EVALUATION OF GLUTATHIONE DISULPHIDE ON OIL PALM SEEDLINGS (*Elaeis guineensis*) AT NURSERY STAGE

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by

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
ANOVA	Analysis of Variance
CRD	Completely Randomised Design
DNA	Deoxyribonucleic acid
DPPH	Diphenyl-2-picrylhydrazyl
FRAP	Ferric reducing antioxidant power
FBA	Fructose-1,6-biphosphatase
GSH	Glutathione
GSSG	Glutathione disulphide
GLS	Glutathione synthetase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HA	Humic acid
OD	Optical density
OTU	Operational Taxanomic Unit
PCS	Phytochelatin synthase
rDNA	Ribosomal DNA
SPSS	Statistic Package for Social Science
TE	Trace element
XRF	X-ray fluorescence
γ-ECS	γ-glutamylcysteine ligase

LIST OF UNITS AND SYMBOLS

cm	Centimeter
g	Gram
h	Hour
mL	Milliliter
mM	Millimolar
min	Minute
μL	Microliter
mg	Milligram
%	Percentage
s	Second
V	Volume
°C	Degree Celcius
p	Alpha value
L	Liter
Psi	Pounds per square inch
rpm	Rounds per minute
μM	Micromolar
π	Pi value = 3.146

PENILAIAN KESAN GLUTATION TEROKSIDA TERHADAP

ANAK BENIH KELAPA SAWIT PADA

PERINGKAT TAPAK SEMAIAN

ABSTRAK

Dalam industri kelapa sawit, peningkatan produktiviti tanpa membuka tanah baru merupakan suatu keutamaan penting bagi syarikat besar dan pekebun kecil. Glutation disulfida (GSSG) mempunyai potensi untuk menggalakkan pertumbuhan tumbuhan dan meningkatkan produktiviti buah sawit. Kajian ini dijalankan bagi menilai kesan GSSG pada pertumbuhan anak benih kelapa sawit di bawah persekitaran tapak semaian dan rumah hijau. Anak benih tersebut telah diuji dengan dua bentuk GSSG iaitu bentuk cecair dan berbutir. Selepas 120 hari pertumbuhan di dalam rumah hijau, anak benih dituai dan biojisim kering, kandungan klorofil dan nutrient ditentukan. Aktiviti antioksidan anak benih telah dibandingkan dengan tiga ujian berbeza iaitu 'DPPH', 'FRAP' dan asai pengkelatan logam. Kesemua data telah dianalisa secara statistik. Genom DNA diekstrak daripada tanah dan penjujukan metagenomik 16S rDNA juga dijalankan untuk menentukan kepelbagaian bakteria dalam tanah kelapa sawit. Analisis statistik menunjukkan bahawa biojisim kering dan isipadu akar anak benih sawit telah meningkat sebanyak 108% dan 140% selepas dirawat dengan cecair GSSG. Anak benih yang dirawat dengan cecair GSSG juga menunjukkan pertumbuhan daun yang lebih besar dan peningkatan ketinggian. Selain itu, aktiviti DPPH adalah lebih tinggi bagi benih yang dirawat GSSG (6.36 mg/mL – 8.04 mg/mL) berbanding dengan benih kawalan (10.64 mg/mL). Analisis metagenomik 16S rDNA mendedahkan kepelbagaian kumpulan bakteria di dalam tanah dan *Proteobacteria* serta *Firmicutes* merupakan kumpulan

terbesar di dalam tanah untuk semua jenis rawatan. Keputusan eksperimen mencadangkan bahawa GSSG mempunyai potensi untuk digunakan di ladang kepala sawit.

EVALUATION OF GLUTATHIONE DISULPHIDE ON OIL PALM SEEDLINGS (*Elaeis guineensis*) AT NURSERY STAGE

ABSTRACT

In the oil palm industry, increasing yield productivity without further land expansion is an important priority for big companies and smallholders. Glutathione disulphide (GSSG) has the potential to promote seedling growth and increase palm fruit productivity. This study was conducted to evaluate the effect of GSSG on the growth of oil palm seedlings grown under nursery and greenhouse conditions. The seedlings were tested with two forms of GSSG that were liquid and granules. After 120 days of growth in the greenhouse, seedlings were harvested and determined for dry weight, chlorophyll content and nutrients content. The antioxidant activities of the seedlings were compared with three different assays namely DPPH, FRAP and metal chelating assay. All parameters measured were then statistically analyzed. Genomic DNA was also extracted from the soil of palm oil and 16S rDNA metagenomic sequencing was carried out to determine bacterial abundance between GSSG-treated and control soil. The results revealed that the top biomass and root volume in oil palm seedlings was effectively enhanced up to 108% and 140% respectively due to exogenous application of GSSG solution. Bigger leaves and height increase was observed for GSSG solution-treated seedlings compared to the control. Besides, DPPH scavenging activity was significantly higher in GSSG-treated seedlings (6.36 mg/mL – 8.04 mg/mL) than the control seedlings (10.64 mg/mL). The 16S rDNA metagenomic analysis revealed diversity of bacterial communities in the soil. Both *Proteobacteria* and *Firmicutes* accounted for the biggest

abundance of between 30% and 60% in the soil for all the treatments observed. The results presented here suggested that GSSG have the potential to be utilized and applied at oil palm nurseries.

