GENETIC VARIATION OF CLONAL *Ananas comosus* var. MD2 PLANTS AFTER MUTAGENIC TREATMENT AT DIFFERENT POST-RECOVERY PERIODS

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ABSTRACT

Studies on genetic variation in plants had become extensively employed in research for plant improvement and conservation. Due to their sessile nature, plants are exposed to various environmental stressors such as exposure to enhanced levels of ultraviolet (UV), ionizing and non-ionizing radiations. Exposure to these harmful radiations will result in various damages, ranging from DNA and chromosomal aberrations to phenotypic abnormalities. However, plants have evolved efficient DNA repair mechanisms to detect and repair any DNA damages caused by the exposure to these harmful stressors, therefore ensuring their survival. In this study, the effect of gamma radiation (as a source of ionizing radiation) on clonal *Ananas comosus* var. MD2 was evaluated. The morphology of the clonal plantlets before and after exposure to gamma radiation was monitored at timely intervals (at different post-recovery period). Moreover, the degree of genetic variation between the samples pre- and post-irradiation was also analyzed by using inter-simple sequence repeat (ISSR) markers. Data analysis revealed that the height of the irradiated plantlets were significantly reduced (compared to control), but improved with recovery period. These observations were also supported by the ISSR analysis, where the genetic dissimilarity between the irradiated samples and control was reduced by 0.1017, after 4 weeks of recovery. Eight of 20 tested ISSR primers provided a total of 4169 bands in 10 non-irradiated and irradiated plantlets (randomly collected). The number of scorable bands per primer varied from 8 to 13 which generated an overall of 303 polymorphic bands, with a mean of 30.3% polymorphism. Our findings indicated that the phenotype recovery undergone by the clonal *A. comosus* var. MD2 plants was contributed by their ability to detect and repair the DNA lesions (as

exemplified by the reduction in genetic dissimilarity after 4 weeks) and hence allow the plantlets to undergo phenotype reversion to normal plant stature.

Keywords: *Ananas comosus* var. MD2, genetic variation, ISSR analysis, DNA repairs, DNA damage.

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ABSTRAK

Kajian mengenai variasi genetik dalam tumbuh-tumbuhan telah banyak digunakan dalam penyelidikan untuk penambahbaikan dan pemuliharaan tumbuhan. Disebabkan sifat semulajadi yang sesil, tumbuhan terdedah kepada pelbagai tekanan persekitaran seperti paras tinggi ultraviolet (UV), pengionan dan radiasi tanpa pengion. Pendedahan terhadap radiasi yang berbahaya tersebut menyebabkan pelbagai kerosakan, bermula dari penyimpangan DNA dan kromosom kepada keabnormalan fenotip. Walaubagaimanapun, tumbuhan mempunyai mekanisme untuk membaiki DNA secara efisyen bagi mengesan dan membaiki sebarang kerosakan DNA yang disebabkan oleh pendedahan kepada tekanan persekitaran yang berbahaya, seterusnya memastikan kemandirian hidup. Dalam kajian ini, kesan radiasi gamma (sebagai sumber radiasi pengionan) kepadatumbuhan klonal *Ananas comosus* var. MD2 telah dinilai. Morfologi anak pokok klonal sebelum dan selepas pendedahan kepada radiasi gamma telah diawasi pada perantaraan masa secara berkala (pada jangka masa pemulihan yang berbeza). Tambahan pula, tahap kepelbagaian genetik diantara sampel sebelum dan selepas pendedahan kepada radiasi juga telah dianalisa menggunakan penanda *inter-simple sequence repeat* (ISSR). Data analisis menunjukkan bahawa tinggi anak pokok yang diradiasi berkurangan secara siknifikan (dibandingkan dengan kontrol), tetapi bertambah baik selaras dengan jangka masa pemulihan. Pemerhatian tersebut turut disokong dengan analisis ISSR, di mana ketidaksamaan genetik diantara sampel yang telah diradiasi dan kontrol telah berkurangan sebanyak 0.1017, selepas 4 minggu pemulihan. 8 dari 20 penanda ISSR primer telah menghasilkan secara keseluruhan 4169 jalur dalam 10 anak pokok tidak diradiasi dan terradiasi. Bilangan jalur yang ternilai

untuk setiap primer berbeza dari 8 sehingga 13 menghasilkan secara keselurahan 303 jalur polimorfik, dengan purata 30.3% polimorfisme. Dapatan kajian menunjukkan pemulihan fenotip dilalui oleh anak pokok klonal *A. Comosus* var. MD2 disumbangkan oleh kebolehan untuk mengesan dan membaik pulih kerosakan DNA (turut dibuktikan dengan pengurangan ketidaksamaan genetik selepas 4 minggu) dan seterusnya membenarkan anak pokok untuk melalui pembalikan fenotip kepada normal.

Kata kunci: *Ananas comosus* var. MD2, genetic variasi, analisis ISSR, pembaikan DNA, kerosakan DNA.

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LIST OF SYMBOLS AND ABBREVIATIONS

°C	:	Degree Celcius
AFLPs	:	Amplified Fragment Length Polymorphisms
BAP	:	6-Benzylaminopurine
BER	:	Base Excision Repair
Bp	:	Base pair
CO_2	:	Carbon Dioxide
CPDs	:	Cyclobutane Pyrimidine Dimers
CSA	:	Cockayne Syndrome Type A
CSB	:	Cockayne Syndrome Type B
DNA	:	Deoxyribonucleic Acid
DSBs	:	Double Strand Breaks
e-	:	Free electrons
EMS	:	Ethyl Methanesulfonate
ERCC1	:	Excision Repair Complementing Defective Repair in Chinese hamster 1
ETP	:	Economic Transformation Programme
g	:	Gram
GGR	:	Global Genome Repair
Gy	:	Gray
$H^{\bullet}$	:	Hydrogen radical
H_2O	:	Hydrogen
HR	:	Homologous Recombinant
IAA	:	Indoleacetic acid
IBA	:	Indole-3-butyric Acid
ISSR	:	Inter Simple Sequence Repeats

kPa	:	KiloPascal
L	:	Liter
Ltd	:	Limited company
mg	:	Miligram
mL	:	miliLiter
mM	:	microMolar
MMR	:	Mismatch Repair
MMS	:	Methyl Methane Sulphanate
MPIB	:	Malaysian Pineapple Industry Board
MS	:	Murashige and Skoog
MSH	:	MutS homologs
NaOH	:	Sodium Hydroxide
NER	:	Nucleotide Excision Repair
ng	:	Nanogram
NHEJ	:	Non-homologous End Joining
NKEA	:	National Key Economic Area
nm	:	Nanometer
OH*	:	Highly reactive hydroxyl radical
$OH^{\bullet}$	:	Hydroxyl radical
PCNA	:	Proliferating Cell Nuclear Antigen
PCR	:	Polymerase Chain Reaction
PGRs	:	Plant Growth Regulators
PMS2	:	Postmeiotic segregation increased 2
PPs	:	Photoproducts
RAPDs	:	Random Amplified Polymorphic DNAs
RFC	:	Replication Factor C

RFLPs	:	Restriction Fragment Length Polymorphisms
RNA	:	Ribonucleic Acid
ROS	:	Reactive Oxygen Species
RPA	:	Replication Protein A
RT	:	Room temperature
SSBs	:	Single Strand Breaks
SSRs	:	Simple Sequence Repeats
TAE	:	Tris-acetate-EDTA
TCR	:	Transcription-coupled repair
TFIIH	:	Transcription factor IIH
U	:	Unit size
UPGMA	:	Unweighted pair group method with arithmetic mean
USA	:	United States of America
UV	:	Ultra Violet
XPA	:	*Xeroderma pigmentosum* Group A
XPB	:	*Xeroderma pigmentosum* Group B
XPD	:	*Xeroderma pigmentosum* Group D
XPF	:	*Xeroderma pigmentosum* Group F
XPG	:	*Xeroderma pigmentosum* Group G
µl	:	Microliter

CHAPTER 1: INTRODUCTION

Ananas comosus is a tropical plant which commonly also known as pineapple belongs to the family Bromeliaceae and ranked as the third among tropical fruits in the world with economically high in demand. Pineapple is a perennial plant that grows best in an arid environment which requires a functional root system for multiple fruiting process (Bartholomew et al., 2002). There are many varieties of pineapple such as Gandol, N36, Mauritius, Josaphine and Sarawak and MD2. Notably, MD2 variety has been selected in Programme of Economic Transformation and recognized as one of the key crops under the National Key Economic Areas (Amar et al., 2015).

Originally, the MD2 variety was developed by Del Monte to produce pineapple fruits with excellent qualities such as a longer shelf life and impervious to internal browning. In addition, the flesh of MD2 fruits are bright-gold in color, very sweet, has low-acidity and has the ability to survive in cold storage for up to two weeks. However, previous report has reported that MD2 variety is defenseless against fruitlet core rot and foliar pathogens (Joy & Anjana, 2016). Despite having superior quality among pineapple varieties, but due to defenseless on pathogens and diseases, it makes MD2 less favored in its qualities.

However, plants are sessile and fully exposed to various environmental stresses such as abiotic and biotic stress factors. Numerous biotic stresses are caused by other living organisms such as bacteria, fungi, nematodes, protists, insects, viruses and viroids while abiotic stress is damage caused by surrounding limitations such as drought and salinity (Mirouze & Paszkowski, 2011). Crop loss by abiotic stress such as through salinity and drought becomes a major limiting factor in plant growth and development as plants are unable to move from one place to another. Being sessile, plants cannot escape and thus face constant exposure from environmental stressors such as ultraviolet (UV), alkylating

agents, low and high temperature as well as ionizing and non-ionizing radiations, which cause injuries that can decrease genetic stability, development of growth and productivity in plant (Gill et al., 2015a). In order to cope with these environmental stressors, evolution of plants with plants have evolved a complex mechanism to effectively detect the DNA damage and rapidly employ DNA repair machineries to detect DNA lesions, thus efficiently remove or replace the affected nucleotides (Gill et al., 2015a).

Among the damaging agents from the environment, radiation is one of the major causes of plant DNA damage. Even though sunlight is complementary in plant for photosynthesis but, it is also a major threat to the plant genome, whereby the radiation of UV-A and UV-B could reach the earth's surface from the atmosphere, leading to formation of highly reactive hydroxyl radicals (Gill et al., 2015a). In this study, gamma radiation was used as the source of ionizing radiation to be inflicted onto the plants, as it has been reported to cause oxidative stress, where the reactive oxygen species (ROS) will interact with DNA and cause oxidative damage such as base modification and double- and/or single- strand breaks (Jiang et al., 1997). The damage of DNA by UV or ionizing radiations can be corrected or repaired by utilizing several pathways such as direct repair, photo reactivation, base excision repair (BER), nucleotide excision repair (NER) or mismatch repair (MMR) to repair the DNA lesions (Zhang et al., 2008).

In response to the various environmental conditions and stressors, plants have developed an important mechanism called phenotypic plasticity whereby they can alter their morphology and phenotype to cope with environmental stress factors. Phenotypic plasticity is defined as an alteration of phenotype by a single genotype in a various environment, and could be genetically controlled (Gratani, 2014). The understanding of phenotypic plasticity in plants is very important to predict changes in species

distribution, community composition, and crop productivity under global change conditions. Phenotypic plasticity is reported to be modulated by complex genetic and epigenetic regulatory mechanisms to allow plants to cope with environmental stresses (Braszewska-Zalewska et al., 2014).

This study was designed to evaluate plant's response towards stress, particularly towards harmful ionizing radiation (eg, gamma radiation) and monitor plant's ability to repair itself (both at gene and phenotype level) after exposure of gamma radiation. Therefore, the objectives of this study are:

1. To determine the degree of genetic variation between clonal *Ananas comosus* var. MD2 plants and control plants after exposure to ionizing radiation (gamma radiation) at different post-recovery periods.
2. To study the effect of exposure to gamma radiation on the morphology of *Ananas comosus* var. MD2 plantlets.

CHAPTER 2: LITERATURE REVIEW

2.1 OVERVIEW OF PINEAPPLES (*Ananas comosus var*. MD2)

Pineapple *(Ananas comosus)* belongs to the bromeliad family and fruit crop solely reflected in the ranking of commercial tropical fruit for worldwide production (Thalip et al., 2015). In the past few decades, pineapple has been commercially used for many purposes, either for table and canned food industries (Thalip et al., 2015). Compared to other pineapple cultivars, MD2 fruits have been prominent in the global market because of its superior characteristics such as better colour quality, flavour, sweetness to acidity balance, juiciness and longer shelf life (Žemlička et al., 2013). MD2 fruits have a longer shelf-life of about 30 days and can be freshly consumed after the arrival in long-distance shipping. Therefore, MD2 pineapple has been stated as a key crop under the National Key Economic Area (NKEA) of the Economic Transformation Programme (ETP) (Thalip et al., 2015).

