQRRYGCRAMMLETVAAVPGMVG 171
Nicotiana tabacum           KHHKPSNFTDKFAYWTVQTLKIPVQLFFQRKHMCHAMLLETVAAVPGMVG 157
Gossypium hirsutum          SLYRFG------------------SITESKVISRCLFLETVAGVPGMVG 133
Vigna unguiculata           SLYRFG------------------SITESKVISRCLFLETVAGVPGMVG 133
Saccharum officinarum       SLYRFG------------------SITESKVISRCLFLETVAGVPGMVG 133
                            .                       : : :   : ::**::*.*****

Triticum aestivum           GMLLHCKSLRRFEQSGGWIKALLEEAENERMHLMTFMEVAKPNVYERALV 245
Zea mays                    GMLLHCKSLRRFEQSGGWIKTLLDEAENERMHLMTFMEVAKPNWYERALV 242
Oryza sativa(japonica)      GMLLHCKSLRRFEHSGGWFKALLEEAENERMHLMTFMEVAKPKWYERALV 210
Oryza sativa                GMLLHLRSLRRFEQSGGWIRALLEEAENERMHLMTFMEVANPKWYERALV 236
Arabidopsis thaliana        GMLLHLRSLRRFEQSGGWIRTLLEEAENERMHLMTFMEVANPKWYERALV 221
Nicotiana tabacum           GMLLHLKSLRRFEHSGGWIKALLEEAENERMHLMTFIELSQPKWYERAIV 207
Gossypium hirsutum          GMLRHLSSLRYMTRDKGWINTLLVEAENERMHLMTFIELRQPGLPLRVSI 183
Vigna unguiculata           GMLRHLSSLRYMTRDKGWINTLLVEAENERMHLMTFIELRQPGLPLRVSI 183
Saccharum officinarum       GMLRHLSSLRYMTRDKGWINTLLVEAENERMHLMTFIELRQPGLPLRVSI 183
                            *** *  *** : :. **:.:** ** ******:**::: :*    :  :

Triticum aestivum           FAVQGVFFNAYFAAYLISPKLAHRIVGYLEEEAVHSYTEFLKELDNGNIE 295
Zea mays                    FAVQGVFFNAYFVTYLLSPKLAHRIVGYLEEEAIHSYTEFLKELDKGNIE 292
Oryza sativa(japonica)      ITVQGVFFNAYFLGYLLSPKFAHRMFGYLEEEAIHSYTEFLKELDKGNIE 260
Oryza sativa                LAVQGVFFNAYFLGYIVSPKFAHRVVGYLEEEAIHSYTEFLRDLEAGRIE 286
Arabidopsis thaliana        ITVQGVFFNAYFLGYLLSPKFAHRVVGYLEEEAIHSYTEFLKDLEAGKID 271
Nicotiana tabacum           FTVQGVFFNAYFLAYVISPKLAHRITGYLEEEAVNSYTEFLKDIDAGKFE 257
Gossypium hirsutum          IITQAIMYLFLLVAYVISPRFVHRFVGYLEEEAVITYTGVMRAIDEGRLR 233
Vigna unguiculata           IITQAIMYLFLLVAYVISPRFVHRFVGYLEEEAVITYTGVMRAIDEGRLR 233
Saccharum officinarum       IITQAIMYLFLLVAYVISPRFVHRFVGYLEEEAVITYTGVMRAIDEGRLR 233
                            : .*.: :   :  *:.**:  **. *******: :**  :. :: * :

Triticum aestivum           -----NVPAPAIAIDYWRLPKD-ATLRDVVLVVRADEAHHRECQPLCI-- 337
Zea mays                    -----NVPAPAIAIDYCRLPKD-STLLDVVLVVRADEAHHRDVNHFASDI 336
Oryza sativa(japonica)      -----NVPAPAIAIDYWQLPPG-STLRDVVMVVRADEAHHRDVNHFASDI 304
Oryza sativa                -----NVPAPRIAIDYWRLPAD-ARLKDVVTVVRADEAHHRDVNHFAADI 330
Arabidopsis thaliana        -----NVPAPAIAIDYWRLPAN-ATLKDVVTVVRADEAHHRDVNHFASDI 315
Nicotiana tabacum           -----NSPAPAIAIDYWRLPKD-ATLRDVVYVIRADEAHHRDINHYASDI 301
Gossypium hirsutum          PTKN---DVPEVARVYWNLSKN-ATFRDLINVIRADEAEHRVVNHTFADM 279
Vigna unguiculata           PTKN---DVPEVARVYWNLSKN-ATFRDLINVIRADEAEHRVVNHTFADM 279
Saccharum officinarum       PTKN---DVPEVARVYWNLSKN-ATFRDLINVIRADEAEHRVVNHTFADM 279
                                   .* :*  *  :. .   : *::  :*****.**  :

Triticum aestivum           --------------------------------------------------
Zea mays                    HYQGQQ-----------------LKDSPAPIGYH---------------- 353
Oryza sativa(japonica)      HYQGRE-----------------LREAAAPIGYH---------------- 321
Oryza sativa                HFQGLE-----------------LNKTPAPLGYH---------------- 347
Arabidopsis thaliana        HYQGME-----------------LKQTPAPIGYH---------------- 332
Nicotiana tabacum           QFKGHE-----------------LKEAPAPIGYH---------------- 318
Gossypium hirsutum          HEKRLQNSVNPFVVLKKNPEE---MYSNQPSGKTRTDFGSEGAKTASNVN 326
Vigna unguiculata           HEKRLQNSVNPFVVLKKNPEE---MYSNQPSGKTRTDFGSEGAKTASNVN 326
Saccharum officinarum       HEKRLQNSVNPFVVLKKNPEE---MYSNQPSGKTRTDFGSEGAKTASNVN 326

Triticum aestivum           ---------------
Zea mays                    ---------------
Oryza sativa(japonica)      ---------------
Oryza sativa                ---------------
Arabidopsis thaliana        ---------------
Nicotiana tabacum           ---------------
Gossypium hirsutum          KHV------------ 329
Vigna unguiculata           KHV------------ 329
Saccharum officinarum       KHV------------ 329
```

Figure 9 (Continued)

2.4 PCR Amplification of *Alternative Oxidase* gene

Degenerated primers for RT-PCR of alternative oxidase cDNA fragments and primer for 26S rRNA gene were designed from the conserved amino acid sequence of *alternative oxidase* genes. cDNA of alternative oxidase was used as template for the PCR. Forward degenerated primer, AOX-F: 5′- GC(CT)GC(AG)GT (GC)CC(GT)GG(AG)ATG -3′, and the reverse degenerated primer, AOX-R: 5′- CG(AG)TG(AG)TG(AT)GC(CT)TC(AG)TC(AG)GC -3′, were used to amplify for 10 μl reaction of partial alternative oxidase gene. The PCR reaction containing 1X reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 1 μM each primers, 0.5 U *Taq* DNA polymerase and ~ 100 ng cDNA template were place in a thermal cycler and followed by 1 min at 94ºC, 1 min at 59 ºC and 1 min at 72ºC for 45 cycles. The PCR of 26S rRNA gene, was amplified by Euca26S F: 5′ -GACTCAGAACTGGTACGG-3′and Euca26S R: 5′- ATAGTAGACAGGGACAGTGG -3′, as same as *alternative oxidase* gene reaction but changed the thermal condition as 1 min at 94ºC, 1 min at 50 ºC and 1 min at 72ºC for 25 cycles. Five microlitres of each PCR product was then analyzed by electrophoresis on 1.5% (w/v) agarose gel, and visualized by staining with EtBr.

2.5 Primers and PCR Amplification of *Alternative Oxidase* Gene from Nucleotide Sequence of *E. camaldulensis*

The degenerated primers of *alternative oxidase* gene, AOX-forward and AOX-reverse, were non-specific. Therefore, two bands with the size of 750 and 540 bp were observed in the PCR product. For transcriptional detection of the expression of *AOX* gene, the PCR product should be a single fragment. Thus, both of the PCR products were sequenced and analyzed at the Bioservice unit (BSU) by an ABI™ Prism™ 377 Genetic Analyzer. The data were recorded by the computer and the results were displayed either as a graph or as a text sequence. The new specific primers for *alternative oxidase* gene were designed from 540 base pairs fragment because this size was the major band and the sequence data of 540 base pairs fragment was more clearly to analyze than 750 base pairs fragment. The new forward and reverse primer used to

amplified *alternative oxidase* gene were EuAOX-F; 5´-CATAGTGGTGGTTGGGTC AAAGCCCTGC-3´and EuAOX-R; 5´-GGTAACCTCCAGTAGTCAATGGCAATA GCCG-3´respectively. The PCR step was performed as following 1 s 94° C, 1 s 55°C and 1 min 72° C for 45 cycles. The PCR product was detected at 354 bp.

```
1    GGAAGAAGCGGAGAATGAAAGGATGCACCTGATGACGATGGTGGAGCTCG
51   TGAAACCGAAATGGTACGAGAGGCTGCTGGTTCTTTCAGTGCAAGGAGTA
101  TTCTTCAATGCTTACTTCGTCCTCTATTTGCTCTCCCCTAAATTAGCACA
151  TAGAGTTGTTGGCTATCTGGAAGAGGAGGCCATTCACTCATATACAGAGT
201  ACTTGAAGGATATTAACAGTGGTGCGATTGAAAATGTTCCTGCTCCGGCT
251  ATTGCCATTGACTACTGGAGGTTACCTAAGGATGCAACTCTGAAGGACGT
301  GATTACAGTTATCCGTGCCGACGAGGCTCACCACCGAATCACTAGTGAAT
351  TCGC
```

Figure 10 Nucloetide sequence of *alternative oxidase* gene of *E. camaldulensis***.**

2.6 Agarose Gel Electrophoresis

Agarose was mixed into 1X Tris-borate EDTA (TBE) buffer (Appendix) with an appropriate concentration for separation the particular size of DNA fragment and heat in a microwave oven until complete solubilization. While the agarose solution was cooling, a clean and dry gel-casting tray was sealed the ends with tape and an appropriate comb was selected for forming the sample slot in the gel. A position of the comb was 0.5-1.0 mm above the plate. The warm agarose (55 °C) was poured into the casting tray. After the gel set completely (30-45 min at room temperature), the comb was removed carefully and the ends of the casting tray were unsealed. The gel is placed in an electrophoresis chamber containing TBE buffer. The DNA sample was mixed with 6X gel-loading buffer (see Appendix) and the sample mixture was loaded slowly into the slots of the submerged gel. Standard DNA marker was loaded into the slots on the right sides of the gel. The lid of the gel chamber was closed and the electrophoresis was carried out in 1X TBE running buffer. The DNA should migrate

toward the positive anode at 100 volts until the bromophenol blue and xylene cyanol FF migrated to an appropriate distance through the gel. Then, the gel was stained in 1 μg/ml ethidium bromide (EtBr) solution for 15 min and destained by soaking it in distilled water for 15 min. The nucleic acid bands were visualized under UV transilluminator and photographed by a Gel Documentary (ImageMaster®VDS)

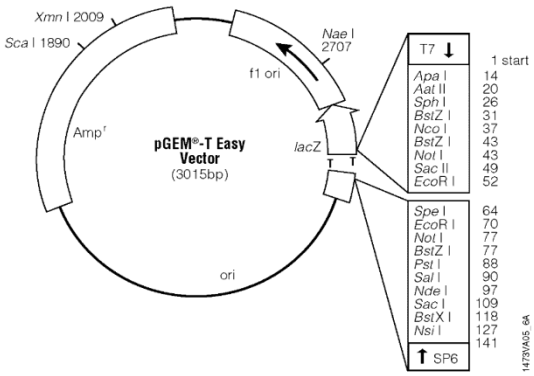
2.7 Purification of DNA Fragment from Agarose Gel

Purification of DNA fragment from agarose gel was performed according to NucleoSpin Gel Extract II kit. In brief, the desired DNA fragments on agarose gel were excised with razor blade and 100 mg of agarose gel containing the band was transferred to a microcentrifuge tube. Three hundred microliters of NT buffer was added to the agarose gel. The sample was incubated at 50 ºC for 10 min or until the agarose gel was completely dissolved. The DNA was transferred to a spin column. The column was centrifuged at 12,000 rpm for 1 min at room temperature. The spined column was removed from the collection tube and discarded the liquid inside the tube. The spin column was placed back into the tube and added 500 μl of NT3 washing buffer. The flow-through was discarded. The spined column was placed back into the tube and centrifuge for an additional 1 min at 12,000 rpm. The spin column was placed into a new microcentrifuge tube. The DNA fragment was eluted by adding 30 μl of NE buffer (10 mM Tris-HCl, pH 8.5) and incubated for 1 min at room temperature. After incubation, the column was centrifuge at 12000 rpm for 1 min. The purified DNA fragment was determined its concentration by gel electrophoresis and stored at –20 ºC until use.

2.8 DNA Ligation

The purified DNA fragment was ligated into pGEM-T vector (Promega) which possessed a single 3′ deoxythimidine (T) overhanged at both ends. This vector allowed easy cloning of PCR product based on the fact that *Taq* polymerase used in PCR tends to add an additional nucleotide, usually a deoxyadenosine (A) to the 3′- end of each strand that it synthesized. Therefore, a double-stranded PCR product has a single adenosine nucleotide overhang. The molar ratio of the insert DNA to the vectors is usually 3:1. The amount of inserted DNA depended on the length of the inserted DNA fragment The 10 µl of each ligation reaction composed of inserted DNA (150 ng), 1 µl of 10X T4 DNA ligase buffer, 1µl of pGEM-T vector (50 ng), and 1 µl of T4 DNA ligase (3 U/µl). Sterile water was added to the final volume of 10 µl. Each mixture was gently mixed by pipetting and then incubated at 16 °C, overnight.

(A)



(B)

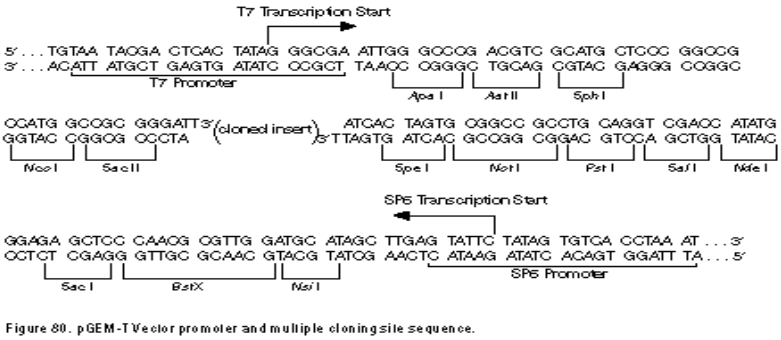


Figure 80. pGEM-T Vector promoter and multiple cloning site sequence.

Figure 11 pGEM-T vector circle map (A) and Promotor and multiple cloningsequence of the pGEM-T (B). The top strand of the sequence corresponded to the RNA synthesized by T7 RNA polymerase. The bottom strand corresponded to the RNA synthesized by SP6 RNA.

2.9 Preparation of Competent *E. coli* Cells

The basic recombinant DNA technique of competent cell preparation, transformation, and isolation of plasmid DNA were conducted on standard protocols of Sambrook and Russell (2000). A single colony of *E. coli*, JM 109, was inoculated into 2 ml of LB broth (see Appendix) and incubated at 37 ºC with shaking (220 cycles/min in a rotary shaker), 12-16 hr. One millilitre of the microbial starter was inoculated into 100 ml of LB broth and the culture was incubated at 37 ºC with vigorous shaking for 3-5 hr until the A_{260} of the cells was reached 0.5-0.7 (about 2-3 hr). The bacterial cells were transferred to a sterile, ice-cold 50 ml polypropylene tube and the tube was chilled on ice for 30 min. The cells were recovered by centrifugation at 4000 rpm for 10 min at 4 ºC then the medium was removed from cell pellets as much as possible. Cell pellet was resuspended by gentle mixing in 10 ml of ice-cold 0.1 M $CaCl_2$ solution and stored on ice for 1 hour. Competent cells were precipitated by centrifugation at 4000 rpm for 10 min. The cells were resuspended in 2 ml of 0.1 M $CaCl_2$, which made by this procedure were preserved at –70 ºC.

