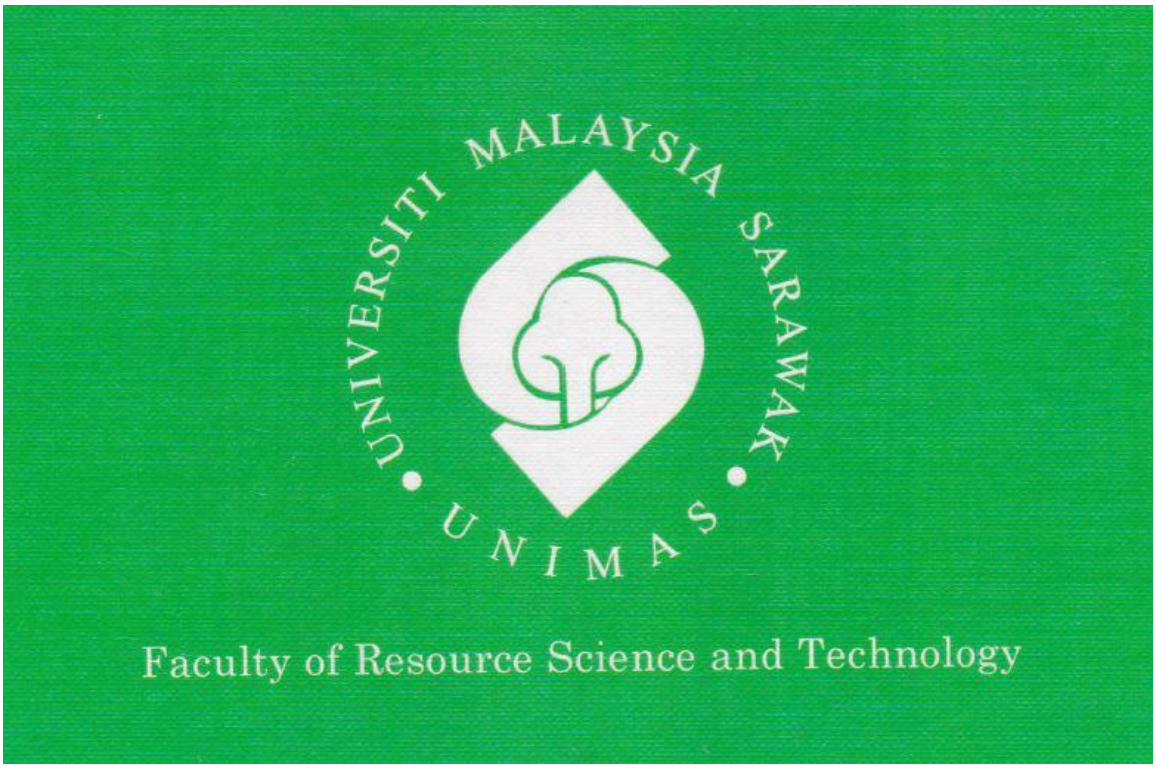

ESTABLISHMENT OF AXENIC CULTURE AND CALLUS INDUCTION OF *SYNSEPALUM DULCIFICUM* DANIEL (MIRACLE BERRY)

TAN HONG WEE

Bachelor of Science with Honours
(Plant Resource Science and Management)
2015

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TAN HONG WEE

This project is submitted in partial fulfillment of the requirements for the Degree of Bachelor of Science with Honours

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2015

APPROVAL SHEET

Name of candidate : Tan Hong Wee

Title of dissertation: Establishment of Axenic Culture and Callus Induction of *Synsepalum dulcificum* Daniel (Miracle Berry)

_____

(Prof Dr. Hamsawi Sani)

Supervisor

_____

(Dr. Rebicca Edward)

Coordinator Plant Resource Science and Management Programme

Department of Plant Science and Environmental Ecology

Faculty of Resource Science and Technology

DECLARATION

I declare that this thesis entitle "Establishment of Axenic Culture and Callus Induction of *Synsepalum dulcifiucm* Daniel (Miracle Berry) is the result of my own research except as cited in the references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

Signature:

Name **:** TAN HONG WEE

Date **:** 15th June 2015

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Establishment of Axenic Culture and Callus Induction of *Synsepalum dulcificum* Daniel

Tan Hong Wee

Plant Resource and Management Programme
Faculty of Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