2.1.1 Background of MD2 (*Ananas comosus* var. MD2)

Pineapple (*Ananas comosus*) is an important fruit crop that belongs to the family of Bromliaceace, which also contained 50 genera and 2500 known species and found in almost all the tropical and subtropical areas (Ko et al., 2009). The genus *Ananas* was reported to be ideal for domestication studies related to the major uses as a food or source of fibers (Joy & Anjana, 2016). Among the pineapple cultivars that are planted in Malaysia include Moris Gajah, Gandul, Yankee, Sarawak, N36, Josapine, and MD2. However, the MD2 is the most popular because of its flavour and aroma which attracts consumers' attention. However, the MD2 variety is also susceptible to serious pineapple diseases such as fungal black rot by *Thielavipos paradoxa* and bacterial heart rot by *Erwinia chrysanthemi* (Thalip et al., 2015). As the market demand for MD2 is higher

compared to other pineapple cultivars, Malaysian Pineapple Industry Board (MPIB) is determined to mass produce MD2 pineapple to lead and drive Malaysia into becoming a prominent pineapple producer (Thalip et al., 2015).

2.1.2 Taxonomic Classification of Pineapple (*Ananas comosus*)

The taxonomic classification of *Ananas comosus* is as shown below:

Kingdom : Plantae
Subkingdom : Viridaeplantae
Division : Tracheophyta
Subdivision : Spermatophytina
Infradivision : Stretophyta
Class : Magnoliopsida
Super Order : Lilianae
Order : Poales
Family : Bromeliaceae
Genus : *Ananas Mill.*
Species : *Ananas comosus* (L.) Merr.

(Joy & Anjana, 2016)

2.1.3 Marketability of MD2

In recent years, MD2 has become one of the most popular fresh pineapple variety compared to other cultivars. According to Bartholomew et al. (2002), many experimental studies have been conducted to identify the appropriate production practices to increase MD2 production over the world. Besides, pineapple growers have been forced to change their cultivar because of the rapid change of consumer acceptance from Smooth Cayenne to MD2 because of its key element of flavour quality and aroma (Bartholomew, 2002). The introduction and development of MD2 cultivar was aimed to consummate fresh pineapples for market demands as rapid expansion through fresh pineapple export markets in the Middle Eastern countries and China since 2015. Furthermore, MD2 pineapple is also more superior than other cultivars due to its 'heterogeneous (Joy & Anjana, 2016). Hence, Malaysia has been driven by MPIB to become a well-known MD2 pineapple producer in support for a brighter economic future (Thalip et al., 2015).

2.2 Genetic variation due to environment stress

Being an immobile, plants are exposed to many stressors that arise from their surroundings, such as abiotic stress and biotic stress. Abiotic stress may cause a series of changes involved with morphological, physiological and molecular which severely affect plant growth and productivity, for example, drought, salinity, extreme temperature, chemical toxicity and oxidative stress (Wang et al., 2003). Consequences of these changes may cause modification of functional and structural protein cause from denaturation process which affect the functionality and development of plant growth system. Abiotic stress such as exposure to radiation can cause a serious damage to plant systems, such as inducing cellular and molecular damages especially in the DNA structure (Rastogi et al., 2010).

Biotic stress in higher plants such as herbivore or pathogen attacks often induce the synthesis and accumulation of similar defense-related secondary metabolites which involved the mechanisms of reactive oxygen species (ROS) generation with lipid oxidation processes resulting in the formation of signalling compounds (Mithofer et al., 2004). The ROS that have been detected in plant pathogen interaction were reported to influence gene expression and affect the induction of plant defense pathways. The wound or injuries caused by insect feeding may lead to the production of ROS in the damaged tissue and induced a shift in the oxidative status of the plant, causing an increase of radical formation in cell tissues (Mithöfer et al., 2004).

2.2.1 Abiotic stress

There are many stress factors classified as abiotic stresses such as drought, salinity, extreme temperature and external genotoxins, such as ultraviolet light, ionizing radiation and chemical mutagens, which become a serious threat to agriculture and natural status of the environment. The threats arise from abiotic stresses had driven research focusing on biotechnology programs in molecular mechanisms to control and monitor the stress in the basis of activation and regulation of specific or stress-related genes (Wang et al., 2003). This is because, these genes are involved in the whole sequence of DNA during stress response such as signalling, transcriptional control, protection of membranes and proteins, free-radical and toxic compound scavenging (Wang et al., 2003). The most commonly encountered injuries to the changes in DNA structure and function are modification to nucleotides and breaks of the phosphodiester bonds (Grossman et al., 1979).

2.2.2 Radiation

The radiation has basic interactions with biological systems which are closely related to human society by various applications in medicine, agriculture, pharmaceuticals and in other technological developments. For example, Keresztes & Kovács (1991) has reported on the effect of irradiation on plant cell and plant food items, whereby the ionizing radiation was absorbed in biological materials and may act directly on critical target in the cells which in turn can cause significant effects to the biological systems of the plants. Scientifically, radiation activate by interaction with other atoms or molecules such as water to produce free radicals which adequately diffuse to reach and damage different sites of attacks and components (Keresztes & Kovács, 2002). The free radicals produced from interaction between molecules in cells create a water radiolysis which has high water content that may influence temperature, pH and dilution of solution by the presence and absence of oxygen. Radiation also might be one of the most important factors in influencing biological systems such as plants structure and growth development because of their exposure towards ionizing radiation from environments. In a study by Jan et al. (2012), the morphological changes induced by radiation were shown to be attributed by the chemical and biological changes of different tissues and cell components. Thus, the functional changes are also quickly affected due to their alteration and modification in biological systems of plants.

Radiation such as gamma ray can affect the physiological and morphological characteristics in plants. Gamma irradiation is one of the stressors which significantly affected biochemical process in plants. For example, the irradiation of seeds with high doses of gamma rays had been reported to interrupt the synthesis of protein (Xiuzhen, 1994), hormone balance, leaf gas-exchange, water exchange and enzyme activity (Stoeva & Bineva, 2001). Moreover, Hameed et al. (2008), has stated that the plant's

morphological, structural and functional change depends on the strength and the duration of the gamma-irradiation stress. In response to environmental stress, protein breakdown and recycling are essential and would occur in order to rebuild and maintain the protein structure (Grudkowska & Zagdanska, 2004). Therefore, the synthesis of new proteins is required in response to degradation of damaged, misfolded and potentially harmful proteins that provides free amino acids. Previous studies have shown the complication in regulation of plant cells by proteolysis, which are involved in protein maturation, degradation and protein rebuild in response to external stimuli and to remove abnormalities such as misfolded proteins (Grudkowska & Zagdanska, 2004).

Degree of response caused by ionizing radiation which is influenced by environmental factors are categorized into many general aspects, such as due to modifying dosage or dose fractionation, types of ionizing radiation, factors that influence growth rate and factors affecting recovery from radiation damage (Ros & Tevini, 1995). Therefore, the degrees of response caused by ionizing radiation are determined by the ability of the plant's sensitivity towards radiation, because different dose rates cause different rate of damage which had been inflicted onto the plants (Feinendegen et al., 2004). However, in a study reported by Esnault et al. (2010), it was shown that repeated exposure to ionizing radiation at low and high dosages allows plants to adapt and exhibit radiation resistance. This adaptive response has affected the genetic structure in population by reducing the genetic variability, which in turn leads to species evolution led by a long-term period of chronic exposure of ionizing radiation (Esnault et al., 2010).

In some cases such as in evergreen forest trees, some clinical studies have reported that usage of ionizing radiation can be beneficial to increase the germination potential and generating useful mutations in agricultural crops (Novak & Brunner, 1992). The

economical and more effective features of gamma rays (due to their high penetration power) widen its application for the improvement of various plant species compared other types of ionizing radiation (Moussa, 2011). Gamma irradiation has an extreme influence on plant growth and by inducing genetic, cytological, biochemical, physiological and morphogenetic changes in cells and tissues development which depend on the levels of gamma dosage (Chandrashekar et al., 2013). Gamma radiation also affects plants' biological processes such as formation of free radicals by hydrolysis of water, resulting in the modulation of antioxidative system, accumulation of phenolic compounds and chlorophyll pigments (Ashraf, 2009). Besides that, treatment of crop varieties with gamma irradiation has been found to alter germination and accumulation of proline content, thus affecting crop yield (Dehpour et al., 2011). A previous study by Donà (2013) has shown that gamma irradiation in Petunia X hybrid cells at different rates had induced different DNA damage responses. Nonetheless, very few information is available in published literature on studies involving DNA damage accumulation and the regulating mechanisms involved in plant recovery from radiation injury.

2.3 Effect of environmental stress onto plants' DNA

Various environmental factors may cause DNA damage in plants such as endogenous and exogenous factors or can be induced by mutagenic agents. There are many mutagenic agents such as chemical and physical mutagens, which caused the plant genome to reduce their stability and have a deleterious effect on plant development, thus, leading to genetic variation (Manova & Gruszka, 2015). The biological impacts of mutagenic agents inflicted by the chemical and physical mutagens have generated genetic variations and diversity, such as inducing lesions in the genetic structure as well as on the efficiency and accuracy of the subsequent repairing processes. The DNA of plants normally suffers damage from chemical mutagens such as alkylating agents;

specifically the monofunctional alkylators such as methyl methane sulphanate (MMS) and ethyl methanesulfonate (EMS) (Manova & Gruszka, 2015). These alkylating agents methylate the DNA bases, mainly at their O- and N- positions, generating small base damage (Shrivastav et al., 2010). According to Manova and Gruszka (2015), the damage from these exogenous factors may cause a DNA lesion from primary category involved with DNA lesion solely at DNA strand for example, the loss and damage of base DNA and single strand breaks (SSBs).

On the other hand, the damage induced by physical mutagens such as ionizing radiation and ultraviolet light are the most frequent injuries encountered by the plant systems and structure. Ionizing radiation such as gamma- and X-rays usually involve with the damaging agents in DNA levels which potentially caused mutagenic effect in plants resulting severe lesions by direct ionization of the DNA molecule (Van Harten, 1998). According to Sax (1963), the effect of these rays on plant growth has been explored by the researchers from past 50 years as the effects may be deleterious and lethal at high doses of exposure. However, plants' sensitivity to radiation varied between different species and largely depends on the dosage of radiation (Van Hoeck et al., 2015). For example, ultraviolet radiation which is a component of sunlight, belongs to electromagnetic radiation spectrum and is the most common damaging agent that can impact or damage plants' genome. UV-A has the longest radiation wavelength ranging from 315 to 400 nm, while UV-B is the main DNA damaging component of the solar light and has a middle range of radiation ranging from 280 to 315 nm, whereas UV-C has the shortest radiation, ranging from 100- 280 nm (Manova & Gruszka, 2015).

The damage affected from UV light may generate two major types of lesions in DNA, which are the formation of cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (PPs), which are induced due to exposure to UV-B. CPDs in plants

occurs when the covalent bonds are formed between C-5 and C-6 carbon atoms of the adjacent pyrimidine bases, usually between TpT and less frequently between TpC and Cpc sequences (Durbeej & Eriksson, 2003). The presence of CPDs has the potential to block the process of transcription, which in turn will alter the pattern of gene expression in plants' genome (Tornaletti et al., 1999). However, the damage caused by UV radiation such as the formation of pyrimidine dimers can be repaired immediately by photoreactivation and nucleotide excision repair (NER) which function to eliminate the oxidative DNA and contribute to the stability in plant genome.

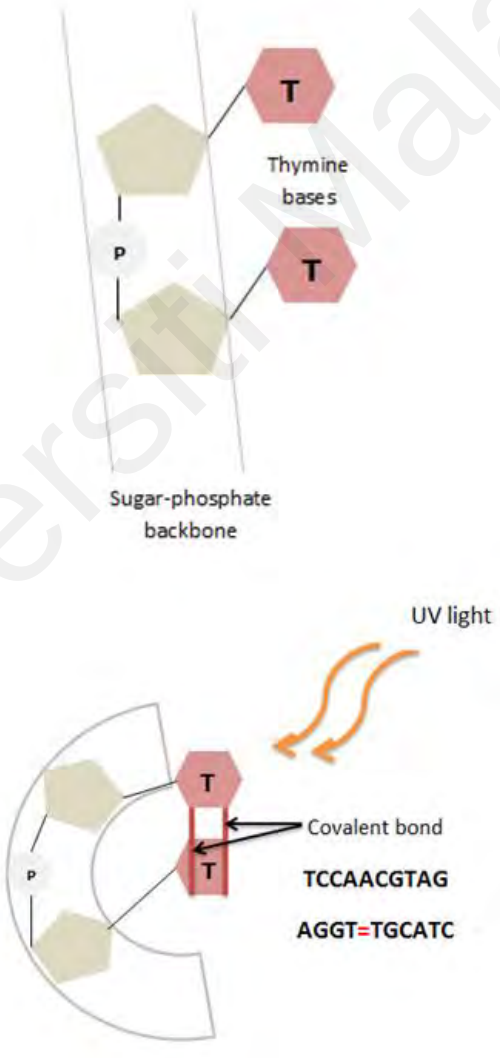

Figure 2.1: The structure of CPDs after exposure of UV radiation. Adapted from Amuzie et al. (2017).

