TISSUE SELECTION AND THE OPTIMIZATION OF THE DNA ISOLATION METHOD FOR THE CONSTRUCTION OF GENOMIC LIBRARY OF RED DRAGON FRUIT (*Hylocereus polyrhizus*)

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Abstract

Genomic library of *Hylocereus polyrhizus* was constructed in bacteriophage Lambda Fix II with 20kb inserts. DNA of the *H. polyrhizus* that was used to construct the genomic library was obtained from young germinated seeds' leaves. The tender and partially expended leaves were selected. Seed germination was also carried out to select the best time to harvest leaves for DNA extraction. Different treatment for seeds affects the germination and growth of the seed and acid washed seeds germinated in light condition the best. DNA extraction was modified in order to obtain pure and high yield DNA for genomic library construction. Restriction enzyme (*Bam*H I) used to digest the selected fragment from the total DNA and analysed on a gel electrophoresis. Phenol-chloroform method used to purify insert DNA successfully yielded high concentration insert DNA which is a very crucial step in constructing genomic library. Plaques formed indicated that the bacteriophage has successfully lysised the cells. In this study, 80 – 100 plaques per Petri dish formed showed bacteriophage has successfully lysised the cells and indicated that a good genomic library has been established. This genomic library can be use for further study such as fish out important gene and study the gene interaction.

Abstrak

Perpustakaan genom bagi *Hylocreus polyrhizus* telah dibina di dalam bateriofaj FIX II dengan kandungan sebanyak 20kb. DNA yang digunakan untuk membina perpustakaan genom adalah didapati dari daun yang muda. Daun yang muda dan belum kembang akan digunakan untuk DNA extraksi. Selain daripada itu, percubaan untuk menkecambah biji benih juga dijalankan dengan beberapa cara, keputusan kita mendapati biji benih yang dicuci dengan asid dan dikecambah di keadaan terang lebih cepat bertumbuh banding dengan cara lain. Kita menggunakan cara extraksi DNA yang telah membuat modifikasi daripada cara tradisional. Keputusan extraksi DNA disemak dengan menggunakan spektrofotometer. Enzim restriksi (*Bam* HI) telah digunakan untuk mencernakan fragmen yang dikehendaki dari DNA, dan seterusnya disemak dengan menggunakan gel elektroforesis. DNA yang telah dicerna seterusnya dimurnikan dengan cara phenol-klorofom supaya mendapat penyelit DNA yang tinggi kepekatannya. Ini merupakan langkah yang sangat penting di dalam projek ini sebab kepekatan akan mempengaruhi bilangan plak and terbentuk dalam pinggan petri. Pembentukan plak bermaksud bakteriofaj berjaya lysised sel. Perpustakaan genom yang dianggap berjaya dibina apabila terdapat 80 – 100 plak terbentuk dalam satu pinggan petri. Kajian yang lebih lanjut boleh dilakukan dengan adanya Perpustakaan genom seperti mencari gen yang penting dan berguna darinya.

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List of Abbreviations

bp	-	Base pair
BSA	-	Bovine serum albumin
cDNA	-	Complimentary deoxyribonucleic acid
cm	-	Centimeters
CTAB	-	Cetylmethylammonium bromide
dATP	-	Deoxyadenosine triphosphate
dCTP	-	Deoxycytidine triphosphate
dGTP	-	Deoxyguanine triphosphate
DNA	-	Deoxyribonucleic acid
dTTP	-	Deoxythymine triphosphate
EDTA	-	Ethylenediaminetetraacetic acid
EtBr	-	Ethidium bromide
FAO	-	Food and Agriculture Organization
GUS	-	beta-glucuronidase
GPI	-	glucose-6-phosphate isomerase
HCl	-	Hydrochloric acids
$MgCl_2$	-	Magnesium chloride
$MgSO_4$	-	Magnesium sulphate
µg	-	Microgram
µl	-	Microliters
ml	-	Milliliters
mm	-	Millimeters
NaCl	-	Sodium Chloride
NEB	-	New England Biolabs
nm	-	Nanometer
OD	-	Optical density
PCR	-	Polymerase chain reaction
pfu	-	Plaques forming units
PVP	-	Polyvinylpyrrolidones

QTL	-	Quantitative trait locus
RAPD	-	Random amplification of polymorphic DNA
RNA	-	Ribonucleic acid
TAE	-	Tris-acetic acid- ethylenediaminetetraacetic acid
TE	-	Tris - ethylenediaminetetraacetic acid
U	-	Unit
UV	-	Ultraviolet
VNTRS	-	Variable number tandem repeat sequence

TISSUE SELECTION AND THE OPTIMIZATION OF THE DNA ISOLATION METHOD FOR THE CONSTRUCTION OF GENOMIC OF RED DRAGON FRUIT (*Hylocereus polyrhizus*)

Chapter 1.0 Introduction

Hylocereus spp and *Selenicereus* spp are veining epiphytic cacti which in their natural state grow under the canopy of, and up tree trunks to which they are anchored by their aerial roots. They are native to the drier tropical and sub tropical forest regions of Mexico and Central and South America. The fruit qualities and characteristics (attractive colours and shape) make them a growing niche in countries such as Vietnam, Colombia, Mexico, Costa Rica and Nicaragua. In Asia, these fruits are often called ‘‘dragon fruit” following their bright red skin which is covered with overlapping bracts. The seeds are small and are consumed with the flesh. The fruit can weigh up to 900 grams, but the average weight is between 350 and 600 grams (Le *et al.,* 2006).

Vietnam is the biggest producer of the *Hylocereus* spp. In Malaysia, only H. polyrhizus and H. undatus are cultivated. These two fruits are commonly differentiated based on the pulp colour. *Hylocereus polyrhizus* and *H. undatus* fruit have red peel but the pulp for *H. polyrhizus* is red while *H. undatus* is white. The red colour is due to the presence of betacyanins, a group of pigments derived from betalains. Betalains are water-soluble pigments which contain violet red betacyanins and the yellow betaxanthins (Strack *et al.*, 2003). *Hylocereus polyrhizus* is a pure source of betacyanin, as betaxanthins are totally absent (Rebecca *et al.*,2008). This explains the red-purple colour of the flesh. Commercially, betacyanin is not only used as colouring agents, but also posseses anti-radical potential (Pedreno and Escribano, 2001).

A number of pests have been recorded in the *Hylocereus* plantation such as ants from the genera of *Atta* and *Solenopsis* which can cause major damage to the plants from the flowers to the fruits. Different fungal (*Gloeosporium agaves*, *Macssonina agaves*, *Dothiorella* sp. and *Botryosphaeria dothidea*), viral (Cactus virus X), and bacterial (*Xanthomonas* sp. and *Erwinia* sp.) diseases have also been reported (Valencia-Botin *et al*., 2003; Liou *et al*., 2004; Luders and McMahon, 2006). These affect farmers by causing poor fruit production.

There are still no known methods to solve the pest and disease issues. At present, growers usually increase the dosage of herbicide and pesticide. This application does not seem to solve the problem in fact may worsen it if the fungal develop resistance toward the chemical. Establishing a genomic library could serve as a platform to study the gene interaction as opposed to the conventional method of cross breeding to obtain disease resistant hybrid which normally involves a longer period.

The objective of this thesis is to take the first step towards understanding the structure and function of the dragon fruit genome by developing a *H. polyrhizus* genomic library through bacteriophage. A consolidated physical and genetic map of dragon fruit would enable gene discovery, studies of gene functions, and comparative genomics with other plant species. The information and resources that will result from this activity will be invaluable to basic research, the dragon fruit industry and ultimately global dragon fruit consumers. The procedures involved in genomic library construction include isolation of genomic DNA, generation of DNA fragments for cloning, packaging and transduction. The most crucial component of the entire procedure is the generation of the desired DNA fragments.

Chapter 2.0 Literature Review

2.1 Botanical description

Pitaya or dragon fruit belongs to the genus *Hylocereus* of the family Cactaceae. *Hylocereus* is a climbing epiphyte (Fig. 2.1) which bear a glabrous berry (Fig.2.2) with large scales. They have triangular, green, fleshy, jointed, stems with many branches (Crane and Balerdi, 2009). *Hylocereus* also use their adventitious roots from the stems to cling on to rocks and trees for support. The aerial roots help to collect water and nutrients from surrounding areas (Luders and McMahon, 2006). The *Hylocereus* is diploid ($2n = 22$) and contains 11 pairs of chromosome (Lichtenzveig *et al*., 2000).

Fig.2.1. A pole is used to support *H. polyrhizus* in the farm

Fig. 2.2. Attractive look of a *H. polyrhizus* fruits

2.1.1 Taxonomy

The edible cacti species classification is based on the nature of the stem habit, colour of the peel and pulp (Crane and Balerdi, 2004 & Tel-Zur *et al*., 2004). Generally, edible cacti are divided into two groups; climbing cacti and columnar cacti based on the nature of the stem habit. The climbing cacti (edible) also belongs to two different genera; *Hylocereus* and *Selenicereus* whilst the columnar cacti species belongs to three genera, namely *Cereus*, *Pachycereus* and *Stenocereus* (Crane and Balerdi, 2004).

The nomenclature of *H. polyrhizus* is as follows:

Kingdom	Plantae (plants)
Subkingdom	Tracheobionta (vascular plants)
Division	Mangoliophyta (flowering plants)
Class	Magnoliopsida (dicotyledons)
Order	Caryophyllales
Family	Cactaceae (cactus family)
Subfamily	Cactodeae
Genus	*Hylocereus* (Berger) Britt & Rose
Species	*Hylocereus polyrhizus* (Haw) Britt & Rose

Sources: Britton and Rose, 1963

2.1.2 Economic importance

Among the edible pitaya species, only three are commercially cultivated namely *H. undatus*, *H. polyrhizus* and *S. megalanthus*. Pitaya is widely consumed in Asia compared to the European market in the mid-1990s, but that trend has changed in the last few years (quantities doubled between 2002 and 2004). The biggest producer in Asia is Vietnam, Nicaragua for Central America and Colombia for South America (Bellec *et al*., 2006). It was also reported that 40% of the fruits imported to the European market comes from Vietnam. According to the Department of Agriculture, Ho Chi Minh city, in 2007 the area for *Hylocereus* sp plantation was about 13,500 hectares with a production of about 211,000 tonnes (Nguyen, 2007).

In Malaysia, dragon fruit was first introduced in large scale at the end of 1990s by Golden Hope Company. In early 1999, the commercial cultivations were developed in Kluang, Kuala Pilah and Setiawan. Since then, the farmers have been cultivating dragon fruit in various lands, such as low and high land, rice-planted land, mined land and even housing yard (Halimi & Satar, 2007). The acreage of dragon fruit-cultivating lands in Malaysia increased from 600 ha in 2005 to almost 1000 ha in 2008. (Fig. 2.3). Recently, this crop has been planted nationwide in Peninsular and East Malaysia (Cheah & Zulkarnain, 2008).

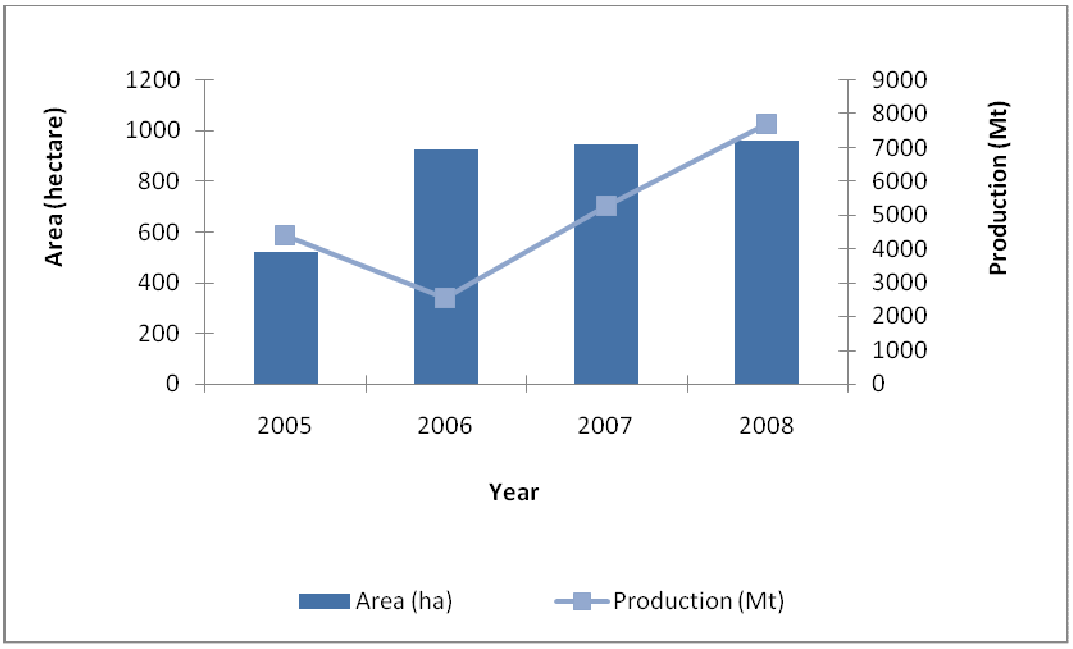


Fig. 2.3: Area of Cultivation and Amount of Dragon Fruit Produced in Malaysia

Source : www.doa.gov.my

2.1.3 Studies conducted on *Hylocerues* species

Hylocereus species began to attract the attention of researchers recently because of the high demand in the market. Most of the studies focus on the cytology and also the postharvest development of that fruit which include studies on betacyanins (Rebecca *et al*., 2008).

In the cytology and mating systems studies, Lichtenzveig *et al*., (2000) confirmed that self-incompatibility (SI) occurs in *H. polyrhizus*, but further molecular study is needed to confirm this result. Many investigations on dragon fruit in Malaysia are particularly aimed to enhance its production technologies as the size of the fruit will determine its economic

value hence development of self-compatible types with valuable fruit size is an important long term goal in climbing cacti breeding (Lichtenzveig *et al*., 2000).

This fruit has also been studied for the betacyanin content. As the public is becoming more aware and concerned about the possible and proven harmful effects of artificial food colorants, *Hylocereus polyrhizus* is a good candidate to replace the artificial food colorants considering its natural origin and stability. (Stintzing *et al*.,2002). Research also showed that *H. polyrhizus* represents a potential commercial source of betalains, since by-products of the fruit can also be exploited. The pectin-like substance could be used in the food industry as a thickening agent or as moisturizer in cosmetics products while the aqueous mesocarp extract as well as the juice from the pulp could serve as a colouring substance for low acid food commodities (Stintzing *et al*., 2002). Furthermore, betacyanins act as antiradical agents (Escribano *et al*., 1998). Studies have also showed that mucilage of *H. polyrhizus* may exert positive influence on cholesterol metabolism (Fernandez *et al*., 1990; Fernandez *et al*., 1992, 1994).

In the ripening and postharvest behavior studies, Nerd *et al.,* (1999) determined the optimum date of harvest for *H. undatus* and *H. polyrhizus* in relation to colour development of pulp and peel. The recommended storage temperature for dragon fruit is 10^{o}C for a maximum 14 days (Chandran, 2010). The irradiated fruit retained visual and compositional quality under these conditions (10^{o}C for 12 days). These would be sufficient to transport to U.S markets, where dragon fruit must be inspected for the presence of mealy bugs and have the sepal removed (Wall and Khan, 2008).

2.2 Introduction to genomic library

There are two types of libraries which are genomic and cDNA library. A genomic library is the total representative of total genes present in an organism, whereas a cDNA library is representative of genes, which are expressed during a particular stage of the cell. cDNA library is a collection of clones containing an insert obtained from cDNA cloning (Sambrook *et. al*., 1989).

Genomic library contains introns which are not represented in the mRNA. The quality and integrity of it are directly correlated with the success of identifying the gene of interest. A good genomic library is supposed to contain a collection of clones carrying all DNA fragments of the organism (Wang *et al.,* 2003).

To construct a genomic library (Fig. 2.4) a high molecular weight genomic DNA must be obtained and is separated and subjected to restriction enzyme digestion by using compatible restriction enzymes (*Bam* HI has been used in this library). The fragments are then fractionated or separated by using agarose gel electrophoresis to obtain fragments of the required size. These fragments are then subjected to alkaline phosphatase treatment to remove the phosphate. This is followed by the dephosphorylation process where the dephosphorylated insert is ligated into a vector which could be a plasmid, phage or cosmid, depending upon the interest of the researcher (phage has been used in this library). Lastly, the recombinant vector is introduced into the host by electroporation and amplified in the host (Zelenin *et al*., 2001).