2.10 Bacterial Transformation

The tube containing the ligation reaction from section 5.8 was centrifuged to collect contents at the bottom of the tube. Five microlitres of each ligation reaction was added to a sterile 1.5 microcentrifuge tube on ice. The tube of JM 109 competent cells (from section 5.9) was placed in an ice bath and then mixed the cells by gently flicking the tube. Fifty microlitres of the cell suspension was mixed with 5µl of ligation mixture. The tube was placed on ice for 2 min. The contents in the tube was heat-shocked in a water bath at exactly 42 ºC (do not shake) for 1 min and then placed immediately on ice for 2 min. The tube containing cells transformed with ligation reactions was added with 950 µl of room temperature LB medium and incubated at 37 ºC for 1 hour with shaking at 220 rpm. Before the transformed mixture was plated on LB ampicillin agar plates (see Appendix), 100 µl of 100 mM IPTG and 20 µl of 50 mg/ml X-Gal were spreaded over the surface of an LB ampicillin plate and allowed to absorb at 37 ºC for 30 min prior to use. The plates were incubated at 37 ºC

for 12-16 hr. Recombinant clones were analyzed by blue/white screening. The transformant colonies were counted and selected randomly to check the transformation of the inserted DNA by PCR and restriction analysis.

2.11 Determination of Recombinant Plasmid DNA by alkaline Method

Plasmid DNA was isolated from small-scale (2 ml) bacterial cultures by treatment with alkali and SDS. A single white colony of X-gal-IPTG-ampicillin plate was cultured in 2 ml of LB broth containing 100 μg/ml ampicillin and incubated at 37 ºC for 12-16 hr with vigorous shaking. The culture was transferred into 1.5 ml microcentrifuge tube and centrifuged at 5000 rpm at 4 ºC for 10 min. After centrifugation, the medium was removed. The bacterial pellet was resuspended in 100 μl of alkaline lysis solution I (see Appendix) by vigorous vortexing. Two hundred microlitres of freshly prepared alkaline lysis solution II (see Appendix) was added to each bacterial suspension and mixed the contents by inverting the tube rapidly five times. After incubating the tube on ice for a few minutes, 150 μl of alkaline lysis solution III (see Appendix) was added and mixed gently by inverting the tube several times. The tube was stored on ice for 3-5 min and then centrifuged at 12000 rpm for 5 min to separate cell debris. The supernatant was transferred into a microcentrifuge tube. An equal volume of phenol : chloroform : isoamyl alcohol (25 : 24 : 1, v/v) was added to the supernatant and mixed by vortexing. The emulsion was centrifuged at 12000 rpm at 4 ºC for 10 min and the upper aqueous phase was transferred to a new tube. Plasmid DNA was precipitated by adding 2 volumes of absolute ethanol then mixed the solution by vortexing. The mixture was allowed to stand at –20 ºC for 20 min. The plasmid DNA was collected by centrifugation at 12000 rpm at 4 ºC for 10 min. The pellet was washed with 70% (v/v) ethanol and inverted the close tube several times. The plasmid DNA was recovered by centrifugation at 12000 rpm at 4 ºC for 5 min. The supernatant was removed by gentle aspiration and the tube was allowed to stand in an inverted position on a paper towel. The open tube was stored at room temperature until the ethanol evaporated completely then the DNA was dissolved in 50 μl of TE buffer. The extracted plasmid DNA was kept at –20 ºC for further analysis.

2.12 Verification of DNA Insertion by Restriction Digestion and PCR

Restriction digestion and agarose gel electrophoresis were used to verify whether insert DNA is cloned at the corrected site in the vector. These methods mainly concentrate on analysis DNA pattern obtained after the restriction enzyme digestion. In this study the pGEM-T vector was used for gene cloning. Vector pGEM-T contains multiple restriction sites within the multiple cloning region. The multicloning region of pGEM-T vector is flanked by recognition sites for the restriction enzyme *EcoR* I, thus producing single-enzyme digestion for release the insert. After digestion of the plasmid DNA with restriction enzymes, electrophoresis was carried out. If a DNA of interest inserts at the site of the vector, the pattern of DNA bands should be as expected. There are two bands which have the same sizes as the insert and vector used for ligation. Approximately 500 ng of the purified plasmid DNA of each clone was digested with restriction enzymes in 10 µl reaction volume containing 1 µl of 10X Buffer, 0.5 mg/ml BSA, 1 U *EcoR* I and incubated at 37 ºC for 3 hours. Three microlitres of loading dye was added to the digested solution, loaded onto 1.5% agarose gel and electrophoresed at 100 volts. The size of inserted DNA was compared with 1 Kb plus DNA ladder marker. The PCR analysis was the second step to selected clones which containing interest inserted plasmid and the specific primers, EuAOX-F and EuAOX-R, were used. Then the clone was selected for sequencing at Macrogen Incorperation (Korea).

3. Semi-quantitative RT-PCR of Alternative oxidase Gene

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was used to perform the expression level (Wang, 1999). In the RT-PCR reaction, amplification starts with mRNA as a template. This template was treated with reverse transcriptase and a specific forward amplifying primer. A cDNA product was made from this RT reaction. This cDNA product then becomes the target of the PCR reactions that follow using forward and reverse primers. In semi-quantitative RT-PCR, the amount of amplified product was detected during successive cycles of the PCR portion of the reaction. This detection was performed to establish when the initial cycle of amplification begins. This system to determine relative expression of genes, since

there was an indirect relationship between the number of mRNA transcripts expressed from a particular gene and the first cycle at which amplification can be observed. The technique is very sensitive to detecting even modestly expressed transcripts since it employs PCR amplification. The most common sources of error are post-PCR manipulations while pipetting samples or loading electrophoretic gels.

One-Step Continuous RT-PCR using MasterAmp^{TM} High Fidelity RT-PCR kit. Reaction containing MastwerAmp 1x RT-PCR premix, MMLV-RT Plus, MasterAmp TAQurate DNA Ploymerase Mix, 12.5 uM gene specific primer and RNA template. The reaction mixture was preheated to 37 ºC 30 min for synthesis first strand

As an expression control for use in quantification, universal 26S primers (David and Aaron, 1999) were performed to determine expression of control gene. Two sets of primers, experimental and control primers were separated to amplified which the primers were able to amplify two different transcripts without contamination with each other.

Densitometry was performed on each cDNA band by application of the Program GelQuant ver 2.7computer software. These experiments were repeated three times independent-experiment.

RESULTS AND DISCUSSION

Results

1. The Growth of *E. camaldulensis* Multiple Shoots under Light Duration

Growth of *E. camaldulensis* under various light periods in the experiment was divided into 4 categories, 0, 8, 16 and 24 hours per day. The experiments were performed for 15 days. According to the experiment, there was visual difference in morphology of *E. camaldulensis*. However, they were normally able to grow up under the 4 different light conditions. The result was shown in Table 3.

Table 3 Morphology of *E. camaldulensis* in light duration periods for 1 day, 3 days, 5 days, 7 days and 15 days

	Dark	Light 8 h./day	Light 16 h./day	Light 24 h./day
1 Day				
3 Days				
5 Days				
7 Days				
15 Days				

2. Isolation of Partial *Alternative Oxidase* genes from *E. camaldulensis*

Because the degenerated primers of *alternative oxidase* gene, AOX-forward and AOX-reverse, were non-specific, therefore, two bands with the size of 750 and 540 bp were observed in the PCR product. For an accurate detection of gene expression at the transcriptional level detection, the PCR product should to be a single fragment**.** Thus, both were sequenced and analyzed at the Bioservice unit (BSU) by an ABI^{TM} Prism^{TM} 377 Genetic Analyzer. The data were recorded by the computer and the results were displayed either as a graph or as a text sequence. The new specific primers for *alternative oxidase* gene were designed from 540 base pairs fragment. The fragment was cloned and sequenced. Specific primer, AOX-F and AOX-R were then designed for the *AOX* gene with the expected size of 354 bp (Figure 12B).

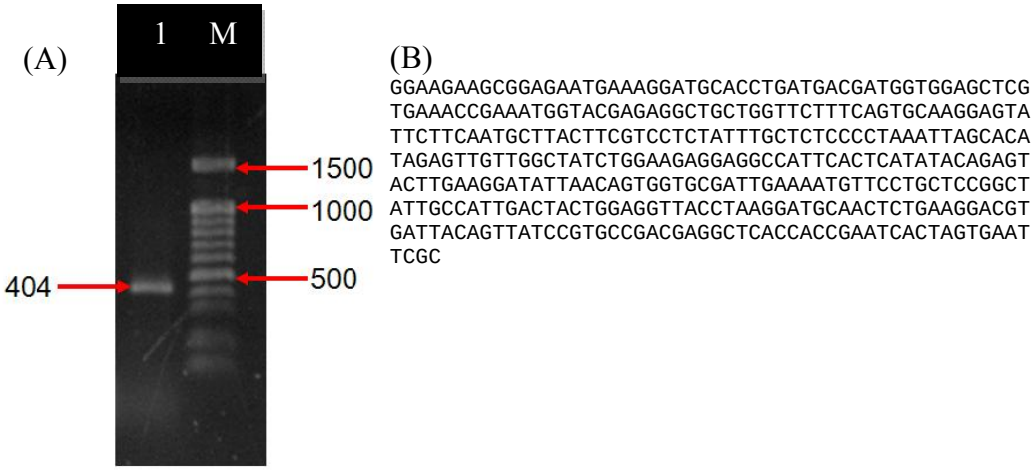


Figure 12 (A) PCR product of *alternative oxidase* gene was amplified by specific primers. Lane M: 100 bp ladder and lane 1: PCR fragments of *alternative oxidase* gene. (B) Partial nucleotide sequence *AOX* gene.

Table 4 Specific primer sequence for Semi-quantitative RT-PCR

Gene	Primer sequence
Euca26S rRNA	Euca26S-F: 5’ GACTCAGAACTGGTACGG 3’
	Eu26caS-R: 5’ ATAGTAGACAGGGACAGTGG 3’
AOX	EuAOX-F; 5’ CAT AGT GGT GGT TGG GTC AAA GCC CTG C 3’
	EuAOX-R; 5’ GGT AAC CTC CAG TAG TCA ATG GCA ATA GCC G 3’

3. Effect of Light Duration on the Expression of *Alternative Oxidase* gene in *E. camaldulensis* Multiple Shoot

The effect of light duration on expression of *alternative oxidase* gene were examined at 1, 3, 5, 7 and 15 days. The expression of *AOX* gene of *E. camaldulensis* in the dark condition obviously increased, especially, when the sample duration of dark was 15 days. The *E. camaldulensis* which were exposed to light for 8 or 16 hours per day for 1, 3, 5, 7 and 15 days, there were no differences the expression of the *AOX* gene under these in periods of light. However, under 24 hour-light a day, it was found that the expression of *AOX* gene decreased and intensity of the 5 bands showed the lowest intensity at the last day (15 days). (Figure 13)

3.1 Expression of *AOX* gene in different duration of the light

The expression of *AOX* gene either in dark or under 8, 16, 24 hour-light a day was shown as the representation of the intensity of the bands (Figure 13A), which was evaluated by the densitormetry in Figue 13B. The result of the effect of duration of light on *AOX* gene expression was summarized in Figure 13

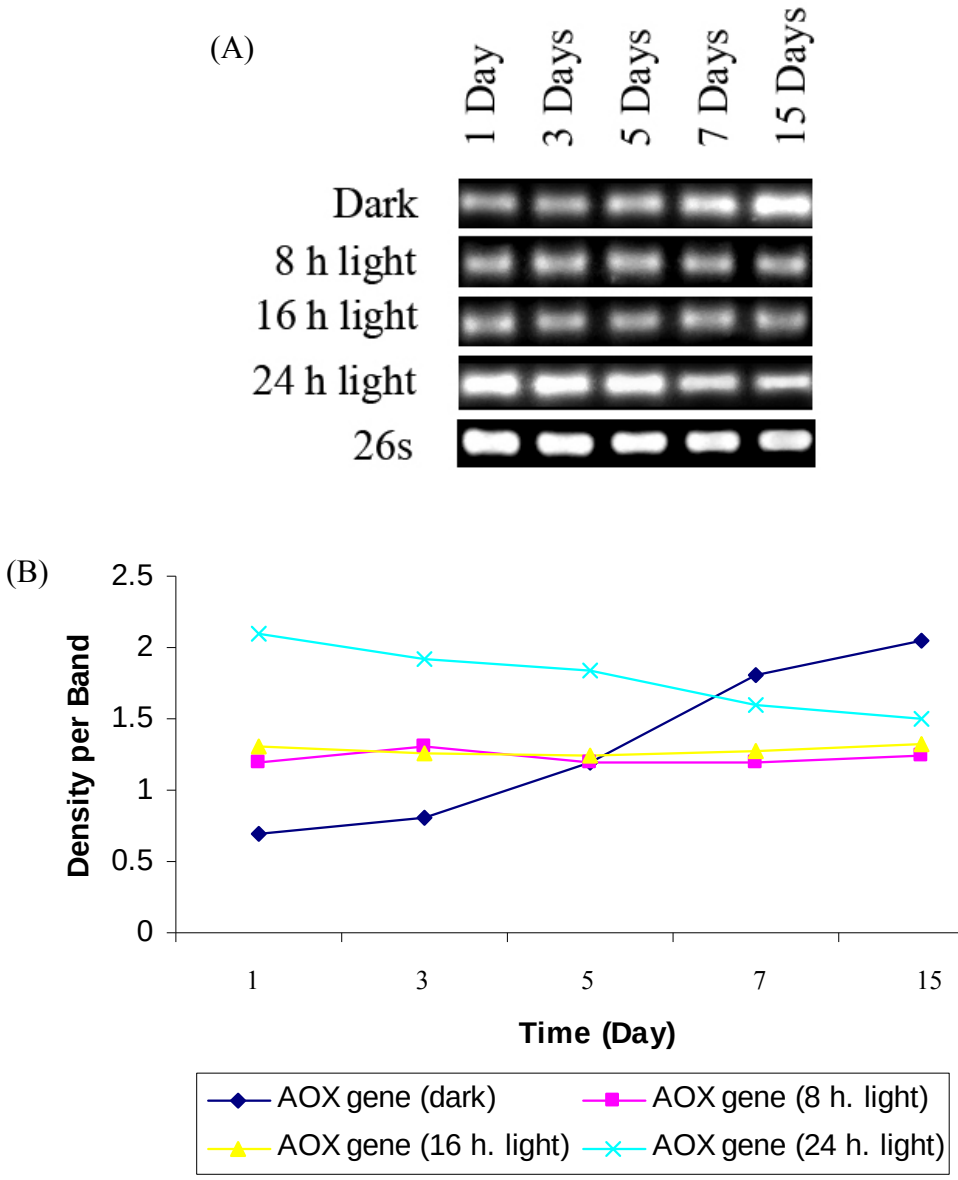


Figure 13 Expression of *AOX* gene in different light duration by the passing time within 15 days. (A) Bands of *AOX* gene expression. (B) Intensity of the (A) bands measured by Densitometry GelQuant 2.7.

4. The Growth Development of *E. camaldulensis* Multiple Shoots under Light Duration and Reduced Sucrose Supplementation

The morphology change was investigated when sucrose supplementation was reduced from the normal condition of 65 mM sucrose to no sucrose. The experiments were performed under various supplementation of sucrose concentrations and different duration of light, concomitantly, of 8, 16 and 24 hour-light a day or in the dark.

The change in morphology of *E. camaldulensis* was observed on the 1, 3, 5, 7 and 15 days. The reason of sucrose reduction is to determine the autotrophic behavior of the eucalypt.