2.3.1 DNA damage due to gamma radiation and irradiation dosage

Ionizing radiation induced by gamma radiation produces a large number of lesions in plants, through direct and indirect interactions with DNA molecule via water radiolysis, which generates highly reactive species such as hydroxyl radicals ($OH^{\bullet}$), free electrons (e-) and hydrogen radicals ($H^{\bullet}$) (Manova & Gruszka, 2015). Water radiolysis generates highly reactive OH* radicals, which are the most reactive compared to all ROS (reactive oxygen species). OH* radical is known to be able to reach all biological molecules such as DNA, lipids, proteins and almost any constituents of cells (Manova & Gruszka, 2015). The risks of of OH* radicals accumulation is greater when excess OH* cannot be removed in the absence of any enzymatic mechanisms. Therefore, the most vulnerable biomolecule to be damaged by both OH* and UV radiation is DNA (Gill et al., 2015b). According to Manova & Gruszka (2015), indirect interaction of ionizing radiation generates double strand breaks (DBSs), which leads to DNA fragmentation and modification of DNA terminus such as hydroxyl, phosphate and glycolate that is important for ligation. Meanwhile, the lesions from direct interaction of ionizing radiation via secondary DNA ionization generates single strand breaks (SSBs), resulting in oxidation products and base loss as the base and phosphodiester backbone may be damaged or modified (Esnault et al., 2010).

The damage responses induced by gamma irradiation depend on dosage rates inflicted onto the plants. In studies on γ-rays by Kovalchuk et al. (2007), on *Populus nigra var. italica* revealed that the regulation of repair mechanisms is necessary for the adaptive response of ionizing radiation and the gene expression of DNA repair mechanisms, which were exhibited in response to chronic or acute exposure of gamma radiation onto the plants. Nevertheless, the information of DNA damage accumulation

and molecular mechanism pertaining to radiation injury at different dosage rate is poorly explored in research (Rastogi et al., 2010).

The interaction with atoms or molecules in the cell, especially water, to produce free radicals is the main biological effect of gamma radiation in plants (Keresztes & Kovacs, 2002). The process of hydrolysis water formed by free radicals may cause in the regulation of antioxidant system, accumulation of phenolic compounds and chlorophyll pigments (Chandrashekar et al., 2013). Gamma irradiation may cause genotoxic damage to DNA strands such as the formation of double strand breaks or DSBs, although these may be repaired by DSB-repair pathways namely homologous recombinant (HR) and the non-homologous end joining (NHR/NHEJ) (Friesner & Britt, 2003). In addition, DSB repairs under chronic radiation conditions can also involve nucleotide excision repair (NER) and double-strand break (DSB) repair pathways. However, limited research of NER and DSB repair pathways have been found especially for irradiation-mediated repairs in plant genome (Boubriak et al., 2008).

The failure to repair a break in DNA leads to chromosome loss and abnormalities such as translocations, deletions and misrepairs (Friesner & Britt, 2003). However, some studies have indicated that gamma radiation can be used as an irradiation treatment to enhance the production of plants with medicinal values and to induce other biochemical changes to influence the regulation of biosynthetic pathways (Jan et al., 2012).

2.4 Types of DNA repair mechanisms

Changes in the genomes of all living things are constantly subjected to damage and decay. The damage occurs consequence from the chemical nature of DNA may be unavoidable to plants, relatively an errors metabolism in plants may happen such as the

donation of methyl group to DNA from its targeted cells, or the presence of stray radicals spontaneously formed during respiration or photosynthesis (Britt, 1996). According to Stapleton (1992), this endogenously generated damage is often termed 'spontaneous' DNA damage, to distinguish it from the damage caused by exogenous sources, such as ultraviolet radiation, ionizing radiations and chemical DNA damaging agents. Changes in plant genome can arise either from intrinsic or extrinsic sources, and may cause changes in plants' morphology by interfering in plant's genome stability. According to Ros and Tevini (1995), the most frequent damage inflicted by radiation exposures is hormonal imbalances, caused by interfering of radiation factors with indoleacetic acid (IAA) metabolism. DNA damage can also be caused by exposure of the organism to atmospheric radiations, heat, desiccation and harmful chemicals. Radiation-induced damage may affect plant's DNA, which in turn result in heritable mutation if not repaired before replication or physiological process (Stapleton, 1992). These mutations are required a mechanisms to repair the damage to maintain the necessary of genetic composition as succession of an individuals or species. The failure in plants to lessen their exposure towards harmful environment further increases the importance of DNA damage avoidance and repair mechanisms (Vonarx et al., 1998).

In contrast to most animal cells, many plant cells are totipotent and do not developmentally migrate in sheets (Vonarx et al., 1998). However, there are primary and secondary responses to external sources in order to repair DNA damage. In primary responses, Vonarx et al. (1998), reported that morphological changes such as thickening of the epidermis, increased production of waxy cuticles and localized necrosis and dessication as in response to UV-B radiation. While, in secondary response, UV light usually has been used as a model genotoxic agent to analyze repair mechanism of DNA damage, for example, photoreactivation, nucleotide excision repair (NER), base excision repair (BER) and transcription-coupled repair (Vonarx et al., 1998).

In summary, nuclear DNA in plant is genetically unstable molecule compared to the animal cells and can be unintentionally damaged by various number of stress factors from the environment as it immobile. Mutagenic agents such as chemical and physical mutagens can cause significant DNA damages in all organisms, especially in plants. The damage arising from chemical mutagens such as methyl methane sulphanate (MMS) and ethyl methanesulfonate (EMS) caused damage to the DNA strands. Whereas, physical mutagens such as UV and ionizing radiation are the major factors that can cause damages such as formation of CPD, 6-4 PPs and double-strand breaks (DBSs). These DNA damage will in turn induce repair mechanisms to take place in order to repair and fix the damage of DNA structure by employing a number of pathways such as Non- Homologous End Joining (NHR/NHEJ), Homologous Recombinant (HR), Base Excision Repair (BER), Nucleotide Excision Repair (NER) or Mismatch Repair (MMR) in order to preserve genetic stability and productivity in plants.

2.4.1 DNA repair mediated to radiation

The major pathway in plants for repairing damage from UV especially UV-B is photorepair which also known as the photoreactivation. This repair mechanism undergoes photoreactivation process which required a DNA repair enzymes called photolyases by absorbing a visible light from the blue light or UV-A for activation purposes to repair the damage caused by ultraviolet light. According to Gill et al. (2015), the enzymes specifically bind to DNA lesions and adequately remove the most of UB induced CPDs and (6-4)-photoproducts directly by absorbing light in 300-600 nm range. However, in order to modulate this repair, it will depend on a number of cofactors such as quality, timing and the quantity of photo reactivating light and damage levels (Sutherland et al., 1996). The effectiveness of this repair has also been reported

by Teranishi et al. (2012), on *Oryza sativa* cultivars, whereby photolyases were shown to be a major factor in modulating the repair of DNA damage due to UV-B radiation. In the same study, it was also reported that the increase in the activity of photolyases in *O. sativa* also resulted in the increase of their resistance towards UV-B radiation.

While, the repair mechanisms caused by physical mutagens can be fixed and repaired by Nucleotide Excision Repair (NER), Base Excision Repair (BER) and Mismatch Repair (MMR). Nucleotide excision repair recognizes and repairs various type of DNA damage caused by UV irradiation and other DNA damaging agents. NER mechanism has four series of reactions which are recognition of DNA damage, excision of the damaged nucleotides and filling of the single stranded gap by DNA synthesis. However, NER mechanism has pathways which can be classified into groups; global genome repair (GGR) that repairs DNA damage anywhere in the genome and transcription-coupled repair (TCR) that specifically restores DNA strands that are being transcribed (Kimura & Sakaguchi, 2006). In GGR, DNA damage recognized by *Xeroderma pigmentosum*, complementation group C/Rad23 (XPC/Rad23) or also known as UV-damaged DNA binding protein (UV-DDB).

In contrast to TCR pathways, the connection between RNA polymerase II with damaged nucleotides triggers recognition of the DNA damage by Cockayne syndrome, type A (CSA) or Cockayne syndrome, type B (CSB). The damage nucleotides that has been recognized in DNA region is unwound by transcription factor IIH (TFIIH) including *Xeroderma pigmentosum*, complementation group B (XPB), *Xeroderma pigmentosum*, complementation group D (XPD), *Xeroderma pigmentosum*, completation group A (XPA) and replication protein A (RPA). The structure-specific endonucleases, *Xeroderma pigmentosum*, complementation group F/excision repair complementing defective repair in Chinese hamster 1 (XPF/ERCC1) and *Xeroderma*

pigmentosum, complementation group G (XPG) excise oligonucleotides of about 20-30 bases containing the damaged part of the DNA. Then, proliferating cell nuclear antigen (PCNA) and replication factor C (RFC)-dependent DNA synthesis is carried out by DNA polymerase δ*/ε to fill the gap formed by the excision. The repair is completed with DNA ligase I by reconnecting the repaired DNA strand (Kimura & Sakaguchi, 2006).

BER also known for correcting damaged DNA by deamination of DNA bases which generate highly mutagenic modifications of DNA bases into uracil, hypoxanthine and xanthine. However, base excision repair is active on a wide range of lesions such as damaged or modified as well as naturally occurring apurinic/apyrimidinic (AP) sites which are initiated by the removal of the damaged bases and incision of AP endonuclease which is mediated by DNA glycosylase. The DNA synthesis following the incision has two pathways which are long-patch BER and short-patch BER. According to Kimura & Sakaguchi (2006), the long-patch BER require the process of synthesized DNA by *DNA polymerase δ*/ε* where PCNA and FEN-1 act in coordination with *DNA polymerase δ*/ε* in the synthesis pf relatively long DNA chains and eventually ligated by DNA ligase I. While, in short-patch BER, the synthesis is catalysed by *DNA polymerase β* where the 5'-dRP residue is removed by the dRP lyase activity of *DNA polymerase β* and eventually ligated by DNA ligase III/X-ray repair complementing defective in Chinese hamster 1 (XRCC1) in order to complete the repair (Kimura & Sakaguchi, 2006). In a study by Choi et al. (2002), it was shown that DEMETER, a DNA glycosylase domain protein was required for endosperm gene imprinting and seed variability in *Arabidopsis,* indicating its role in transcriptional regulation of the gene is required for gene imprinting. This indicates that in BER, some of the genes that are involved may have other functions in addition to DNA repair.

Besides, DNA damage can be resulted from a mutation due to mismatches, as well as insertion or deletion of the nucleotides, whereby these can give rise to the slip-mispairing during replication and recombination of DNA. MMR has been reported to conserve the genomic stability via correction of DNA replication errors, antagonizing homologous recombination and responding to various DNA lesions (Leonard et al., 2003) to increase the constancy of DNA replication, to decrease the rate of mutations, to control the dynamics of short repetitive sequences and to maintain genome integrity. Moreover, this repair system is mentioned by Leonard et al. (2003) to be extremely preserve the species where the error rate in targeted DNA chain decrease after polymerase proofreading removes majority (99.9%) of the error remaining from the damage. Mismatch repair (MMR) systems have the biological role to correct and fix the errors during DNA replication such as mismatches, deletion or insertion of nucleotides. MutS homologs (MSH) protein subunits are the proteins involved in MMR mechanisms to recognize the rare mismatches during replicating DNA and to prevent the establishment of mutations (de Wind & Hays, 2001). The MMR in *Arabidopsis* was shown by Bray & West (2005), in initial recognition of the mismatch lesion via MutS homolog isolated from *Arabidopsis thaliana* (AtMSH) heterodimer which involves an enzyme complex, MutL1 homolog (AtMLH1). Postmeiotic segregation increased 2 (PMS2) are then discriminates between the nascent and template DNA strands, promoting unwinding of the DNA helix and culminating in resynthesis and eventually proceeding with the ligation steps via DNA ligase to fill the gap in the nascent DNA strand.

2.5 Phenotypic plasticity

Phenotypic plasticity is defined by a change in the phenotype, expressed by a single genotype where new stress factors were involved or introduced when plants are exposed

to different condition in the environment. Plasticity plays an important role in plant response towards global change conditions, therefore it is involved in predicting changes in species distribution, community composition and crop productivity (Lande, 2009). In order words, inability and loss of functionality in plant genome can interfere with biological systems, which reduces and minimize the productivity and developmental process the plants. Hence, plasticity has been reported as a significant mode of phenotype adaptation which served as an important aspect of organism development, functionality and evolutionary in their environment. The term 'norm of reaction' also has been recognized in phenomenon of phenotypic plasticity where the specific traits can be measured by a particular phenotype in response of particular environment stress factor (Sultan, 2003).

Apart from that, Bradshaw (1965) have stated that phenotypic plasticity could itself be under genetic control and therefore subjected to selective pressure. The pressure driven by harsh exposure from environments may change the phenology and morphology of the plants due to a number of genetic alterations and modifications. Besides, according to Pigliucci et al. (2006), plasticity also can be defined by appearances with possible induced novel phenotype from new environment. Thus, plasticity could ease the expression of adaptive phenotypes under novel conditions which improve the performance of the population by genetic assimilation of the trait in the new environment. These interesting properties possessed by plants have the potential to explain a variety of evolutionary ecological process (Pigliucci et al., 2006).