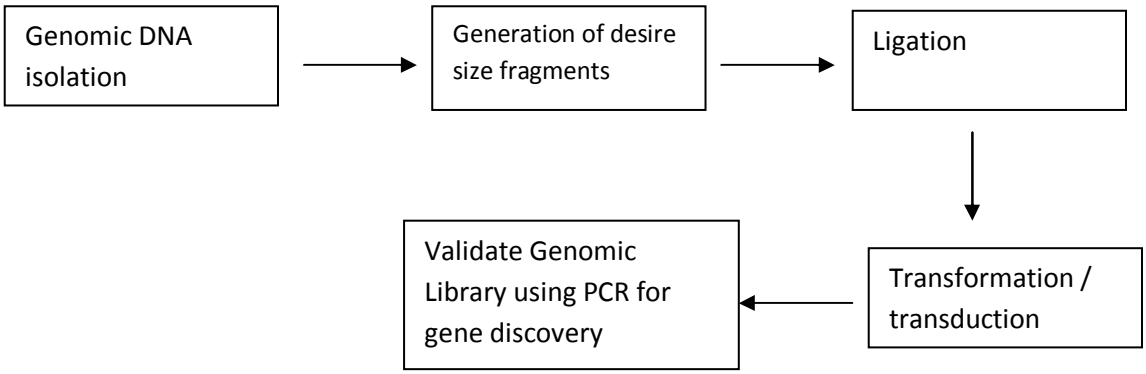


Fig. 2.4 The steps in constructing genomic library

2.2.1 Extraction, purification and quantification of DNA

Extraction of usable nucleic acids from plants has been difficult due to some chemical composition in an organism. A good extraction should yield good amount and intact DNA of reasonable purity (Puchooa, 2004). Polysaccharides and tannins pose a major problem in plants because they are difficult to separate from the organism DNA. The presence of polysaccharides- like contaminants will cause viscosity in the extract. Therefore selection of starting materials for DNA extraction is a crucial part and will further influence the purity of the DNA. This problem can be solved by using young, tender and unbruised leaves where the content of polysaccharides is lesser compared to old leaves (Puchooa, 2004).

The extraction of DNA from plant cell involves disruption of the cell membranes by grinding with liquid nitrogen to release the DNA into the extraction buffer. Extraction buffer normally contains detergents such as sodium dodecly sulphate (SDS) or Cetylmethylammonium bromide (CTAB). EDTA is normally included in the extraction buffer to chelate magnesium ions, a necessary co-factor for nucleases. The extracted DNA normally contains high amount of proteins, polysaccharides, tannins and pigments,

chloroform:isoamyl alcohol are used to denature and precipitate the proteins. The aqueous phase which contain DNA will then be heat-treated with RNase to remove RNAs in the DNA extract(Puchooa, 2004). β-mercaptoethanol is used to reduce oxidation and Polyvinyl pyrrolidone (PVP) is added into the extraction buffer to remove polyphenols and higher concentration of ascorbic acid (Maliyakal, 1992; De La Cruz *et al*., 1997)

DNA pellet is formed at the bottom of the tube and TE buffer is used to dissolve the pellets. Clear or white pellets should be obtained in order to prejudge the quality of the DNA before proceeding to DNA quantification with other methods.

2.2.2 Gel electrophoresis

Agarose gel electrophoresis is used to preliminary determine the quality of the DNA. Conventional gel electrophoresis separation of DNA relies on several factors including gel pore size, electric, ionic moiety, buffer composition and conductivity. Agarose gels are characterized with large pore size, high mechanical strength and biologically inert separation matrices. Agarose gel is normally used to analyze larger double stranded DNA fragments from few hundred base pairs to more than a thousand base pairs (Guttman, 1999).

DNA mixed with loading dye is loaded into the agarose gel with an electric current. These negatively charged DNA molecules will migrate towards the positive end field in a buffer bath. The DNA molecules are attracted to the positive end by the current but they will encounter resistance from the agarose mesh where the smaller molecules are able to navigate the mesh faster than the large molecules. The smaller molecules will appeared at the bottom of the gel compared to the large molecules which will stay at the top

(Westermeier, 1993) . The gel is stained with ethidium bromide so it can be visualized according to their size. Ethidium bromide will intercalate will nucleic acids and fluorescent under UV light. The bands are normally run parallel with a marker with a known size and this will approximately indicate the size of the DNA. The intensity of the bands also serve an indication of the quality of the extracted DNA by referring to the marker guide. Although the intensity of the DNA is used to estimate the concentration of the DNA but it is not really accurate. A further estimation should be run to confirm the concentration.

2.2.3 Spectrophotometer quantification of DNA

Most biological molecules do not intrinsically absorb light in the visible range, but they do absorb ultraviolet light. UV absorbance can be used to quickly estimate the concentration and purity of DNA, RNA and proteins in a sample. It can also be used to quantify the amount of DNA in a sample by measuring at its absorbance at a wavelength of 260nm or 280nm.

Proteins have two absorbance peaks in the UV region, one between 215nm – 230nm, where peptide bonds absorb, and another at about 280nm due to light absorbance by aromatic amino acids (tyrosine, tryptophan and phenylalanine). Certain subunits of nucleic acids (purines) have an absorbance maximum slightly below 260 nm while others (pyrimidines) have a maximum slightly above 260 nm. Although it is common to use the absorbance peak of nucleic acids at 260 nm, the maximum absorbance still vary depending on their subunit composition. Therefore to check the purity of DNA obtained, measurement is carried out at 260 nm and 280nm and the ratio of A260/A280 should be 1.8 – 2.0. When purifying nucleic acid, a ratio less than 1.7 means there is probably a contaminant in the solution, typically either protein or phenol.

2.2.4 Vector selection

Plasmids are covalently closed, circular-supercoiled molecules of a few kilobases in size. Although plasmids occur naturally, mainly in bacteria, those employed in the construction of DNA libraries are usually designed synthetic constructs. These cloning vectors contain restriction sites for which a given enzyme may have only a single unique size on a plasmid into which foreign DNA may be inserted. The sites for many different enzymes may be clustered in a "multiple-cloning site" or "polylinker".

Plasmids additionally contain one or more "marker" genes, such as gene conferring antibiotic resistance, which enable the growth of only plasmid-containing host cells, and genes for an enzyme, such as beta-galactosidase, which enable insert-containing recombinant molecules to be distinguished from nonrecombinants. The disadvantages of plasmids as cloning vectors are that the limitation for small fragments of only a few kilobases and they have a relatively low frequency in transformation of host cell (Theophilus, 1998).

Bacteriophages are viruses that infect bacteria. They could be circular or linear double-stranded DNA, and they are contained within a protein coat that mediates their entry into a host cell.

Bacteriophage λ is one of the most popular virus-based cloning vectors used in the construction of genomic libraries. The linear double stranded DNA with the size of 50-kb will for a double stranded circular DNA when it infects *E. coli*. During replication in the "lytic" cycle, concatamers of λ genomes are produced that are subsequently cleaved at specific sequences (COS sites) to produce λ monomers that are packaged into preformed

protein heads. The bactriophage can accept up to 20 kb of insert DNA, λ vectors are normally obtained as left and right "arms" to which insert DNA is ligated (Theophilus, 1998; Wang *et al*., 2000).

Single-stranded bacteriophage vectors, such as M 13 are often used for library construction. They have circular genomes that replicate initially as a double-stranded molecule following bacterial infection, and subsequently produce single-stranded genomes for further cycles of infection (Karcher, 1995).

Cosmids are plasmid vectors that contain the *cos* sites of λ (Collins and Hohn, 1978). Cosmids can therefore be packaged into λ particles at high efficiency. The difference between cosmids from plasmids is that cosmids can hold relatively higher genomic DNA inserts of ~40-45 kb in size. Although cosmids can accept larger fragments than phage λ, rearrangement of the inserted DNA may occur (Collins and Hohn, 1978; Theophilus, 1998; Wang *et al*., 2003) .

Yeast artificial chromosomes (YACs) are linear cloning vector based on natural yeast chromosome structure (Burke *et al*., 1987). They can be cleaved into two fragments or chromosome arms, which may accept very large fragments up to 1000 kb. YAC libraries have been used for mapping large regions of human DNA. Pulsed field gel electrophoresis (PEGE) have to be used because of its large size(Schwartz and Cantor, 1984).

The disadvantages of YACs include the low cloning efficiency of some system and the instability of the insert which makes it difficult to manipulate as compared to the bacterial system.

The bacterial artificial chromosome system is based on the F-plasmid factor, which can replicate in *E.coli* with inserts more than300 kb in size (Shizuya *et al*., 1992). BAC recombinants are introduced into bacterial cells by electroporation.The permeability of the membrane is increased by applying a high voltage to the cells. BACs are useful for library construction, mapping and genome analysis because they have a high cloning efficiency, and the inserts are stable and easy to manipulate.

2.2.5 General criteria for a cloning vector

The Lambda FIX II vector is a replacement vector used for cloning large fragments of genomic DNA. The Lambda FIX II system takes advantage of spi selection. Lambda phages containing active *red* and *gam* genes that are unable to grow on host strains that contain P2 phage lysogens. Lambda phages without these genes are able to grow on strains lysogenic for P2 such as XL1-Blue MRA (P2), a P2 lysogen of XL1-Blue MRA. The *red* and *gam* genes in the Lambda FIX II DNA are located on the stuffer fragment; therefore, the wild-type Lambda FIX II phage cannot grow on XL1-Blue MRA (P2). Replacing the stuffer fragment with an insert DNA, the recombinant Lambda FIX II vector becomes Red–/Gam–, and the phage is able to grow on the P2 lysogenic strain. Therefore, by plating the library on the XL1-Blue MRA (P2) strain, only recombinant phages are allowed to grow. Furthermore, the unique arrangement of the polylinker for the Lambda FIX II vector permits the isolation of the insert and flanking T3 and T7 bacteriophage promoters as an intact cassette by digestion with *Not* I. T3 and T7 promoters flanking the insertion sites can be used to generate end-specific RNA probes for use in chromosomal walking and restriction mapping (Sambrook J *et al.*,1989 and Ausubel FM *et al.*,1987).

2.2.6 **Screening of genomic library**

DNA cloned into plasmid or YACs produces colonies when the transformed cultures are spread onto an agar plate of growth medium and incubated under appropriate conditions. Bacteriophages lyses the cells they infect and produce "plaques" circulae (2-3 mm) zones of clearing on a background bacterial lawn.

A clone containing the particular sequence of interest may be identified by colony hybridization. A small amount of the transformation or plaque is transferred onto a nitrocellulose or nylon filter, which has been overlaid onto the agar plate. The DNA is denatured and fixed onto the filter by baking, and then hybridized in a buffer containing a radioactively labelled probe comprising sequence that is complementary to a part of the sequence to be identified. For example, this may be a synthetic oligonucleotide probe; it can be derived from partial genome DNA, cDNA, or a protein sequence, or PCR product. Sometimes a probe may be based on a sequence derived from a homologous gene of another species and hybridized at low stringency with the expectation of sufficient sequence conservation to enable cross-hybridization. Excess probe is washed away and the filter exposed to X-ray film. By orientating the developed film with respect to the original agar plate, it is then possible to match the actual colonies with corresponding positively hybridizing colonies on the X-ray film (Eshed and Zamir, 1994; Theophilus, 1998; Zelenin *et al*., 2001).

Prior to the completion of a working draft of the human genome on June 26, 2000 (Meldrum, 2000), there was a strong drive to advance genetic and molecular biology genome analysis methods. Traditional methods depended on the protocol by Benton and Davis, (1977) for phage plaques and by Grunstein and Hogness, (1975) for *Escherichia coli*

colonies. New methods have been established over the decades and these included PCR-based method, hybridization method and also combination of other methods (Campbell and Choy, 2002).

Hybridization-based screening can be performed against high-density gridded micro arrays of a whole library using either a single type or mixture of different types of radioactively labelled-probes. The most common used probes include sub-cloned DNA fragments, PCR amplified products or DNA oligonucleotides (Han *et al*., 2000). Several new approaches have been developed over the years beside the common theme of hybridization. These include "hybridization fingerprinting" (Craig *et al*., 1990), pulsed-field gel southern blot analysis of pooled clones (Mendez *et al*., 1991) and two-dimensional overgo hybridization (Han *et al*., 2000)

Polymerase chain reaction is one of the most widely used techniques in molecular biology. PCR-based strategies now rival hybridization –based strategies as the method of choice when screening yeast and bacterial libraries. A single PCR can be used to determine insert presence, size and orientation without the need for purification, restriction digestion, or hybridization (Campbell and Choy, 2002). Numerous PCR-based screening protocols have showed that crude cell lysate from whole cells or phage is sufficient for analysis (Güssow and Clackson, 1989; Bloem and Yu, 1990 ; Campbell and Choy, 2002). As with hybridization-based library screening methods, PCR-based techniques have benefited from increased automation.The advances in thermal cyclers, for example have focused on scaling sample volume down, increasing the number of samples run simultaneously, and decreased overall required cycling time (Meldrum, 2000).

Aside from PCR and hybridization, other screening methods have been used. For example, one such method is “fingerprinting” (some referred to as “restriction mapping”). However, fingerprinting is rarely used as the lone screening method, but is instead usually part of a multi-faceted screening approach to ensure data validity and reliability (Campbell and Choy, 2002).

Chapter 3.0 Seed germination

3.1 Introduction

Seeds are one of the important components for plant propagation. *Hylocereus* fruits contain thousand of seeds with variations in form, size, structure, embryo characteristics, and colour of the testa. In general, cactus seeds are divided into parts which consist of testa, embryo and endosperm (Rojas-arehchiga and Vahzquez-yanes, 2000). *Hylocereus polyrhizus* fruit, contains more than a thousand seeds per fruit. When seeds are dispersed in an unfavorable condition, the seeds will not germinate, a condition which is called seed dormancy. One important function of most seeds is delayed germination, which prevent germination of all the seeds at the same time.This usually occurs when there is competition from other plants for light and water. Another factor that causes seed dormancy is when the external environment conditions become too dry or warm or cold for germination which can delay germination from many weeks to years (Fenner and Thompson, 2005).

Currently, *H. polyrhizus* is propagated through stem cutting where the stem is removed and tied onto a supportive material for it to grow. The supportive material can be man-made pillars or consists of dead organic material such as tree bark or a living tree which provide the support. Stem cutting is the preferred way as it can produce fruit rapidly in a year compare to seed germination which takes three years to bear fruits (Le Bellec *et al*., 2006). There is no natural variation and genetic uniformity in crops which use vegetative propagation.This tends to make the crop vulnerable to disease outbreak and environmental disasters resulting in yield loss. Although seed germination takes a relatively longer time for the plant to produce fruit compared to stem cutting, it is useful in providing the starting material for DNA extraction. As the fruit contains thousand of seeds, seed germination can

be used to produce material for DNA extraction by harvesting the seeds from the mature fruit.

The longevity of seeds in most crops in storage is extended by reducing temperature, moisture content or both. However, some studies have also reported that seeds of a number of crop species were adversely affected by desiccation and therefore cannot be stored for a long time (Roberts, 1973). Nobel (1988) showed that seeds of most desert plants germinate in a wide range of temperatures, but reach their maximum germination percentage between 15 ^{o}C and 25^{o}C. Simao *et al*., (2007), carried out a study of *H. setaceus* seed germination whereby the effect of light and temperature were investigated. In that study, it was found that the optimum temperature is between 25 ^{o}C and 30 ^{o}C. The minimum temperature for germination was between 5 ^{o}C to 10 ^{o}C whereas the maximum was between 45 ^{o}C to 50 ^{o}C. The study also shows that the seeds do not germinate in complete darkness. The experimental design for this chapter was adapted from Simao *et al.,* (2000). The aim of this chapter is to establish an efficient seed germination protocol, this method can help us in the next step which is the get the desired DNA materials for construction of genomic library.

3.2 Materials and Methods

3.2.1 Selection of material

Three fully ripened and defect free *Hylocereus* fruits were obtained from a local market and deployed for experiment immediately.

3.2.2 Separation of seed from pulp

The fruits were cut into half and then the peels were removed revealing the pulps. Seeds were separated from the flesh using a strainer. All the seeds were first placed in water to separate floaters from the sinkers. The seeds that were partly hollow or decayed would float, while the solid seeds would sink. The sinkers were then divided into three groups which consist of control (Group 1), acid washed (Group 2) and air-dried (Group 3).