4.1 The reduced sucrose supplementation under the dark condition

The supplementation of sucrose was reduced from 65 mM sucrose (normal condition) to no sucrose (0 mM sucrose). The eucalypt were cultured in the dark within 15 days. With no added sucrose, the chlorosis of leaves were observed, while 15, 30, and 65 mM sucrose supplementation, all were still green within 15 days (Table 5)

Table 5 Morphology of *E. camaldulensis* in the dark with different concentrations of sucrose for 1 day, 3 days, 5 days, 7 days and 15 days

	Dark 0 mM Sucrose	Dark 15 mM Sucrose	Dark 30 mM Sucrose	Dark 65 mM Sucrose
1 Day				
3 Days				
5 Days				
7 Days				
15 Days				

4.2 The reduced sucrose supplementation under 8 hour-light a day condition

When the *E. camaldulensis* were cultured either various concentrations of sucrose supplementation or no added sucrose under 8 hour-light a day within 15 days, no difference in morphology change and growth were observed (Table 6)

Table 6 Morphology of *E. camaldulensis* in the 8 hour-light a day with different concentrations of sucrose for 1 day, 3 days, 5 days, 7 days and 15 days

	Light 8 h./day 0 mM Sucrose	Light 8 h./day 15 mM Sucrose	Light 8 h./day 30 mM Sucrose	Light 8 h./day 65 mM Sucrose
1 Day				
3 Days				
5 Days				
7 Days				
15 Days				

4.3 The reduced sucrose supplementation under 16 hour-light a day condition

When the *E. camaldulensis* were cultured either with various concentrations of sucrose supplementation or no added sucrose under 16 hour-light a day within 15 days, no difference in morphology change and growth were observed (Table 7)

Table 7 Morphology of *E. camaldulensis* in the 16 hour-light a day with different concentrations of sucrose for 1 day, 3 days, 5 days, 7 days and 15 days

	Light 16 h./day 0 mM Sucrose	Light 16 h./day 15 mM Sucrose	Light 16 h./day 30 mM Sucrose	Light 16 h./day 65 mM Sucrose
1 Day				
3 Days				
5 Days				
7 Days				
15 Days				

4.4 The reduced sucrose supplementation under 24 hour-light a day condition

When the *E. camaldulensis* were cultured either with various concentrations of sucrose supplementation or no added sucrose under 24 hour-light a day within 15 days, no difference in morphology change and growth were observed (Table 8)

Table 8 Morphology of *E. camaldulensis* in the 24 hour-light a day with different concentrations of sucrose for 1 day, 3 days, 5 days, 7 days and 15 days

	Light 24 h./day 0 mM Sucrose	Light 24 h./day 15 mM Sucrose	Light 24 h./day 30 mM Sucrose	Light 24 h./day 65 mM Sucrose
1 Day				
3 Days				
5 Days				
7 Days				
15 Days				

5. Effect of Light Duration and Reduced Sucrose Supplementation on the Expression of *Alternative Oxidase* gene in *E. camaldulensis* Multiple Shoot

The experiment was performed for the expression of *AOX* gene when reduced sucrose supplementation from normal condition of 65 mM sucrose, until no added sucrose, 0 mM sucrose either in different durations of light 8, 16, 24 hour-light a day or in the dark. The observation was on the designated day, 1, 3, 5, 7, 15.

5.1 The reduced sucrose supplementation under the dark condition

When reduced sucrose supplementation to 30 mM instead of the normal condition of 65 mM sucrose, the expression of *AOX* gene increased. However, either added 15 mM sucrose or no added sucrose condition the expression did not change or decreased, respectively. (Figure 14)

5.1.1 Expression of *AOX* gene in the dark with different concentrations of sucrose supplementation

Summarizing the result, it was found that the expression of the gene was induced when 65 mM or 30 mM sucrose was supplemented, even in the dark condition. However, either added 15 mM sucrose or no added sucrose condition, the expression did not change or decreased, respectively.

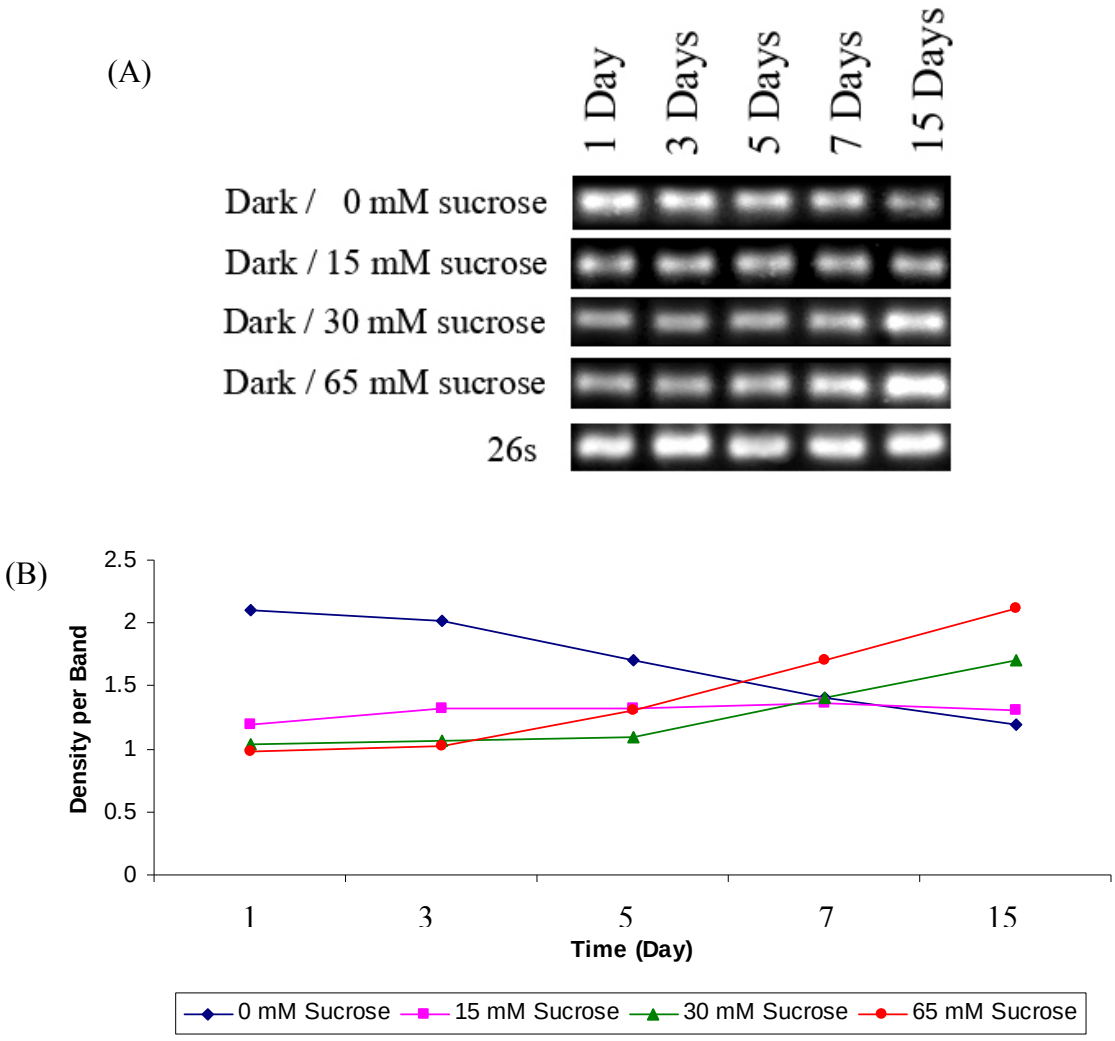


Figure 14 Expression of *AOX* gene in the dark with different concentrations of sucrose supplementation in various periods of time within 15 days. (A) Bands of *AOX* gene expression. (B) Intensity of the (A) bands measured by Densitometry GelQuant 2.7.

5.2 The reduced sucrose supplementation under 8 hour-light a day

The experiments were performed for the expression of the *AOX gene* in 8 hour-light a day and with various concentrations of sucrose supplementation. The expression was reduced when either added 15 mM sucrose or no sucrose added. However, no change of the expression when 30 mM or 60 mM sucrose supplementation.

5.2.1 Expression of *AOX* gene in 8 hour-light a day with different concentrations of sucrose supplementation

Summarizing the result, it was found that the expression of *AOX* gene did not change when the supplementation of 30 mM and 65 mM sucrose in 8 hour-light a day. However, the expression of *AOX* gene decreased remarkably either in 15 mM sucrose supplementation or no added sucrose by the passing time within 15 days.

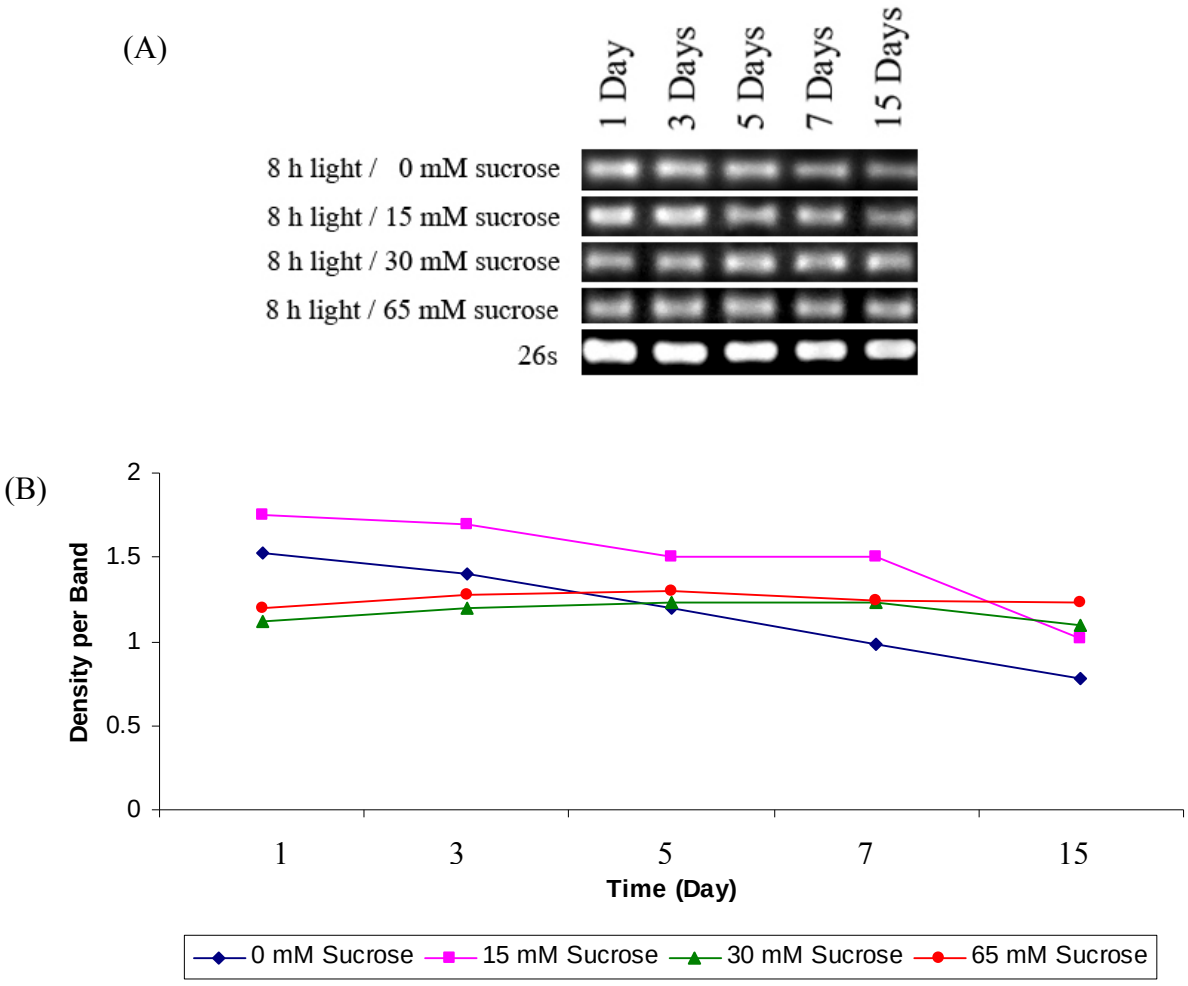


Figure 15 Expression of *AOX* gene in the 8 hour-light a day with different concentrations of sucrose supplementation in various periods of time within 15 days. (A) Bands of *AOX* gene expression. (B) Intensity of the (A) bands measured by Densitometry GelQuant 2.7.

5.3 The reduced sucrose supplementation under 16 hour-light a day

The experiments were performed for the expression of *AOX* gene in 16 hour-light a day and with various concentrations of sucrose supplementation. Expression of *AOX* gene reduced when no added sucrose and did not change by the sucrose suppementation.

5.3.1 Expression of *AOX* gene in 16 hour-light a day with different concentrations of sucrose supplementation

Summarizing the result, it was found that the expression of *AOX* gene did not change when supplementation of sucrose was reduced or no sucrose added in 16 hour-light a day by the passing time within 15 days.

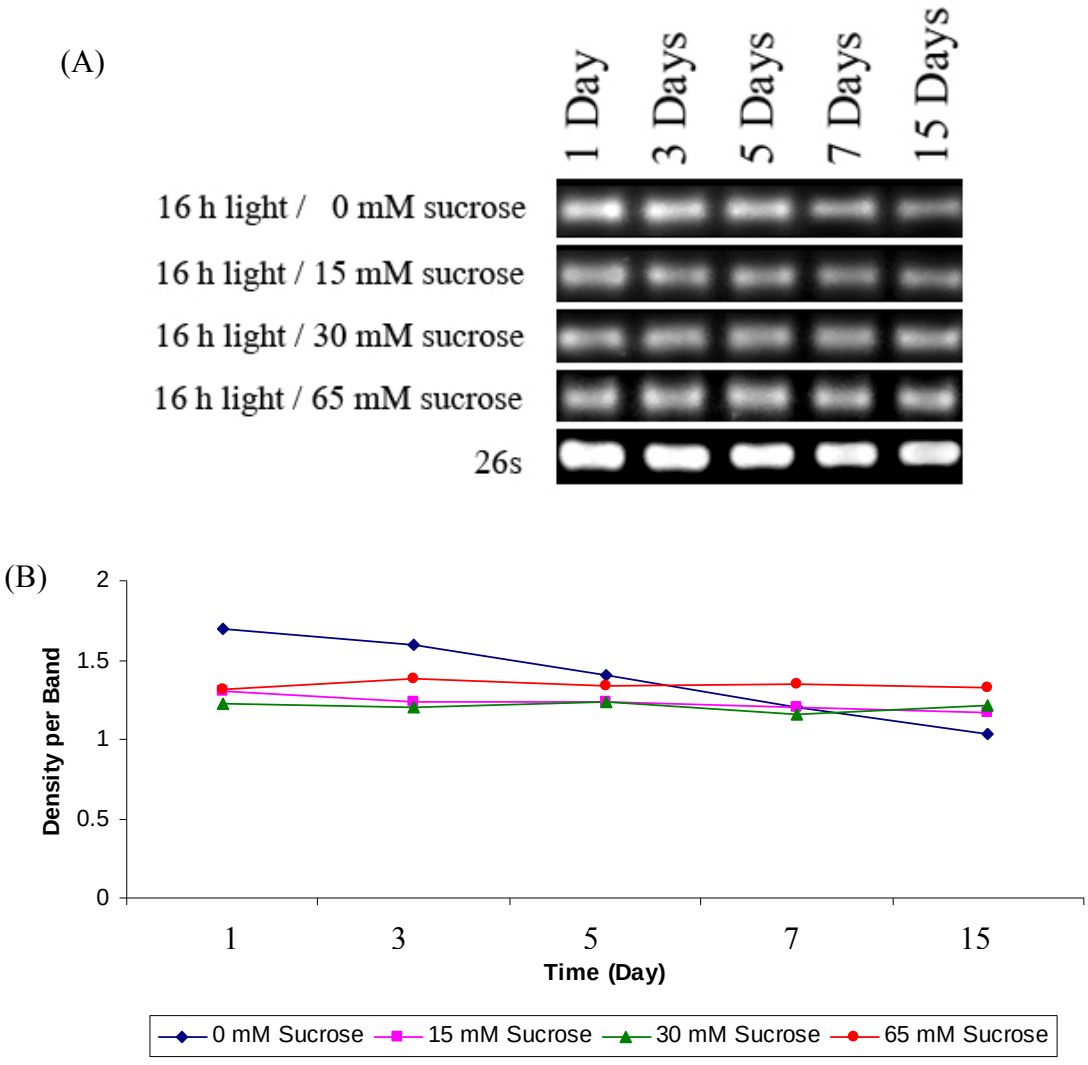


Figure 16 Expression of *AOX* gene in the 16 hour-light a day with different concentrations of sucrose supplementation in various periods of time within 15 days. (A) Bands of *AOX* gene expression. (B) Intensity of the (A) bands measured by Densitometry GelQuant 2.7.