In terms of ecology, plasticity is fundamental to ecological development because it influences the natural process and growth of an organism which leads to successful survival and fitness in their environment conditions. Naturally, this plasticity also leads to a variation in plant genotype which can be measured by the changes of phenotype in

plant structure and characteristics. Hence, plants having plasticity express some degree of variation after phenotype response to various exposures to the stressful environment. Bradshaw (1965) stated that phenotypic responses to different environments may also include highly specific developmental, physiological and reproductive adjustments that enhance function in those environments. Moreover, the plasticity by each individual that is affected by harsh environments has a huge effect and contributes to the degree of survival and evolutionary diversification. Therefore, plasticity has been recognized to play a major role in ecological distribution of organism which affects their patterns of evolutionary diversification (Sultan, 2000).

In genetic terms, plasticity extends beyond simple effects, but also influences key life-history traits such as sex expression and breeding system, reproductive allocation and phenology (Sultan, 2000). Plants can respond to environmental conditions not only by adjusting their own phenotypes but also by altering those of their offspring through changes in the quantity and quality of flower production conditions. For example, the proportions of staminate and hermaphroditic flowers in an andromoneocious *Solanum* were shown to depend on plant resource status, confirming a long-standing ecological hypothesis (Diggle, 1994). This indicates that normally self-incompatible plants can change to self-fertilization in response to floral age and lack of prior fruit development (Vogler, 1998). This plasticity for self-incompatibility results in a 'delayed selfing' strategy that covers reproduction if outcrossing fails. This implies that a plasticity change can occur in response to a plant's internal environment as well as to resource availability or other external signals. The parental environment can also alter the phenotypic plasticity of progeny as the offspring structure, development and morphology can be influenced in unusual specific ways by the parent's environment. In the case of increased or decreased carbon dioxide (CO_2) concentration in the offspring, there is significant effect from parental (CO_2) environment that affect the fertilization or

growth response in plants which alters the offspring quality thus, in ways, affect a population's stability to fertilize. Such specific plasticity changes to seedling growth patterns might allow offspring to survive at any critical aspects of function (Sultan, 2000).

However, changes in phenotypes under global changes have led to plant adaptation towards the environment. Plastic responses involve with the timing of plant development from unavoidable effect of environment which limit the growth and physiology. Therefore, the plastic response cause an adaptive adjustment in an organism that may enhance their success in environment that induce them (Sultan, 1995). In fact, physiological, morphological and anatomical plasticity may have a different role in plant adaptation to environmental changes. In terms of altering the physiology of plants, the new traits may allow plants to grow and reproduce in spatially or temporally variable environments allowing adaptation in plants (Kuiper & Kuiper, 1988). Hence, the plasticity expressed in plant growth and development system may characterized by changes of certain trait in genotype through adaptation towards environment responses.

2.6 Methods to detect genetic variability

For the past decade, there are several DNA markers that are widely used for detecting genetic variability such as restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs) and simple sequence repeats (SSRs). However, these molecular markers has their limitations based on their technical differences in terms of cost, effectiveness, amount of DNA needed, technical labour, precision of genetic distance estimations and degree of polymorphism detection (Garcia et al., 2004). According to Garcia et al. (2004), RAPDs were stated to lack in reproducibility for generating polymorphism due to mismatch annealing. Besides, the study also showed

that AFLPs and RFLPs techniques are reliable and able to detect a large number of polymorphic bands, but these techniques are laborious and consume more time compared to other DNA markers (Gerber et al., 2000). Meanwhile, Rongwen et al. (1995), stated that the limitation of microsatellites (SSRs) limits the use of SSR in detecting genetic variability because the effectiveness of this technique depends on the availability of suitable microsatellite markers, as the flanking sequences will need to be known in order to develop specific primers to detect any polymorphism.

2.6.1 Advantages of ISSR technique

In recent years, some molecular markers have evolved for genetic analysis studies especially based on microsatellite repeats which has led in producing a large number of amplified DNA, detection of high level of polymorphism, highly reproducible and reliable (Li et al., 2009). ISSR is one of the simplest and widely used technique among PCR based marker techniques involving the amplification of DNA segments. This technique has been stated to be more stable and reproducible compared to RAPD even though ISSR markers are dominant like RAPD technique because these properties have been found widely used in fingerprinting, phylogenetic analysis, population structure analysis, line identification, genetic mapping analysis and marker-assisted selection (Vijayan, 2005). Moreover, ISSR has been used in various studies, for example in analyzing phylogenetic relationship among cultivated mulberry varieties (Vijayan, 2005).

2.6.1.1 Source of Genetic Variability

ISSR technique associates with amplification process of DNA segments between two identical short tandem repeats which use primers, usually 16-25 bp long, of di-nucleotide, tri-nucleotide, tetra-nucleotide or penta-nucleotide repeats to target multiple

DNA loci. After the loci is targeted, polymorphism is generate from primer used after one genome passes the repeat sequence or has a deletion or translocation that alter the gap between the repeats (Vijayan, 2005).

2.6.1.2 Template DNA

The creation of DNA variability can occur during DNA replication by slipping of DNA polymerase which subsequently causes failure to repair mismatches (Levinson & Gutman, 1987). The polymorphism might occur in the presence of mutation at priming site such as deletion and insertion events within the SSR region (ISSR) which, depending on the variability in number of nucleotides within the microsatellite repeats result in length polymorphisms when using a 5'- anchored primer (Reddy et al., 2002a).

2.6.1.3 Characteristics of primers used

The degree of polymorphism varies with the nature of the repeat sequences which consist of three types of primers naturally known as unanchored, 3'-anchored and 5' anchored (Reddy et al., 2002a). In order to serve as priming sites, the anchor only enable a group of microsatellites to produce amplified product such as microsatellites sequences and their variation in length in genome which subsequently generate high polymorphism in DNA sequences (Reddy et al., 2002a). However, the type of anchors gives different result of polymorphism in the sequence repeats. Based on previous studies, the di-nucleotide repeats, anchored either at 3' or 5' end revealed high polymorphism (Blair et al., 1999; Joshi et al., 2000; Nagaoka & Ogihara, 1997). However, the primers anchored at 3' end was stated to give clearer banding pattern compared to those anchored at 5' end (Blair et al., 1999; Nagaoka & Ogihara, 1997; Tsumura et al., 1996). Besides, according to (Reddy et al., 2002a), the higher polymorphism was shown from primers with (AG), (GA), (CT), (TC), (AC) and (CA)

repeats compared to other primers from di-, tri-, or tetra- nucleotides repeats. The previous studies were showed the based primers of (AG) and (GA) amplify clear bands in rice whereas, primers based on (AC) di-nucleotide repeats were found more useful in wheat (Kojima et al., 1998; Nagaoka & Ogihara, 1997)

2.7 Novelty of study

This study was conducted because inadequately information about genetic variation among plants after mutagenic treatment. Besides, the used of ISSR marker as a marker tool to determine genetic variability also poorly discover by researchers, thus made this study as a novel technique and good information for other researchers and people for future references. In this study, ISSR marker was used as a new and most effective marker to generate high reproducible polymorphisms in order to determine genetic variation between irradiated and control samples after exposure of gamma radiation. This study was conducted to prove that the plants were capable to repair the damage and injured cells by itself within time interval because of the 'norm of reaction' in nature during encounter harsh environment such as gamma radiation. The adaptive plasticity developed in plant systems proved their ability to cope and altered itself by DNA repair through time for survival.

Moreover, this study also investigated the changes in plant characteristics and growth after mutagenic treatment to detect the effect caused by gamma radiation. The changes caused by gamma radiation give severe effect to plants characteristics which led to reduction in plant height and stunted the plant growth. Therefore, this study was conducted to observe the morphology after effect of gamma radiation within different post recovery hence, the DNA repair takes place to repair the damage occurred by DNA repair mechanism.

CHAPTER 3: METHODOLOGY

The process of research carried out during the study was based on the flow chart shown in **Figure 3.1**.

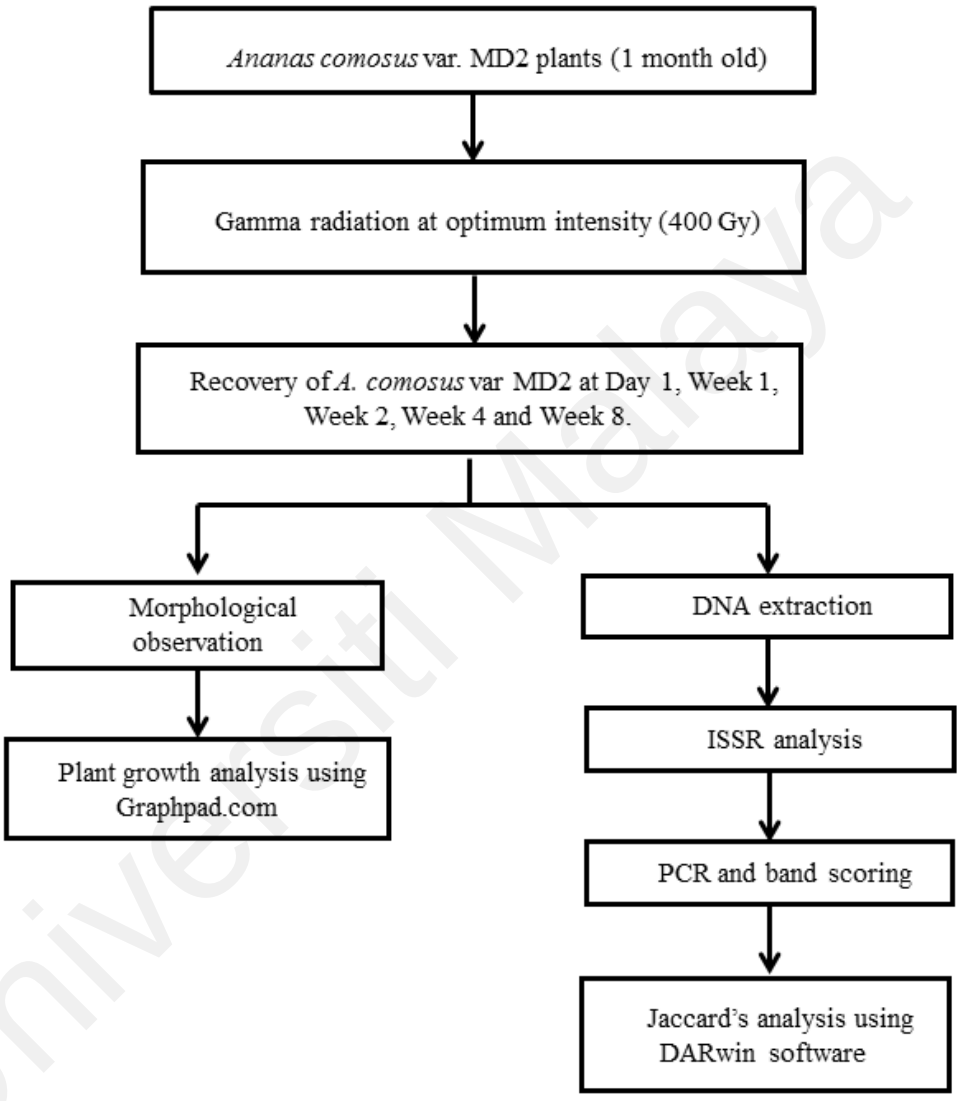

Figure 3.1: Flow chart of research methodology.

3.1 Plant tissue culture

3.1.1 Choices of clonal explants

Two-month-old *in vitro* grown *Ananas comosus* var. MD2 plantlets were obtained from Integrated Plant Research Laboratory, University of Malaya. The plantlets were used as the starting material for this study, where the leaf base explants were harvested and used to induce direct regeneration of this species. The leaf base explants were cultured on Murashige and Skoog (MS) media supplemented with 1.0 mg/L IBA (Indole-3-butyric acid) and 3mg/L BAP (6-Benzylaminopurine), which is the optimum concentration of IBA and BAP, as had been previously determined (Halim et al., 2017). The cultures were maintained at 25± 1°C with a photoperiod of 16 hours light and 8 hours dark, under light illumination of 1000 lux. In this experiment, all of the explants were harvested and cultured from the same mother plant in order to produce genetically identical (clonal) plantlets.

3.1.2 Media preparation

3.1.2.1 Stock hormone preparation

The hormones used in the plant tissue culture experiments were auxin (IBA) and cytokinin (BAP) (Duchefa Biochemie, Netherlands). Each of the hormones was prepared with 30 ml of distilled water and 0.05 gram of hormone in a Schott bottle. The hormones were then dissolved in diluted NaOH and added with distilled water to make up the volume to 50 ml. The stock hormones solution was placed in the Schott bottle and wrapped with aluminium foil before being stored under -5 degree celcius (°C) for further usage.

3.1.2.2 Preparation of culture media

For preparation of 1 L of culture media, 800-900 mL of distilled water was filled in 1 L Schott bottle, and added with 30 g sucrose (SYSTERM ChemAR, Shah Alam) and 4.4 g Murashige and Skoog (MS) powder. All the media components were well-mixed by using HTS-1003 magnetic stirrer (Labmart, USA). The media was then supplemented with plant growth regulators (PGRs) which are cytokinins and auxins; namely 1.0 mg/L of Indole-3-butyric acid (IBA) and 3.0 mg/L of 6-Benzylaminopurine (BAP). The pH of the culture media was adjusted to 5.7 - 5.8 (Bhojwani & Dantu, 2013) using LE438 pH meter (METTLER TOLEDO, Malaysia). Then, the media was solidified using 2 g of gelrite powder (Duchefa Biochemie, Netherlands).