CHAPTER I INTRODUCTION

1.1 Introduction

Malaysia is one the world's biggest exporter of palm oil. In 2012 alone, Malaysia's palm oil exports account for about 30% of worldwide palm oil production. Palm oil is mainly processed as cooking oil for food industry. Besides, the oil is also utilized to produce oleochemical fuel and other non-edible products such as plywood, cosmetics and paper. Nowadays, increase in population growth and economic development drives the demand for palm oil higher than before. As the result, there is a need to increase the production of oil from the oil palm seedlingations. Stepping up the oil yield production of commercial oil palms is the biggest challenge in the industry. Genetic hybridization, molecular cloning and application of new compound fertilizers are some of the methods to increase oil yield but little have so far produce convincing results.

Glutathione (GSG), a non-protein thiol compound is found in most organisms ranging from multicellular organisms such as seedlings and animals to unicellular organisms like bacteria or protozoa. The compound is made up of three amino acids namely glycine, glutamic acid and cysteine linked by covalent bond. The bond between glutamic acid and cysteine is assumed to offer stability to the molecule and allow it to undergo degradation by specific enzymes.

Compared to animals, the role of glutathione in seedlings is not so extensively studied. It is known that the compound play some important functions including roles in growth development, biosynthetic pathways, detoxification, antioxidant reaction and redox homeostasis. The earliest recognition of glutathione's function is the thiol-disulphide exchange that acts as an antioxidant barrier to remove harmful oxygen

radicals in the cells. Redox reaction will convert glutathione into oxidized forms that include disulfides. For example, glutathione can link with another glutathione residue to form glutathione disulphide (GSSG) when oxidized. Since the presence of glutathione had also been discovered to affect seedling development, researchers have great interest to know whether increasing the content of glutathione or glutathione disulphide in the seedlings will enhance the growth of seedlings or not. Initial studies (data not reported) had shown that glutathione disulphide exhibit growth promoting effect and applying this compound to seedlings such as ornamental seedlings and fruit bearing seedlings will lead to an increase of yield and size of fruits being produced.

All the initial studies represent efforts to support the idea that glutathione disulphide possess growth promoting effect on seedlings. None, however, have been reported to study the growth promoting effect of GSSG on oil palms.

1.2 Objective

Thus, the objectives of this study were:

1. To evaluate the growth of oil palm seedlings at nursery stage with and without GSSG.
2. To optimize the application method of GSSG at different concentration on the oil palm seedlings.
3. To evaluate the antioxidant potential between GSSG treated and control seedlings.
4. To study the effect of GSSG on oil palm soil microbe diversity through DNA metagenomic approach.

CHAPTER II LITERATURE REVIEW

2.1 Oil Palm Ecology and Uses

Oil palm is a monoecious seedling under the Arecaceae family. This family includes other palm trees such as coconut, date and rattan. There are two species of oil palm; *Elaeis guineensis* and *Elaeis oleifera* (Obahiagbon, 2012). Unlike *Elaeis oleifera* which is only exploited locally, *Elaeis guineensis* is seedlinged commercially and have become the principal source of palm oil worldwide. Recently, archaeological evidence had proven the origin of oil palm from West Africa with the discovery of jar containing palm oil from an Egyptian tomb in Abydos. The jar dates back to 3000 BC. It was believed that Arab traders were the ones selling palm oil between Gulf of Guinea and ancient Egypt and this were about 5000 years before the palm oil starts to commercialize globally (Sowunmi, 1999; Kiple, 2001; Obahiagbon, 2012).

Oil palms can grow up to 30 metres in height and take 3 to 4 years before the fruits can be harvested. Surprisingly, oil palm is in fact a giant grass and not a tree (Rival and Levang, 2014). Being an equatorial seedling, oil palms requires high temperature of between 24˚C to 28˚C and adequate rainfall requirement of about 2000 mm and more per year (Obahiagbon, 2012).

Both species of oil palm (*Elaeis guineensis* & *Elaeis oleifera*) are seedlinged for its oil. The oil is extracted from the fruits of oil palm (Figure 2.1). The fruit contains two types of oil that are found in different parts of the fruit. The main product is the oil that accumulates in the mesocarp of the fruit which is also commonly known as crude palm oil or mesocarp oil. Mesocarp oil contains 49% of saturated fatty acids (composed mainly of palmitic acid and stearic acid) and another 49% unsaturated fatty acids (oleic acid and linoleic acid) (Hilditch, 1949; Rival and Levang, 2014). The

remaining 2% are minute quantities of myristic and lauric acid. The other type of oil is found in the hard seed of the fruit or kernel. Often known as palm kernel oil (PKO), this oil has a different fatty acid composition. A large proportion of the kernel oil (82%) consists of saturated fatty acids. Another 18% of the oil is unsaturated (Hilditch, 1949; Rival and Levang, 2014). Palm kernel oil makes up about 10% of the total oil fraction of palm oil (Murphy, 2014).

Figure 2.1 Cross sections showing the internal structure of oil palm fruits. The mesocarp provides the palm mesocarp oil and the kernel provides palm kernel oil after harvesting.