5.4 The reduced sucrose supplementation under 24 hour-light a day

The experiments were performed for the expression of *AOX* gene in 24 hour-light a day and with various concentrations of sucrose supplementation. Expression of *AOX* gene reduced whether supplementation of sucrose or not. It could be noticed by the decrease in the intensity of bands

5.4.5 Expression of *AOX* gene in 24 hour-light a day with different concentration of sucrose supplementation

Summarizing the result, it was found that the expression of *AOX* gene decreased either no sucrose or sucrose supplementation in 24 hour-light a day by the passing time within 15 days.

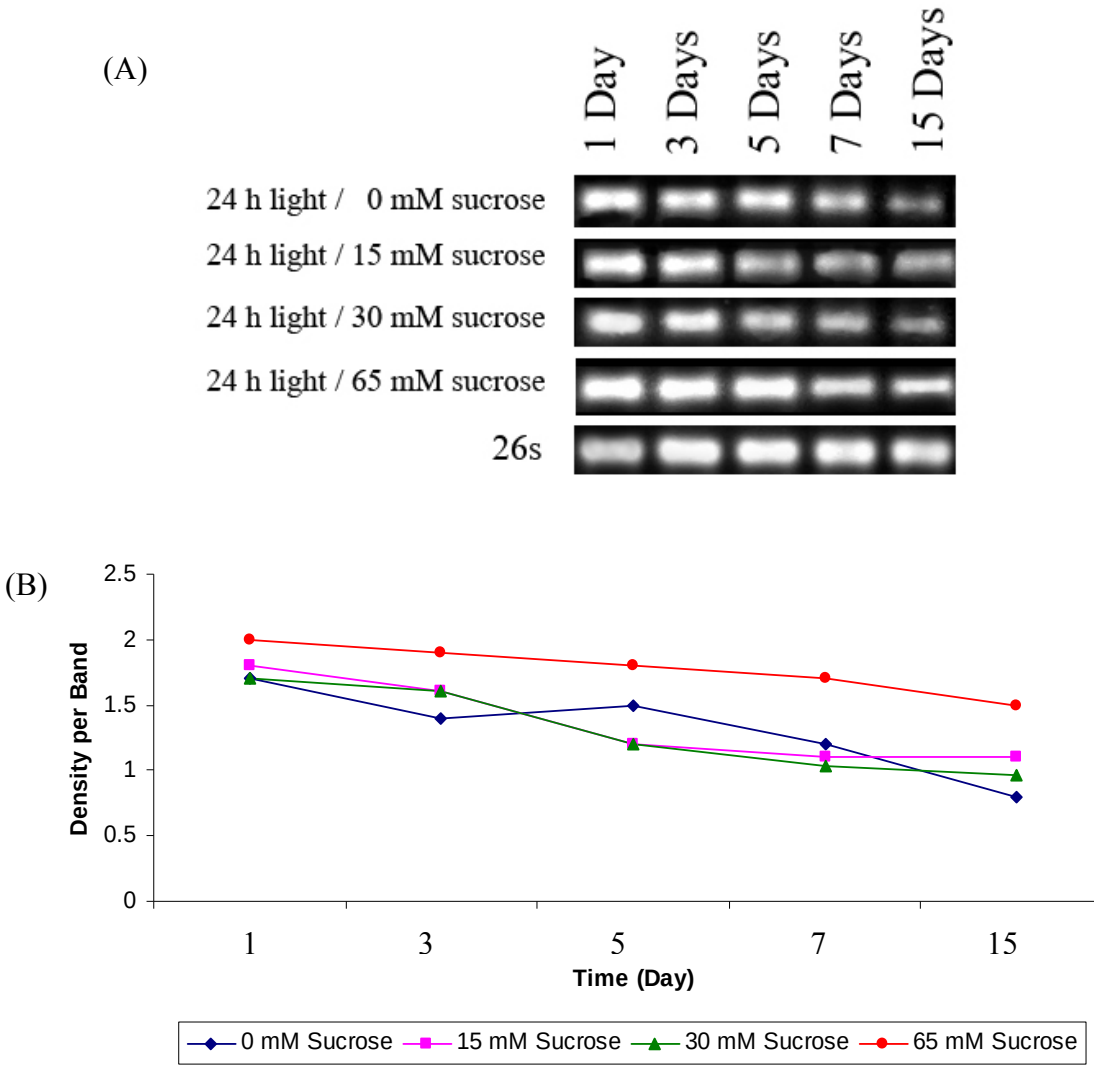


Figure 17 Expression of *AOX* gene in the 24 hour-light a day with different concentrations of sucrose supplementation in various periods of time within 15 days. (A) Bands of *AOX* gene expression. (B) Intensity of the (A) bands measured by Densitometry GelQuant 2.7.

6. The Growth Development of *E. camaldulensis* Multiple Shoots under Light Duration with Different Concentrations of Sucrose and KCN

To sum up, the morphology change of *E. camaldulensis* which was cultured in 3 environmental factors: durations of light, reducing sucrose and added KCN was observed on the 1,3,5,7, and 15 days. The results showed that *E. camaldulensis* could not been able to endure with KCN-stress because its leaves changed to yellow and parched.

6.1 Morphology of *E. camaldulensis* grown in the dark with different concentrations of sucrose and 5 mM KCN

The *E. camaldulensis* could endure when the 5 mM of KCN was added until the fifth day. After that, the leaves changed to yellow color and parched. However, when sucrose of 30 or 65 mM were added, the *E. camaldulensis* did not become to the adverse condition when compared to the one without sucrose added (Table 9). The *E. camaldulensis* with clearly appearance in morphology change were chosen and shown separately in Figure 18 and 19.

Table 9 Morphology of *E. camaldulensis* in the dark with different concentrations of sucrose and 5 mM KCN for 1 day, 3 days, 5 days, 7 days and 15 days.

	Dark 0 mM Sucrose. 5 mM KCN	Dark 15 mM Sucrose 5 mM KCN	Dark 30 mM Sucrose 5 mM KCN	Dark 65 mM Sucrose 5 mM KCN
1 Day				
3 Days				
5 Days				
7 Days				
15 Days				

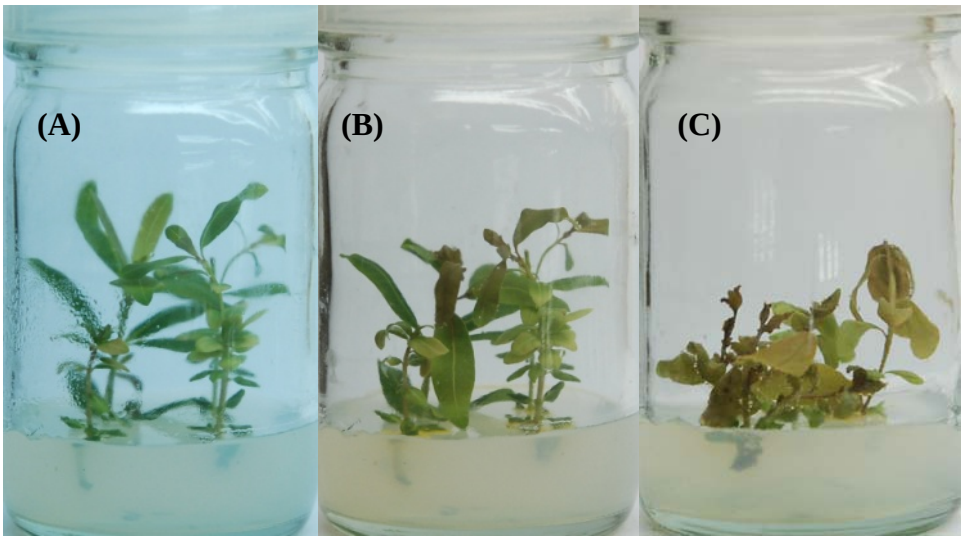


Figure 18 Comparison of *E. camaldulensis* morphology in the dark with 0 mM sucrose and 5 mM KCN. (A) for 1 day (B) for 5 days (C) for 15 days.

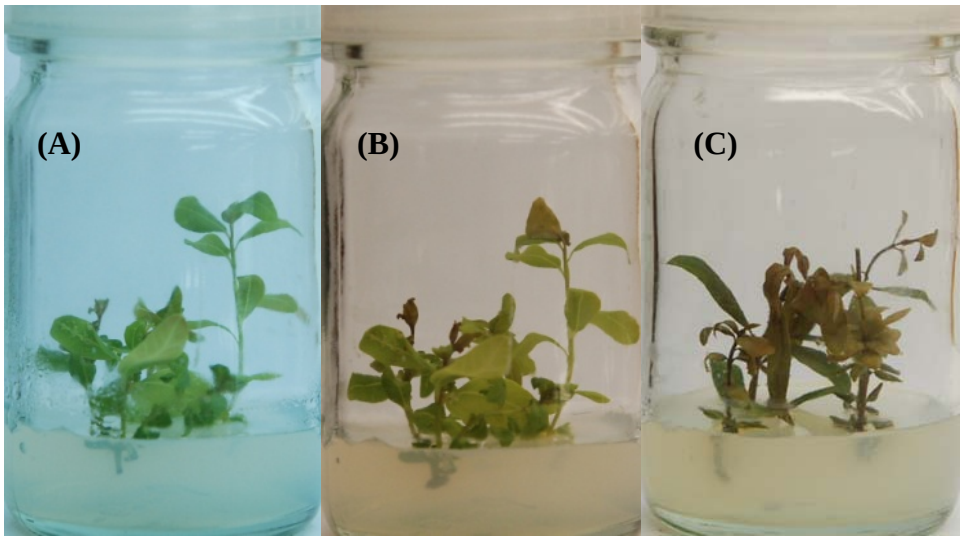


Figure 19 Comparison of *E. camaldulensis* morphology in the dark with 15 mM sucrose and 5 mM KCN. (A) for 1 day (B) for 5 days (C) for 15 days.

6.2 Morphology of *E. camaldulensis* grown in the dark with different concentrations of sucrose and 10 mM KCN

The results were similar to those with added 5 mM KCN. After the fifth day, the leaves faded and changed to be yellow color and parched. However, when sucrose concentration of 30 or 65 mM were added, the *E. camaldulensis* did not become to the adverse condition when compared to the one without sucrose added (Table 10). The *E. camaldulensis* with clearly appearance in morphology change were chosen and shown separately in Figure 20 and 21.

Table 10 Morphology of *E. camaldulensis* in the dark with different concentrations of sucrose and 10 mM KCN for 1 day, 3 days, 5 days, 7 days and 15 days.

	Dark 0 mM Sucrose. 10 mM KCN	Dark 15 mM Sucrose 10 mM KCN	Dark 30 mM Sucrose 10 mM KCN	Dark 65 mM Sucrose 10 mM KCN
1 Day				
3 Days				
5 Days				
7 Days				
15 Days				

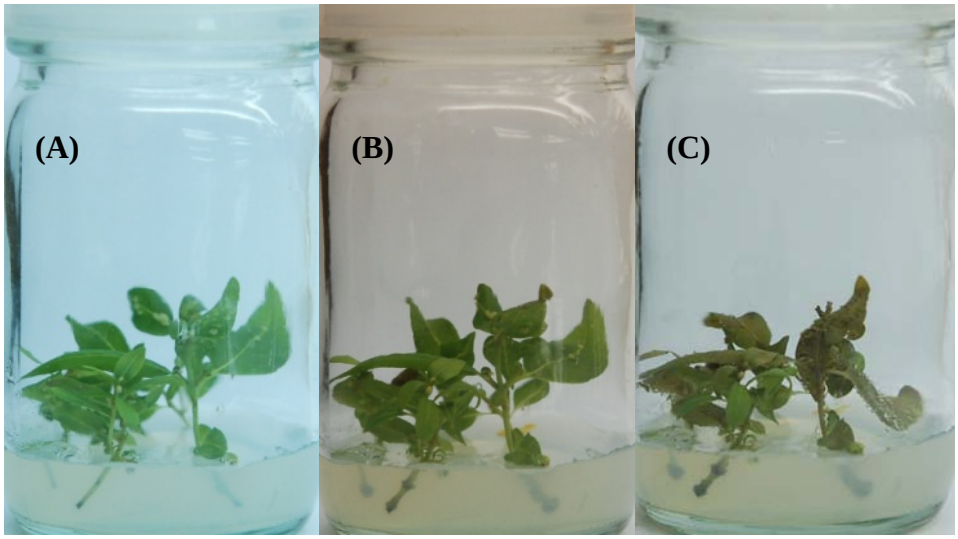


Figure 20 Comparison of *E. camaldulensis* morphology in the dark with 0 mM sucrose and 10 mM KCN. (A) for 1 day (B) for 5 days (C) for 15 days.

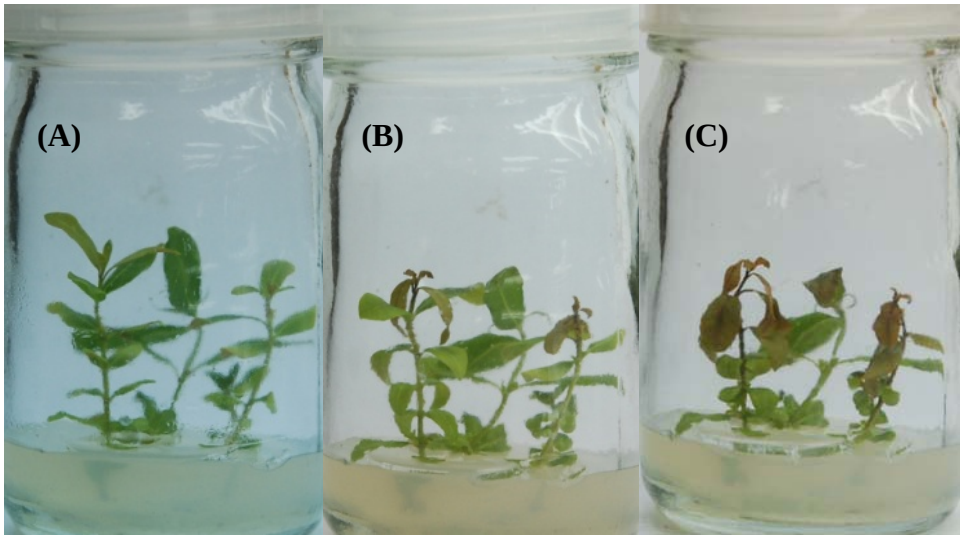


Figure 21 Comparison of *E. camaldulensis* morphology in the dark with 30 mM sucrose and 10 mM KCN. (A) for 1 day (B) for 5 days (C) for 15 days.

6.3 Morphology of *E. camaldulensis* grown in the 8 hour-light a day with different concentrations of sucrose and 5 mM KCN

The leaves of *E. camaldulensis* changed to be yellow color and parched after the fifth day. In addition, the low concentration of sucrose supplementation, the *E. camaldulensis* became to the adverse condition more than the one with higher concentration of sucrose added (Table 11). The *E. camaldulensis* with clearly appearance in morphology change were chosen and shown separately in Figure 22 and 23.