3.1.2.3 Sterilization of media

The prepared media was subjected to sterilization process in order to eliminate or deactivate all forms of life and other biological agents that might contaminate the media. The media was sterilized by autoclaving it at 121° C and 1 kPa for 2 hours using SX500 High-Pressure Stream Sterilizer (Tomy Seiko, Japan). The caps of the jam jar were loosened before autoclaving to reduce the pressure. The media was then sealed with parafilm after sterilization to prevent from contamination and stored at room temperature (24 ± °C) for further usage.

3.1.3 Subculture

3.1.3.1 Culture condition

The leaf base of the stock plants was cut in lateral about 1.0 ± 0.1 cm using a sterilized scalpel with blade and forceps. All the small and damaged leaves were removed. Each meristem was placed upright in universal containers filled with MS

media. All cultures were placed inside a sterile culture room and were exposed under white light for 16 hours light and 8 hours dark at 25± 1°C with 1000 lux intensity of white light.

3.2 Recovery of *Ananas comosus* var. MD2

3.2.1 Gamma irradiation

The clonal plantlets were irradiated with gamma using the radiation facility (Gamma Cell 220, Canada) at Physics Laboratory, Department of Physics of University Malaya (**Figure 3.2**). The plantlets were irradiated with gamma at a previously determined optimum radiation dosage of 400 gray (Gy) while, the control plantlets were not irradiated. The optimum radiation dosage was determined based on the LD50 (lethal dose for 50% of the plants tested, or radiation intensity that causes 50% mortality rate). After the plant samples were irradiated, the samples were kept inside the culture room and let to recover.

Figure 3.2: Gammacell 220 at Physics Laboratory used to irradiate the plantlets.

3.2.2 Morphological observation

The morphology of the plantlets were observed and recorded at different recovery period; day 0 (non-irradiated; control), day 1, week 1, week 2, week 4 and week 8 after irradiation. For this purpose, the morphological characters of the plantlets such as the height of the plantlets leaf width (based on the D-leaf) and chlorophyll variegation were observed and recorded.

3.2.3 DNA extraction

Total DNA was extracted from leaf materials obtained from 10 regenerated clonal plantlets (randomly collected) at each recovery period (day 0 (non-irradiated; control), day 1, week 1, week 2, week 4 and week 8). Briefly, 100 mg (0.100 gram) of plant leaves were weighed and subjected to DNA extraction. DNA was purified from the fresh leaves using the Gene Matrix Plant and Fungi DNA Purification Kit (EURx Ltd., Poland) according to the manufacturer's specification. The leaves were ground in liquid nitrogen using a micropestle in 1.5 µl collection tube. The ground leaves were lysed with 400 µl of Lyse P added with 3 µl of RNAase A and 10 µl proteinase K. The cells were mixed by vortexing or inverting the collection tube and incubated for 30 minutes at 65 °C.

Following that, the samples were added with 130 µl of Buffer AC and mixed by inverting the sample tubes. The samples were then incubated for 5 minutes at -20 °C. The lysate was centrifuged for at 15 000 × g (times gravity) for 10 minutes at room temperature (RT). Then, 400 µl of supernatant produced from the centrifugation process was transferred into new 1.5 µl collection tubes and added with 350 µl buffer Sol P and 250 µl of 96% ethanol. The transferred supernatant was then centrifuged at 14 000 × g for 1 minute at RT. After the lysate was centrifuged, 600 µl of supernatant was

transferred into a spin column with new 1.5 µl collection tube and centrifuged at 14 000 ×g for 1 minute at RT. Then, the spin column was taken out and the flow through in the collection tubes was discarded. The collection tube was then placed back together with the spin column and the remaining supernatant was transferred into the spin column. The steps were repeated until the remaining supernatant were all transferred and the flow through was discarded before being washed with 500 µl of wash PX. After being washed with wash PX, the spin column was centrifuged again for 2 minutes at 14 000 × g. The spin column was then placed in a new 1.5 µl collection tube and 100 µl of elution buffer was added into the spin column. However, the elution buffer must be heated to 70 °C before being added to the spin column. After the elution buffer was added into the spin column, the samples were incubated for 5 to 30 minutes at room temperature before centrifuged for 1 minute at 14 000 × g. This step was repeated twice and the samples collected in the collection tube were stored at -80 °C in Ultra Low Temperature Freezer (New Brunswick, USA) for further usage.

3.2.4 DNA quantification

The extracted DNA was then quantified to determine their concentration by using Gen 5.0 Microplate Reader and Image Software, BioTek Gen 5 (BioTek Instrument, USA). The microplate reader was set first with 2 µl of distilled water as a blank before DNA samples were read. After that, the DNA samples were quantified with 2 µl at 50 ng/µl concentration.

3.2.5 ISSR analysis

In this experiment, the primers used were universal primers from Eurx Molecular Biology Product (EURx Ltd.). The sequences of 12 tested ISSR primers in this analysis (**Table 3.1**) are UBC 807, UBC 809, UBC 829, UBC 834, UBC 836, UBC 840, UBC

841, UBC 845, UBC 851, UBC 855, UBC 856 and UBC 873 (Viehmannova et al., 2014).

Table 3.1: ISSR primers

Primers code (UBC)	Sequence 5'-3'	Annealing temperature (°C)
UBC 807	$(AG)_8T$	46.5
UBC 809	$(AG)_8G$	48.0
UBC 829	$(TG)_8C$	52.5
UBC 834	$(AG)_8YT$	52.0
UBC 836	$(AG)_8YA$	48.0
UBC 840	$(GA)_8YT$	46.5
UBC 841	$(GA)_8YC$	52.0
UBC 845	$(CT)_8RG$	47.5
UBC 851	$(GT)_8CT\ G$	52.5
UBC 855	$(AC)_8YT$	53.0
UBC 856	$(AC)_8YA$	54.0
UBC 873	$(GACA)_4$	46.5

In this ISSR analysis, twelve primers (Integrated DNA Technologies, USA) were tested by using Polymerase Chain Reaction (PCR) method. PCR was performed in a 20 µl reaction volume containing the following components:

Table 3.2: Component in each total reaction volume.

Components	Concentration	Volume added
Template DNA	50 ng/µl	x µl
Primer (Integrated DNA Technologies, USA)	2 µM	1 µl
Buffer C (Eurx Ltd.)	10 x	2 µl
$MgCl_2$ (Eurx Ltd.)	25 mM	2 µl
BSA (Invitrogen, USA)	4 mg/µl	1 µl
Taq DNA Polymerase (Eurx Ltd.)	1.25 U/µl	1 µl
dNTPs (Eurx Ltd.)	4 mM	1 µl
ddH_2O	-	x µl
Total reaction volume		**20 µl**

As shown in **Table 3.2**, the PCR reaction was prepared in 20 µl of total reaction volume containing 50 ng/µl of template DNA, 2 µl 10x Pol Buffer C, 2 µl $MgCl_2$ (mM), 1 µl BSA (Invitrogen, USA) at 20 µl/mg concentration, 1 µl of 2 µM primers, 1 µl of 4 mM dNTPs, 1 µl of 1.25U Taq polymerase and PCR H_2O. Amplifications were performed in a Mastercycler Nexus Thermal Cycler (Eppendorf, North America). The PCR was carried out at the pre-determined optimum annealing temperatures of each individual primer. The ISSR PCR reactions were performed starting with an initial denaturation process at 94 °C for 5 minutes followed by 35 cycles consisting of 1 minute at 94 °C for denaturation, 1 minute for annealing process and 2 minutes at 72 °C for extension process. The PCR reaction was ended with 1 cycle of final extension for 10 minutes at 72 °C followed by storage or cooling process at 10 °C. The PCR products produced from the PCR reactions were stored at -80 °C for further usage.

The PCR products were then subjected to an electrophoretic separation using EPS-300X (C.B.S. Scientific Company, United State) with 8 µl of amplified products and on 1.5 % Agarose Electrophoresis Grade (EURx Ltd., Poland) in 1x TAE buffer for about 90 minutes at 50 V. DNA amplification products were stained with Red Safe Nucleic Acid Staining Solution (Intro Biotechnology, Korea). The gels were then visualized under UV light by AlphaImager™ Gel Imaging System (Alpha Innotech, Germany).

3.2.5.1 Band scoring

The banding profiles generated by all the ISSR primers were visualized and scored in order to calculate the genetic dissimilarity between the samples, to determine the genetic variation that has been resulted due to gamma radiation. The bands were scored 1 as presence of band and 0 as the absence of band for each irradiated and control plant samples. The scored bands were then transformed into a binary character matrix to calculate the Jaccard's distances using Darwin 6.0 software.

3.3 Statistical analysis

3.3.1 Jaccard analysis by DARwin software

The binary matrix produced from the ISSR analysis was used to calculate the Jaccard's distances to determine the genetic dissimilarity and variation between the irradiated and clonal plants of *Ananas comosus* var. MD2 at each recovery period, using the DARwin 6.0 software.

3.3.2 Plant growth analysis

All morphological data were analysed using Student's t-test analysis in Graphpad.com to compare the means between non-irradiated and irradiated samples at $p<0.05$.

CHAPTER 4: RESULTS

4.1 PRODUCTION OF CLONAL PLANTLETS

In this study, clonal *Ananas comosus* var. MD2 plantlets were used as the samples. For this purpose, the leaf base explants from two-month-old *in vitro* grown A. *comosus* plantlets were harvested and sub-cultured onto optimum regeneration media, composed of MS media added with 1.0 mg/L IBA and 3.0 mg/L BAP (Halim et al., 2017). The cultures were incubated in the culture room for 1 month, and their growth was monitored weekly. **Figure 4.1** shows the clonal A. *comosus* var. MD2 plantlets after 1 month of incubation in the culture room.

Figure 4.1: One-month-old clonal *A. comosus* var. MD2 plantlets, produced through direct regeneration from leaf base explants.

4.1.1 Assessment of Genetic Similarity between Clonal Plantlets

Following successful generation of clonal *A. comosus* var. MD2 plantlets, the leaves of the plantlets were harvested and used for ISSR analysis, to assess the genetic similarity between the plantlets. This experiment was conducted to prove the clonal identity of all samples, before being used in subsequent experiments.

In this experiment, 12 ISSR primers were tested, whereby it was observed that only 8 of the 12 primers yielded clear and scorable bands with decent intensity. Meanwhile, the other 4 primers, namely UBC_836, UBC_845, UBC_851, and UBC_873 did not generate any bands. 10 clonal plantlets were randomly selected, used for DNA extraction and subjected to PCR analysis using the 8 chosen ISSR primers. The banding profile generated by the primers were observed and scored, whereby score of 1 indicated the presence of the band and 0 as the absence of the band. The scored bands were then transformed into a binary character matrix and used to calculate the Jaccard's distances using Darwin 6.0 software. The Jaccard's dissimilarity distance table between the clonal samples is as shown in Figure 4.2.

Jaccard's dissimilarity distance displays the level of dissimilarity between samples, whereby the genetic distance is provided in decimal forms, ranging between zero (0) indicating no genetic variability, to one (1) indicating the highest genetic variability. In this study, the genetic distance between all the clonal samples (Figure 4.2) ranged from 0.02914 – 0.16281, implying that all samples were of clonal origin, and have very low genetic variability.

The Jaccard's dissimilarity distances were then used to compute a phylogenetic tree using Darwin 6.0 software. For this purpose, a cluster analysis was performed on the basis of dissimilarity coefficients generated from the ISSR data of the 62 scorable bands (generated using the 10 randomly selected clonal plantlets), and used to generate a UPGMA dendogram (Figure 4.3). Based on the dendogram, it can be observed that 8 out of the 10 clonal plantlets were grouped together in a single cluster with 90% similarity. Nevertheless, plantlets H and I were grouped in another single cluster, but at a very low rate of polymorphism with only 0.16281 dissimilarity indices. Therefore, it

can be deduced that plantlets H and I were also of clonal origin, with very low genetic variability compared to the other plantlets.