Both mesocarp and kernel oil have been used in many ways (Figure 2.2). In food sector, the oil is utilized as cooking or frying oil, margarines and other types of food preparation. This accounts for 80% from the total palm oil usage. About 19% of the oil usage is in non-food sector. For example, the kernel oil which is enriched with saturated fatty acids is used in the production of oleochemical such as cosmetics, soaps, surfactants and agrochemicals. Besides, a small percentage (about 1%) of the total oil is used to produce biofuel (Murphy, 2014; Rival and Levang, 2014). Since year 2000, more and more palm oil has been processed into methyl ester compounds to produce biodiesel for vehicles (Rosillo-Calle *et al.*, 2009). The oil palm seedling

itself can also be used to produce biomaterials such as papers and thin wood (Sulaiman *et al.*, 2012).

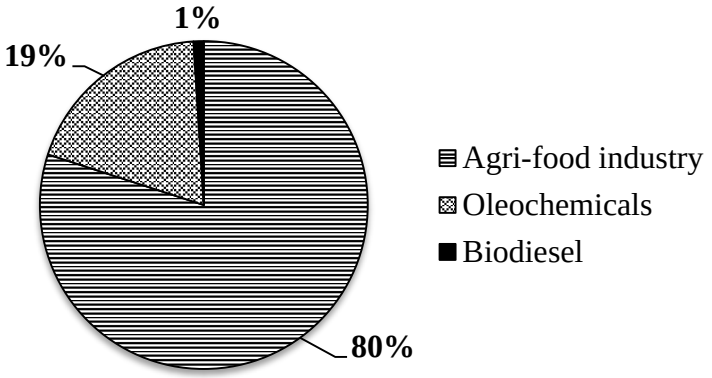

Figure 2.2 Usage of palm oil in different sectors (Rival and Levang, 2014).

The usage of palm oil as vegetable oil continues to grow and has overtaken soybean number one spot in terms of vegetable oil production. Compared to other oil producing crops such as soybean, rapeseed and sunflower, oil palm yields the highest production of vegetable oils whereby it accounts nearly a third of the oil produced globally (Figure 2.3) (Murphy, 2014; Rival and Levang, 2014). This is because oil palm produces exceptional high amount of oil yield of about 4 tonnes per hectare as average compared to other crops.

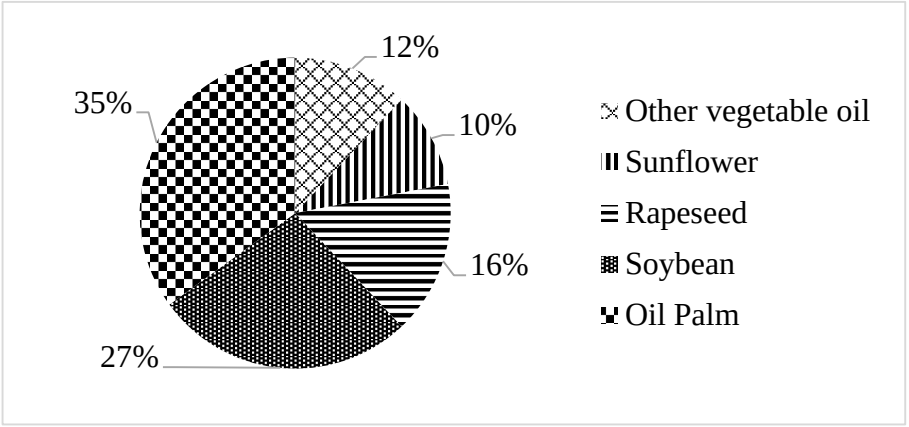

Figure 2.3 Usage of oil producing crops in vegetable oil production (FAOSTAT, 2015).

2.2 Oil Palm Industry

In the early 20th century, oil palm from Africa continent was brought into Peninsular Malaysia and Sumatra during the British colonization era. Oil palm was first seedlinged as ornamental seedlings in Malaysia during the British occupation. After independence, Malaysian government took the approach to convert old rubber seedlingation into oil palm seedlingation leading to an increase in commercial cultivation of oil palm (Rival and Levang, 2014). From 1960s to 2005, Malaysia is the largest palm oil producer before it was overtaken by Indonesia. By 2015, both Malaysia and Indonesia produced about 53 million tonnes of oil accounting about 85% of the world palm oil production (Figure 2.4).

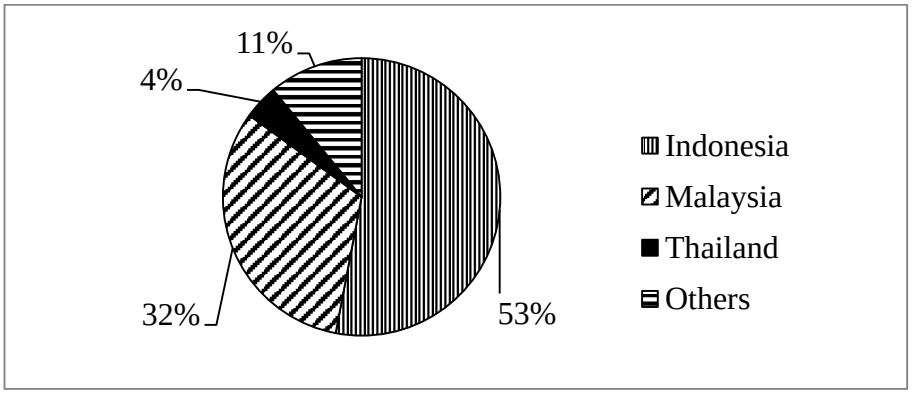

Figure 2.4 Palm oil producing countries (IndexMundi, 2003).