Table 11 Morphology of *E. camaldulensis* in the 8 hour-light a day with different concentrations of sucrose and 5 mM KCN for 1 day, 3 days, 5 days, 7 days and 15 days

	Light 8 h./ day 0 mM Sucrose. 5 mM KCN	Light 8 h./ day 15 mM Sucrose 5 mM KCN	Light 8 h./ day 30 mM Sucrose 5 mM KCN	Light 8 h./ day 65 mM Sucrose 5 mM KCN
1 Day				
3 Days				
5 Days				
7 Days				
15 Days				

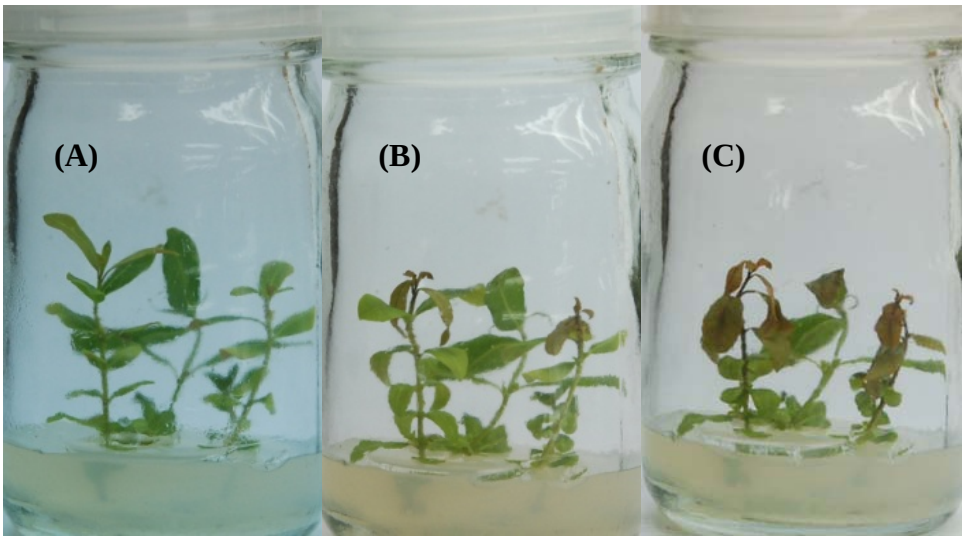


Figure 22 Comparison of *E. camaldulensis* morphology in the 8 hour-light a day with 0 mM sucrose and 5 mM KCN. (A) for 1 day (B) for 5 days (C) for 15 days.

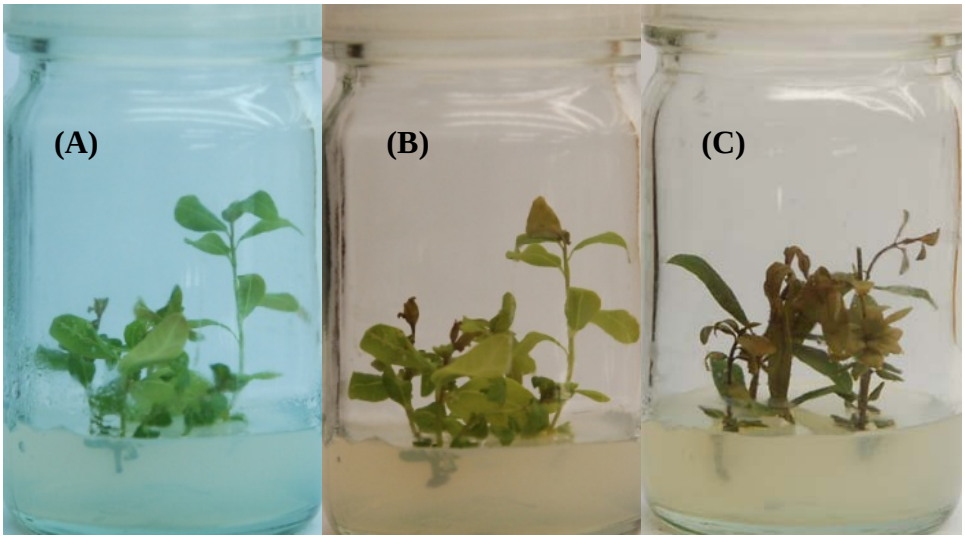


Figure 23 Comparison of *E. camaldulensis* morphology in the 8 hour-light a day with 65 mM sucrose and 5 mM KCN. (A) for 1 day (B) for 5 days (C) for 15 days.

6.4 Morphology of *E. camaldulensis* grown in the 8 hour-light a day with different concentrations of sucrose and 10 mM KCN

The result obviously came out the same as section 6.3. After the fifth day, the leaves faded and parched. Likewise, with low concentration of sucrose supplementation the eucalypt became to the adverse condition more than the one with more concentration of sucrose added (Table 13). The *E. camaldulensis* with clearly appearance in morphology change were chosen and shown separately in Figure 24 and 25.

Table 12 Morphology of *E. camaldulensis* in the 8 hour-light a day with different concentrations of sucrose and 10 mM KCN for 1 day, 3 days, 5 days, 7 days and 15 days

	Light 8 h./ day 0 mM Sucrose. 10 mM KCN	Light 8 h./ day 15 mM Sucrose 10 mM KCN	Light 8 h./ day 30 mM Sucrose 10 mM KCN	Light 8 h./ day 65 mM Sucrose 10 mM KCN
1 Day				
3 Days				
5 Days				
7 Days				
15 Days				

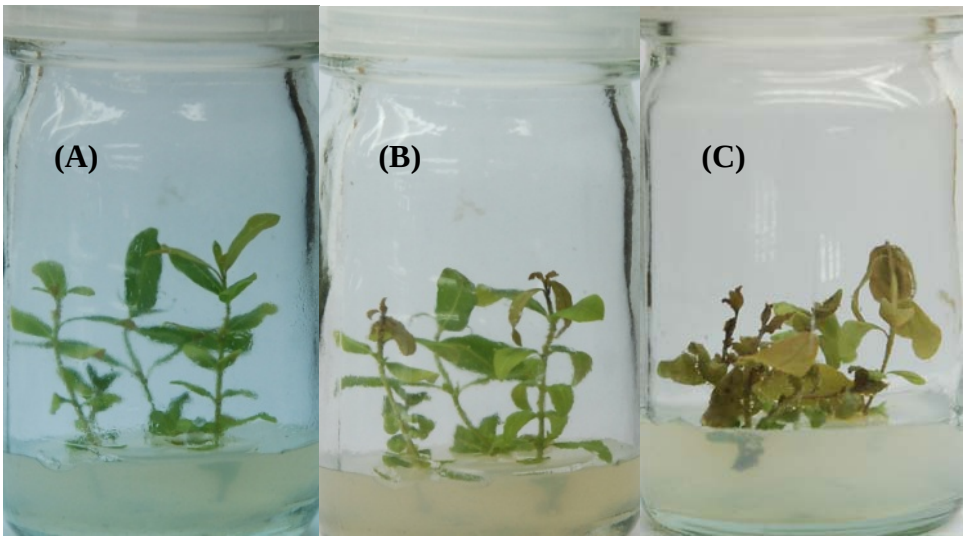


Figure 24 Comparison of *E. camaldulensis* morphology in the 8 hour-light a day with 15 mM sucrose and 10 mM KCN. (A) for 1 day (B) for 5 days (C) for 15 days.

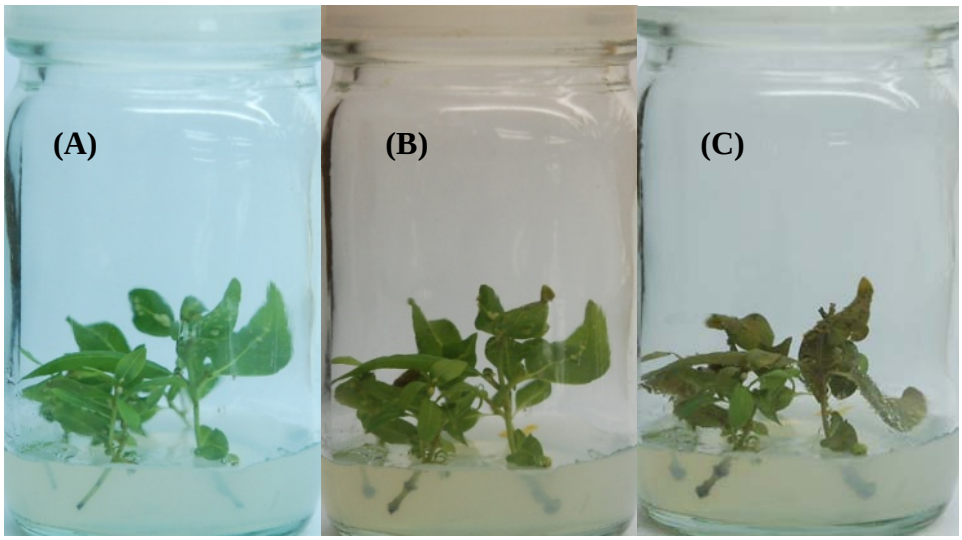


Figure 25 Comparison of *E. camaldulensis* morphology in the 8 hour-light a day with 30 mM sucrose and 10 mM KCN. (A) for 1 day (B) for 5 days (C) for 15 days.

6.5 Morphology of *E. camaldulensis* grown in the 16 hour-light a day with different concentrations of sucrose and 5 mM KCN

The leaves of *E. camaldulensis* became yellow and parched which were begun on the fifth day and could be clearly noticed on the seventh day. By the supplymentation of more concentration of sucrose, the leaves seem not to be in the adverse symptom better than the one with less sucrose added (Table 13).

Table 13 Morphology of *E. camaldulensis* in the 16 hour-light a day with different concentrations of sucrose and 5 mM KCN for 1 day, 3 days, 5 days, 7 days and 15 days

	Light 16 h./ day 0 mM Sucrose. 5 mM KCN	Light 16 h./ day 15 mM Sucrose 5 mM KCN	Light 16 h./ day 30 mM Sucrose 5 mM KCN	Light 16 h./ day 65 mM Sucrose 5 mM KCN
1 Day				
3 Days				
5 Days				
7 Days				
15 Days				

6.6 Morphology of *E. camaldulensis* grown in the 16 hour-light a day with different concentrations of sucrose and 10 mM KCN

It was found that leaves of *E. camaldulensis* became faded and parched which began on the fifth day and it could be clearly noticed on the seventh day. By the supplymentation of more concentation of sucrose, the leaves seem not to be in the adverse symptom better than the one with less sucrose added (Table 14).

Table 14 Morphology of *E. camaldulensis* in the 16 hour-light a day with different concentrations of sucrose and 10 mM KCN for 1 day, 3 days, 5 days, 7 days and 15 days

	Light 16 h./ day 0 mM Sucrose. 10 mM KCN	Light 16 h./ day 15 mM Sucrose 10 mM KCN	Light 16 h./ day 30 mM Sucrose 10 mM KCN	Light 16 h./ day 65 mM Sucrose 10 mM KCN
1 Day				
3 Days				
5 Days				
7 Days				
15 Days				

6.7 Morphology of *E. camaldulensis* grown in the 24 hour-light a day with different concentrations of sucrose and 5 mM KCN

The *E. camaldulensis* could stand with the 5mM KCN for 5 days. After that the leaves became faded and it could be clearly noticed on the seventh day. By the supplementation of more concentration of sucrose, the leaves seem not to be in the adverse symptom better than the one with less sucrose added (Table 15).

Table 15 Morphology of *E. camaldulensis* in the 24 hour-light a day with different concentrations of sucrose and 5 mM KCN for 1 day, 3 days, 5 days, 7 days and 15 days

	Light 24 h./ day 0 mM Sucrose. 5 mM KCN	Light 24 h./ day 15 mM Sucrose 5 mM KCN	Light 24 h./ day 30 mM Sucrose 5 mM KCN	Light 24 h./ day 65 mM Sucrose 5 mM KCN
1 Day				
3 Days				
5 Days				
7 Days				
15 Days				

6.8 Morphology of *E. camaldulensis* grown in the 24 hour-light a day with different concentrations of sucrose and 10 mM KCN

The result came out the same as section 6.7. The *E. camaldulensis* could stand with the 10mM KCN for 5 days. After that the leaves became faded and it could be clearly noticed on the seventh day. By the supplementation of more concentration of sucrose, the leaves seem to be the adverse symptom less than the one with less sucrose added (Table 16).

Table 16 Morphology of *E. camaldulensis* in the 24 hour-light a day with different concentrations of sucrose and 10 mM KCN for 1 day, 3 days, 5 days, 7 days and 15 days

	Light 24 h./ day 0 mM Sucrose. 10 mM KCN	Light 24 h./ day 15 mM Sucrose 10 mM KCN	Light 24 h./ day 30 mM Sucrose 10 mM KCN	Light 24 h./ day 65 mM Sucrose 10 mM KCN
1 Day				
3 Days				
5 Days				
7 Days				
15 Days				

7. Effect of Light Duration, Sucrose Deprivation and KCN on the Expression of *Alternative Oxidase* gene in *E. camaldulensis* Multiple Shoot

When either 5 mM or 10 mM KCN were added into media, the expression of *AOX* gene increased within the periods of 5 days. After that the expression decreased. However, the expression of *AOX* gene decreased since the third day in some experiments.

7.1 Expression of *AOX* gene in the dark with different levels of sucrose concentration and 5 mM KCN

The expression of *AOX* gene in either no sucrose added or with 15 mM sucrose supplementation together with 5 mM KCN, was increased to the maximum on the fifth day. Nevertheless, expression of *AOX* gene in 30 mM and 65 mM sucrose supplementation continually increased within the period of 15 days.

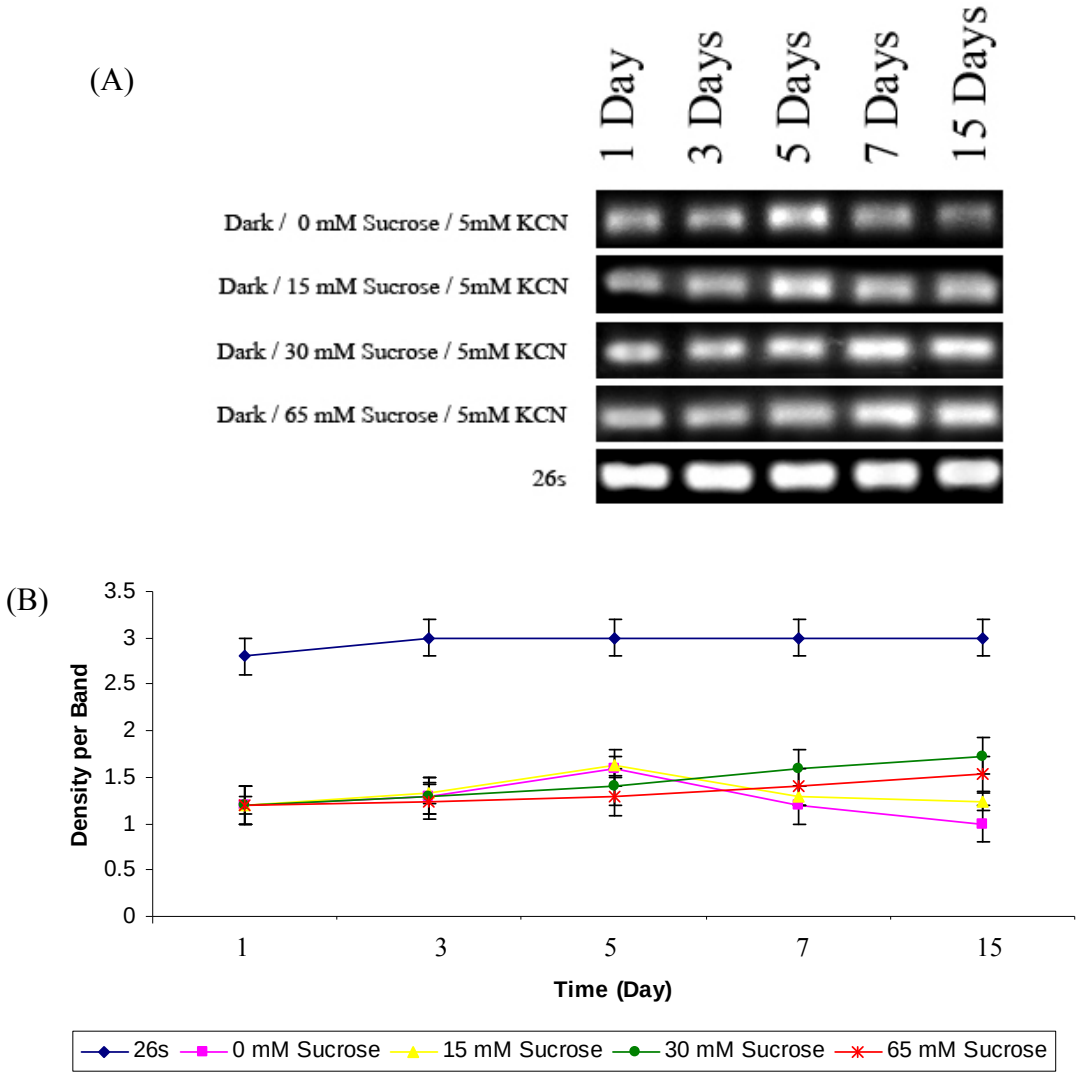


Figure 26 Expression of *AOX* gene in the dark with different levels of sucrose concentration and 5 mM KCN for various periods of time within 15 days. (A) Bands of *AOX* gene expression. (B) Intensity of the (A) bands measured by Densitometry GelQuant 2.7.