3.2.3 Acid wash treatment

Seeds were treated in a 1L beaker which contained concentrated sulphuric acid (Systerm) (1.25% v/v). The mixtures were then stirred using a magnetic stirrer for 45 minutes, rinsed with running water and dried at $25^{o}C \pm 2\ ^{o}C$ using a hand towel for 24 hours. The seeds were then placed in the sterilized petri dishes and labelled respectively.

3.2.4 Air-dried treatment

The seeds were air-dried at $25^{o}C \pm 2\ ^{o}C$ for 24 hours. The seeds were then placed in their sterilized petri dishes and labelled accordingly.

3.2.5 Untreated

The seeds that were not given any treatments were directly extracted out from the flesh and arranged carefully on the sterilized petri dishes and labelled accordingly.

3.2.6 Germination

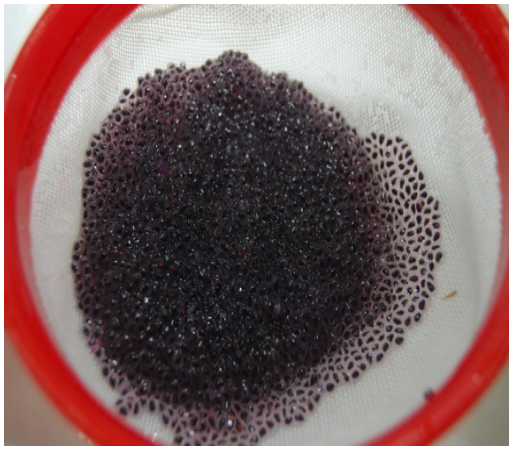
One hundred seeds from each treatment (3.2.3, 3.2.4 and 3.2.5) were arranged on two layers of Whatman filter-paper in each of 50mm diameter petri dishes and carried out in triplicates. The filter papers were moistened with distilled water and care was taken to ensure there was no free water in the dish to prevent fungal contaimination. To maintain moisture, 1ml of sterile distilled water were added to wet the filter papers every two days interval. Seeds with at least 1mm long root were considered as germinated (Simao et al., 2007). For each treatment, three petri dishes were placed under fluorescent light (100 lux) whereas another three petri dishes were placed in a dark room. The entire experiment was observed for 14 days and was carried out in an air-conditioned room where the temperature was kept at 26^{o}C. Figure 3.1 and Figure 3.2 shows graphical representation of the germination process whereas Figure 3.3 shows a pictorial representation of seed germination carried out in this chapter.

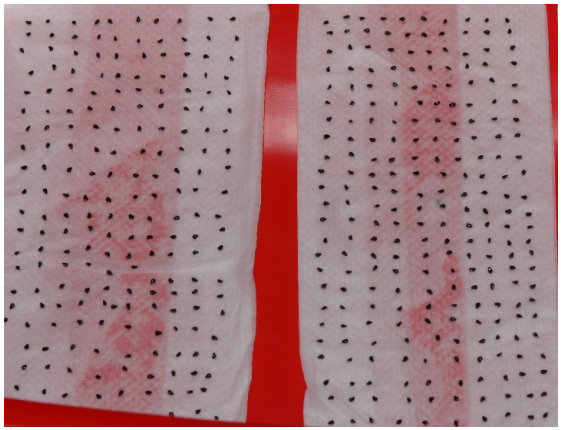
a. *Hylocereus polyrhizus*

b. Seeds were separated from flesh and deployed for experiment

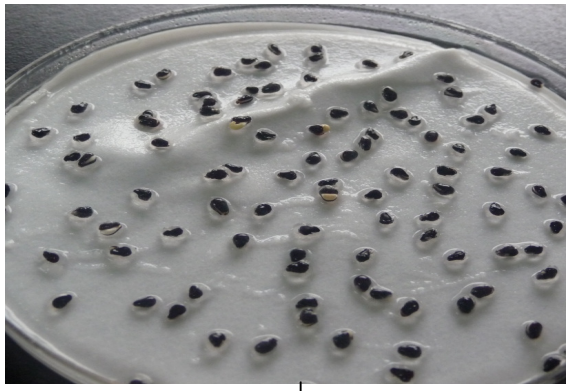
c. Seeds were acid washed

d. Seeds were separated from flesh and air-dried on hand towel





e) Seeds from (b), (c) and (d) were placed for germination (day 0)



f) Germination (Day 2)

g) Young leaves appeared at Day 6

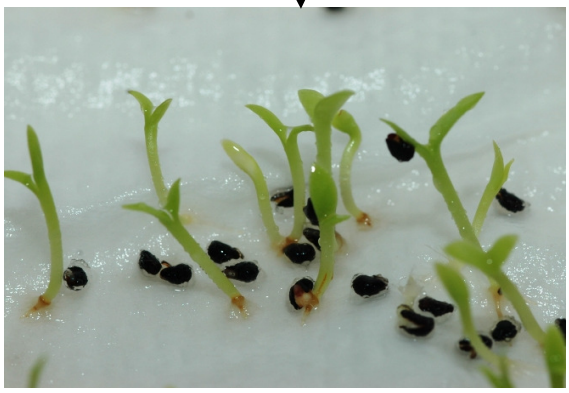


Fig 3.1: Pictorial representation of seed germination. Seeds from both groups were subjected to 2 treatments (acid wash and air-dried) with a control. Treated seeds were then placed into petri dishes and allowed to germinate.

3.3 Results

Hylocereus polyrhizus seeds were subjected to different treatments with the end point being 100% germination or after 14 days of observation.

3.3.1 Germination percentage of sinker seeds taken directly from fresh fruits under fluorescent light and dark room

Figure 3.2 shows that seeds exposed to light germinated faster compared to seeds germinated in the dark. Seeds exposed to light also showed 100% germination rate in a short period of time compared to dark room seeds which did not reach 100% germination for the entire germination period.

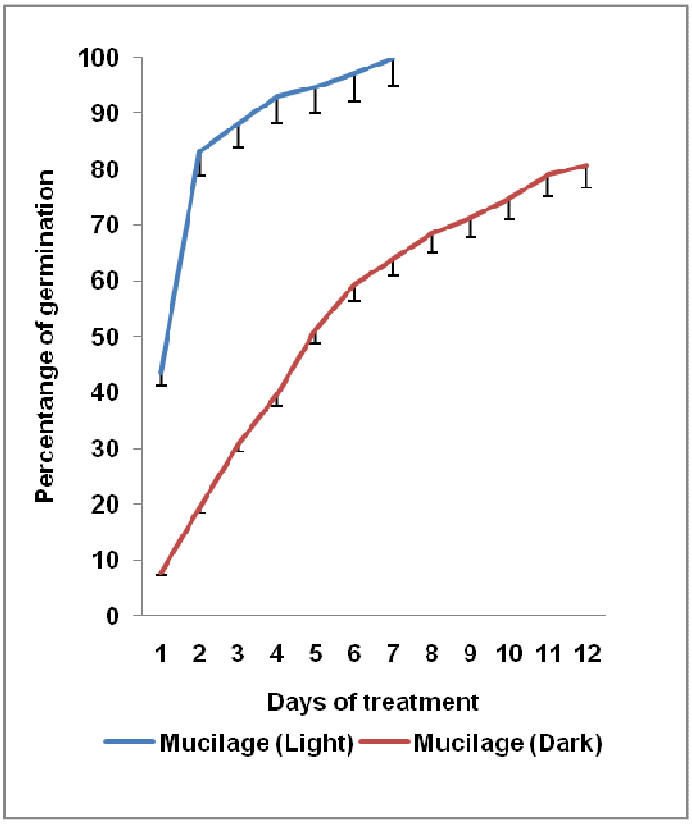


Fig 3.2: Germination percentage of seed harvested from fresh fruit under fluorescent light and in the dark. Both groups of seeds germinated but only the seeds that exposed to light achieved 100% germination.

3.3.2 Germination percentage of acid washed seeds harvested from fresh fruit under fluorescent light and in the dark

Figure 3.3 showed that both group of seeds germinated 100% but acid washed seeds exposed to light reached 100% germination faster than acid washed seeds kept in the dark. The acid washed seeds exposed to light achieved 100% germination on Day 6 while the acid washed seeds kept in the dark achieved 100% germination on Day 12.

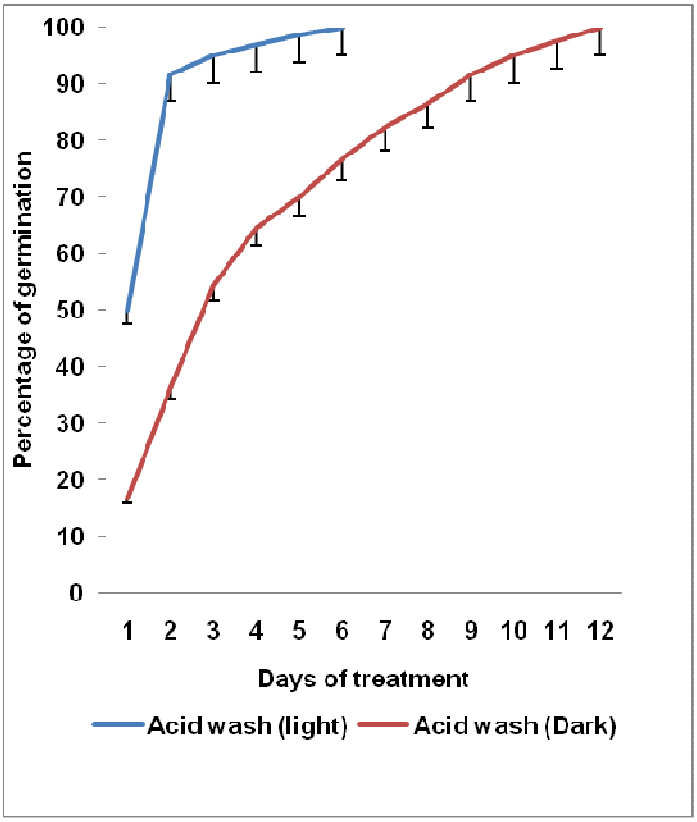


Fig 3.3: Germination percentage of acid washed seeds under fluorescent light and in the dark room. Both groups of acid washed seeds achieved 100% germination. However, the acid washed seeds that were kept in the dark were two times slower as compared to the acid washed seeds that exposed to light.

3.3.3 Germination percentage of acid dried seeds under fluorescent light and in the dark.

Figure 3.4 shows that air dried seeds exposed to light and placed in the dark both achieved 100% germination. However air dried seeds exposed to light achieved 100% germination faster than in the dark. The air dried seeds exposed to light reached 100% germination on Day 7 while seeds germinated in the dark only reached 100% germination on Day 14.

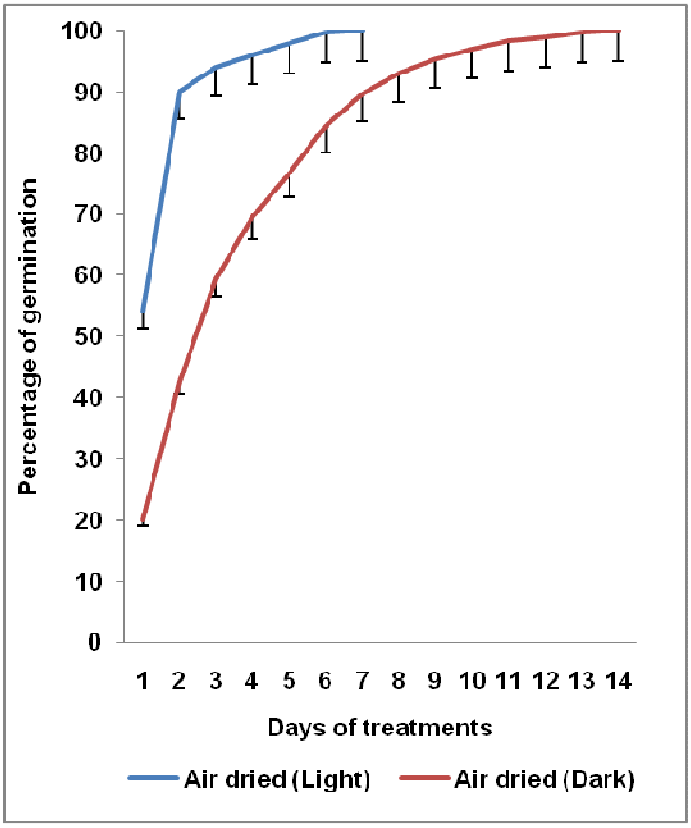


Fig 3.4: Germination percentage of air dried seeds under fluorescent light and in the dark room. Both group of air dried seeds achieved 100% germination. However, the air dried seeds exposed to light achieved 100% germination in a very short period as compared to the air dried seeds kept in the dark.

3.3.4 Germination percentage of seeds harvested from fresh fruit under fluorescent light

Figure 3.5 shows that untreated, acid washed and air dried seeds under fluorescent light achieved 100% germination. Acid washed seeds achieved 100% germination one day earlier as compared to untreated and air-dried seeds under fluorescent light. Acid washed seeds reached 100% germination on Day 6 while untreated and air dried seeds reached 100% germination on Day 7. There were no significant differences among the three groups with different treatments; they reached 100% eventually at almost the same time.

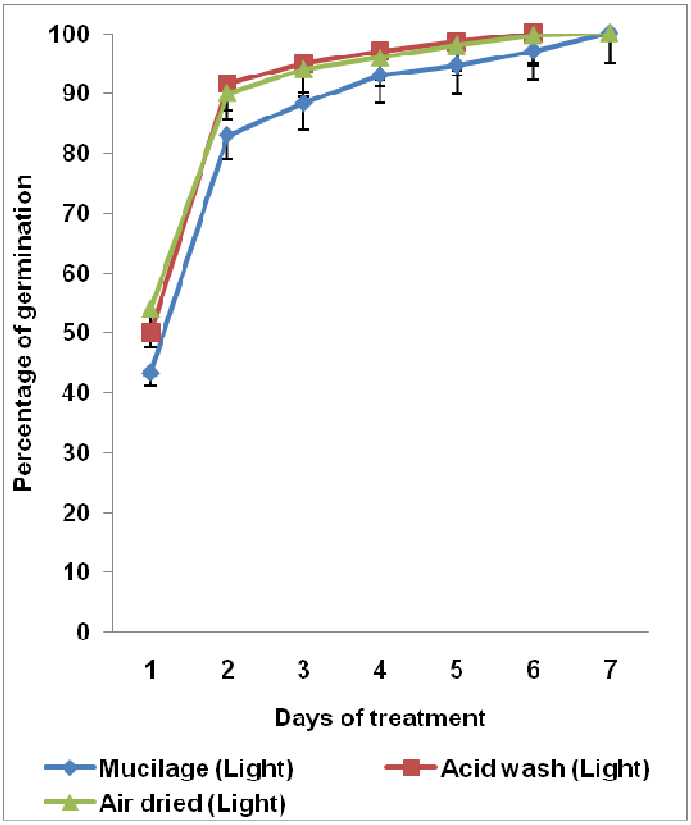


Fig 3.5: Germination percentage of seeds from fresh fruits under fluorescent light. All three groups of seeds reached 100% germination. Acid washed seeds under light achieved 100% germination one day earlier at Day 6 as compared to untreated and air dried seeds.

3.3.5 Germination percentage of seeds harvested from fresh fruit placed in the dark Fig 3.6 shows that both seeds from acid washed and air dried treatments reached 100% germination eventually as compared to the untreated seeds placed in the dark which only managed to reach approximately 80% germination in a period of 14. Figure 3.6 also shows that acid washed seeds reached 100% germination fastest in the dark (Day 12) compared to air dried seeds which only managed to achieve 100% germination on Day 14.