The main reason behind the success of oil palm in establishing itself as a major global vegetable oil are population growth and economic development in developing countries (Murphy, 2014). These two factors spur the demand for palm oil. Countries such as China and India are among the main importers for palm oil due to the reason that the population in these countries are increasing rapidly. This generally results in higher demand for food products like vegetable oil. However, demographic factor is

only the small reason behind the increase of demand in palm oil as population increase has now starts to reach a plateau especially in China (Murphy, 2014).

This brings us to the role of economic development as the main driver for higher demand of palm oil around the world. The use of fats during cooking greatly enhance the taste and flavour of the food compared to non-fatty food. This is because during heating, volatile fats and oils become solubilise and the mixture produce complex and attractive flavour compounds. As the result, humans tend to crave lipidic food such as food cooked with vegetable oil (Murphy, 2007).

Murphy (2007) reported that in 1990s, vegetable oil consumption increases in several countries such as China (64%), Indonesia (65%) and India (94%). It was also found that the income level in these developing countries also rises at that time. As people have more money, they tend to consume foods that contain more fats and oils and the reverse of this aspect is also interestingly true (Murphy, 2007). For example, during the fall of Soviet Union, the demand for edible oil decreases by 35% due to economic meltdown.

In Malaysia, oil palm industry is one of the significant contributors to Malaysia's economy. By 2011, this sector had contributed RM 53 billion to the country's Gross National Income (GNI) and became the fourth largest contributor to Malaysia's economy (Malaysian Palm Oil Board, 2010). The development of this industry had created a new dimension in the economy field by pumping income to the country. The excitement is even higher when this industry is utilized as a tool to reduce poverty in the country. In the 1970s, poverty among agricultural farmers recorded the highest rate of 68.3% and the introduction of planting oil palm by the Malaysian government arrived at the right time (Simeh and Ahmad, 2001). Through rural development

agencies established by the government such as Federal Land Development Authority (FELDA) poor landless people are given support and loans to open oil palm estate. Besides, the government built new infrastructures such as house, clinic, community hall and road to facilitate their life. When the oil palms have matured, these smallholders can pay back the loans by selling off revenue. As such, the government has used oil palm to inject a different future for these smallholders.

As the conclusion, oil palm industry in Malaysia will continue to assume a significant role in the country's development. New socio-economy's opportunities arises from the demand of palm oil will make sure the journey is equally exciting for both industry and academia sector related to oil palm.

2.3 Oil Palm Nursery

The production of high quality D×P seeds is the main objective of seed breeding programme in the past few decades. These seeds are usually raised in nursery with the primary aim to sustain its large potential of oil yield for 25 years and more. Oil palm seedlings are typically seedlinged in the nursery for about 12 months to 18 months before it is seedlinged in the outside field. Even though the nursery stage is quite short considering the long living period of oil palm, it is important to know that good nursery management would be essential to produce healthy seedlings in order to get early fruiting and high yield in bigger seedlingations.

Theoretically, for oil palm planting, nursery stage can omitted by planting the seeds directly into the field. However, this practice is not practical because seedlingers cannot provide enough care to the young seedlings in a large area especially in terms of pest control. Direct seeding into the field also does not allow seedlingers to do culling of abnormal seedlings. Moreover, young oil palm seedlings are expected to have low living rate if seedlinged directly into the field.

An oil palm nursery can be of single stage or double stage nursery. In a single stage nursery system, the germinated seedlings are seedlinged directly into soil-filled large polybags (46 cm × 41 cm) and cared in the same polybags until the seedlings are sufficiently old to be transferred into the open field. This usually takes about 12-15 months. On the other hand, in a double stage nursery system, the seedlings are first seedlinged in soil-filled small polybags (23 cm × 15 cm) for the first four months. Later, the seedlings will be transferred into larger polybags for another eight to eleven months before transplanting into outside field (Heriansyah and Tan, 2005). Some oil palm companies such as IOI have replace the use of small polybags with plastic trays

as more seedlings can be seedlinged in to save space (Mathews and Chong, 2007; Mathews *et al.,* 2010). However, due to smaller growth area in the plastic tray, the growth of the roots are restricted compared to polybags causing the seedlings to grow smaller in size. In fact, Mathews et al. (2010) had reported that the roots of the seedlings became harder when grown in plastic trays. Hence, the use of small polybags are still popular among the seedlingers till now.

The practices of oil palm nursery covers a wide range of aspects such as distance between seedlings, sunshine availability, soil media, watering, nutritional requirement of the seedlings and control of pests and diseases. Only a few of these aspects will be reviewed here.