7.2 Expression of *AOX* gene in the dark with different levels of sucrose concentration and 10 mM KCN

In the dark, when 10 mM KCN was added, the expression of *AOX* gene in either 30 mM or 65 mM sucrose increased to the maximum on the fifth day.

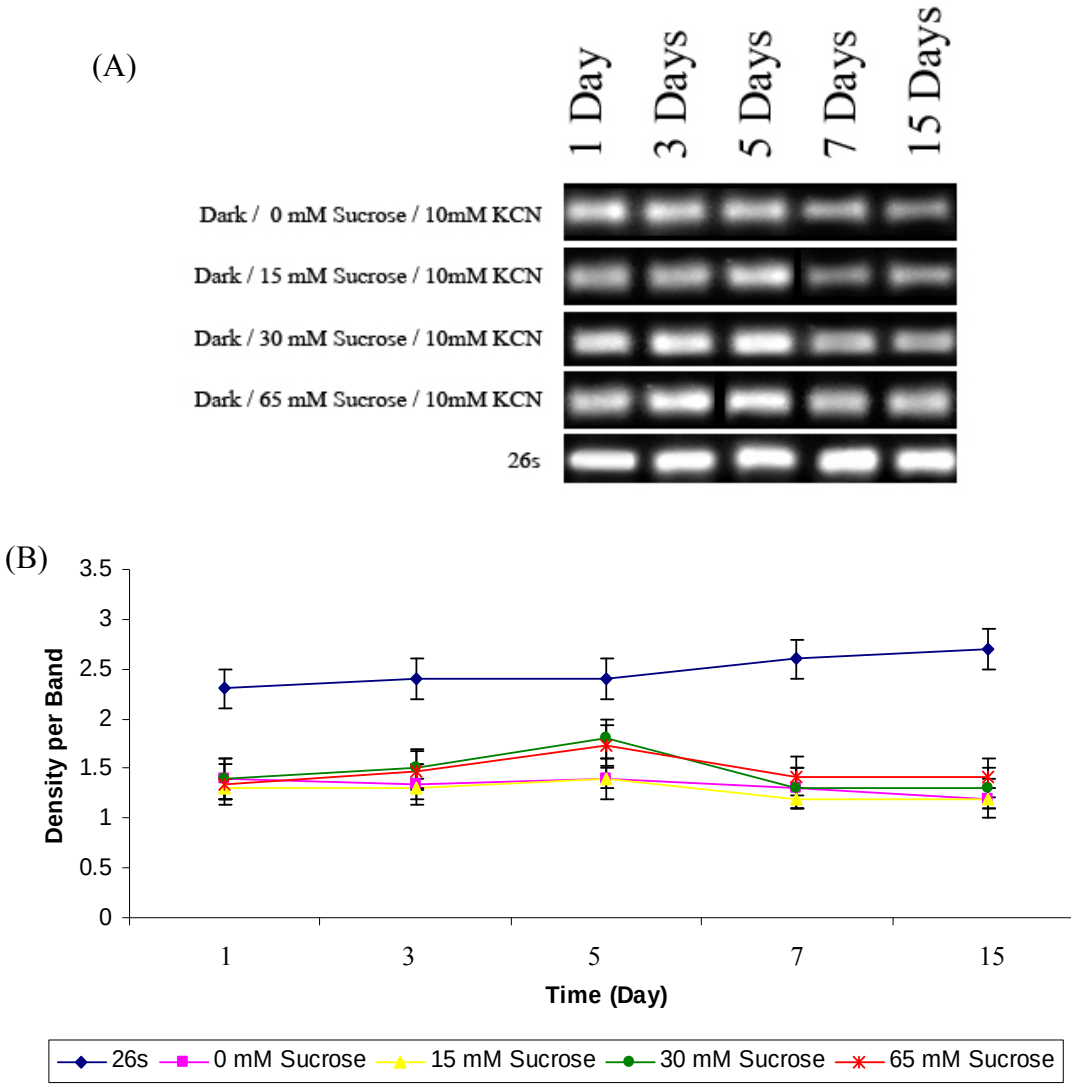


Figure 27 Expression of *AOX* gene in the dark with different levels of sucrose concentration and 10 mM KCN for various period of time within 15 days. (A) Bands of *AOX* gene expression. (B) Intensity of the (A) bands measured by Densitometry GelQuant 2.7.

7.3 Expression of *AOX* gene in the 8 hour-light a day with different levels of sucrose concentration and 5 mM KCN

Under 8 hour-light a day when 5 mM KCN was added, the expression of *AOX* gene in different levels of sucrose concentration increased to the maximum on the fifth day.

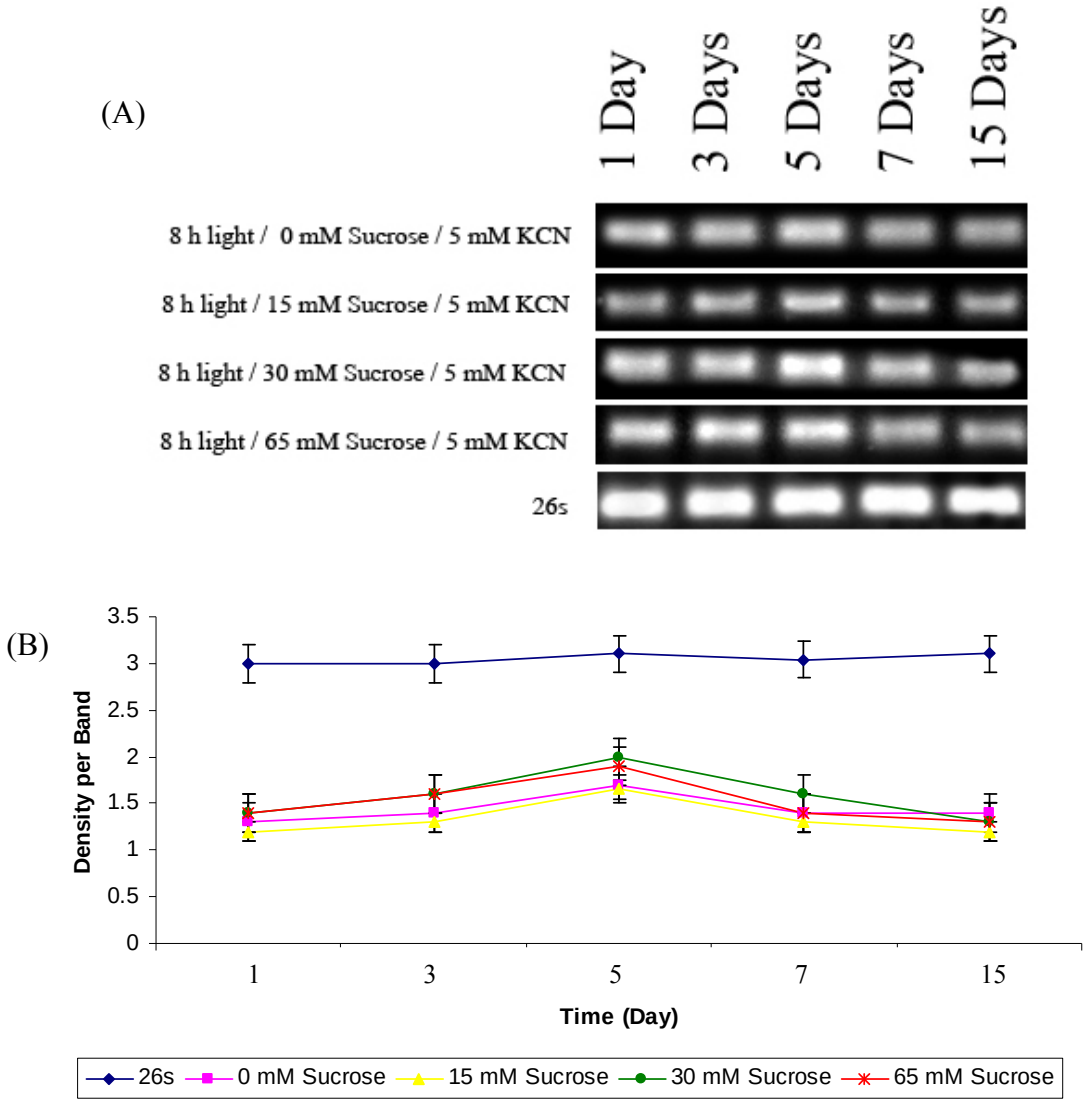


Figure 28 Expression of *AOX* gene in the 8 hour-light a day with different levels of sucrose concentration and 5 mM KCN for various periods of time within 15 days. (A) Bands of *AOX* gene expression. (B) Intensity of the (A) bands measured by Densitometry GelQuant 2.7.

7.4 Expression of *AOX* gene in the 8 hour-light a day with different levels of sucrose concentration and 10 mM KCN

Under 8 hour-light a day when 10 mM KCN was added, the expression of *AOX* gene in different levels of sucrose concentration increased to the maximum on the fifth day.

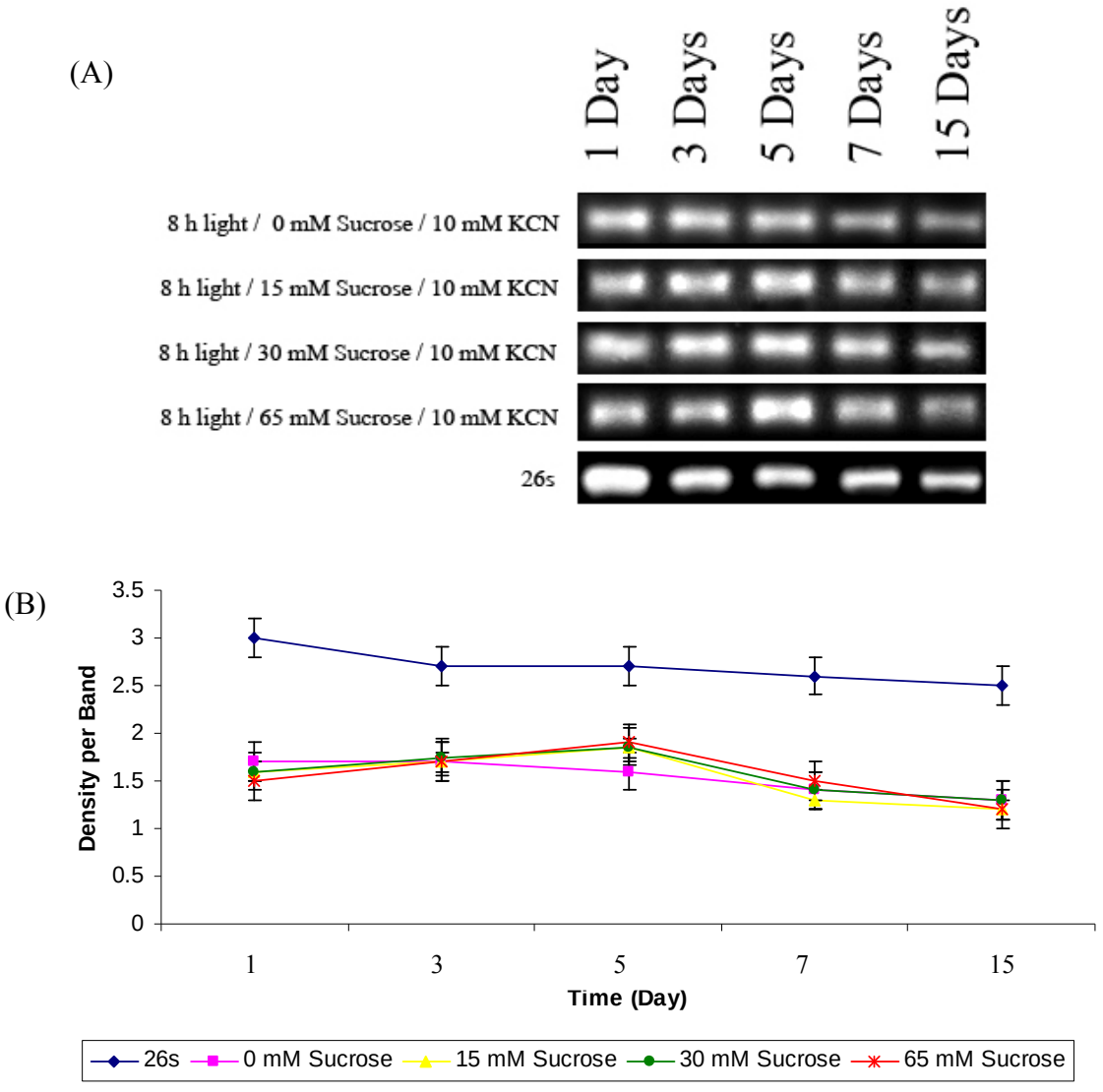


Figure 29 Expression of *AOX* gene in the 8 hour-light a day with different levels of sucrose concentration and 10 mM KCN for various period of time within 15 days. (A) Bands of *AOX* gene expression. (B) Intensity of the (A) bands measured by Densitometry GelQuant 2.7.

7.5 Expression of *AOX* gene in the 16 hour-light a day with different levels of sucrose concentration and 5 mM KCN

Under 16 hour-light a day, the expression of *AOX* gene increased to the maximum on the fifth day when supplementation with 65 mM sucrose together with 5 mM KCN.

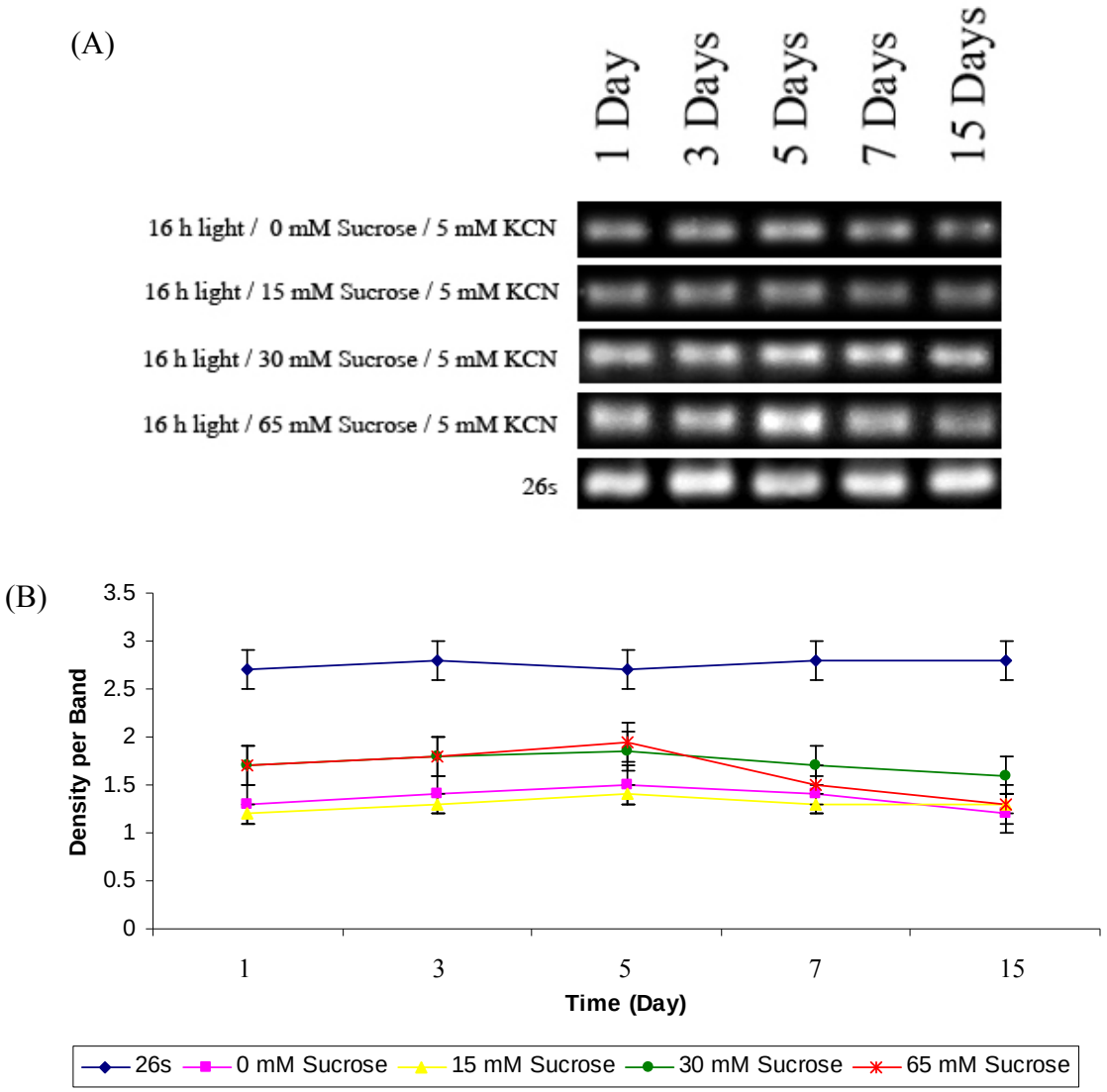


Figure 30 Expression of *AOX* gene in the 16 hour-light a day with different levels of sucrose concentration and 5 mM KCN for various periods of time within 15 days. (A) Bands of *AOX* gene expression. (B) Intensity of the (A) bands measured by Densitometry GelQuant 2.7.