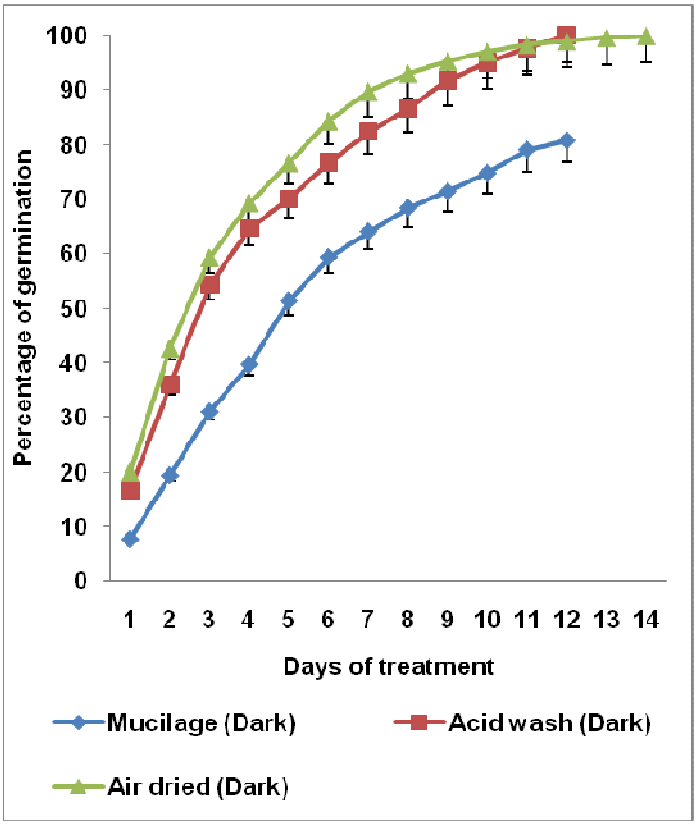


Fig 3.6: Germination percentage of seeds from fresh fruits in the dark. Seeds with acid washed and air dried showed 100% germination as compared to untreated mucilage seeds which only achieved about 80% germination. Acid washed seeds placed in the dark achieved 100% germination on Day 12 which was one day earlier than air dried seeds placed in the dark which reached 100% germination on Day 13.

3.4 Discussion

Seed germination depends on both internal and external conditions. Similar work has been done by Simão *et. al*., (2007) and Rojas-Aréchiga *et al*., (1997) using *Hylocereus setaceus* and seven different types of cacti species seeds. Hence, this experimental design was adapted from the studies with slight modifications. Sulfuric acid was used in this study to remove mucilage layer from the seeds. Simão *et al*., (2007) used a series of temperature to germinate the seeds and resulted in 25^{o}C being the most suitable temperature for germination of *H. setaceus* seeds. This study used optimum temperature (~26^{o}C) to germinate *H. polyrhizus* seeds. Simão *et al*.,(2001) also mentioned that germination of cactacean seeds is a fast event and it can be shortened with the presence of water in arid environment.

Most seeds are not affected by light or darkness, but many seeds including species found in forest settings, will not germinate until an opening in the canopy allows sufficient light for growth of the seedling (Raven *et al*., 2005). In this study *H. polyrhizus* seeds germinated faster under light compared to dark environment. This showed that fluorescent light played an important role in hastening germination process. Seed germinated under fluorescent light took only 7 days to reach 100% germination rate compared to seeds placed in the dark which took 14 days to reach 100%. This further proves the importance of light in germination process. However, light is not a prerequisite for germination but rather an enhancement for germination of *H. polyrhizus* seeds. Seeds can be germinated in the dark which means that they could germinate below the soil or without light induction (Lopez & Sanchez, 1989).

Different times of germination occurred for the control and the treated seeds. Fig. 3.5 showed that the growth of the control seeds were at a slower pace compared to treated seeds. This might be caused by the mucilage which slowers the germination process. Witztum *et al*., (1969 showed that the mucilage of *Blepharis persica*'s seeds acts as an oxygen barrier during germination. This can further explain the role that mucilage plays in lowering the germination process. In a work done by Walter *et al*., (1968), it was found that the mucilage which was formed under wet conditions creates a barrier which prevents the transfer of oxygen to the embryo. Under these conditions the rate of oxygen supply may not reach the threshold level required for germination. In this work, acid-washed is the best way to clean the mucilage from the seeds as air drying couldn't successfully clean the mucilage. This is evident as control seeds grown in the dark room poorly germinated as compared to the other two treatments (Fig. 3.6).

Overall, air-dried seeds and acid-washed seeds showed very good germination rate not only under light but also under dark condition. The increase in germination after acid treatment could be related to the observation by Barnea *e. al*., (1990) who suggested that seeds ingestion by birds generally favour germination because the acidic environment in the digestive system may reduce the thickness of the seed coat or remove the mucilage (waxy layer surrounded the seed). This may enhance seeds' permeability and probably improve gas exchange and water intake which eventually lead to a higher rate of germination.

In the germination process, the seeds starts to rapidly take up water that causes the seed coat to swell and eventually lead to radical emergence. the mechanism for the radicle

protrusion is yet to be concluded. However, it may be due to the osmotic potential of the radicle cells that become more negative. This would lead to increased water uptake and cell extension, expansion of radicle cell wall and breakdown of the seed tissues surrounding the radicle tips (Mei and Song, 2008). Interaction between the embryonic radicle tip and the enclosing endosperm cap control the germination of tomato and tobacco as reported by Koornneef *et al*.,(2002). The radicle protrusion was caused by the weakening of the endosperm cap during enzymatic hydroloysis. Enzymes responsible for this process are expansin and endo-ß-mannanase, which are specifically expressed in the endosperm cap of tomato.

Enzymes are hydrated during the rapid uptake of water. This subsequently leads to hydrolysis of the storage reserves in order to provide energy for cell expansion. Mei and Song, (2008) showed that contents of soluble sugars and starch gradually decreased. Activities of α- , and ß-amylase increased with imbibition which resulted in the decrease in soluble starch content. As the cotyledons become exhausted, the seedling is faced with the need to maximize the production of above ground biomass in order to achieve an optimal resource foraging balance. During this period energy demands are high, resulting in the emergence of thin nitrogen-rich leaves with a high photosynthetic capacity (Ishida *et al*., 2005).

The results obtained from this chapter showed that generally, all seeds germinated in light environment but were not entirely germinated under dark condition. Successful seed germination process required water, light and temperature. Adjusting the

parameters mentioned above can further shorten the time for germination of *H. polyrhizus* seeds.

Chapter 4.0 Selection of best plant tissues for DNA extraction of *Hylocereus polyrhizus*

4.1 Introduction

In plant molecular biology, the preparation of a long and pure DNA has been a major concern. Recombinant DNA techniques have been widely used (Murray and Thompson, 1980). Extraction of DNA from plant tissue is a critical step as it involves many procedures and is also a time-consuming step (Fulton *et al*., 1995).

Generally, most studies use young leaves as starting material to obtain high quality DNA (Amani *et al*. ,2011; Lodhi *et al*., 1994; and Hanania *et al*., 2004). However, due to the characteristic of *Hylocereus polyrhizus*, the leaves have been modified to needle like structure, which make it difficult to obtain a large number of cells per unit volume resulting in low concentration of nuclei. Therefore, *H. polyrhizus* leaves are not suitable as starting material for DNA extraction.

In a previous study for obtaining DNA from *H. polyrhizus,* Tel-Zur *et al.,* (1999) used roots (Fig 4.1) as starting material because it produced lower viscosity extract relative to other plant tissues such as stems, floral buds and vegetative buds. (Abolghasem *et al.,* (2010), used callus as the starting material to obtain an average DNA yield of 100µg/g which is a relatively good yield. However, generating callus requires time, space, expertise in tissue culture and most importantly it involves high cost. The advantage of using callus as starting material for DNA extraction is because of the absence of the cell differentiation in callus which prevents the synthesis of secondary metabolites that causes contamination (Diadema *et al.* ,2003).

Young, partially expanded leaf (Fig. 4.1) was preferred for DNA extraction as compared to roots (Fig. 4.2) because it contained high number of actively dividing cells and the tender leaf was relatively soft and easy to be homogenised. The modification done in this DNA extraction was the addition of Sodium Bis-sulphite, Sodium Sarkosyl and Sorbitol to the extraction buffer. The function of Sodium Bis-sulphite and Sodium Sarkosyl are to prevent the oxidation of phenolic compounds and precipitate high levels of polysaccharides respectively (Hanania *et al.,* 2004 and Sharma *et al.,* 2002). The presence of contaminants such as polyphenolic compounds (Katterman and Shattuck, 1983; Couch and Fritz, 1990; Howland *et al.,* 1991 and Collins and Symons, 1992) and polysaccharides (Murray and Thompson,1980 and Fang *et al.,* 1992) have been reported to cause the samples to be viscous and render DNA unrestrictable in endonuclease digestion. These powerful oxidizing agents aere the main factors that reduce the productivity and purity of the extracted DNA. Therefore, ß-mercaptoethanol and Polyvinylpyrrolidone (PVP) were used as antioxidants during DNA extraction. EDTA was used to chelate magnesium ions, a co-factor for nucleases which degrades the released DNA.

The aims of this chapter are to:

i. choose the best starting material for DNA extraction
ii. establish an efficient DNA extraction method

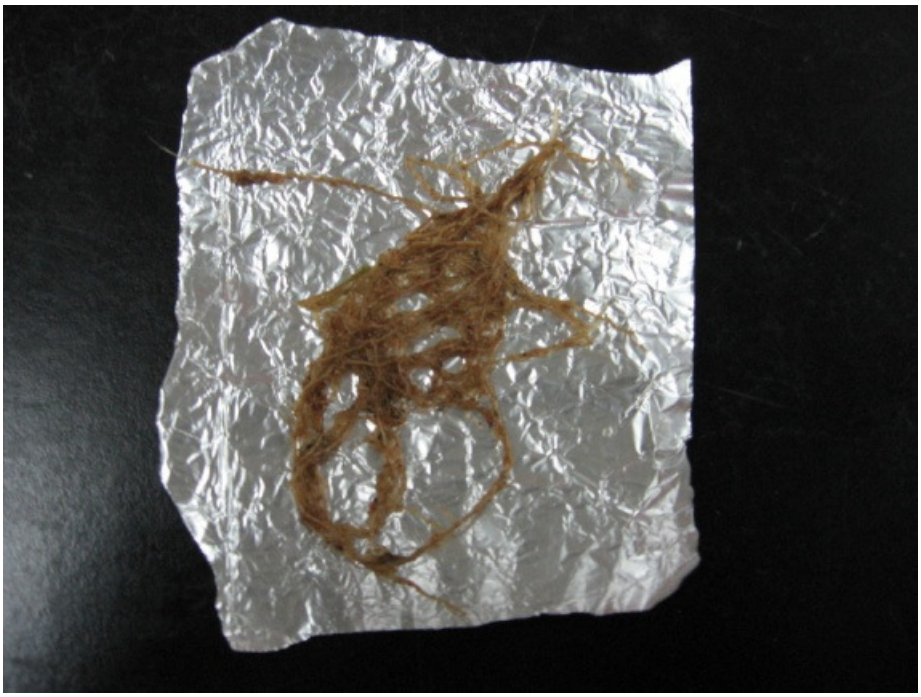


Fig 4.1: Roots for DNA extraction

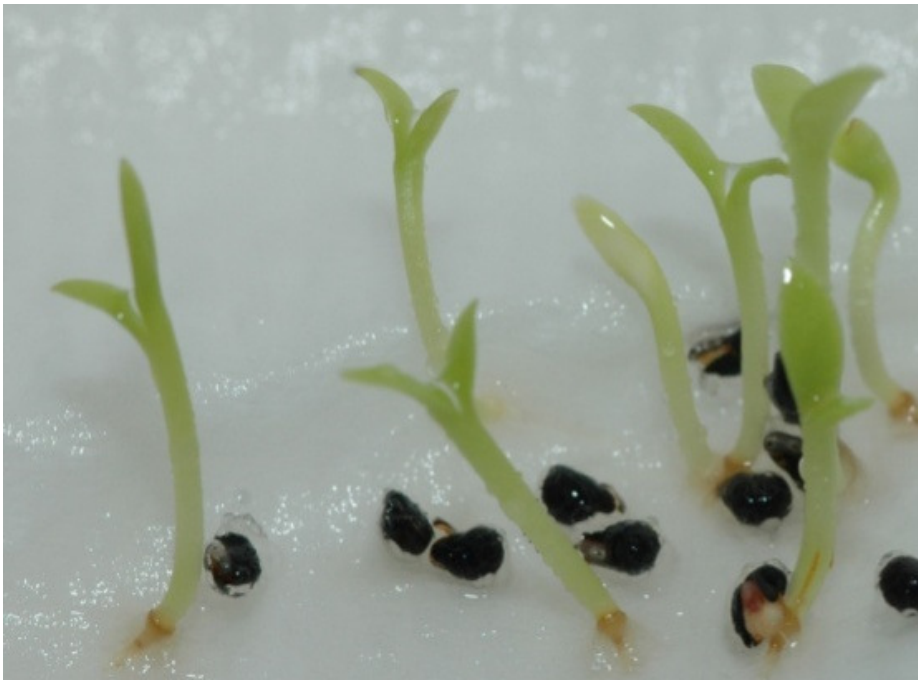


Fig 4.2: Young partially expanded leaves for DNA extraction

4.2 Materials and Methods

4.2.1 Plant material

Plant material was obtained as described in Section 3.2

4.2.2 Preparation of reagents

4.2.2.1 DNA isolation buffer (Modified method)

a) 1.37g of Sorbitol (Sigma)
b) 0.76g of Tris-base (Sigma)
c) 0.73g of EDTA (BDH)
d) 2.43g of Sodium Chloride (Sigma)
e) 0.42g of Hexadecyltrimethyl-ammonium bromide (CTAB) (Sigma)
f) 0.42ml of Sodium Sarkosyl (BioWhittaker)
(a) - (f) were dissolved in 50ml of sterile distilled water, autoclaved and stored at room temperature. 0.5g of Polyvinylpyrrolidone (PVP) (MW 40,000) (Research Organic) and 100µl of β-mercaptoethanol (AppliChem) and 57mg of Sodium Bis-sulphite (R&M) were added fresh before DNA extraction.

4.2.2.2 Extraction buffer (Tel-Zul *et al.*, 1999)

a) 12.76g of Sorbitol (Sigma)
b) 100ml of (200mM Tris-HCl, pH 8.0)
c) 2ml of (0.5M EDTA, pH 8.0)
(a)-(c) were added to 50ml of sterile distilled water and volume made up to 200ml with sterile distilled water. The solution was autoclaved and stored at room temperature. 2 ml of β-mercaptoethanol (AppliChem) was added fresh before use.

4.2.2.3 High-salt CTAB buffer (Tel-Zul *et al.*, 1999)

a) 11.69g of Sodium Chloride (Sigma)
b) 0.9g of Hexadecyltrimethyl-ammonium bromide (CTAB) (Sigma)
(c) 5ml of (200mM Tris-HCl, pH 8.0)
(d) 2.5ml of (0.5M EDTA, pH 8.0)
(a)-(d) were to 25ml of sterile distilled water and volume made up to 50ml with sterile distilled water. The solution was autoclaved and stored at room temperature.

0.5M EDTA

14.61g of EDTA (BDH) was dissolved in sterile distilled water; pH was adjusted to 8.0 with Sodium hydroxide pellets (Merck) and volume made up to 100ml with sterile distilled water. The solution was autoclaved and stored at room temperature.

200mM Tris-HCl

12.12g of Tris-base (Sigma) was dissolved in sterile distilled water; pH was adjusted to 8.0 with Sodium hydroxide pellets (Merck) and volume made up to 500ml with sterile distilled water. The solution was autoclaved and stored at room temperature.

Chloroform : isoamyl alcohol (24:1)

2ml of Isoamyl alcohol (R&M) was added to 48ml of Chloroform (R&M). The solution was stored at room temperature.

5M Sodium Chloride

14.61g of Sodium Chloride (Sigma) was dissolved in 40ml of sterile distilled water and the volume made up to 50ml with sterile distilled water. The solution was autoclaved and stored at room temperature.

70% Ethanol

70ml of pure Ethanol (BDH) was added to sterile distilled water and the volume made up to 100ml with sterile distilled water. The solution was stored at 4°C.

75% Ethanol

75ml of pure Ethanol (BDH) was added to sterile distilled water and the volume made up to 100ml with sterile distilled water. The solution was stored at 4°C.

TE buffer (10mM Tris-Cl, 1mM EDTA)

10ml of 1M Tris-Cl pH 7.5 and 2ml of 500mM EDTA pH 8.0 were added to sterile distilled water and the volume made up to 1000ml with sterile distilled water. The solution was autoclaved and stored at room temperature.

3M Sodium acetate (pH 5.2)

40.8g of Sodium acetate (Sigma) was added to 80ml sterile distilled water, pH was adjusted to 5.2 with glacial acetic acid and the volume made up to 100ml with sterile distilled water. The solution was autoclaved and stored at room temperature.

Phenol : Chloroform (1:1)

20ml of liquid phenol (Gen-Apex) was added to 20ml of Chloroform (R&M). The solution was stored in amber bottle at 4°C.

DNeasy Plant Mini Kit (Qiagen)

4.2.3 DNA isolation method

4.2.3.1 DNA isolation using fresh roots

0.5-1.0g of fresh roots was rinsed with distilled water to remove foreign material. The fresh roots were ground in liquid nitrogen using a mortar and pestle. The powder was transferred to a 50ml centrifuge tube containing 20ml of extraction buffer and centrifuged at 10,000g at 4°C for 10 minutes. The supernatant was decanted and upon centrifugation the pellet was dissolved in 20ml extraction buffer by inverting the tube. The mixture was then spun at 10,000g, 4°C for 10 minutes. This step was repeated one more time.