2.3.1 Oil palm seedlings

In Malaysia, the oil palm seedlings seedlinged in the nursery are mainly the *tenera* variety, which is a hybrid between the *dura* and *pisifera*. The seeds are usually produced by companies such as Felda Agricultural Services Sdn. Bhd. which obtain certification from Malaysia Palm Oil Board (MPOB) and Scientific and Industrial Research Institute of Malaysia (SIRIM). A hybrid seedling is a cross product between two different types of parental source. For oil palm, *Dura* fruits is the mother palm and *Pisifera* is the father palm. *Dura* fruits has a thick shell gene and produce a thick kernel shell. On the other hand, *Pisifera* has shell-less gene and do not a kernel shell. When these two varieties cross, *Tenera* hybrid is produced which has thin kernel shell and contain 30 % more oil than *Dura* and *Pisifera* fruits (Woittiez *et al*., 2016). Kok et al. (2011) also reported that *Tenera* fruits have higher fat and fiber content in the kernel compared to clonal fruits derived from it.

2.3.2 Planting media

The soil content in the planting media is important to nurture healthy seedlings throughout the nursery stage. The planting media should contain 30 to 60% of sand, 25 to 45% of clay, free from any chemical contamination and disease inoculum. Too much sand content in the planting media can cause growth retardation of the seedlings due to low water retention. As the results, the seedlings will not have enough access to water. In an experiment conducted by Mathews et al. (2010) using plastic tray, they reported that the growth of seedlings become retarded when the sand content is too high.

Besides, according to FELDA, peat soil is not suitable as the planting media for oil palm seedlings in the nursery stage as it can cause growth retardation and seedlings chlorosis. This is due to the lack of cuprum and iron element in the peat soil.

2.3.3 Nutrients requirement

Oil palm needs a lot of nutrients and this fact is not surprising due to its high dry biomass production. In the nursery, adequate fertilization is required to provide enough nutrients to the seedlings for healthy growth development. Fertilizers are only added when the first leaf of the seedling is fully developed that is about 1 month after planting. This is to help the developing seedlings when at that time the endosperm can no longer provide enough nourishments (Lucas *et al*., 1979).

Along with other photosynthesizing seedlings, oil palm has the same nutrient requirements such as nitrogen, phosphorus, potassium and other secondary macronutrients (sulphur, calcium and magnesium) as well as micronutrients. In the nursery, NPK is usually provided to the seedlings as inorganic composites and the

exact ratio of N, P and K provided actually depends on the growth stage and development of the seedlings. According to FELDA fertilization recommendation for oil palm seedlings in the nursery stage, in the first six months after planting, the seedlings were provided with nitrogen (N), phosphorus (P), potassium (K) and magnesium (Mg) at a ratio of 15:15:6:4. After the 6^{th} month until the end of nursery stage, NPK(Mg) were provided to the seedlings at ratio of 12:12:17:2 (Goh *et al*., 1999)

During the first six months after planting, nitrogen and phosphorus were added at a higher ratio to facilitate active growth of the seedlings. The seedlings require more nitrogen to produce new leaves and phosphorus to promote new root development. As the seedling gets older (after the sixth month), higher ratio of potassium is provided for the seedlings because potassium is needed to produce more fruit clusters and makes them bigger (Goh *et al*., 1999)

2.4 Challenges in oil palm nursery

The prime aim of these nurseries husbandries as described before is to produce high quality seedlings for planting in the outside field. However, before achieving this aim, there are still few aspects that remain as major concerns in the oil palm industry.

Firstly is the high labour requirement. Labour force is basically required for all activities concerning the nursery husbandries. For example, culling, watering, soil filling, fertilization, weeding and control of pest and diseases. Availability of migrant workers in Malaysia is declining. This is because many oil palm companies tend to open up more seedlingations instead in neighboring countries like Indonesia due to more land space (Mathews *et al.*, 2010).

Secondly is pest attack and disease infections. One of the major pests in the nursery is red spider mite which attacks oil palm seedling and cause patches of green-yellow areas on the leaves. There are many types of diseases affecting the seedlings and the most common one is seedling blight caused by fungus *Curvularia eragrostidis*. These pest and disease infection can be controlled by application herbicide and fungicide. However, there is a risk that after treatment, the seedlings will experience leaf abnormalities such as collante and crinkled leaf (Mathews *et al.*, 2010).

Lastly is the cost of fertilizers used. Oil palm industry rely heavily on the usage of inorganic fertilizers to produce high yielding fruits. Surprisingly, fertilizers cover nearly half of the total production cost. Studies were actively carried out recently to reduce the usage of fertilizers without compromising the production of good fruits with high oil yield. For example, the substitution of inorganic fertilizers with organic material such as palm sludge waste (POME) and palm trunk manure.

2.5 Glutathione (GSH)

In the past decades, GSH has received much attention from researchers due to their biological importance in reactions to remove radical and harmful species from the cells, making them as an essential part of the antioxidant system in seedlings and animals. Glutathione is a thiol tripeptide found in various organisms. It is made up of three amino acids that are glycine, cysteine and glutamic acid (Figure 2.5) (Lushchak, 2012). The peptide bond that links the γ-carboxyl group of glutamate and amino group of cysteine confers stability to this molecule because intracellular peptidase can generally only cleave peptide bonds between α-carboxyl groups of amino acids (Noctor *et al.*, 2011; Lushchak, 2012). Thus, this bond distinguishes it from the other peptide bonds normally found in proteins.