7.6 Expression of *AOX* gene in the 16 hour-light a day with different levels of sucrose concentration and 10 mM KCN

The expression of *AOX* gene in 16 hour-light a day with no added sucrose and10 mM KCN, reduced by the passing time within 15 days. However, the expression of *AOX* gene increased with added 65 mM sucrose to the maximum on the fifth day.

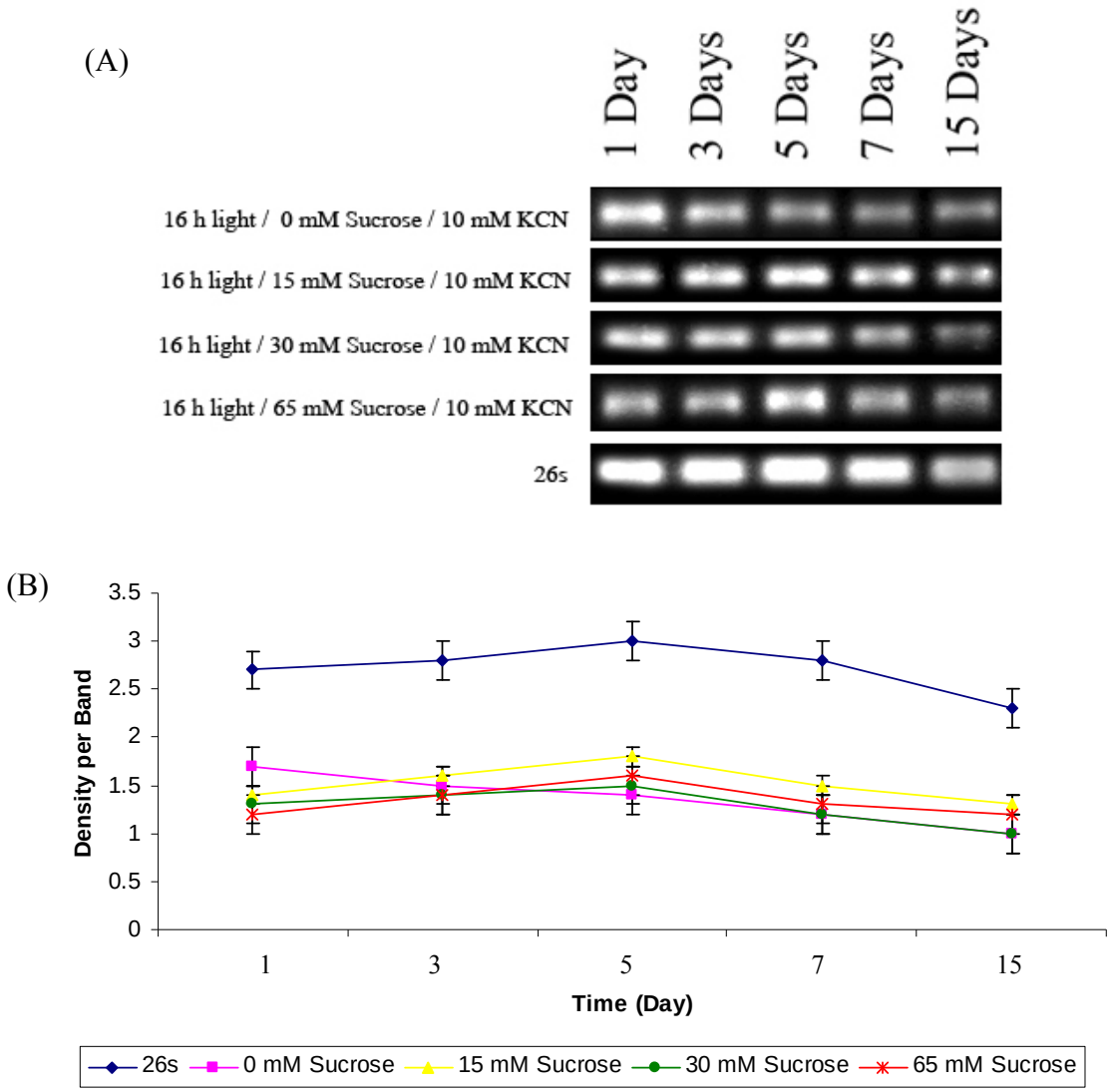


Figure 31 Expression of *AOX* gene in the 16 hour-light a day with different levels of sucrose concentration and 10 mM KCN for various period of time within 15 days. (A) Bands of *AOX* gene expression. (B) Intensity of the (A) bands measured by Densitometry GelQuant 2.7.

7.7 Expression of *AOX* gene in the 24 hour-light a day with different levels of sucrose concentration and 5 mM KCN

Under 24 hour-light a day, the expression of *AOX* gene in different level of sucrose concentration together with 5 mM KCN supplementation increased to the maximum on the fifth day.

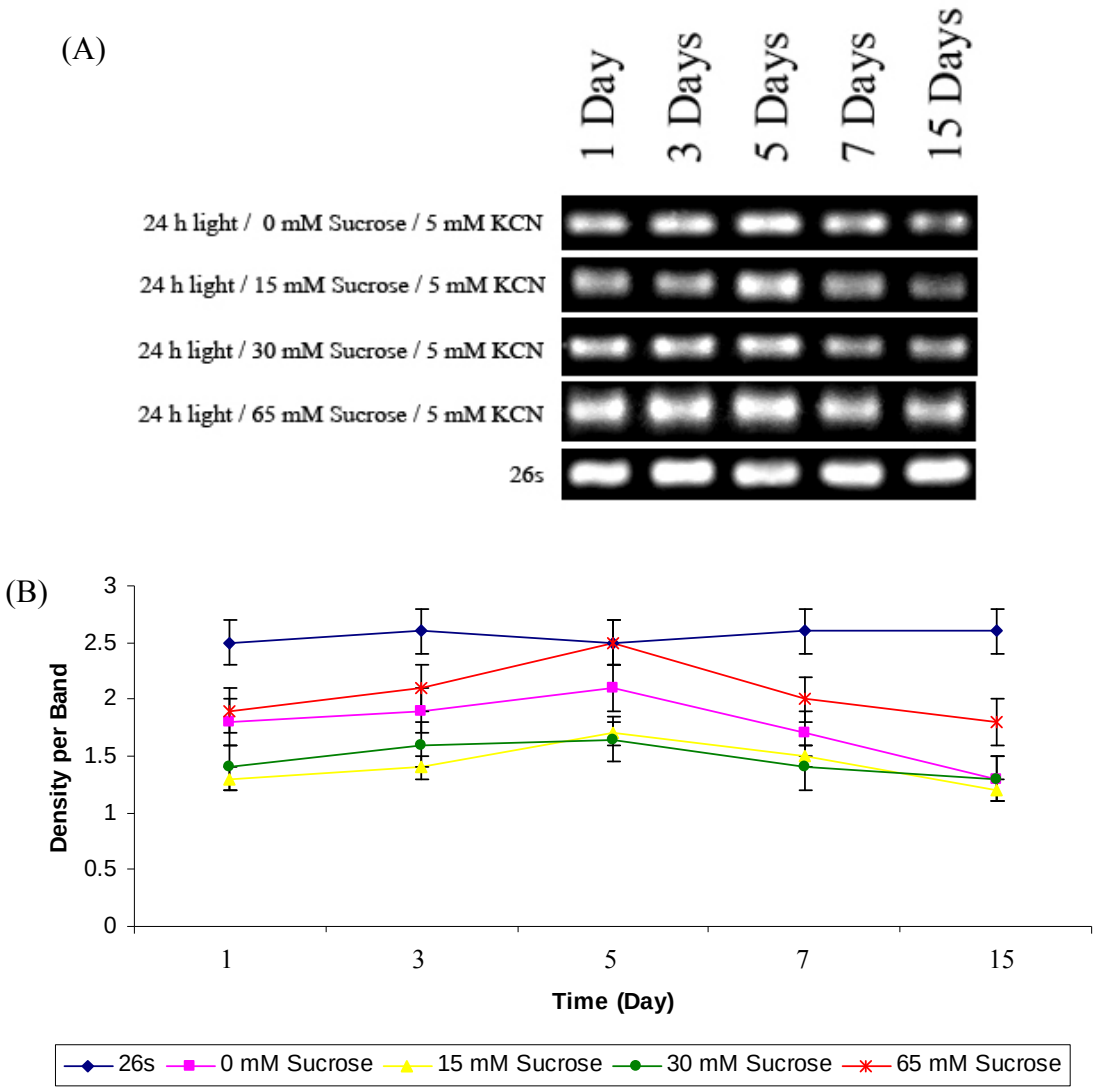


Figure 32 Expression of *AOX* gene in the 24 hour-light a day with different levels of sucrose concentration and 5 mM KCN for various periods of time within 15 days. (A) Bands of *AOX* gene expression. (B) Intensity of the (A) bands measured by Densitometry GelQuant 2.7.

7.8 Expression of *AOX* gene in the 24 hour-light a day with different levels of sucrose concentration and 10 mM KCN

Under 24 hour-light a day, the expression of *AOX* gene with 10 mM KCN was similar to the previous experiment that it increased to the maximum on the fifth day.

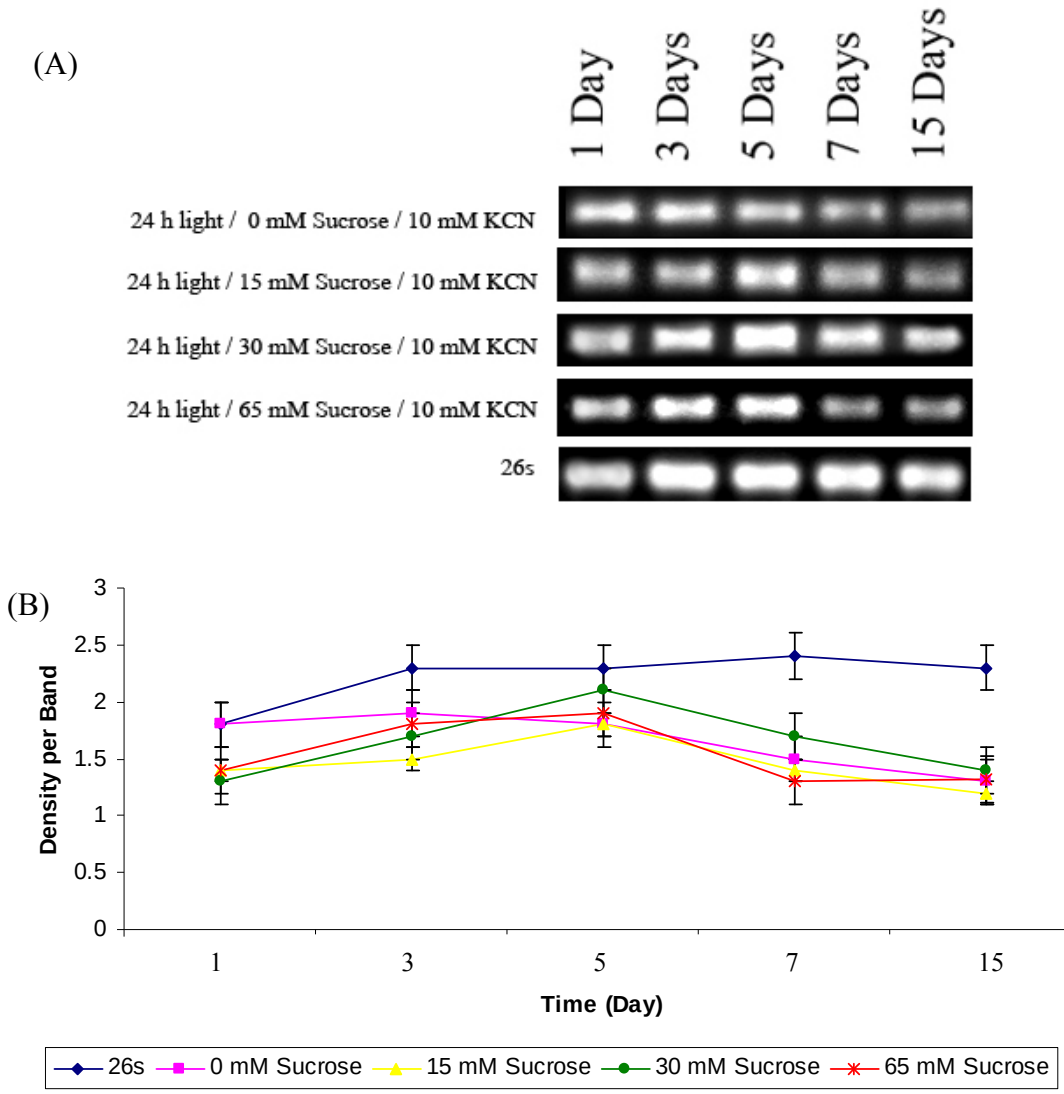


Figure 33 Expression of *AOX* gene in the 24 hour-light a day with different levels of sucrose concentration and 10 mM KCN for various periods of time within15 days. (A) Bands of *AOX* gene expression. (B) Intensity of the (A) bands measured by Densitometry GelQuant 2.7.

Discussion

Eucalyptus camaldulensis clone T5 was selected to investigate the effect of light duration, sucrose deprivation and KCN on the expression of *AOX* gene within 15 days.

1. Effect of Light Duration on the *AOX* gene Expression

Figure 13 shows the effect of light duration on *AOX* gene expression of *E. camaldulensis* grown either in day light of 8, 16 and 24 hours a day or in the dark. The result showed that the expression of *AOX* gene in the *E. camaldulensis* grown in the dark increased, but not in the *E. camaldulensis* grown in 8 or 16 hour-light a day. However, the decrease in *AOX* gene expression of the *E. camaldulensis* grown in 24 hour-light a day, was observed. The results were in agree with those reported by McDonnell and Farrar (1992) and Millenaar *et al.*(2000). In the former report, they grew plants in the dark and measured the rate of photosynthesis and respiration of root. They found that the ratio of those in the dark to those in the light decreased to 28 and 39 in order. AOX protein is a branching component from the main electron transport chain in mitochondria. Therefore, if the photosynthesis flaw or not enough light exists, the level of electron flow in electron transport chain in both processes will be reduced. Then *E. camaldulensis* adapts by an increase in the expression of *AOX* gene otherwise the alternative oxidase pathway will be activated. This may be the reason of the increase of the *AOX* gene expression in the dark.

According to the experiment when the *E. camaldulensis* were in 8 hours and 16 hours a day of light, the expression of *AOX* gene did not change. This may be because 8 hours a day of light is enough to maintain rate of photosynthesis. The 16 hours a day of light may be also not sufficient to activate the *AOX* gene expression (Figure 13). Millenaarr *et al*. (1998) reported the gene expression in term of the relationship between abundance of AOX protein and AOX station state in which the certain cysteine is in oxidized form (inactive form). AOX protein will be activated if the activity of AOX protein decreased or high abundance of pyruvate as an activator.