	Plantlet A	Plantlet B	Plantlet C	Plantlet D	Plantlet E	Plantlet F	Plantlet G	Plantlet H	Plantlet I
Plantlet B	0.07639213504396								
Plantlet C	0.07639213504396	0.02914285714286							
Plantlet D	0.07639213504396	0.07639213504396	0.07639213504396						
Plantlet E	0.07639213504396	0.07639213504396	0.07639213504396	0.07639213504396					
Plantlet F	0.07639213504396	0.07639213504396	0.07639213504396	0.07639213504396	0.07639213504396				
Plantlet G	0.07639213504396	0.07639213504396	0.07639213504396	0.07639213504396	0.07639213504396	0.07639213504396			
Plantlet H	0.16280814204616	0.16280814204616	0.16280814204616	0.16280814204616	0.16280814204616	0.16280814204616	0.16280814204616		
Plantlet I	0.16280814204616	0.16280814204616	0.16280814204616	0.16280814204616	0.16280814204616	0.16280814204616	0.16280814204616	0.06089552238806	
Plantlet J	0.07639213504396	0.07639213504396	0.07639213504396	0.07639213504396	0.07639213504396	0.07639213504396	0.07639213504396	0.16280814204616	0.16280814204616

Figure 4.2: The Jaccard's dissimilarity distances generated from 10 randomly selected clonal plantlets**.**

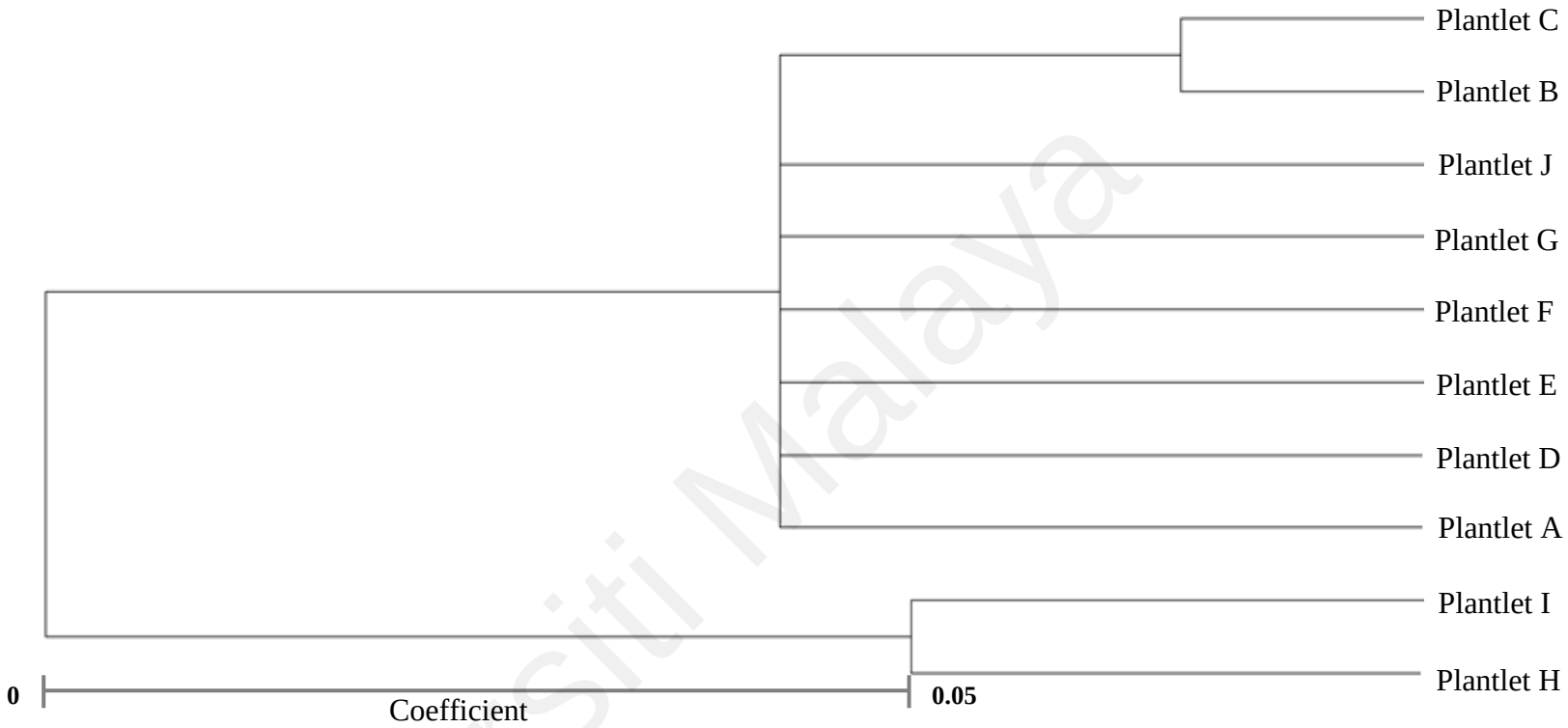

Figure 4.3: UPGMA dendogram generated from 10 randomly selected clonal plantlets.

4.2 GAMMA IRRADIATION

4.2.1 Morphology of Plantlets Following Gamma Irradiation

The clonal *Ananas* comosus var. MD2 plantlets were irradiated with gamma at a previously determined optimum radiation dosage of 400 Gray (Gy), while the control plantlets were not irradiated. The radiation dosage was determined based on the LD50 value (lethal dose for 50% of the plants tested, or radiation intensity that causes 50% mortality rate). Following exposure to gamma radiation, all samples (irradiated and non-irradiated) were monitored for 8 weeks and the morphological characteristics (plant height, leaf width, leaf colour and leaf variegation) of the plantlets were observed and recorded.

4.2.1.1 Morphology of *Ananas comosus* var. MD2 Plantlets (Control)

Figure 4.4: Morphology of the non-irradiated (control) *Ananas comosus* var. MD2 plantlet.

The morphology of non-irradiated (control) plantlets were observed and used as comparison with the irradiated plantlets. As shown in **Figure 4.4,** non-irradiated (control) *A. comosus* var. MD2 plantlets have a sword-like and bear sharp, up-curved leaves. The colors of the leaves are uniformly green and the leaf surfaces were waxy.

4.2.1.2 Morphology of Irradiated Plantlets Compared to Control

As indicated previously, the growth and morphology of the clonal plantlets were monitored for 8 weeks following gamma radiation treatment and were compared to the morphological characteristics of non-irradiated (control) plantlets. Based on data analysis, it was revealed that plantlets irradiated with gamma at 400 Gy showed a reduction of plantlet height compared to the non-irradiated plantlets (**Figure 4.5**). It was observed that the height of the non-irradiated (control) plantlets continuously increased during the 8 weeks of observation. However, plantlets irradiated with gamma at 400 Gy were observed to be stunted and showed significant height differences compared to non-irradiated (control) plantlets after 1, 2, 4 and 8 weeks of post-recovery periods. Nevertheless, the plant height of the irradiated samples also increased with recovery period (although at a much slower rate than the control plantlets), implying that the irradiated plantlets had undergone phenotype repair and were slowly recovering after exposure to gamma irradiation.

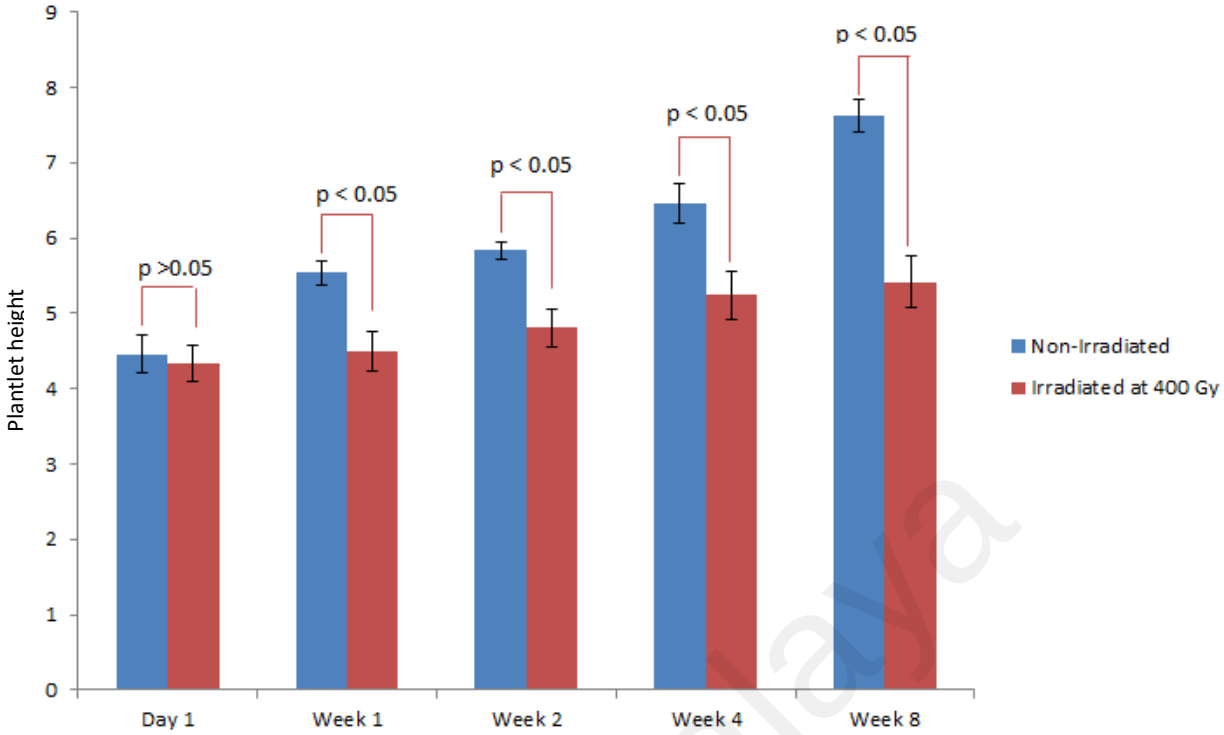

Figure 4.5: Plantlet height at five different post-recovery periods; at Day One, Week One, Week Two, Week Four and Week Eight after being irradiated with gamma at 400 Gy.

Other than plant height, the width of the leaves of irradiated and non-irradiated (control) plantlets were also measured and compared. Data analysis revealed that the leaf width of both irradiated and non-irradiated (control) plantlets were not significantly different ($p>0.05$, when observed at all post-recovery periods) and also increased with time (**Figure 4.6**).

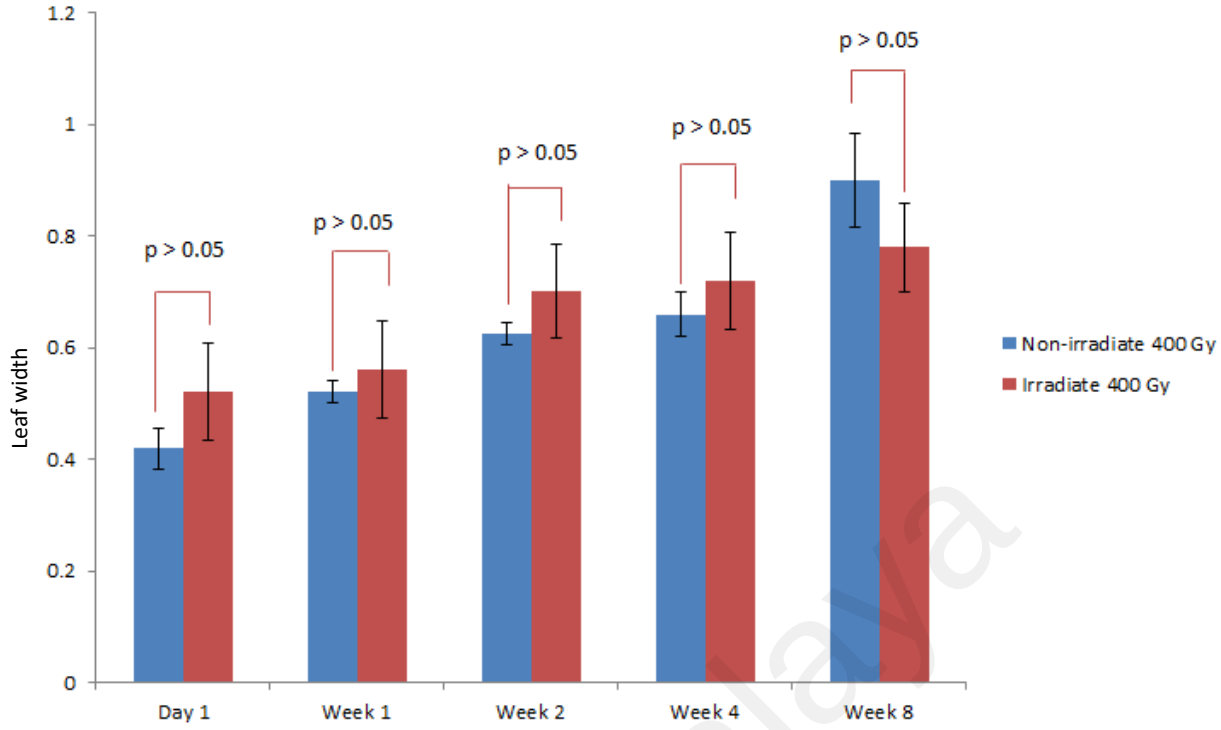

Figure 4.6: Leaf width at five different post-recovery periods; at Day One, Week One, Week Two, Week Four and Week Eight after being irradiated with gamma at 400 Gy.

Moreover, the leaf color and any occurrence of leaf variegation on the samples were also monitored for 8 weeks. **Figure 4.7** shows the gamma-irradiated plantlets after 2 weeks of post-recovery period. It was observed that at 2 weeks post-irradiation, some leaves of the gamma-irradiated plants started to undergo senescence and showed patterns of leaf variegation or differential chlorophyll distribution (**Figure 4.7a** and **4.7b**).