The supernatant was decanted, the pellet was resuspended in 5ml extraction buffer, and 3.5ml high-salt CTAB and 0.3ml Sarkosyl 30% were added to the tubes accordingly. The tubes were incubated in a water bath at 55°C for 60 minutes. An equal volume of chloroform : isoamyl alcohol was added to the tube and centrifuged at 10,000g for 10 minutes. The resulting supernatant was transferred to a 50ml centrifuge tube where 2/3 volume of cold absolute isopropanol and 1/10 volume sodium acetate were added to the tubes accordingly and centrifuged at 10,000g at 4°C for 20 minutes. The supernatant was decanted andthe pellet was washed with 75% cold ethanol. The supernatant was decanted, the pellet was air-dried and resuspended in 200 µl of TE buffer. 10µl of RNase stock solution was added to the tube and incubated in a water bath at 37°C for 40 minutes. The solution was transferred to a 1.5ml microcentrifuge tube where an equal volume of phenol : chloroform was added and centrifuged at 14,000 rpm for 10 minutes at room temperature.

The upper aqueous phase was transferred to a new 1.5ml microcentrifuge tube, an equal volume of cold chloroform was added and centrifuged at 14,000 rpm for 10 minutes at room temperature. The upper aqueous phase was transferred to a 1.5ml microcentrifuge tube, 2 volumes of absolute cold 100% ethanol and 1/10 volume of sodium acetate solution were added. The microcentrifuge tube was then kept at -20°C for 30 minutes. Finally, the pellet DNA was obtained by centrifugation at 14,000 rpm for 15 minutes, rinsed with cold 75% ethanol, air-dried and dissolved in 30-50µl of TE buffer.

4.2.3.2 Modified DNA isolation method using young leaves

In this method, 0.1 grams of young leaves of germinated seeds were ground to fine powder in liquid nitrogen using a mortar and pestle. The powder was transferred to a microcentrifuge tube containing 700µl of DNA isolation buffer, gently inverted and incubated in a water bath at 65°C for 45 minutes. The mixture was extracted twice using equal volumes of chloroform:isoamyl alcohol and the tubes were centrifuged at 10,000 rpm for 15 minutes. The supernatant was transferred to a new tube and 0.5 volumes of 5M NaCl and 2 volumes of absolute cold isopropanol were added. The tube was gently inverted and incubated on ice for 15 minutes. The tube was centrifuged at 10,000 rpm for 5 minutes and the resulting pellet was washed twice with 500µl of 70% ethanol. Finally, its pellet was air dried and dissolved in 30-50µl of TE buffer.

4.2.3.3 DNeasy Plant Mini (Qiagen) using young leaves

DNA was extracted from 0.1 grams of sample material. The sample was ground to fine powder in liquid nitrogen using a mortar and pestle. The powder was transferred to a microcentrifuge tube, 400µl of Buffer AP1 and 4 µl RNase A were added, vortexed and incubated in a water bath at 65°C for 10 minutes. The tube was inverted 2-3 times

during incubation. 130µl of Buffer AP2 was added to the mixture, mixed and incubated on ice for 5 minutes. The lysate was centrifuged at 14,000rpm for 5 minutes and the lysate was pipetted into a QIAshedder Mini spin column in a 2ml collection tube, centrifuged at 14,000rpm for 2 minutes. The flow-through fraction was transferred into a new tube without disrupting the pellet. 1.5 volume of Buffer AP3/E was added to the flow-through and mixed by pipetting. The 650µl of mixture was transferred into a DNeasy Mini spin column in a 2 ml collection tube, centrifuged at 8,000rpm for 1 minute and the flow-through was discarded. The spin column was placed into a new 2 ml collection tube and500µl Buffer AW was added to the spin column.The tube was then centrifuged at 8,000rpm for 1 minute and the flow-through was discarded. Another 500µl Buffer AW was added to the spin column and centrifuged at 14,000rpm for 2 minutes. The spin column was transferred to a 1.5ml microcentrifuge tube, 50µl Buffer AE was added to spin column, incubated for 5 minutes at room temperature and centrifuged at 8,000rpm for 1 minute. 50µl Buffer AE was added to the spin column again, incubated for 5 minutes at room temperature and centrifuged at 8,000rpm for 1 minute.

4.2.4 DNA quantification

A 1000X dilution was made by adding 2900µl of distilled water to the cuvette followed by 3µl of sample DNA (4.2.3.1, 4.2.3.2 and 4.2.3.3) and 97µl of distilled water. It was thoroughly mixed by pipetting. 3ml of distilled water was used as a blank. The absorbance was recorded at 260nm and 280nm. DNA concentration was calculated using the following equation: DNA concentration (µg/µl) = OD_{260} x dilution factor x (50µg DNA/ml). Purity was calculated by taking the ratio of reading of OD_{260} / OD_{280}

4.2.5 0.8% Agarose gel electrophoresis

0.4g of agarose (AppliChem) was added to 1ml of 50X TAE buffer and 49ml of sterile distilled water. The solution was boiled until the agarose was completely dissolved. The solution was removed after boiling and allowed to cool to 60°C. 1.5µl ethidium bromide was added and mixed by gently swirling the solution. The solution was poured into a casting tray containing a sample comb and was allowed it to solidify at room temperature. The tape and comb were removed after the gel has solidified. Aliquots of DNA (4.2.3.1, 4.2.3.2 and 4.2.3.3) was mixed with a 6X loading dye (EURx) and loaded into the wells. Electrophoresis was carried out at 60V for 4 hours. The migration pattern was captured with a camera.

4.3 Results

4.3.1 DNA from using fresh roots

Genomic DNA was isolated using the described method, and 57.5µg/g of DNA was yielded from the root material. The pellet was brown and the spectrophotometer readings gave a A_{260} nm/ A_{280} nm ratio of 1.28 (Table 4.1). The band from gel electrophoresis showed DNA degradation as shown in Figure 4.3.

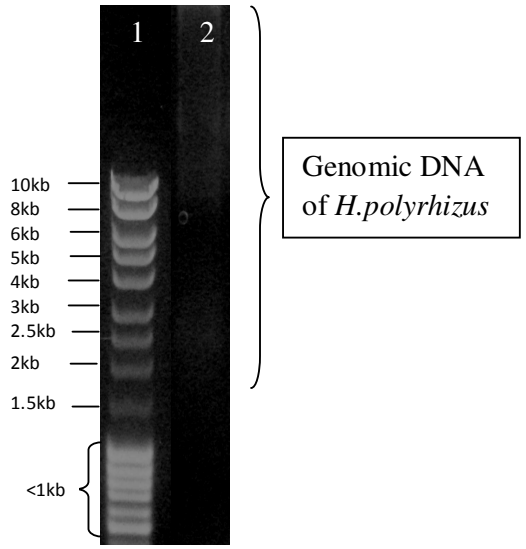


Fig 4.3: Electrophoresis of *H.polyrhizus* genomic DNA from fresh roots on 0.8% agarose gel. Lane 1: Mass ruler DNA ladder mix (Fermentas) and Lane 2 Genomic DNA of *H.polyrhizus* extracted from roots.

4.3.2 Modified DNA isolation method using young leaves

Isolation of genomic DNA using the described method resulted in 232.5µg/g of DNA obtained leaves material. The colour of the pellet was whitish and the spectrophotometer readings gave a A_{260} nm/ A_{280} nm ratio of 1.90 (Table 4.1). The band from gel electrophoresis showed a sharp and thick band as shown in Figure 4.4 which indicates that the DNA material is pure and clean.

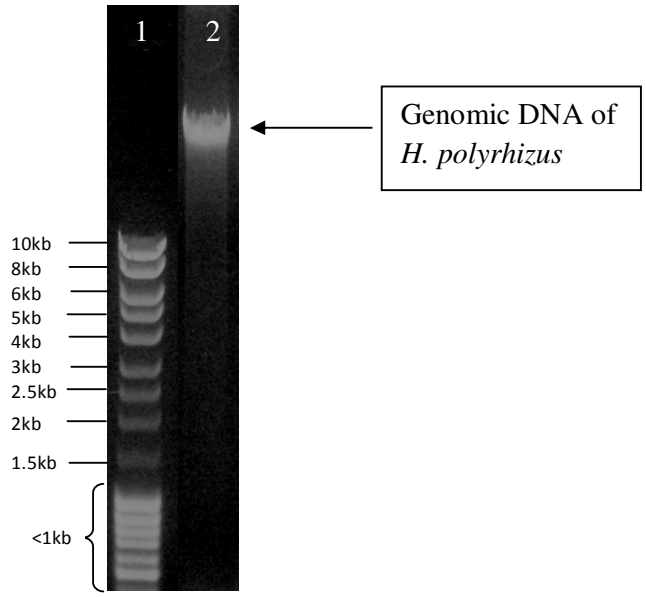


Fig 4.4: Electrophoresis of *H.polyrhizus* genomic DNA from young leaves on 0.8% agarose gel. Lane 1: Mass ruler DNA ladder mix (Fermentas) and Lane 2 Genomic DNA of *H.polyrhizus* from leaves using modified DNA isolation protocol.

4.3.3 DNeasy Plant Mini kit (Qiagen) using young leaves

Isolation of genomic DNA using the described method resulted in 20µg/g of DNA from the leaves. The pellet was white and the spectrophotometer readings gave a A_{260} nm/ A_{280} nm ratio of 1.60 (Table 4.1). The band from gel electrophoresis showed a sharp and thin band as shown in Figure 4.5 which indicates the amount of the DNA is too little for the construction of genomic library.

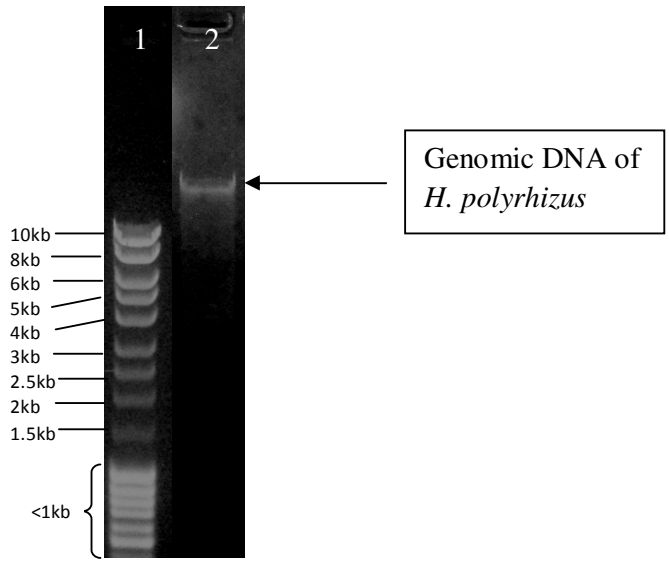


Fig 4.5: Electrophoresis of *H.polyrhizus* genomic DNA from young leaves using Qiagen kit on 0.8% agarose gel. Lane 1: Mass ruler DNA ladder mix (Fermentas) and Lane 2 Genomic DNA of *H.polyrhizus* from leaves using DNeasy Plant Mini Kit (Qiagen).

Table 4.1: DNA analysis using UV-spectrophotometer. Table comparing purity using different starting materials from *H.polyrhizus*.

Plant materials	A_{260}	A_{280}	A_{260}/A_{280}
Young leaves (Commercial kits)	0.008	0.005	1.60
Roots	0.023	0.018	1.28
Young leaves	0.093	0.049	1.90

4.4 Discussion

DNA isolation using young leaves according to the modified DNA extraction protocol produced high purity DNA as compared to the method by Tel-Zur *et al*., (1999) and DNeasy Plant Mini DNA kit (Qiagen). The isolation of DNA using the modified DNA protocol gave the highest yield of DNA (232.5µg/g) compared to the method by Tel-Zur *et al*.,(1999) and the DNeasy Plant Mini DNA kit (Qiagen) which only gave a DNA yield of 57 µg/g of root material and 20 µg/g of leaf material respectively. This yield is much better compared to both methods and the purity of the DNA was within the desired range of 1.8 – 2.0. The result obtained using the Tel-Zur *et al*.,(1999) method using roots material could not be reproduced in this experiment. This may be due to the different source of roots as the study used root cuttings as opposed to using roots from the ground. Soil found on the roots may be another factor causing DNA degradation.

Both the extraction methods which used young leaves as starting material were able to produce intact DNA. However the DNA yield from the modified DNA extraction protocol was 10 times that of the yield obtained from DNeasy Plant Mini DNA kit (Qiagen). This can be observed in Figure 4.4 and Figure 4.5.

Hylocereus polyrhizus contains high amounts of polysaccharides which bind to the DNA making it viscous and glutinous after the precipitation step during extraction. This

will affect the downstream application such as PCR or restriction digest as the enzyme cannot access the DNA (Tel-Zur *et al*., 1999; Barnwell *et al*., 1998 and Puchooa, 2004). Presence of polysaccharide is easily detected during elution step where the DNA extracts tend to be sticky and viscous.

The DNA extraction using the commercial kit resulted in the lowest DNA yield.This may be caused by the column in the kit. In the process of isolation of DNA using the kit (as described in 4.2.3.3), the spin column was used. This may have caused DNA to be trapped in the column. This will render the DNA unsuitable for further process such as restriction digest.

The DNA extraction method was adapted from Lodhi *et al*., (1994) with slight modifications. The modifications include the addition of Sodium Bis-sulphite, Sodium Sarkosyl and Sorbitol into the extraction buffer. The addition of Sodium Bis-sulphite into the extraction buffer is to prevent oxidation of phenolic compounds whereas the addition of Sodium Sarkosyl is to precipitate high levels of polysaccharides (Havania *et al*., 2004 and Sharma *et al*., 2002). Lodhi *et al., (*1994) carried out tissues extraction with CTAB in high salt condition which suppresses the co-precipitation of polysaccharides and DNA. In that study, i ratio of 1:1 extraction buffer to leaf material was used. (Murray and Thompson, 1980 and Lodhi *et al*.,1994). However, in this modified extraction protocol, only 0.7ml of isolation buffer was used for 0.1g of leaves which is sufficient to yield high quality DNA. Preheating the isolation buffer to 65^{o}C before use also helps in obtaining a betterquality DNA as it can shorten the time for

increasing the temperature of the frozen tissues extracted at -80°C to 65°C (Puchooa, 2004).

The phenolic compounds in plant cells are rapidly oxidized to form covalently linked quinines that vigorously bond nucleic acids and cause insoluble RNA and DNA pellets (Loomis, 1974). Gehrig *et al*., (2000) also mentioned that polysaccharides also are problematic as they coprecipatate with nucleic acids in buffer with low ionic strength. This will have an impact on the activity of enzymes such as restriction enzymes. By adding low molecular weight polyvinyl pyrrolidone (PVP) and ß-mercaptoethanol into the isolation buffer, oxidation of phenolic compounds is reduced and contamination is also removed from the pellets.

Selection of the starting material is a very crucial stage in the extraction of high quality DNA. Young and partially expanded leaf material is the best starting material for DNA isolation as compared to fully expanded leaves. Fully expanded leaves are heavily loaded with polysaccharide and polyphenols which will become contaminants during the DNA isolation process, making the pellets brown in colour and insoluble (Abolghasem *et al*., 2010). Furthermore, mature leaves are tougher making the grinding process difficult (Couch and Fritz, 1990).

It only took one week for seeds to germinate to produce partially expanded young leaves. It is therefore a time-saving way of producing the most suitable material for DNA isolation of *H. polythizus*. The germination process is also cheaper and easier as compared to generating callus.

In the process of homogenizing the tissues, tissues should be thoroughly ground but not into very fine powder because DNA will be sheared if the tissue is ground too vigorously. According to Couch and Fritz, (1990), pulverised tissue should be transferred to tubes containing buffer immediately to reduce the chances of DNA getting thawed as thawing of DNA will cause polyphenolic compounds to build up. Hence care must be taken during this process.

The results in this chapter are reproducible using young partially expanded leaves with the modified protocol. It consistently gave high yield of DNA. This process only requires leaves from seed germination which is inexpensive and easy to obtain. The DNA can be stored in TE buffer at -20^{o}C for months without degradation.