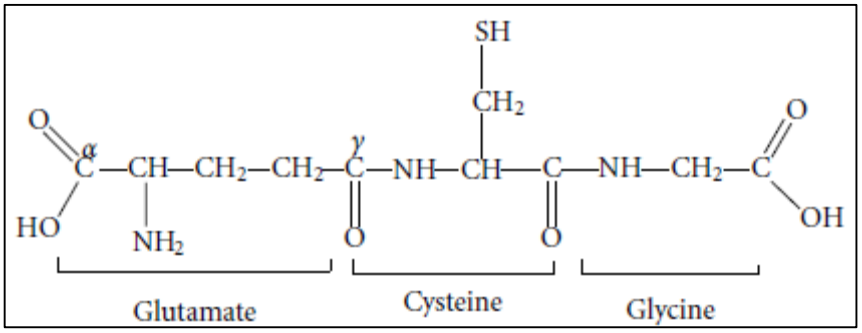

Figure 2.5 Structure of glutathione (GSH) (Lushchak, 2012).

GSH is found in seedlings and animals under millimolar concentrations typically ranging from 0.5 to 10 mM (Maher, 2005; Lushchak, 2012). Despite the fact that it exists in a very small amount, GSH still remains the most abundant thiol in both animals and seedlings. For seedlings, instead of glycine, the tripeptide contains other C-terminal amino acids giving rise to different homologues of glutathione in certain taxa. Examples of amino acids are serine, β-alanine and are glutamate (Klapheck, 1988; Klapheck *et al.*, 1992; Meuwly *et al.*, 1993). Homoglutathione (Glu-Cys-β-Ala)

found in legume seedlings have the same functions as GSH but the two homologs are in fact encoded by different genes in the genome (Macnicol, 1987; Frendo *et al.*, 2001).

The major oxidized form of glutathione is known as glutathione disulphide (GSSG) (Figure 2.6). Two resides of GSH is oxidized and connected by intermolecular disulpide bond.

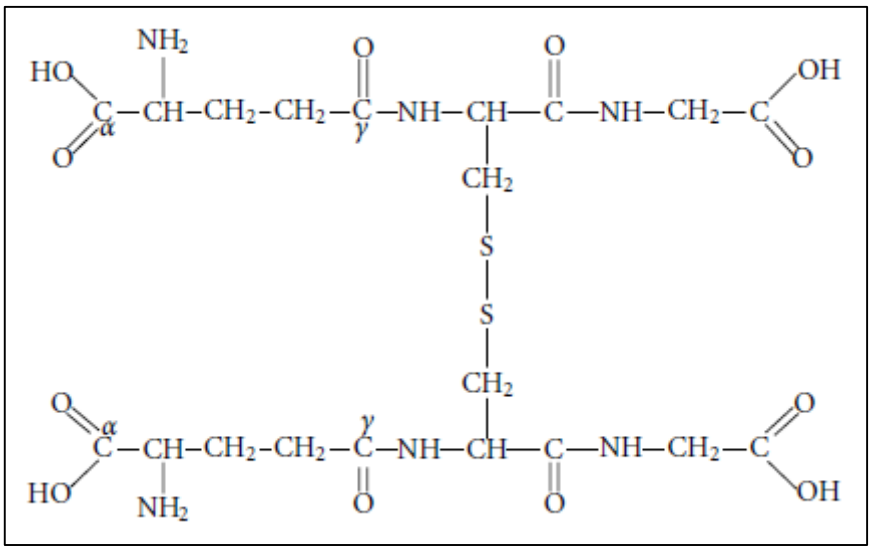

Figure 2.6 Structure of glutathione disulphide (GSSG) (Lushchak, 2012)

In two ATP-dependent steps, glutathione is synthesized in both animals and seedlings from its constituent amino acids (Noctor *et al.*, 2002; Mullineaux and Rausch, 2005). In the first step, the enzyme γ-glutamylcysteine ligase (γ-ECS) links the γ-carboxyl group of glutamate and the amino group of cysteine to form γ-glutamylcysteine (γ-ECS) using the energy from ATP hydrolysis. Next, enzyme glutathione synthetase (GLS) combines both the dipeptide and glycine to produce a glutathione molecule under the expenditure of energy from ATP hydrolysis.

2.6 Functions of Glutathione

Glutathione has been reported to involve in various important functions in signalling mechanisms, glutathione-dependent biosynthetic pathways, metabolic functions in defence against diseases or biotic stress and seedling growth development. The functions of glutathione are broad and complex and it is still not well understood especially on the roles that GSH plays in growth development for seedlings and animals. Only a few of the important metabolic functions of glutathione will be discussed in the following section.

2.6.1 Glutathione in detoxification

Glutathione is a strong nucleophile that can react with weak electrophile. Detoxification is one of the examples of the major function of glutathione. In seedlings, glutathione is involved in detoxification of xenobiotics and heavy metal through several mechanisms. One of it is through synthesis of phytochelatin. Glutathione is used as the precursor by enzyme phytochelatin synthase (PCS) to synthesize phytochelatins when the seedling cells are exposed to heavy metals (Figure 2.9). Phytochelatin is a group of compounds that sequester the heavy metal and form complexes. These complexes are then transported into the vacuole for further removal of the metal complexes (Cobbett and Goldsbrough, 2002; Rea *et al.*, 2004). PCS is encoded by two genes which are *PCS1* and *PCS2* (Cazale and Clemens, 2001). Besides being the precursor, glutathione glutathione could also involve in heavy metal resistance; for example removal of heavy metals from bound sites of enzymes or molecules (Noctor *et al.*, 2011).