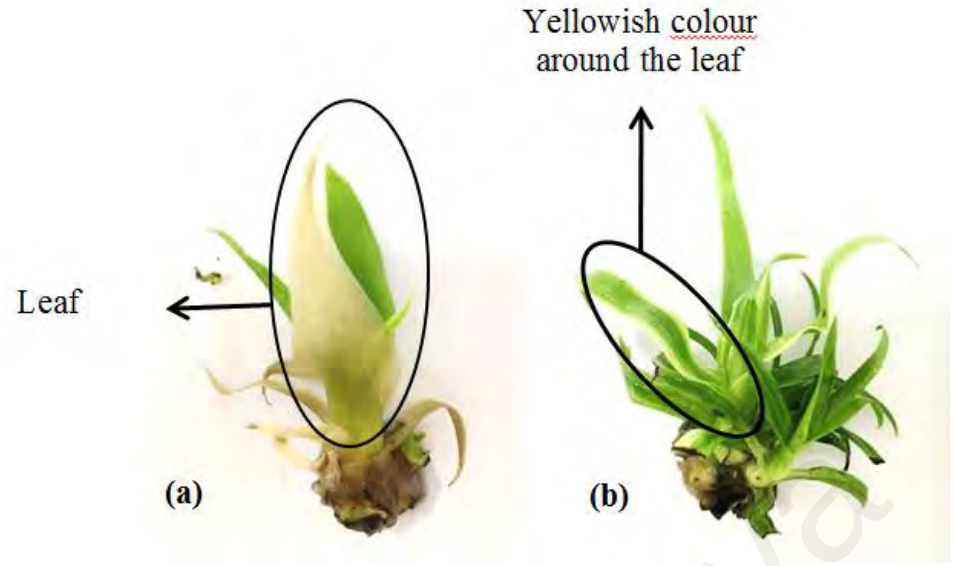

Figure 4.7: (a) Gamma-irradiated plantlet after 2 weeks of recovery period showing leaf senescence, (b) Gamma-irradiated plantlet after 2 weeks of recovery period showing leaf variegation.

4.2.2 ISSR Analysis of Gamma-Irradiated Plants Compared to Control

4.2.2.1 Gel Images Showing Polymorphic and Monomorphic Bands

Following gamma radiation treatment, the gamma-irradiated and non-irradiated (control) plantlets were let to recover for 8 weeks. The leaves of 10 randomly selected gamma-irradiated and non-irradiated (control) plantlets were harvested at each post-recovery period, used for DNA extraction and subjected to PCR analysis using 8 ISSR primers, as previously mentioned. The gel images were captured and used in bands scoring analysis, whereby the absence of a band was scored '0' and the presence of a band was scored '1'. These scores were then used to generate a binary character matrix and used to calculate the Jaccard's distances using Darwin 6.0 software. **Figures 4.8** and **4.9** show the gel images obtained from PCR reactions using ISSR primers UBC_840 and UBC_841.

Figure 4.8 shows the presence of polymorphic bands generated using ISSR primer UBC_840, from 10 randomly selected irradiated and non-irradiated (control) clonal *Ananas comosus* var. MD2 plantlets at various post-recovery periods (Day 1 until Week 8). As observed in the gel picture, a mean number of 12 scorable bands per primer were obtained, and this primer amplified a total of 667 bands from all samples. The size of the amplification fragments ranged from 400 bp to 2000 bp.

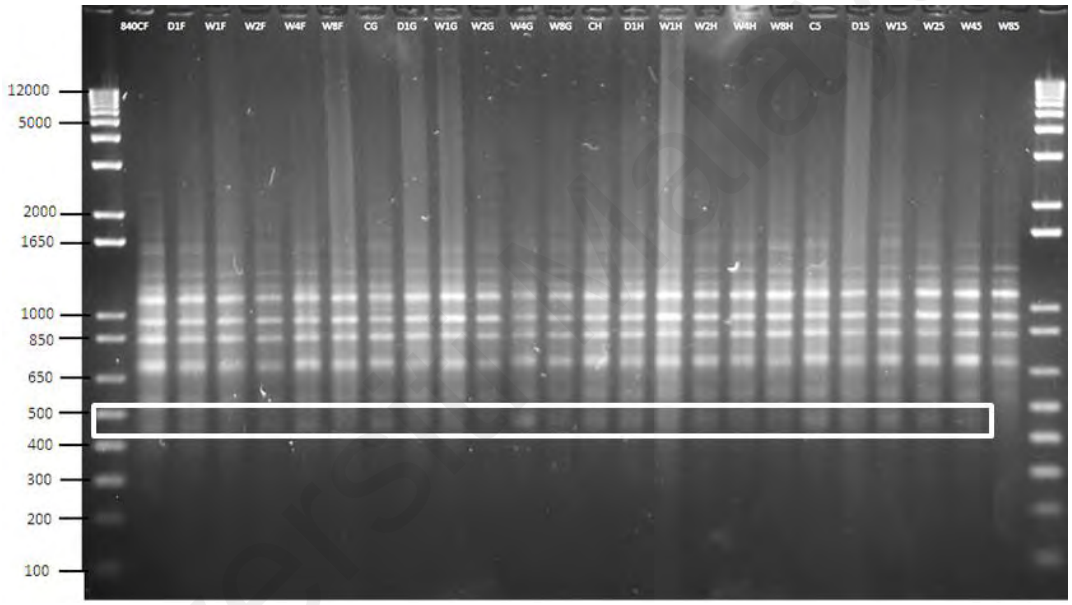

Figure 4.8: Gel image obtained using ISSR primer UBC_840, where the highlighted portion of the image shows the presence of polymorphic bands. C: Control plantlet, A-J: Plantlets A-J, D1: Day 1, W1-8: Week 1-8.

Figure 4.9 shows the presence of monomorphic bands generated using ISSR primer UBC_841, from 10 randomly selected irradiated and non-irradiated (control) clonal *Ananas comosus* var. MD2 plantlets at various post-recovery periods (Day 1 until Week 8). This primer generated 12 scorable bands, and amplified a total of 653 bands from all samples. The size of the amplification fragments ranged from 400 bp to 2000 bp.

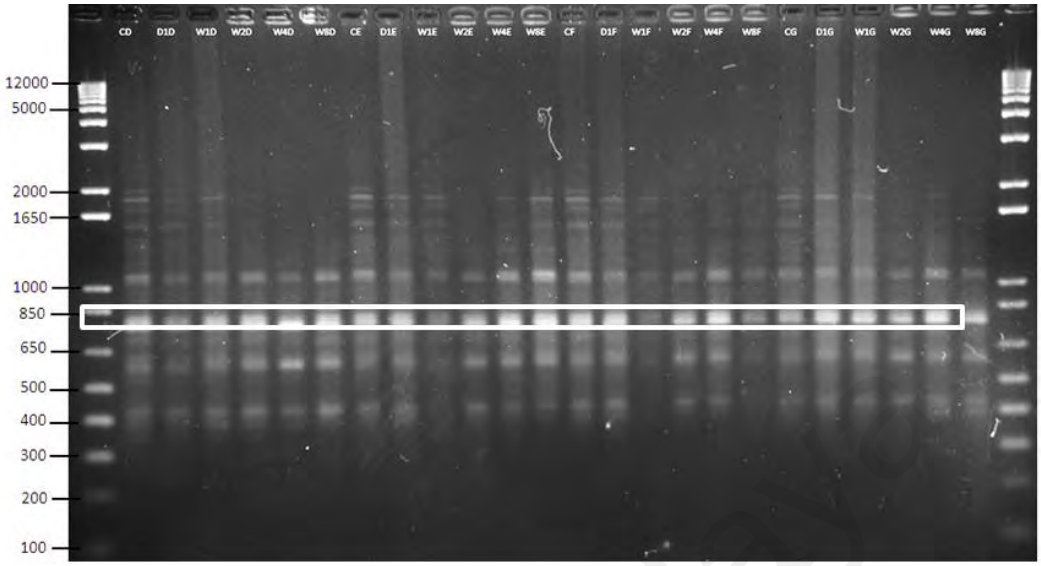

Figure 4.9: Gel image obtained using ISSR primer UBC_841, where the highlighted portion of the image shows the presence of monomorphic bands. C: Control plantlet, A-J: Plantlets A-J, D1: Day 1, W1-8: Week 1-8.

4.2.2.2 Summary of ISSR Analysis Results

Overall, a total of 4169 bands were amplified from the control and gamma-irradiated plants (Table 4.1). The size of the amplified fragments ranged from 400 to 5000 bp (Table 4.1). The mean number of scorable bands per primer varied from 7 to 13 (**Table 4.1**). The details of the DNA bands amplified for each of the ISSR primer were tabulated in Table 4.1:

Table 4.1: Primers used in ISSR polymorphism analysis, number and size of amplified fragments from 10 randomly selected gamma-irradiated and non-irradiated plantlets.

Primers code (UBC)	Sequence 5'-3'	Annealing temperature (°C)	Total number of bands amplified	Mean number of scorable bands per primer	Range of amplification (bp)
UBC_807	$(AG)_8T$	46.5	249	8	500-2000
UBC_809	$(AG)_8G$	48.0	300	8	500-1650
UBC_829	$(TG)_8C$	52.5	346	7	500-2000
UBC_834	$(AG)_8YT$	52.0	754	13	400-1650
UBC_840	$(GA)_8YT$	46.5	667	12	400-2000
UBC_841	$(GA)_8YC$	52.0	653	12	400-2000
UBC_855	$(AC)_8YT$	53.0	736	13	650-5000
UBC_856	$(AC)_8YA$	54.0	464	8	650-1650
Total			**4169**	**81**	

As shown in Table 4.2, both primers UBC_807 and UBC_809 generated the highest number of polymorphic bands, generating 67 polymorphic bands in total (83.75%) and 13 monomorphic bands. The size of amplification of fragments for primer UBC_807 ranged from 500 bp to 2000 bp, while the size of the amplification fragments for primer UBC_809 ranged from 500 bp to 1650 bp (**Table 4.1**). In contrast, primers UBC_834 only produced 17 polymorphic bands (13.08%) and 113 monomorphic bands in total, from 10 randomly selected gamma-irradiated and non-irradiated plantlets.

In this study, it was shown that all 8 ISSR primers were able to detect the occurrence of polymorphism in the samples. Out of 4169 amplified bands produced by all primers, the highest polymorphic bands were produced from primers UBC_807 and UBC_809, where an average of 6.7 out of the 8 scorable bands per primer was polymorphic. Those

primers were detected to be the most reproducible, as they resulted in the highest polymorphism between all samples tested.

Table 4.2: Total, mean number and frequency of monomorphic and polymorphic bands for each ISSR primer from 10 randomly selected gamma-irradiated and non-irradiated plantlets.

Primers code (UBC)	Mean number of scorable bands per primer	Total number and mean number of polymorphic bands per primer	Total number and mean number of monomorphic bands per primer	Frequency of polymorphic bands
UBC_807	8	67 (6.7%)	13 (1.3%)	83.75
UBC_809	8	67 (6.7%)	13 (1.3%)	83.75
UBC_829	7	42 (4.2%)	28 (2.8%)	60
UBC_834	13	17 (1.7%)	113 (11.3%)	13.08
UBC_840	12	23 (2.3%)	97 (9.7%)	19.17
UBC_841	12	39 (3.9%)	81 (8.1%)	32.5
UBC_855	13	34 (3.4%)	96 (9.6%)	26.15
UBC_856	8	14 (1.4%)	66 (6.6%)	17.5
Total	**81**	**303 (37.4%)**	**507 (62.6%)**	

4.2.3 Jaccard's Distance Analysis after Gamma Irradiation.

The binary character matrix generated from gamma-irradiated and non-irradiated (control) samples at various post-recovery periods were also used to calculate the Jaccard's distances using Darwin 6.0 software. The Jaccard's dissimilarity distance table between the clonal samples is as shown in **Figure 4.10**. As indicated previously, Jaccard's dissimilarity distance displays the level of dissimilarity between samples, whereby the genetic distance is provided in decimal forms, ranging between zero (0) indicating no genetic variability, to one (1) indicating the highest genetic variability.

Data analysis revealed that the genetic distance between all samples ranged from 0.0821 to 0.2073. As shown in **Figure 4.10**, the highest genetic distance (0.2073) was observed between the non-irradiated (control) plants and gamma-irradiated plants after 1 day to 2 weeks post-irradiation. Interestingly, the genetic distance between the control and irradiated plants was observed to decrease after 4 weeks of recovery, resulting in genetic distance of 0.1017.

	Control	Day 1	Week 1	Week 2	Week 4
Day 1	0.20727560811520				
Week 1	0.20727560811520	0.20727560811520			
Week 2	0.20727560811520	0.20727560811520	0.20727560811520		
Week 4	0.10167148419887	0.20727560811519	0.20727560811519	0.20727560811519	
Week 8	0.10167148419887	0.20727560811519	0.20727560811519	0.20727560811519	0.08211340206184

Figure 4.10: The Jaccard's dissimilarity distances between clonal *Ananas comosus* var. MD2 plantlets after gamma radiation treatment at different post-recovery periods, compared to control.

4.2.4 UPGMA Dendogram of Gamma-Irradiated and Non-Irradiated Samples at Various Post-Recovery Periods

The Jaccard's dissimilarity coefficients were also used to construct a UPGMA dendogram to show the genetic relationship between the samples at different post-recovery period. Based on the UPGMA dendogram shown in **Figure 4.11**, the samples were divided into three main clusters, with irradiated plantlets at post-recovery period of Week 4 and Week 8 being the closest to the control plantlets. On the other hand, irradiated plantlets at post-recovery period of Day 1 until Week 2 were grouped together and were the most distant from the control. This observation further supports earlier findings whereby exposure to gamma irradiation had caused a certain degree of mutation to occur, thus resulting in an increase in the genetic distance between the irradiated samples and the control, although they were all clonal in origin. However, plants are also capable of undergoing phenotype and DNA repair, and as indicated by the outcomes of this study, the genetic distances between the irradiated samples and the control were shown to reduce after 4 weeks of recovery.