Chapter 5: Preparation of Insert DNA, Ligation, Packaging and Titering the Library

5.1 Introduction

A genomic library is a collection of genes or DNA sequences created using molecular cloning method. Clones of bacteria or yeast are normally used as host for population of vectors that contain partially digested DNA (Robert *et al.*, 2001). The bacteria and yeast are normally grown in culture and the vector will then be replicated when the microorganisms replicate their genome (Susan *et al*., 2005). Theoretically, the collection of clones should contain all the sequences found in the original source including the sequence of interest. Genomic libraries can be constructed using various commercially available hosts, depending on the insert size of the DNA. Thus identifying the size of the insert is one of the crucial step. For DNA insert size up to 15 kb, plasmid can be used as a host, for DNA insert size up to 20 kb, bacteriophage lambda can be used as a host. Cosmids serve as a host for DNA inserts up to 45 kb, YACs is for DNA inserts up to 2,000 kb and many more which can also be used as a host for different type of DNA insert size (Susan *et al*.,2005).

In order to obtain a representative genomic library, some criteria need to be fulfilled. The number of recombinants needed to obtain a desired gene in a library can be calculated from the following formula:

$$N = \ln (1-P) / \ln(1-f)$$

Where,

N= the number of recombinants (clones) in a gene library

P= desired probability of gene represented in library

f= fraction of genome in one insert

For example, *E. coli* genome contained 4.6 million base pairs and to calculate a probability of 0.99 (99% chance of desired gene in the library) with an average insert size of 20kb, the number of recombinants can be calculated as below:

$$N_{E.coli} = \frac{\ln (1-0.99)}{\ln[1-(2x10^4/4.6x10^6)]}$$

$= 1.1 \times 10^3$ recombinants.

From the example above, 1.1×10^3 recombinants are needed to qualify as a representative gene library (Lodish *et al*., 2000).

Bacteriophage lambda is the most extensively studied bacterial virus, it has a head which contains viral DNA genome and a tail which functions in infectiong *E.coli* host cell. It will undergo either lytic or lysogenic growth when lambda DNA enters the host-cell cytoplasm. In the lytic growth, the viral DNA is replicated and produced more than 100 progeny virions in each infected cell. Lysogenic pathway and other viral genes are removed from viral DNA and replaced with the DNA to be cloned, thus ~25kb of foreign DNA can be inserted into the lambda genome. Infection by lambda phage is about a thousand times more efficient than transformation with plasmid vectors, resulting in high efficiency of lambda phage cloning. This makes bacteriophage more suitable for this project. Figure 5.1 shows the the multiple cloning sites that are present in a lambda vector. This vector is commercially available which simplifies the construction of genomic library (Lodish *et al*.,2000).

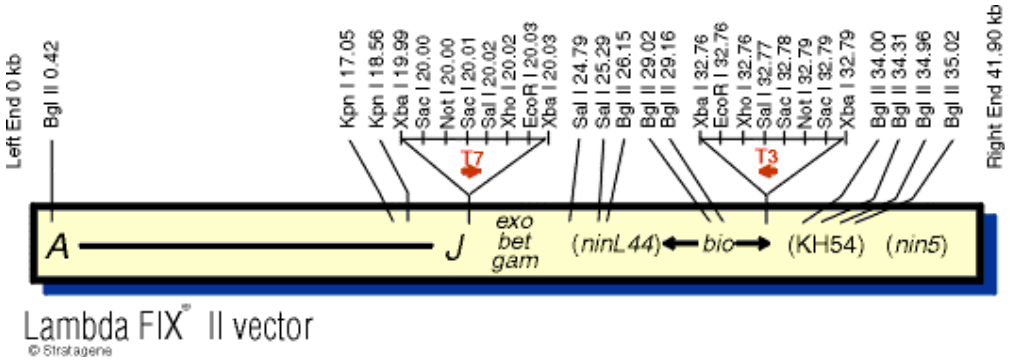


Fig. 5.1: Lambda FIX II vector with multiple cloning sites (from commercially Stratagene kit).

The aims of this chapter are to

i. Prepare the insert DNA

ii. Ligate the insert DNA into Lambda FIX II vector

iii. Assembly of bacteriophage lambda virions using the Gigapack III XL Packaging extract

iv. Titer the library

5.2. Material and Methods

5.2.1. Preparation of reagents

10mM dGTP

10µl of 100mM dGTP (Finnzymes) was added into 90µl sterile distilled water. The solution was stored at -20°C.

10mM dATP

10µl of 100mM dATP (Finnzymes) was added into 90µl sterile distilled water. The solution was stored at -20°C.

TE buffer (10mM Tris-Cl, 1mM EDTA)

This buffer was prepared according to Section 4.2.

Phenol-chloroform

This solution was prepared according to Section 4.2.2

10 X STE buffer

5.84g of NaCl (Sigma) and 2.92g of EDTA (Sigma) was dissolved in 50ml of sterile distilled water. 20ml of Tris-HCL (1M, pH7.5) was added to the solution and volume made up to 100ml with sterile distilled water. The solution was autoclaved and stored at room temperature.

1 X STE buffer

0.1ml of 10X STE buffer was added to 0.9ml of sterile distilled water. The solution was stored at room temperature.

70% (v/v) Ethanol

This solution was prepared according to Section 4.2.2

Tris-Cl (1M, pH7.5)

30.28g of Tris-base (Sigma) was dissolved in 200ml sterile distilled water, pH was adjusted to 7.5 using concentrated hydrochloric acid and volume made up to 250ml with sterile distilled water. The solution was autoclaved and stored at room temperature.

Gelatin (2% w/v)

2g of Bovine gelatine (Merck) was dissolved in 10ml of sterile distilled water. The solution was stored at room temperature.

SM buffer

2.9g of Sodium Chloride (Sigma) and 1g of Magnesium Sulfate Heptahydrate (Merck) were dissolved in 300ml of sterile distilled water. 25ml of Tris-Cl (1M, pH 7.5) and 2.5ml of Gelatin (2% w/v) were added into the solution and made up to 500ml with sterile distilled water. The solution was autoclaved and stored at room temperature.

NZY broth

10g of NZ Amine (Merck), 5g of Yeast Extract (Merck), 5g of Sodium Chloride (Sigma) and 2g of Magnesium sulphate heptahydrate (Merck) were added into a beaker containing 100ml sterile distilled water, pH was adjusted to 7.5 with Sodium hydroxide pellets (Merck) and volume made up to 1000ml with sterile distilled water. The solution was autoclaved.

NZY agar plate

15g of bactoagar powder (BD) was added to 1000ml of NZY broth and autoclaved. Solution was cooled to 60°C and poured into plates. The plates were then placed in a plastic and sealed. The sealed plastic containing the plates were stored at 4°C.

NZY supplemented with 0.2% maltose

250ml of NZY broth was autoclaved and allowed to cool down to 40°C before adding 2.5ml of sterile 20% maltose. The solution was then stored at room temperature.

20% Maltose

20g of Maltose (Merck) was dissolved in 100ml of sterile distilled water and filter sterilized using 0.2µm pore size (Sartorius). The solution was then stored at room temperature.

10mM $MgSO_4$

0.2465g of Magnesium sulphate heptahydrate (Merck) was dissolved in 100ml of sterile distilled water. The solution was then stored at room temperature.

NZY top agarose

1.75g of agarose powder (Promega) was added into 250ml of NZY broth and autoclaved.

5.2.2. Preparation of the insert DNA

5.2.2.1. Pilot scale partial digestion

Each tube (Table 5.1) containing 2.5µg of DNA was mixed with 10X *Bam*HI buffer, 10mg/ml BSA, 10mg/ml RNase A, sterile distilled water and different ratios of enzyme (Table 5.2); 0.05U/µg, 0.10U/µg, 0.15U/µg, 0.20U/µg, 0.25U/µg and 0.30U/µg. The contents were gently mixed by pipetting before being dispensed into each tube. The tubes were incubated in a 37°C water bath for 90 minutes. 5µl of loading dye (EURx) was added into the tubes to stop the reaction at the end of incubation period.

Table 5.1: DNA master mix cocktail. The following table shows the quantity of component added into each tube.

Tube	Per reaction	For 6 reactions
2.5µg DNA (µl)	2.4	14.4
10X *Bam*HI buffer (µl)	0.50	3.0
10mg/ml BSA (µl)	0.15	0.9
10mg/ml RNase A (µl)	0.15	0.9
Sterile distilled water (µl)	Made up to 5µl	Made up to 30µl

Table 5.2: Serial dilution of *Bam*HI to genomic DNA of *H. polyrhizus*.

Tube	1	2	3	4	5
DNA(µg)	2.5	2.5	2.5	2.5	2.5
Dilution (U/µg DNA)	0.05	0.10	0.15	0.20	0.25
Unit of restriction enzyme	0.25	0.50	0.75	1.00	1.25
Volume of 0.1U/µl restriction enzyme (µl)	2.5	5	7.5	10.0	12.5

5.2.2.2 Agarose gel electrophoresis

The best dilution of unit of enzyme per microgram of DNA that yielded the highest intensity of fragments between 9-23 kb was identified through agarose gel electrophoresis. Samples from Section 5.2.2.1 were loaded to agarose gel. The agarose gel was prepared as described in Section 4.2.5.

5.2.2.3. Full scale partial digestion

In this study, 0.25U/µg yielded the highest intensity of fragments between 9-23 kb (Figure 5.2). Hence the ratio was used for full scale partial digestion. Ten tubes (Table 5.3) containing 5µg of DNA was mixed with 10X *Bam*HI buffer, 10mg/ml BSA, 10mg/ml RNase A and 0.25U of *Bam*HI enzyme (Table 5.4). The contents mentioned above were gently mixed by pipetting before being dispensed into each tube. The tubes were incubated at 37°C for 90 minutes and at 65°C for 20 minutes in a water bath to denature the *Bam*HI enzyme. The content from the 10 tubes were pooled to make 1 tube after the *Bam*HI enzyme was denatured. The pooled 50µg genomic DNA insert was precipitated by mixing with 1ml of 100% (v/v) ethanol and centrifuged for 10 minutes at 14,000rpm. The supernatant was decanted and the DNA pellet was washed with 1ml of 70% (v/v) ethanol. The mixture was further centrifuged for 1 minute at 14,000rpm.

The supernatant was then decanted and the pellet was air-dried and resuspended in 100µl of TE buffer.

Table 5.3: DNA master mix cocktail for full scale digestion. The following table shows the quantity of components added into each tube.

Tube	Per reaction	For 10 reactions
5µg DNA (µl)	4.8	48.0
10X *Bam*HI buffer (µl)	1.0	10.0
10mg/ml BSA (µl)	0.1	1.0
10mg/ml RNase A (µl)	0.1	1.0
Sterile distilled water (µl)	Made up to 10µl	Made up to 100µl

Table 5.4: Dilution of *Bam*HI to genomic DNA of *H. polyrhizus* prepared for 1 tube. 12.5 µl of 0.1U/µl restriction enzyme were added to all 10 tubes.

Contents	Tube
DNA(µg)	5
Dilution (U/µg DNA)	0.25
Unit of restriction enzyme	1.25
Volume of 0.1U/µl restriction enzyme (µl)	12.5

5.2.2.4. Partial end fill with dGTP, dATP and Klenow polymerease

Samples (Section 5.2.2.3) was partially filled-in according to Stratagene fill-in kit instruction; 30µl of 10X fill-in buffer, 5µl 10mM dATP, 5µl 10mM dGTP, 15U of Klenow polymerase and the volume made up to 300µl with sterile distilled water. The mixture was added into a tube and incubated at room temperature for 15 minutes.

150µl of 1X STE buffer, 50µl of 50X STE buffer and 500µl of phenol-chloroform were then added into the tube, vortexed and spun for 2 minutes at 14,000 rpm. The upper aqueous phase was transferred into a new tube. The addition of 500µl of phenol-chloroform were then added into the tube, vortexed and spun for 2 minutes at 14,000 rpm. This step was repeated if there is an interface. 500µl of chloroform was added into the tube, vortexed and spun for 2 minutes at 14,000rpm. The upper aqueous phase was transferred into a new tube and 1ml of 100% (v/v) ethanol was added and incubated at -20°C for 30 minutes. The tube was then centrifuged for 10 minutes at 4°C at 14,000rpm. The supernatant was decanted and the DNA pellet was mixed with 1ml of 70% (v/v) ethanol. The mixture was further centrifuged for 1 minute at 14,000rpm. The supernatant was decanted and the pellet was air-dried and resuspended in 25µl of TE buffer.

5.2.3. DNA quantification

The purity and the concentration of the DNA sample was determined as described in Section 4.2.4.

5.2.4 Ligation of the insert DNA to lambda phage DNA

The ligation mixture containing 1.0µg of the Lambda Fix II *Xho I* predigested DNA (Stratagene), 1µg insert, 0.5µl of 10X ligase buffer (NEB), and 1µl of T4 DNA Ligase (NEB) was prepared and volume made up to 5µl with sterilized distilled water. The ligation mixture was incubated overnight at 4°C and is then ready for use in packaging.

5.2.5. Packaging

About 2.5 µl of the ligated DNA (5.2.4) was added to the packaging extract by gentle pipetting. The tube was incubated at room temperature (22ºC) for 2 hours after which 500µl of SM buffer was added followed by 20µl chloroform. The tube was spun briefly and the supernatant, containing the phage and ready for tittering, was stored at 4°C.

5.2.6. Titering of the Library

5.2.6.1. Growth and Preparation of Host Strain

Three microliter of *E. coli* strain XL-1 Blue MRA (P2) (Stratagene) was streaked on an NZY agar plate. The plate was inverted and incubated overnight at 37°C. Single colony of E. coli strain XL-1 Blue MRA (P2) (Figure 5.2) was isolated into 5ml of NZY supplemented with maltose (0.2% w/v) and grown overnight at 30°C with shaking at 225rpm by using Shellab orbital shaking incubator (S14). Upon incubation, the cells were spun down at 1000rpm for 10 minutes. The medium was decanted and the cell pellet was resuspended in 2.5ml of 10mM $MgSO_4$ and diluted till OD_{600}=0.5.

5.2.6.2. Preparation of the λ bacteriophage

Two hundreds microliter of XL-1 Blue MRA (P2) at OD_{600}=0.5 was added to individual sterile culture tube in a test tube rack. Ten microliter of λ bacteriophage was added into each sterile culture tube containing the XL-1 Blue MRA (P2) cells. The test tube rack was placed in a 37°C water bath for 20 minutes. Three mils of NZY top agarose was poured into each tube containing the λ phage and E. coli strain XL-1 Blue MRA (P2) after the incubation period. The tube was removed from the rack and quickly flicked before pouring onto the NZY plates. The top agarose was allowed to solidify before inverting and overnight incubation at 37°C.

The Lambda FIX II system operates based on spi (sensitive to P2 inhibition) selection. Lambda phages containing active *red* and *gam* genes on the stuffer fragment are unable to grow on host strains that contain P2 phage lysogens such as XL1-Blue MRA (P2). When the stuffer fragment is replaced by an insert, the recombinant Lambda FIX II vector becomes Red–/Gam–, and the phage is able to grow on the P2 lysogenic strain. Hence, in the Lambda FIX II system, only recombinant phages are allowed to grow. The titer was determined by using the following formula (Rahman *et al.,* 2007):

$$\frac{\text{Number of plaques (pfu)} \times \text{Dilution factor} \times 1000\ (\mu l/ml)}{\text{Volume of the } \lambda \text{ bacteriophage added to the cells } (\mu l)}$$

5.3 Results

5.3.1 Pilot scale partial digestion

In the pilot scale partial digestion, total DNA of 2.5µg was digested with different ratios of enzymes; 0.05U/µg, 0.10U/µg, 0.15U/µg, 0.20U/µg, 0.25U/µg and 0.30U/µg to obtained the DNA fragments of 9-23Kb. This step was crucial as the length had to be fitted within the range of the Lambda FIX II vector. The ratio of 0.30U/µg of *Bam*HI enzyme that yielded majority of fragments in the 9-23Kb range as compared to other enzyme ratio (Figure 5.2). Therefore the ratio of 0.30U/µg of *Bam*HI enzyme was chosen to proceed to full scale partial digestion.