2.6.2 Protein *S*-Glutathionylation and gluthathionylation targets

In addition to the well described roles of glutathione in the previous sections, glutathione (GSH) is also involved in some of the signalling mechanisms that occur in the cells. One of them is a posttranslational modification process known as protein *S*-glutathionylation.

Protein *S*-glutathionylation is the formation of disulphide bond between glutathione and a cysteine of the protein target (Rouhier *et al.*, 2008; Noctor *et al.*, 2012). This reaction will then bring a change to the structure, stability and activity of the target protein (Noctor *et al.*, 2012). In theory, glutathionylation can occur by other reaction mechanisms but it is widely accepted the mechanism occur in the presence of glutathione or glutathione disulphide.

Studies on glutathionylation have been extensively done in mammalian cells. By using biotinylated glutathione, researchers have discovered about 150 proteins that undergo glutathionylation and this reaction affect various processes such as glycolysis, signal transduction and protein folding (Rouhier *et al.*, 2008). Glutathionylation can increase or decrease the activity of target proteins and also protect them from activation due to oxidative stress. On the other hand, glutathionylation of seedling proteins are not so well known. Some of the known proteins that undergo glutathionylation are thioredoxin f (TRXf), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and fructose-1,6-biphosphatase (FBA) (Ito *et al.*, 2003; Michelet *et al.*, 2005; Zaffagnini *et al.*, 2007). These proteins are relatively abundant in seedling and involve in primary metabolism.

Thioredoxin f (TRXf) is one of the 4 types of thioredoxin found present in chloroplast. The others are m, x and y thioredoxin. However, only TRXf is known to

undergo able to undergo glutathionylation (Michelet *et al.*, 2005). The TRXf is reduced by ferredoxin or ferredoxin-thioredoxin reductase (FTR) under the presence of light. This in turn regulates enzymes involved in the Calvin cycle (Lemaire *et al.*, 2007). When glutathione (GSH) binds to thioredoxin f, the protein is less reduced by FTR enzyme and this increases the protein ability to activate several other enzymes in a light-dependent regulated reaction. Most of the proteins that undergo glutathionylation are usually involved in various metabolic processes such as photosynthesis, oxidative metabolism and protein folding. This shows how wide and diverse the functions of glutathione in facilitating these processes.

2.7 Potentials of glutathione disulphide (GSSG) in oil palm growth

It has long been known that GSSG is required for critical seedling development in various seedling organs. For example, few researches have reported its critical functions in embryo and seedling meristem development (Vernoux *et al.* 2000; Cairns *et al.* 2006; Bashandy *et al.* 2010). Besides, the production of glutathione is also important for pollen germination and pollen tube growth (Zechmann *et al.* 2011). While the essentiality of GSSG on the development of certain seedling organs was already known for some time but the potential function of GSSG on promoting seedling growth is still quite new and under active research. Hatano-Iwasaki and Ogawa (2012) reported the findings that *Arabidopsis* seedlings with up-regulated glutathione content have higher biomass content. As the results, the seedling appears bigger in size. Unpublished experiments conducted by KAKEKA Company further found out that exogenous application of GSSG on onions in a field trial had increased the average yield significantly for about 17% compared to control onions. This is among earliest trials carried out to consolidate the fact that GSSG can be exploited as a seedling growth promoter for crops. So, the question is can GSSG be used to improve growth of the oil palm at the nursery stage instead? Undeniably, the potential is there.

One of the concerns in the nursery is to shorten the holding period (nursery phase) of seedlings in the nursery (Heriansyah and Tan, 2005; Mathews *et al*. 2010). The nursery phase of oil palm seedlings is usually around 1.5 years and there are many factors that affects the holding period of seedlings in the nursery. One of it is the growth rate of the seedlings. GSSG has the potential to increase the growth rate of these seedlings and shorten the nursery phase. As the result, those oil palm seedlings

can achieve faster growing and more seedlings can be transseedlinged into the outside field sooner.

As discussed in Section 2.6 (Functions of glutathione), glutathione and glutathione disulphide plays an important role in detoxification of compounds detrimental to the seedling growth and development such as heavy metals and formaldehyde-based compounds. This is because GSH and GSSG is part of the antioxidant system that functions to remove free radicals and toxic compounds from the cells (Noctor *et al*., 2011).

Most of the seedlings seedlinged in Malaysia are high oil yielding seedlings produced from cross fertilization of different parent seedlings. These new seeds are more sensitive and susceptible to the use of herbicides and fungicides. The use of these chemicals are normal in the nursery to treat diseases and pest attacks on the seedlings. However, oil palm seedlings can experience chemical toxicity from overdose and frequent application of fungicides such as hexaconazole. This in turn can lead to collante or crinkled leaf symptoms (Mathews *et al.,* 2008). GSSG has the potential such that after application, the seedlings will have better antioxidant capacity which will increase their resistance to chemical toxicity due to herbicides and fungicides use.