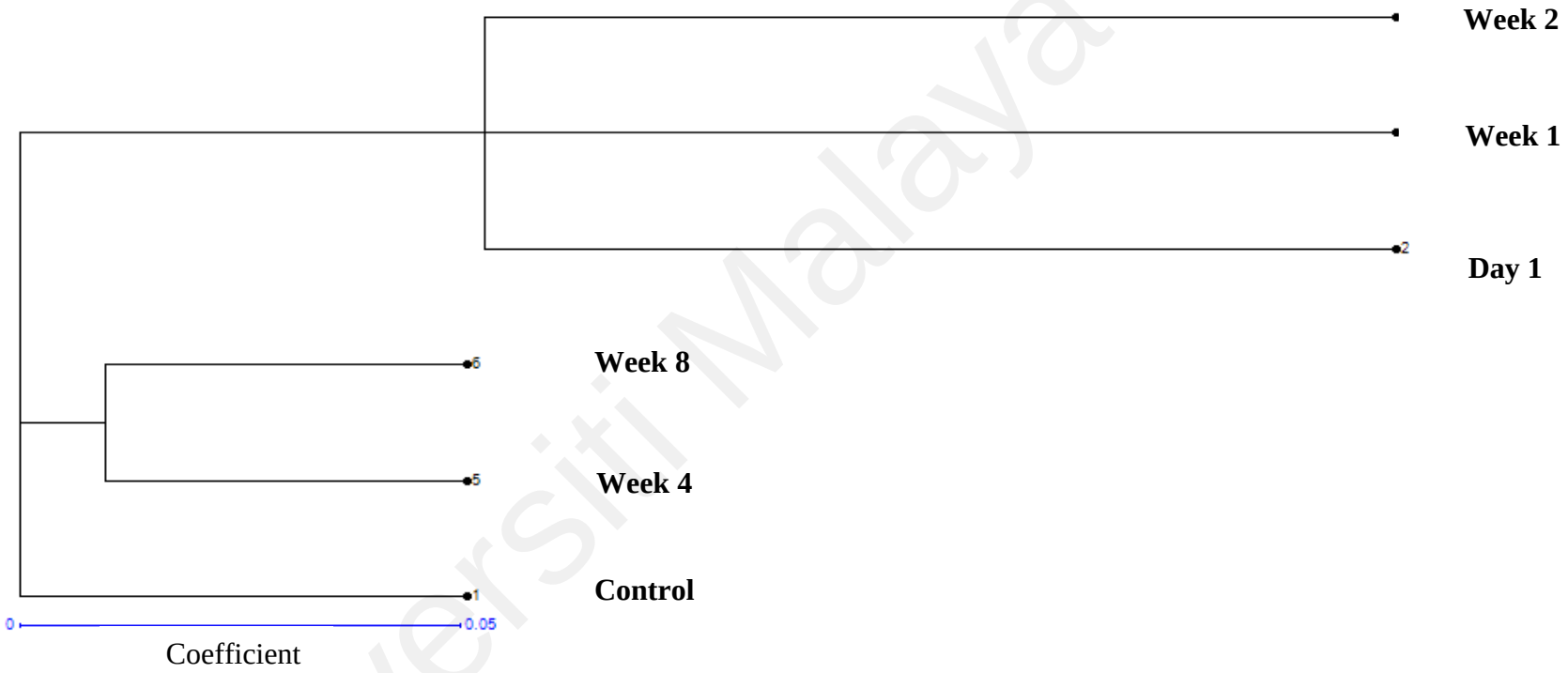

Figure 4.11: UPGMA dendogram generated from 10 randomly selected irradiated and non-irradiated (control) clonal plantlets at various post-recovery periods.

CHAPTER 5: DISCUSSION

5.1 EFFECT OF GAMMA IRRADIATION ON PLANTLET MORPHOLOGY

Gamma radiation is an example of ionizing radiation which had been used in crop improvement and generation of new cultivars. A reported study by Amjad & Anjum (2002), exposure at higher doses of gamma irradiation had led severe and drastic effects, and relatively lower doses often result in changed plant growth characteristic and genetic variability in plant genome. According to Amjad & Anjum (2002), the study in seedling growth of onion (*Allium cepa* L.) to investigate the effect of gamma radiation exposure showed a difference between irradiated and control samples. The study indicated that gamma irradiation at higher doses caused severe reduction in seedling length compared to lower doses of gamma exposure. A study by Al-Safadi & Simon (1996) reported also that the survival rate of carrot plants were observed to reduce almost 50% at higher doses compared to lower doses because of the delayed germination growth. The regeneration of carrot cultures inhibited after the exposure of the gamma radiation which damage the DNA structure and morphological characteristics such as root and shoot length (Al-Safadi & Simon, 1996).

Although plants immobile and cannot be moved under exposure of gamma irradiation, they have developed a mechanism for itself to survive after exposed under harsh or in a new environment which known as phenotypic plasticity. The plant characteristics can be maintained by repairing a damage and alteration in genetic composition (Sultan, 2003). However, the effects of exposure to gamma radiation on plant plasticity and plant's DNA damage repair has not been fully understood.

In this study, gamma radiation was used as a stress factor to induce DNA damage onto plants (clonal *Ananas comosus* var. MD2). The effect of gamma irradiation on the plantlets was monitored at timely intervals, at various post recovery periods (for 8 weeks). The morphology of the plantlets was observed and compared to the control plant (non-irradiated) and their genetic variability was also assessed by ISSR analysis.

Several morphological characters such as plantlet height, leaf width and leaf color were observed and recorded for 8 weeks following exposure to gamma irradiation. The height and leaf width of plantlets were tested using Student t-test to determine the difference of means between irradiated and control plantlets. The test compares the difference in means related to the variations occurred at various post-recovery periods. In this study, the test assumed that there is degree of correlation between the two measurements of both control and irradiated plantlets which indicate by the assumption of Student t-test (Cherry et al., 2012). The variation occured among both control and irradiated plantlets were observed at different post-recovery periods as plant recovers through time after damaged. Therefore, among these three morphological characters, the change in plantlet height was found to be the most prominent.

As observed in **Figure 4.5**, the height of the plantlets at Day 1 post-irradiation were not significantly different, as the starting samples (clonal plantlets) used in this experiment were of similar height. However, following exposure to gamma radiation, the height of the irradiated plantlets were observed to be significantly lower than the control (non-irradiated), as the exposure to gamma irradiation at 400 Gy had caused the plantlets to be stunted, but the plantlets continued to grow as they recover with time. Songsri et al. (2011) also reported similar observation in physic nut (*Jatropha curcas* L.), where the exposure to gamma radiation had caused the growth of irradiated plantlets to be stunted (Songsri et al., 2011).

According to Datta (2009), the reduction of plantlet height could be due to the damage in plant tissues which induced the inhibitory effect towards the exposed plants. Hence, it was observed that the irradiated plantlets become stunted when exposed to gamma radiation compared to the non-irradiated plantlets. This study also supported by previous study from El-Aishy et al. (1976), that gamma radiation caused interference with the growth of rice seedlings. The results showed increased number of stunted roots after exposure of gamma radiation at higher doses which inhibit the growth and had altered the biological system at genome level.

5.2 EFFECT OF GAMMA IRRADIATION ON GENETIC VARIABILITY

In this study, ISSR markers were used to detect any occurrence of genetic variability between the gamma-irradiated samples and the control plant. The degree of genetic variability at various post-recovery periods was also monitored. In this study, 8 of 20 ISSR primers tested had produced reproducible and satisfactory results, and were able to detect polymorphism in the samples. The clonal nature of the sample was initially confirmed through ISSR analysis (Figures 4.2 and 4.3). Then, the same ISSR markers were subsequently used to determine the genetic variability between the irradiated plantlets with the control (non-irradiated).

As mentioned in previous chapter, the Jaccard's dissimilarity distance table was generated based on the data matrix obtained from the ISSR analysis. The outcome of this study revealed that the highest genetic distance (0.2073) was observed between the non-irradiated (control) plants and gamma-irradiated plants after 1 day to 2 weeks post-irradiation (Figure 4.10). Interestingly, the genetic distance between the control and irradiated plants was observed to decrease after 4 weeks of recovery, resulting in genetic distance of 0.1017. The UPGMA dendogram also revealed similar findings, where the

irradiated plantlets at post-recovery period of Day 1 until Week 2 were grouped together and were the most distant from the control (Figure 4.11).

This observation further supports earlier findings whereby exposure to gamma irradiation had caused a certain degree of mutation to occur, thus resulting in an increase in the genetic distance between the irradiated samples and the control, although they were all clonal in origin. A study conducted by Al-Safadi & Simon (1996) to determine the genetic variation after gamma radiation exposure in carrot (*Daucus carota* L.) cultures was showed a presence of chromosomal abnormalities between irradiated cultures. The experiment showed that there is variation in irradiated cultures; relatively control cultures have no significant variation which indicated that gamma irradiation was proved to cause variation in carrot cultures. Therefore, it was concluded that gamma irradiation had caused changes in plants characteristics and genetic composition in plant genome.

However, plants are also capable of undergoing phenotype and DNA repair, and as indicated by the outcomes of this study, the genetic distances between the irradiated samples and the control were shown to reduce after 4 weeks of recovery. The plants were gifted as they recover itself in an interval of time to survive by repairing the damage via DNA repair mechanisms such as photoreactivation, Nucleotide Excision Repair (NER), Base Excision Repair (BER) and Mismatch Repair (MMR) (Kimura & Sakaguchi, 2006). A study reported by Gichner et al. (2000), to determine DNA repair in tobacco seedlings after gamma radiation showed the damage in tobacco leaf was repaired by DNA repair mechanism after four weeks period of recovery. This is because, the result showed reduction of damage in leaf after a recovery period (Gichner et al., 2000). As well as a study by Kariuki et al. (2019), they found that the effect of gamma radiation exposure in rice (*Oryza sativa* L.) seedlings were observed to recover

after two weeks which indicate that plants are able to revive itself from ionizing radiation. Therefore, it was showed that plants were well evolved and capable to recover itself through time to survive in new environment.

5.3 BENEFITS ISSR MARKERS

According to Vijayan (2005), ISSR is one of the quickest marker systems with high reproducibility, which could detect more polymorphism than other markers such as mtDNA, cpDNA and RAPD in a closely related plant group. ISSR primers could be used to detect the misses of sequence repeat and deletion or insertion of one genome which can modify the distance between the repeats, resulting in polymorphisms in DNA fragments. In recent years, ISSR markers have been widely used by researchers to assess plant's genetic stability, which determines the presence of genetic variation in plant genome. Besides, ISSR markers had also been widely used to assess the genetic fidelity of plants produced through tissue culture system, in order to observe the variation in genetic composition.

In a previous study by Huang et al. (2009), ISSR markers had been successfully used to determine the genetic stability iof *Platanus acerifolia*. In the study, 86 out of 103 generated bands were polymorphic. From this result, it is proven that the ISSR markers can generate higher polymorphism compared to other markers as it can produce a high degree of sensitivity for detecting genetic variability (Huang et al., 2009). Besides that, ISSR primers had also been used in genetic diversity assessments of many plant species such as *Ananas comosus* (L.) Merr (Popluechai et al., 2007; Vanijaviva, 2012) and mulberry (Reddy et al., 2002b).

Besides that, ISSR markers can also be used to locate and isolate the presence of mutation for genetic discrimination purposes (Jin et al., 2008). ISSR analysis is

beneficial for detecting polymorphism in living cellular organisms, as it is able to locate the variation in DNA fragments. In a study conducted by Rathore et al. (2011), ISSR markers was found to be a reliable method that enables rapid evaluation of occurrence of somaclonal variation between samples, by fast scanning of the whole genome. Moreover, according to Viehmannova et al. (2014), ISSR marks had also been successfully used to reveal somaclonal variation in yacon *(Smallanthus sonchifolius),* thus can aid in yacon improvement, especially when low sexual reproductive capacity inhibits classical ways of breeding.

CHAPTER 6: CONCLUSION

In this study, it can be concluded that there is genetic variation between clonal *Ananas comosus* var. MD2 and control plantlets after exposure at different post-recovery period. Based on the results in Jaccard's dissimilarity, the genetic distances showed a high variability of genetic composition between clonal and control plantlets.

The result indicated that exposure to gamma irradiation had caused mutation to occur at a certain degree which resulting a genetic distance to be varied between irradiated samples and the control.

However, it was also evidenced that the plants were capable undergoes phenotype and DNA repair after exposure to gamma irradiation based on the result in Jaccard's distance at different post-recovery period from Day 1 until Week 8. The genetic distance between the control and irradiated plants was observed to decrease after 4 weeks of recovery.

Lastly, our findings also proved that ISSR marker was the quickest marker systems with high reproducibility of polymorphisms which can detect the presence of genetic variation in the plant genome. ISSR marker can be used by researchers as an effective marker tool to assess a plant's genetic stability and fidelity to observe the variation in genetic composition.

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