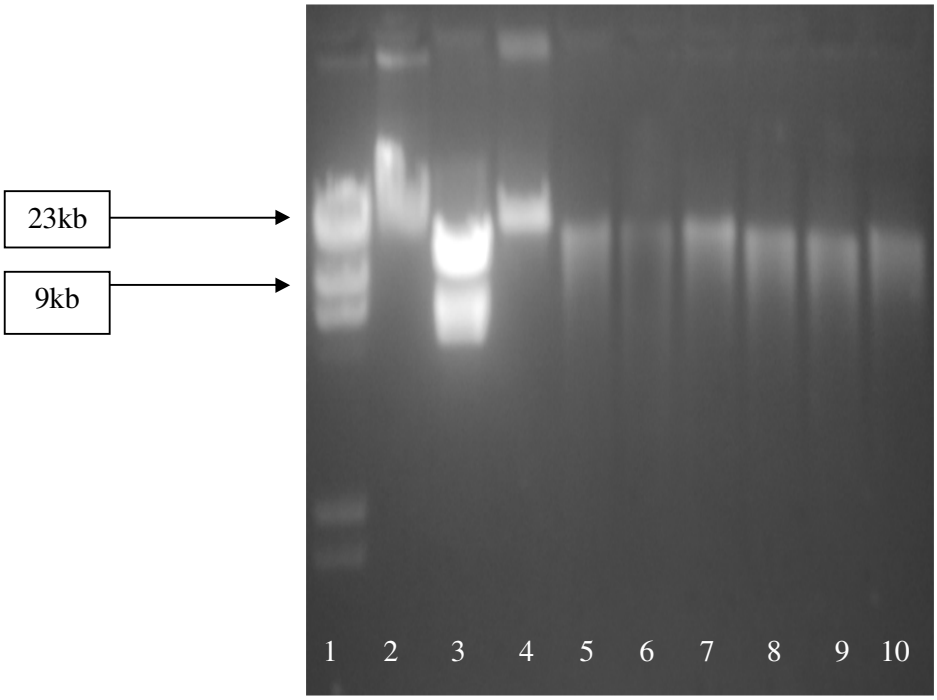


Fig. 5.2: Restriction enzyme digestion of *H. polyrhizus* genomic DNA using different units of *Bam*H1. Lane 1: *HindIII* digest ladder; Lane 2: lambda DNA; Lane 3: lambda DNA digested with *Bam*HI; Lane 4: genomic DNA; Lane 5-10: Genomic DNA of *H.polyrhizus* digested with *Bam*HI with 0.05, 0.10, 0.15, 0.20, 0.25 and 0.30U/µg respectively. The ratio of 0.30U/µg of *Bam*HI enzyme that yielded majority of fragments in the 9-23Kb range as compared to other enzyme ratio.

5.3.2 DNA quantification of purified DNA insert

After the partial fill-in, DNA was quantified using a spectrophotometer. Table 5.2 showed that the different spectrophotometer readings for insert DNA and different concentration was used before proceeding to the ligation step and packaging step. This chapter used two different insert DNA, with different concentrations which were 1.1µg/µl and 0.5µg/µl. The purity of both insert DNA were also different as shown in Table 5.2.

Table 5.2: Spectrophotometer reading for two inserts DNA used for ligation.

Sample	260nm	280nm	A_{260}/A_{280}	Concentration
Insert DNA A	0.022	0.013	1.69	1.1 µg/µl
Insert DNA B	0.009	0.007	1.28	0.5 µg/µl

5.3.3 Growth and Preparation of Host Strain

For the growth and preparation of the host strain, a single colony was successfully obtained as shown in Figure 5.3. The single colony was then need as the host strain.

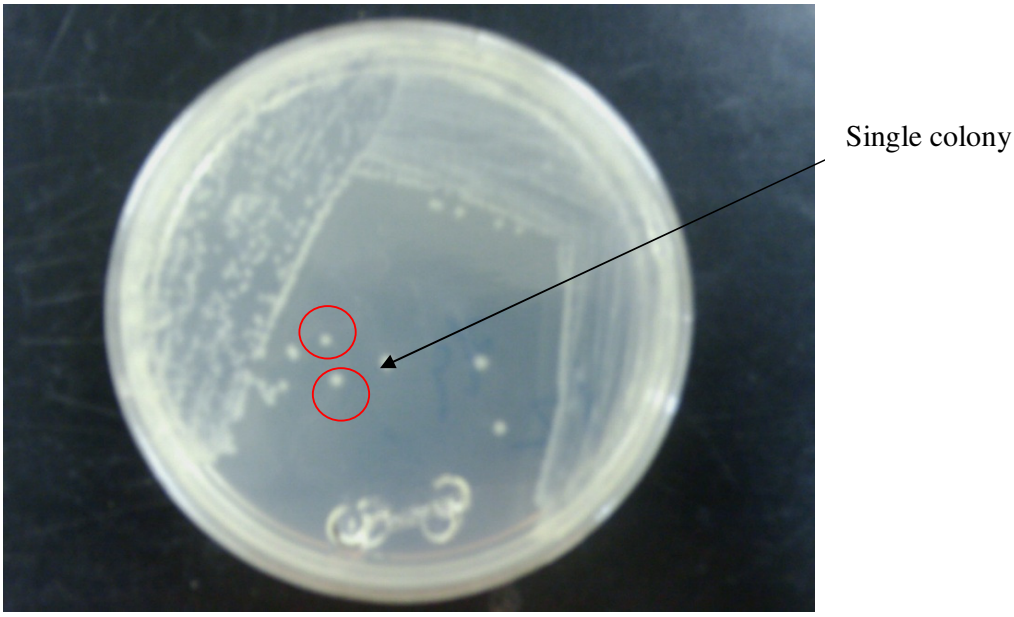


Fig. 5.3: Image of the growth of the host strain XL1-Blue (circled) on NZY plate after overnight incubation at 37°C in order to obtain the single colony.

5.3.4 Titering of library

There were approximately 300 plaques (Figure 5.4) and less than 10 plaques (Figure 5.5) formed in the petri dish using 10µl of bacteriophage from using insert A and insert B as packaging. The titer for *H. polyrhizus* genomic library using two different concentration of the insert DNA for packaging was 3 x 10^4pfu/ml and 8 x 10^2 pfu/ml respectively using the formula below:

$$\frac{\text{Number of plaques (pfu) x Dilution factor x 1000 (µl/ml)}}{\text{Volume of the lambda bacteriophage added to cells (µl)}}$$

$$= \frac{300 \text{ pfu x 1 x 1000 µl/ml}}{10 \text{ µl}}$$

$$= 3 \times 10^4 \text{ pfu/ml}$$

$$\frac{8 \text{ pfu x 1 x 1000 µl/ml}}{10 \text{ µl}}$$

$$= 8 \times 10^2 \text{ pfu/ml}$$

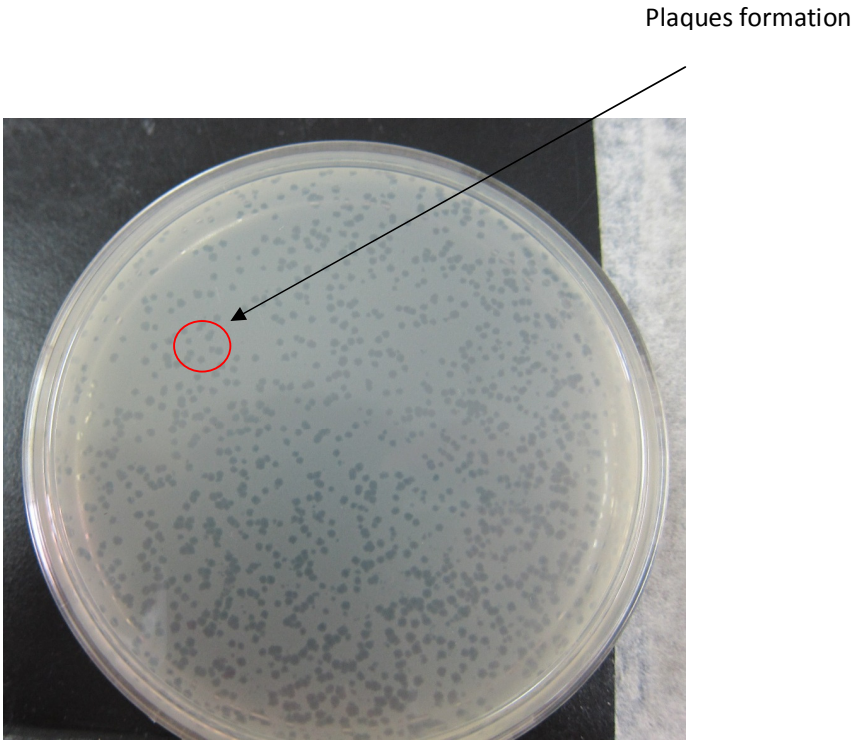


Fig. 5.4: Plaque formation on the NZY plate using 10µl bacteriophage and using insert DNA A which contained higher concentration after overnight incubation at 37°C. The red circle shows the plaques that were formed in the plate.

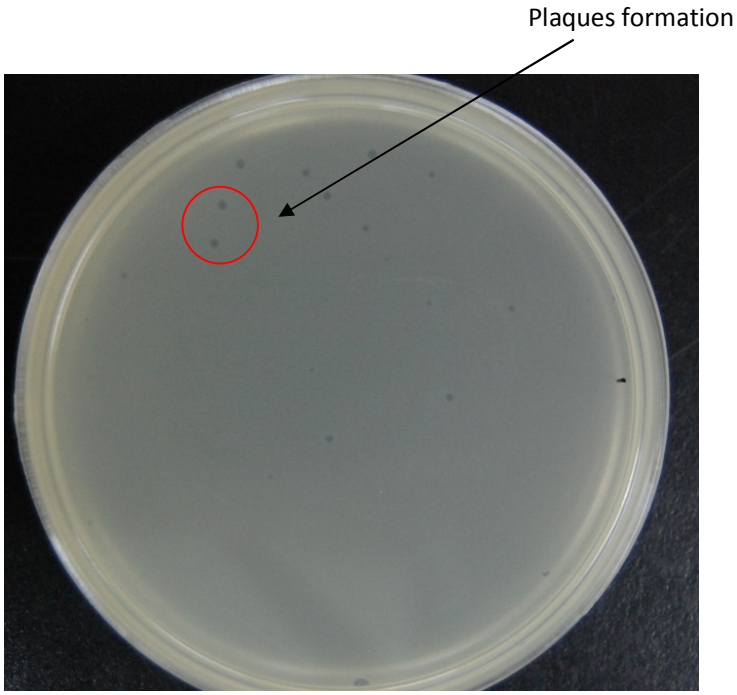


Fig. 5.5: Plaque formation on the NZY plate using 10µl bacteriophage and using insert DNA B which contained lower concentration after overnight incubation at 37°C. The red circle shows the plaques that were formed in the plate.

5.4 Discussion

After obtaining pure and good concentration of DNA in Chapter 4, the next step in the library construction was to digest the total DNA into desired fragments (in this project is 9-23kb)(Fig. 5.2) using compatible restriction enzymes such as *Mbo*I, *Sau*3A or *Bam*H I. As this insert DNA is going to be ligated into the Lambda FIX II vector , the lambda DNA was digested with *Xho*I.

In this work, *Bam*H I was used to partially digest the total DNA as it provided the fragments that was desired which is between 9-23kb. It also has the recognition site

(G'GATCC), leaves a sticky end and a GATC overhang which is compatible with the vector. The vector and the insert DNA must have compatible ends in order to construct recombinant molecule. A method by Rahman *et al*., (2007) was carried out using *Sau*3A for partial digestion. However, it resulted in small size DNA which could not be used for ligation as it is less that the desired size.

The pilot digestion was carried out using different enzymes in order to select the enzyme that gave the desired fragment in this work. Pilot digestion was also used to select the best dilution for full scale partial digest. The best dilution was selected by running the electrophoresis gel which ran parallel with the lambda DNA digested with *Hind* III as a ladder, the fragments that fall between the first and the second band from the top of the ladder was selected (Fig.5.2). 50µg of DNA was digested in ten different centrifuge tubes instead of one single tube because DNA in a single tube was difficult to be fully dissolved and the solution became very viscous. Furthermore, the restriction digest did give good results. In contrast, when the DNA was separated into ten different tubes, the DNA is easily dissolved and restriction digest can be performed successfully (Van Buuren *et al*.,1992) .

After the full scale partial digestion, partial fill-in process was carried out as described above. This step is essential where the dATP and dGTP was used because the Lambda FIX II from the Stratagene's kit has been digested with *Xho*I enzyme and already partially filled in with dCTP and dTTp leaving 3'-CT-5' overhang. This will then generate a compatible end to prevent it from self ligation.

The purification of the insert was the the next step. In this work phenol extraction was employed because it is a common technique used to purify DNA sample. This is the standard and preferred way to remove proteins from nucleic acid solutions. Concentrated ethanol was then added to the DNA sample to recover the precipitated DNA. Most nucleic acids may be precipitated by addition of monovalent cations and two to three volumes of cold 95% ethanol, followed by incubation at 0 to -70^{o}C. After centrifugation, the pellet was washed with 70% ethanol followed by brief centrifugation to remove residual salt and moisture. After the TE buffer elution step, the DNA insert was tested spectrophotometerically. This step is essential in genomic library construction at the later stage as it will directly influence the result of the tittering of the library.

In this chapter two different concentrations of the insert DNA was used which are 1.1µg/µl and 0.5µg/µl. Both of the concentrations were further used for packaging and tittering the library. Fig. 5.4 (1.1µg/ul insert DNA) and Fig. 5.5 (0.5 µg/ul insert DNA) shows the difference in the amount of plaques forming in the plate. With a higher concentration of insert DNA, more plaques were formed compared to the less concentrated. This is a crucial step as the amount of plaques determines the coverage area of the whole genome (Lodish *et al*.,2000).

Lambda vector used in this study is the simplest procedure where the genomic DNA was cleaved with restriction enzyme (*Bam*H I) and is broken into fragments. The donor DNA is then ligated between the right and left arm of the genomic DNA. Generated donor DNA is then added to either sides of the new fragment that is present followed by the final DNA molecule added to *E. coli* (P2 strain) and recombinant phages are grown

as plaques (Fig 5.4 and Fig 5.5). Beside its simplicity, the vector used in this study was chosen because of the fragment size as vectors used for genomic cloning are very size selective. For example, when cloning a large fragment of DNA, and only lambda to work with, generating a library will not be as successful. Conversely, if only a small fragment of DNA is available and YAC vectors are used, the library construction will be very troublesome (Boulnois, 1987).

Bacteriophage is a virus that infects bacteria. The initial stage of the bacteriophage method involves the isolation of a small number of phage from a bacterial culture (*E.coli* strain XL-1-Blue MRA (P2) was used in this work). The bacterial culture was then cultured on the surface of a petri dish containing nutrient medium (NZY was used) and on such medium the bacteria grow until they cover the dish's surface. Plaques were formed on the surface of the nutrient medium because phage-infected bacterial would eventually lyse, releasing new viruses that begin another cycle of infection and clear areas containing lysed cells and freed bacteriophage are soon seen on the surface which called plaques (Fig 5.4 and Fig 5.5).

When ligating inserts containing the DNA of interest, the volume of the insert size should be less than 2.5µl. The Lambda FIX II vector can accommodate inserts ranging from 9 – 23kb. As shown in chapter 5.3,the concentration of insert DNA plays an important role in determining the formation of plaques in the petri dishes. This may be because the higher concentration insert has a higher probability to attach to the vector.

Packaging extracts were used to package recombinant lambda phage with high efficiency. Ligation was carried out at DNA concentrations of 0.2µg/µl or more, which

favours concatemers and efficient packaging. Care was taken in ensuring the plaque forming units were the same for every packaging extracts used with the same dilution. The *Hylcereus polyrhizus* library was around 3 x 10^4 pfu/ml to 3.5 x 10^4 pfu/ml in different tubes with the same ligation product.

In this chapter, it can concluded that, a successful library is a library which can be reproducible. The concentration of the insert DNA was also the main factor that influences the plaque form. The purity of the DNA also played an important role in construction of a successful DNA library.

Chapter 6 General Discussion

Red dragon fruits (*Hylocereus polyrhizus*) are of high demand and fetch very high prices in the market. Currently it is one of the economically important fruit crops in Malaysia. However, it has been reported that many of the farms have been affected by fungal, bacterial and viral infections which resulted in huge losses for the farmers. In this project, it is hoped that the construction of a genomic library could serve as a platform to preserve and to store the germplasm so as to have a readily available source for analysis and manipulation.

The prerequisite of constructing a library is to obtain genomic DNA. The isolation of high molecular weight DNA from plants is difficult due to several reasons; the plant cell wall is not easily removed by physical means without damaging the contents of the cell and the vacuole which is full of degradative enzymes such as DNases and secondary metabolites such as phenolic which can damage DNA. Furthermore, *Hylocereus polyrhizus*, a dicotyledonous plant belonging to the Cactaceae family, contains high amount of polysaccharides, therefore posing a challenge in obtaining good quality genomic DNA.