CHAPTER III MATERIALS AND METHOD

3.1 Experiment 1: Enhancement of growth of oil palm seedlings by GSSG under field nursery condition

In order to assess the effect of GSSG on oil palm seedlings, an experiment was carried out on the seedlings under field nursery condition. GSSG was applied on 1 month old seedlings and the growth response of seedlings was recorded at the end of nursery stage.

3.1.1 Field nursery condition

The experiment was conducted at Guan Soon Nursery, Bagan Serai, Perak (Longtitude - 100˚36’51’’N, latitude - 5˚2’56’’E) (Figure 3.1) using Customized Random Design (CRD) as the design layout. The planting of oil palm seedlings was done in double stage nursery practice. Firstly, pre-germinated seedlings (DxP) were sown into black polythene polybags (10 × 15cm) each containing non-sterile nursery soil and drainage holes. The polybags were kept under shade and cool. Nitrogen based nutrient solution was applied as NPK yellow (15:15:6:4Mg) according to the seedling requirement (Appendix A). After 4 months, the seedlings were transseedlinged into bigger polythene polybags (38 × 48 cm) containing the soil prepared in the same manner as for the pre-nursery. Nitrogen based fertilizer was applied as NPK Blue Composite (12:12:17:2) according to the seedling requirement until the end of the experiment which is day harvest 300 (D_{300}) (Appendix A). All the seedlings were watered twice daily using overhead sprinklers.

Figure 3.1 Guan Soon Nursery, Bagai Serai, Perak.

3.1.2 Observation of GSSG-treated seedlings under field nursery condition

Two types of GSSG (1% GSSG granules & 30% GSSG powder) obtained from KANEKA (Tokyo, Japan) were used in this study (Figure 3.2). 1% GSSG granules were applied onto the soil surface at once per week for a total of 36 weeks after the seedlings reach 1 month old (Figure 3.3). The respective treatments for the seedlings consisted of: (1) Control (untreated seedlings receiving NPK fertilizer); (2) Treated seedlings (+ 0.23 g of GSSG and NPK fertilizer); (3) Treated seedlings (+0.56 g of GSSG and NPK fertilizer); (4) Treated seedlings (+1.18 g of GSSG and NPK fertilizer); (5) Treated seedlings (+2.36 g of GSSG and NPK fertilizer) (Table 3.1). The amount used in this study was recommended by the KANEKA Company which was based on the results obtained from unpublished experiments on other crops.

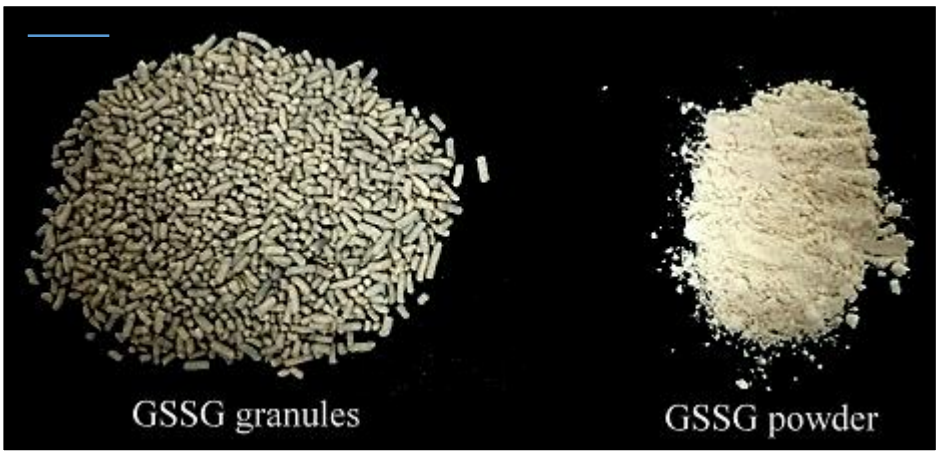

Figure 3.2 Two forms of GSSG used in the study. Bar indicates 1 cm in length

Figure 3.3 GSSG granules were applied on the soil surface. The seedling shown is one month old. Bar indicates 2 cm in length.

Table 3.1: 1% GSSG granule treatments for the seedlings. The amount used is the weight of 1% GSSG granules.

Label	Treatment (g GSSG/seedling)
Control	No GSSG
A	0.23 g GSSG
B	0.56 g GSSG
C	1.18 g GSSG

30% GSSG powder was dissolved in distilled water. The solution was stirred ten times clockwise and anticlockwise to ensure the powder dissolved homogenously. GSSG solution was applied onto the seedlings through foliar spraying at once per month for a total of 9 months after the seedlings reach 1 month old. The respective treatments for the seedlings consisted of: (1) Control (untreated seedlings receiving NPK fertilizer); (2) Treated seedlings (+ 1 g/L of GSSG and NPK fertilizer); (3) Treated seedlings (+ 2 g/L of GSSG and NPK fertilizer); (4) Treated seedlings (+ 4 g/L of GSSG and NPK fertilizer); (5) Treated seedlings (+8 g/L of GSSG and NPK fertilizer) (Table 3.2).