Therefore, eventhough the AOX protein is in active form (reduced form of the certain cysteine), this may not correlate with the activity of the AOX enzyme.

Expression of *AOX* gene of the *E. camaldulensis* in 24 hours a day of light decreased (Figure13). The *E. camaldulensis* might sense something unusual such as photooxidative stress so that would cause the plant to get an adaptation which made the expression of *AOX* gene reduced at that moment. Photosynthesis continually occured and caused an increase in the sugar level which resulted in more energy generated from the main electron transport chain. That may be why the expression of *AOX* gene decreased.

Expression of the *AOX* gene could be clearly seen if there is a large difference of duration of light providing, such as in the dark and 24 hour-light a day (Figure 13). However, when the eucalypt got the close duration of light of 8 hours and 16 hours a day (Figure 13), the results could not be clearly identified. The light may influence the alteration of plant mechanism but the plant still need some time to adapt.

2. Effect of Light Duration and Sucrose Deprivation on the *AOX* gene Expression

The MS5 media included a number of nutritions that are more than what plant needs. Besides, it consists of 65 mM sucrose. In order to study the relationship between duration of the light and concentration of sucrose in the culture media, the plants were grown in different duration of light, along with decrease the sucrose from 65 to 30, 15, and 0 mM. According to the experiment, the level of sucrose influenced on the expression of *AOX* gene. In Figure 14, the expression of *AOX* gene in the dark and reduced sucrose supplementation from 65 to 30, 15 and 0 mM, the different expression levels were shown. The expression of *AOX* gene reduced in the absence of sucrose, remained at the same level at 15 mM sucrose and increased when growing plants in 30 mM and 65 mM sucrose (Figure 14). It is suggested a positive correlation between sucrose concentration and *AOX* gene expression when the *E. camaldulensis* plant was cultured in the dark.

When the plant was grown in 8 hours of light a day, the expression of *AOX* gene decreased along with the time when the concentration of sucrose in the culture media was lower than 30 mM (Figure 15). Higher sucrose concentration (30 to 65 mM) caused no change in *AOX* gene expression. Growing *E. camaldulensis* in 16 hours a day of light with various concentrations of sucrose supplemented in culture media revealed similar result to the one with 8 hours light a day except the stable expression of *AOX* gene was found when sucrose concentration in culture media was higher than 15 mM sucrose (Figure 16). Interestingly, the expression of the *AOX* gene dramatically reduced along the time of growing *E. camaldulensis* in 24 hours a day of light in the culture media supplemented with various concentrations of sucrose (Figure 17).

Taken the results together, they showed the relationship between light duration and sucrose supplementation on the expression of *AOX* gene in *E. camaldulensis* plant. There was no correlation between light duration and *AOX* gene expression. A positive correlation between concentration of sucrose and *AOX* gene expression was detected. These results was agree with the results reported by Millenaar *et al.* (2000). They found that the activity of the alternative oxidase in the annual grass *Poa annua* L root were not changed under severe light deprivation but increased upon addition of exogenous sugars to the root cultures. The concentration of sucrose higher than 30 mM has no influence on the AOX pathway in *E. camaldulensis* plant. According to our experiments, it might be concluded that the transcriptional expression of *AOX* gene in *E. camaldulensis* plant was regulated by exogenous sugar and light duration. However, repiratory rate and ubiquinone concentration are needed to be determined in order to understand the important of these factors to the activity of the alternative pathway in this plant.

3. Effect of Light Duration, Sucrose Deprivation and KCN on the *AOX* gene Expression

It is general accepted that the ability of AOX protein to endure at a level of cyanide. By this reason, it makes AOX pathway, cyanide-insensitive pathway, be of interest because it helps cytochrome C pathway, cyanide-sensitive pathway when there is something flawed to main electron flow under unfavarable condition (Schonbaum *et al*., 1971). Millenaar *et al.* (2001) found that the cyanide activated the AOX activity. The concentration of 5 and 10 mM KCN were chosen to observe the expression of *AOX* gene.

In plants, programmed cell death is thought to be activated during differentiation and in response to biotic and abiotic stresses. Although its mechanisms are far less clear (Ma´rcia et al., 2007)

The morphology of *E. camaldulensis* cultured in cyanide containing media were significantly changed compared to control. Obviously, the leaves became yellow and parched on the fifth day (Table 9-16). Gray *et al.* (2004) reported the relationship between *AOX* gene and reactive oxygen species so to avoid the effect of program cell death that may interfere the AOX expression, the effect of KCN in combination with light duration and sucrose concentration on the level of *AOX* gene expression will be considered from day 0 to day 5.

Growing *E. camaldulensis* in the dark, with 5 mM of cyanide and with different concentration of sucrose, it was found that at 30 mM and 65 mM sucrose, the expression of *AOX* gene unchanged. At the 0 mM and 15 mM sucrose, the expression of *AOX* gene increased in the first five days of the experiment. This may be because 30 mM and 65 mM of sucrose is sufficient and growing in the dark that reslting in the expression of *AOX* gene unchanged. However, 0 mM and 15 mM sucrose in normal state (without KCN) should result the expression to be reduced or unchanged in the order but if added cyanide, the expression of gene in the first 5 days increased. This may be the inhibition of cyanide to cytochrome pathway and lead to activate the AOX

pathway procedure so the expression of *AOX* gene increased. However, after that, the expression of *AOX* gene decreased. If looked at the morphology of the eucalypt, it was found that the *E. camaldulensis* leaves on the fifth day obviously changed to yellow, parched and closed to the state of death. Besides, the level of RNA in order to test the the expression of *AOX* gene, got from the filter was less than the normal state, less than 100 ng/ul. This may be possible that the expression of *AOX* gene after the date of 5 reduced because the plant was nearly dead. (Figure 26 and 27)

Similarly, if added cyanide to 10 mM, according to the experiment (Figure 27), the result revealed that after the fifth days of the treatment, the expression of *AOX* gene unchanged from the normal state but when added 15, 30 mM and 65 mM sucrose, the expression of *AOX* gene increased in the first 5 days. Expression of *AOX* gene without sucrose was also indifferent from that of 5 mM cyanide added.

If growing the *E. camaldulensis* in 8 hours and 16 hours a day of light, it was found that expression of *AOX* gene in 0 mM sucrose and 10 mM of cyanide that the expression of *AOX* gene increased in the first five days. It could be clearly seen when it got sufficient sucrose, 30 mM and 65 mM sucrose. Contrastingly, when it got insufficient sucrose, 0 mM and 15 mM sucrose, expression of *AOX* gene would have an unclear result. (Figure 28-31)

According to the experiment in 24 hours a day of light, the expression of *AOX* gene with 5 mM and 10 mM of cyanide increased in the first five days. This may be because of the sufficient light for the activity of photosynthesis. The cyanide could extremely affects to cytochrome pathway therefore, the *AOX* gene may extremely expresse (Figure 32 and 33), especially, when comparing to those in previous light conditions (Figure 28-31) or in the dark (Figure 26 and 27). However, after the fifth day, the *E. camaldulensis* would not be able to tolerate the stress and it got parched and died at last.

According to the experiment of Millenaar *et al*. (2002) which measured the alternative oxidase activity in 0.5,1,2,4,25,40,400,500,1000 μM KCN, in the first 7

days of the experiment, no report about the morphology of *Poa annua* whether it could endure the cyanide or not. It could be assumed that it could endure until 1000 μM of cyanide so that they did not report of anything abnormal. However, from the experiment, growing *E. camaldulensis* at the concentration of 5 mM KCN, it was found that the quantity of cyanide was too much for *E. camaldulensis* to stand even though increase in the expression of *AOX* gene could be evaluated in the first five days. However, the RNA quantity which got from the filtrate was less than usual. This may be a sign that *E. camaldulensis* was close to state of death.

Expression of *AOX* gene in different conditions given the different result might be the ability of AOX to attenuate cell death might relate either to its capacity to generate low levels of ATP (Veiga *et al.*, 2002) or to its ability to dampen the excessive mitochondrial ROS by preventing over-reduction of electron transport chain components (Maxwell *et al.*, 1999). However, it is also possible that the signalling pathway for the induction of AOX is more complex.

According to the 2 sets of experiment, light duraton and sucrose deprivation, the later may be more influence on the alteration of the expression of *AOX* gene. And when determination of the 3 factors; light duration sucrose deprivation and cyanide content, it was found that even though reducing sucrose but increasing cyanide, the expression of *AOX* gene still increased. This may be that the quantity of cyanide affected the expression of *AOX* gene more than the others.

In summary, the integral regulation of *AOX* gene expression in eucalypt by 3 environmmental factors; light duration, sucrose deprivation and KCN, revealed that sucrose may be the more stronger factor than light duration while KCN may be the most effective factor.

CONCLUSION AND RECOMMENDATIONS

Conclusion

1. The morphology change of *E. camaldulensis* clone T5 within 15 days was investigated. It was found that *E. camaldulensis* could endure in the state of 24 hour-light a day or in the dark. It could also grow in the media with no sucrose. Moreover, it might not stand in concentration of cyanide, 5mM and 10 mM KCN.

2. Duration of light affected the expression of *AOX* gene. When the *E. camaldulensis* were exposed 24 hour-light a day, the expression of *AOX* gene reduced. Contrastingly, when they were grown in the dark, the expression of *AOX* gene increased.

3. Reduction of sucrose concentration affected the expression of *AOX* gene. The reduced concentration of sucrose in the same duration of light receiving at 24, 16, 8, 0 hours a day, the expression decreased.

4. The concentration of sucrose in the medium was a more influential factor to *AOX* gene expression than duration of light.

5. Concentration of cyanide at 5 mM and 10 mM KCN, enhanced the expression of the *AOX* gene in the first 5 days of treatment but after that, the expression of the *AOX* gene decreased.

6. In the same duration of light, even though lower concentration of sucrose, cyanide added caused an increased in the expression of *AOX* gene within 5 days after treatment and reduced in the later time.

7. Upon the relation of light duration, reduction of sucrose and potassium cyanide, it was found that cyanide may be the most effective factor to the expression of *AOX* gene, the second one is quantity of sucrose and duration of light, in order.

Recommendations

1. According to Millennar's report, it was found that the relation among expression of *AOX* gene, AOX protein, and AOX activity might be incorrelation. Therefore, in order to study AOX, the research in the expression of *AOX* gene, active from of AOX protein and AOX activity in the same condition are supposed to be done.

2. The concentration of KCN used in the research, 5 mM and 10 mM KCN, was very high. At these concentrations of KCN, the *E. camaldulensis* was able to endure only 5 days. Therefore, the suitable concentration of KCN for further research may be in between 1 to 5 mM KCN.

3. All data report in the research must be measurable, for example dry weight, leaf area, growth rate.

4. Change in concentration of sucrose in the media may effect not only photosynthetic rate but also may induce osmotic stress in plant.

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APPENDIX

Chemical and Chemical Analysis

1. Reagent for molecular cloning

1.1 DNA extraction buffer

100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, pH 8.0, 2 % CTAB, 0.3 % v/v β-mercaptoethanol

1.2 10X TBE buffer (stock)

Tris 108 g
Boric acid 55 g.
EDTA 9.5 g.

The chemicals were dissolved in distilled water, and adjusted the final volume to 1 liter with distilled water. (working solution is 1X)

1.3 TE, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0)

1 M Tris-HCl, pH 8.0 1.0 ml
1 mM EDTA, pH 8.0 0.2 ml

The mixture was mixed thoroughly and adjusted to the final volume of 100 ml with distilled water.

1.4 3 M Sodium acetate, pH 5.2

Sodium acetate 24.2 g

The chemical was dissolved in distilled water, adjusted to pH 5.2 with glacial acetic acid. The final volume was adjusted to 100 ml with distilled water.

1.5 Chloroform : Isoamyl alcohol (24 : 1, v/v)

Chloroform (Merck) 24.0 ml

Isoamyl alcohol 1.0 ml

Both reagents were mixed together and stored in a dark bottle at room temperature.

1.6 Ethidium bromide (10 mg/ml)

Ethidium bromide 1.0 g

Distilled water 10.0 ml

The solution was stored in a dark bottle at room temperature.

1.7 6X gel-loading dye buffer

0.25 % bromophenol blue,

0.25 % xylene cyanol FF

30 % glycerol in water

The chemicals were dissolved and adjusted to the final volume of 100 ml with distilled water.

1.8 DNA Tracking Dye

Glycerol 500 µl

0.1 M EDTA 200 µl

1 % xylene Cyanol FF 60 µl

1 % bromophenol blue 60 µl

1 M Tris HCl, pH 7.5 180 µl

1.9 1X Reverse transcription buffer

10 mM Tris-Cl pH 9.0, 50 mM KCl, and 0.1 % Triton®X-100

1.10 1 M IPTG

Isopropylthio-β-D-galactoside 2.38 g

Distilled water 100 ml

The solution was sterilized by filtration through a 0.2 ฅm filter and dispensed the solution into 1 ml aliquot tube and stored at –20 °C.

1.11 20 mg/ml X-gal

5-bormo-4-chloro-3-indolyl-β-D-galactoside 100 mg

The chemical was dissolved in 2 ml of dimethyl-formamide. The solution was stored in a tube covered with aluminum foil and stored at -20 °C.

1.12 Ethidium bromide (10 mg/ml)

Ethidium bromide 1.0 g

Distilled water 10.0 ml

The solution was stored in a dark bottle at room temperature.

1.13 Proteinase K (20 mg/ml)

Proteinase K 0.2 g

Distilled water 10.0 ml

The solution was stored at –20 °C.

1.14 RNase A (10 mg/ml)

RNaseA 10.0 mg

RNase was dissolved in 10 mM Tris-HCl (pH 7.5), 15 mM NaCl and stored at -20 °C.

1.15 10X ligation buffer

250 mM Tris-HCl (pH 7.8)

50 mM $MgCl_2$,

5 mM DTT

5 mM ATP

25 % w/v polyethylene glycol (MW 8000)

2. Reagent for alkaline lysis method

2.1 Solution I (50 mM glucose, 25 mM Tris-Cl, and 10 mM EDTA, pH 8.0)

1.0 M glucose 5.0 ml

1.0 M Tris-HCl (pH 8.0) 2.5 ml

0.5 M EDTA (pH 8.0) 2.0 ml

Distilled water 90.5 ml

The mixture was sterilized by autoclaving for 15 minutes and stored at 4 °C.

2.2 Solution II (0.2 M NaOH, 1 % SDS)

1.0 M NaOH 2.0 ml

10 % SDS 1.0 ml

Distilled water 7.0 ml

2.3 Solution III (3 M Potassium acetate, Glacial acetic acid)

5 M Potassium acetate 90.0 ml

Glacial acetic acid 11.5 ml

Distilled water 28.5 ml

3. Media for bacterial culture

3.1 Luria-Bertani medium (LB medium per liter)

Tryptone (Difco) 10 g
Yeast extract (Difco) 5.0 g
NaCl 10 g
Adjust pH to 7.0 with NaOH. Then the solution was adjusted to the final volume of 1000 ml with distilled water and sterilized by autoclaving.

3.2 LB plates with ampicillin

Fifteen grams of agar was added to 1 litre of LB medium then the media was sterilized by autoclaving. The medium was allowed to cool to 50°C before adding ampicillin to a final concentration of 100 μg/ml. The medium (20-25 ml) was poured into 85 mm petri dishes. The agar was allowed to harden. Agar plates were stored at 4°C for up to 1 month or room temperature for up to 1 week.

3.3 LB plates with ampicillin / IPTG / X-gal

The LB with ampicillin was prepared then supplement with 0.5 mM IPTG and 80 μg/ml X-Gal and pour the plates. Alternatively, 100 μl of 100 mM IPTG and 20 μl of 50 mg/ml X-gal may be spread over the surface of an LB ampicillin plate and allowed to absorb for 30 minutes at 37 °C prior to use.

CIRRICULUM VITAE

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