Lambda bacteriophage genomic library for *H. polyrhizus* was constructed using the commercial kit, Stratagene Lambda FIX II / *Xho* I Partial Fill-in vector kit. The crucial part of this study was to obtain the ideal tissues as starting material which can produce the best DNA to be used for the construction of a genomic library for *H. polyrhizus*. The first objective was to obtain the best starting material from the entire *H. polyrhizus* fruit for the construction of a genomic library.

The selection of suitable tissues as starting material for DNA extraction was based on two methods previously reported in literature. The first method used in this study was as reported by Tel-Zur *et al*., (1999) using fresh roots. However, the method yielded insufficient DNA in this study. Furthermore, the DNA was also of bad quality when tested using spectrophotometer (Table 4.1). The second method used in this study was based on a study carried by Diadema *et al*., (2004) who worked on DNA extraction from succulent plants. This method also failed to yield sufficient and good quality DNA.

In order to solve the problems of sufficient yield and quality, young leaves were used instead of the roots and the whole fruit. Germination of *H. polyrhizus* seeds was found to be a probable method to obtain young leaves for DNA isolation. Experiments were carried out with two treatments. Seeds that were obtained directly from the pulp itself were labeled as control while the two treatments were acid wash and air-dry in two conditions (light and dark). In Chapter 3, the results obtained showed that acid washed seeds grown under light gave the highest and fastest germination rate. This result provided information on when the leaves can be harvested for DNA extraction.

DNA extraction method for the young leaves was modified from the method by Lodhi *et al*., (1994). PVP and beta-mercaptoehanol were added to the isolation buffer which was reported to effectively removing polyphenols from mature, damage and improperly stored leaf tissues (Rogers and Bendich, 1995; Howland *et al*., 1991; Dawson and Magee, 1995; Clark, 1997). Sodium bis-sulfite which acts as an antioxidant and sodium sarkosyl acts as a detergent, were also added into the extraction buffer. This method proved to be successful in obtaining good yield & quality DNA. The Lodhi protocol

was a macro-method which uses 5ml of extraction buffer for 0.5g of leaves with a ratio of 1ml for every 1g of leave. However, in this extraction only 0.7ml of isolation buffer was used for 0.1g which is sufficient to yield high quality DNA. Tissue extraction with CTAB in high salt condition suppressed the co-precipitation of polysaccharides and DNA (Murray and Thompson, 1980 and Lodhi *et al.,* 1994).

Previous studies have shown that different growth stages of leaf results in different quality of isolated DNA. For example, rapidly expanding leaves were found to be most suitable for the DNA extraction of Theobroma *cacao*. In contrast, Lodhi *et al*., (1994) suggested that partially expanded leaves were the best starting material for grapevine cultivar and *vitis* species. In this thesis, both fully and partially open leaves were used. Results obtained showed that partially expanded leaves were better. This could be because partially expended leaves have smaller cell density in succulent tissues. Furthermore, the older the leaves the higher the contaminants (Porebski *et al*., 1997).

The next step was digesting the total genomic DNA in order to obtain a desired range of fragments which can be further ligated to the selected cloning vector. Lambda FIX II vector (Stratagene) which had been digested with *Xho* I and partially filled in with dCTP and dTTP, leaving 3'-CT-5' overhangs was used. This vector can withhold 9-23kb DNA fragments. As a result of the compatible end, the genomic DNA can be digested with a few types of restriction enzymes which are *Mbo* I, *Sau* 3A, *Bgl* II and *Bam* HI.

All the restriction enzymes except for *Bgl* II were used to digest the *H. polyrhizus* genomic DNA. Only *Bam* HI gave the best result with digested fragments that fell

between the 9-23kb range. The other restriction enzymes resulted in fragments below 9kb which is not suitable to be ligated into the vector. After selecting the suitable enzyme, a pilot scale of partial digestion was set up using 2.5µg of DNA at different digestion times. A ratio of 0.1U of *Bam* HI per 1µg of genomic DNA was used. The result of the pilot partial digestion showed that the best incubation time to produce the most desired fragments was 90 minutes. After the pilot digestion, a full scale partial digestion was done using 50µg of total genomic DNA. The sample was divided into 10 tubes in order to avoid viscosity in one tube.

After the full partial digestion step, the digested DNA was then partially filled-in with dATP and dGTP. As the vector from the kit has been digested with *Xho* I and already filled in with dCTP and dTTP, this will then generate a compatible end and also prevent self ligation. This step has to be carefully done as the results can only be checked at the end of the next step.

Generally, partial fill-in is followed by the removal of the desired fragments from the agarose gel. Subsequently, phenol purification is carried out. However in this thesis, the removal of the desired fragments failed to generate satisfying results as the insert DNA was degraded in the process of excising the gel for purification. Therefore, direct purification using phenol purification of the insert DNA was carried out instead. By using the phenol protocol, high purity and concentration of insert DNA was obtained. The purity of the insert DNA l directly affects the result of packaging.

In this thesis, two different concentrations of DNA were used for ligation which were 0.5µg/ µl and 1.1µg/ µl. These two different concentrations of insert resulted in two

different packaging results. The success of the library was determined by the formation of plaque unit per ml. Rahman *et al., (*2007) postulated that the success of constructing a genomic library depends on efficient ligation. The higher the number of pfu/ml of the library, the higher the efficiency of the ligation process. The titer was determined using the formula: Number of plaques (pfu) × Dilution factor × 1000 (µl/ml) / Volume plated (µl). The titer was 3 x 10^{4} pfu/ml. Fig. 5.4 and Fig. 5.5 clearly showed that the concentration of the insert DNA and the purity of insert DNA determined the titer result. Thus, obtaining a good insert DNA was a crucial step in obtaining a good genomic library.

With the successful construction of the genomic library, gene of interest can be identified using probes by hybridization. This work can set as a platform for the future studies where gene of interest can be used for direct transformation to investigate the effects of these genes on the plant traits.

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Appendix

Obtaining Genomic DNA for a Genomic Library

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Keywords: Genomic library, *Hylocereus*, restriction digest, high molecular weight DNA, Cactaceae

Abstract

The isolation of pure genomic DNA from *Hylocereus undatus* and *Hylocereus polyrhizus* (Cactaceae) for the construction of a genomic library is described. These vine cacti originate from northern South America, Central America and Mexico, and are cultivated as new exotic fruit crops in SE Asia. Successful establishment of a genomic library serves as a platform for molecular studies, such as gene characterization and gene analysis, but intact and high molecular weight DNA of good quality can be difficult to obtain. Furthermore, presence of polysaccharides in DNA extracts interferes with downstream processes such as restriction digest and polymerase chain reaction (PCR). Genomic DNA can be extracted using various plant parts from 2–3-week-old seedling leaves, roots and stems. DNA quality is checked using a spectrophotometer and digested using *Bam*H1. Genomic DNA so isolated had the highest quality following restriction digestion analysis.

INTRODUCTION

Dragon fruits of the genera Hylocereus and Selenicereus which originate from northern South America, Central America, and Mexico, are currently being grown as new exotic fruit crops in SE Asia. The fruit 'Flesh' largely includes a mucilaginous placenta in which is found thousands of small seeds (Le Bellec *et al.,* 2006).

At present, only three species are being cultivated on a commercial scale which is *Hylocereus undatus, H. polyrhizus* and *Selenicereus megalanthus* in Colombia, Israel, Vietnam and Nicaragua (N. Tel-Zur *et al.,* 2005). Vietnam is the biggest producer of the *Hylocereus* spp. However, only *H. undatus* and *H. polyrhizus* are being cultivated in small scale in Malaysia.

These three species are distinguished by peel and pulp colour. *H. polyrhizus* fruit has red peel and red pulp while *H. undatus* fruit has red peel and white pulp. *S. megalanthus* has yellow peel and white pulp. The red colouration is due to the presence of betalain pigments. Plantations of such non-native fruit species are affected by various diseases that lead to poor fruit yield, threatening the income of small-scale farmers who resort to application of pesticides and herbicides. This problem can be solved by establishing a genomic library that serve as a platform to study the gene interaction. The overall procedure involved in genomic library construction includes isolation of genomic DNA, generation of DNA fragments for cloning, packaging and transduction.

MATERIALS AND METHODS

Plant material

Red and white dragon fruits were obtained from a local market. Their seeds were separated from the pulp and air-dried. The seeds were allowed to germinate for about two weeks and seedling leaves were used for DNA extraction.

Reagents and solutions

DNA isolation buffer [0.15M Sorbitol, 0.125M Tris-base, 0.05M EDTA, $8.3x10^{-3}$M Sodium Bis-sulfide, 0.83M NaCl, 2% w/v hexadecyltrimethly-ammonium bromide (CTAB), 0.83% v/v Sodium Sarkosyl, 1% w/v Polyvinylpyrollidone (PVP) (MW 40,000), 1% v/v β-mercaptoethanol)] Chloroform: isoamyl alcohol (24:1), 5M NaCl, Isopropanol, 70% ethanol, TE buffer [10mM Tris-HCl (pH 8.0), 1mM EDTA (pH 8.0)], RNase A (0.1µg/µl)

DNA isolation

A total of 400µl of DNA isolation buffer was added into each 1.5-ml microfuge tube. Leaf fragments from 2-week-old seedlings were weighed and pulverized in liquid nitrogen. 0.20g of leaf sample was transferred into each buffer-added microfuge tube. Microfuge tubes were quick-spun. An additional 300µl of DNA isolation buffer was added. All tubes were gently flicked and inverted to thoroughly mix the sample and incubated at 65°C for 40 minutes (tubes were gently flicked and inverted at 10-minute intervals). 700µl of chloroform: isoamyl alcohol (24:1) was added to each microfuge tube and tubes were gently inverted until an emulsion formed. Samples were centrifuged at 10,000 rpm for 15 minutes. The aqueous phase was gently transferred into new 1.5ml microfuge tubes. The chloroform: isoamyl alcohol procedure was repeated. Half volume of 5M NaCl was added and microfuge tubes were gently inverted. Two volumes of cold isopropanol (-20°C) was added into each microfuge tube and tubes were gently inverted before being incubated on ice for 10 minutes. Samples were centrifuged at 10,000 rpm for 5 minutes. The supernatant was discarded. DNA pellets were washed twice with 500µl of cold 70% ethanol (-20°C). Samples were centrifuged at 10,000 rpm for 1 minute. The supernatant was discarded and the pellet washing procedure repeated. DNA pellets were air-dried at room temperature, and then dissolved in 30 to 50µl of TE buffer (depending on their solubility). RNase A was added to each sample (1µl/10µl DNA sample), which was then incubated at 37°C for 1 hour. After RNase A treatment, total DNA was run on a 0.8% agarose gel against a marker of known molecular weight, and stained with ethidium bromide for visualization (Figure 1). The samples were stored at -20°C.

Analytical analysis

Extracted DNA samples were quantified using a spectrophotometer at A_{260nm} while the purity of the DNA was checked through A_{260nm}/A_{280nm} (Table 1).

Partial digestion of the Genomic DNA

Partial digestion of the genomic DNA was carried out with the enzyme *Bam*H1 according to the Stockinger Lab 06/01/05 protocol. After digestion, the product was analyzed through 0.8% agarose gel electrophoresis (Figure 2).

RESULTS AND DISCUSSION

There are several published works that have reported on the use of different tissues for obtaining genomic DNA, such as callus tissue and roots. Roots were chosen as the source tissue due to the lower viscosity of the extracts relative to that from other tissues. Leaf callus, essentially an unorganized and undifferentiated cell mass, lacks

synthesis of secondary metabolites and so may be more easily dealt with (Diadema *et al.,* 2003 and N. Tel-Zur *et al.,* 1999).

Hylocereus undatus and *H. polyrhizus* contains high amounts of polysaccharides in their tissues generally. The polysaccharides bind to the DNA, rendering it viscous and glutinous after precipitation of the DNA. This makes the DNA unsuitable for downstream applications such as PCR and restriction digest, as the applied enzyme cannot access the DNA (N. Tel-Zur *et al.,* 1999, Barnwell *et al.,* 1998 and Puchooa, 2004). However, tissues extraction with CTAB in high salt condition suppresses the co-precipitation of polysaccharides and DNA (Murray and Thompson, 1980; Lodhi *et al.,* 1994).

In plant cells, there is an abundance of secondary metabolites. To prevent the interaction of these compounds with DNA, β-mercaptoethanol and/or polyvinyl pyrrolidone (PVP) is often added to the buffer solution. PVP forms complexes with polyphenolics through hydrogen bonding and β-mercaptoethanol reduces oxidation of phenolic compounds (Micheils *et al.,* 2003, De la Cruz *et al.,* 1997 and Maliyakal, 1992).

High yield and quality of DNA from leaf extractions were attributed to active cell division, which produces high cell density and little or no synthesis of secondary metabolites. In addition, the extracted genomic DNA can be restricted using enzyme *Bam*H1.

Table 1 show that DNA extraction using leaves provides higher purity compared to other parts of the plants. Figure 1 also shows that DNA extraction using young leaves yielded a higher amount of DNA. However, when the same extraction protocol was carried out on stem tissue, the extract was viscous throughout the extraction process due to the presence of high amount of polysaccharides (N. Tel-Zur *et al.,* 1999). The root extraction carried out in this study follows the protocol by N. Tel-Zur *et al.,* 1999 and resulted in a brown pellet. This may be caused by the binding of secondary metabolites to the DNA and the fibrous root structure which results in low yield of DNA. Furthermore, Figure 2 confirms that the DNA extract from leaves is of high quality, implying that the enzyme efficiently digested total DNA into smaller fragments.

CONCLUSION

From the experiment conducted, young seedling leaves gave the best quality of DNA and yield. This was confirmed through spectrophotometer and gel electrophoresis assessments. The ratio of A_{260nm}/ A_{280nm} fall between 1.8-2.0 range and the extracted genomic DNA can be restricted.

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Tables

Table 1. DNA purity and DNA yield using different plant materials

Plant material	A_{260nm}	A_{280nm}	Ratio [A_{260nm}/ A_{280nm}]	DNA yield [µg/g]
Stem	0.008	0.007	1.14	20.0
Root	0.023	0.018	1.28	57.5
Leaves (*Hylocereus undatus*)	0.081	0.042	1.92	202.5
Leaves (*Hylocereus polyrhizus*)	0.093	0.049	1.90	232.5

Figures

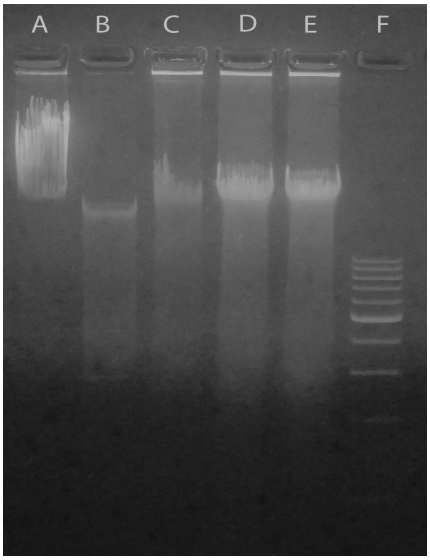

Figure 1. Electrophoresis of genomic DNA extracted using different plant materials. A- Lamda DNA ; B- stem ; C- Root; D- Leaves *Hylocereus undatus* ; E- Leaves *Hylocereus polyrhizus* ; F = 1kb DNA ladder

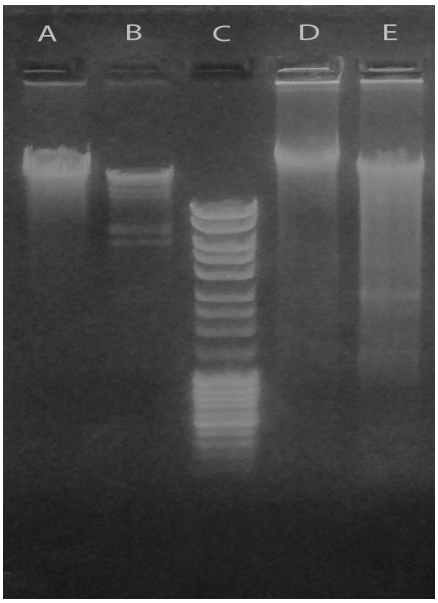

Figure 2. Electrophoresis of *Bam*H1 restricted genomic DNA extracted from *Hylocereus* leaves. A- Lambda DNA ; B- Lambda DNA digested with *Bam*H1; C- 1kb DNA ladder; D- Total DNA *Hylocereus* ; E- Total DNA *Hylocereus* digested with *Bam*H1