Synsepalum dulcificum is a slow growing shrub from family Sapotaceae. The fruits contain high medicinal values which aid in chronic diseases due to the presence of active component Miraculin, a taste modifying properties. The current research emphasizes on the axenic cultures establishment and callus induction of *Synsepalum dulcificum* from leaf and seed explants. The sterilized leaf explants were obtained from both 25% Clorox® solution (Sodium hypochlorite) which produced 91.7% axenic cultures and 25% CaOCI solution (Calcium hypochlorite) that produced 94.4% axenic cultures with 20 minutes exposure time upon addition of 1% Benomyl solution respectively. The sterilized leaf explants were cultured onto Murashige & Skoog (MS) media supplemented with various concentrations of 2,4- Dichlorophenoxyacetic Acid (2,4-D), and Picloram. No callus was induced despite some of the leaf explants remained viable after certain period of time and most of the leaf explants results in browning conditions whereas callus was induced from seeds supplemented with 2.0 mg/L of 2,4-D after 3 weeks of culturing. The poor callus induction from leaves was highly due to the presence of high poly-phenolic compounds within the miracle berry plants. Future work should utilize different PGR's at different concentrations and also the use of different explants.

Key words: *Synsepalum dulcificum*, Miraculin, Axenic Culture, Callus Culture, Plant Growth Regulators (PGR)

ABSTRAK

Synsepalum dulcificum adalah sejenis pokok renek yang berasal dari keluarga Sapotaceae. Tumbuhan ini mengandungi nilai perubatan yang tinggi dengan adanya komponen aktif dalam Pokok Ajaib yakni Miraculin yang membantu dalam penyakit kronik dan turut berfungsi sebagai agen pengubahsuai rasa. Penyelidikan ini menekankan aspek penghasilan kultur arsenik dan pembentukan kalus dari bahagian daun dan biji Synsepalum dulcificum sebagai sumber eksplan. Daun eksplan yang steril terhasil dari penambahan 1% Benomyl dalam kedua-dua kepekatan larutan iaitu 25% Clorox® (Natrium hipoklorit) yang menghasilkan 91.7% aresenik dan 25% CaOCI (Kalsium hipoklorit) yang menhasilkan 94.4% arsenik dengan pendedahan masa selama 20 minit. Daun eksplan yang steril dikulturkan dalam media Murashige & Skoog (MS) yang ditambah dengan variasi kandungan kepekatan 2,4-Dichlorophenoxyacetic acid (2,4-D), dan Picloram. Walaubagaimanapun, tiada kalus terbentuk walaupun sebahagian daun eksplan masih dalam keadaan sihat tetapi kebanyakkan eksplan berubah warna menjadi perang manakala kalus terbentuk dari biji yang dikulturkan dalam 2.0 mg/L kepekatan 2,4-D. Kekangan pembentukan kalus daripada bahagian daun adalah disebabkan faktor utama iaitu kandungan kimia poli-phenolik yang tinggi dalam pokok ajaib. Kajian yang akan datang seharusnya menggunakan pelbagai PGR's dalam kepekatan berbeza dan juga jenis eksplan yang lain.

Kata kunci: *Synsepalum dulcificum, Miraculin, Kultur Arsenik, Kultur kalus, Hormon Pertumbuhan Tumbuhan*

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LIST OF ABBREVIATIONS

PGRs	Plant Growth Regulators
2,4-D	2,4-Dichlorophenoxyacetic Acid
MS media	Murashige and Skoog media
KOH	Potassium hydroxide
HCl	Hydrochloric Acid
ANOVA	Analysis of variance
CaOCI	Calcium hypochlorite
CRD	Completely Randomized Design
Picloram	4-amino-3,5,6-trichloro Picolinic Acid
FDA	Foods and Drugs Administration

1.0 INTRODUCTION

Miracle berry (*Synsepalum dulcificum*) or its vernacular name "Buah Ajaib" is an important source of medicinal plant which belongs to Sapotaceae family. This plant has its origin in Tropical West Africa which is a slow growing shrub that produced fruits occasionally throughout the year. As stated by Ogunsula and Ilori (2008), the fruits are small with approximately 2 to 3 cm long ellipsoid berries. The fruits are surrounded by edible pulp covering an elongate ovoid shape seed which changes colour into bright red upon ripens (Chen *et al*., 2012). The interesting facts about this fruit lie within the ability of its flesh pulp that is able to modify sour taste foods into sweet upon consumption (Yong, 2011). This unordinary effect was first documented in the scientific literature by Kurihara and Beidler (1968). The effect will usually last for about 30 minutes and beyond thus making it an extraordinary fruit that brings about all sour foods such as lemon to be tasted sweet when being consumed (Ogunsula and Ilori, 2008). This is due to the presence of Miraculin compound which is a type of glycoprotein that is able to alter our sweet receptor buds by manipulating the configuration of proteins on our tongues.

At present, *Synsepalum dulcificum* has been treated as an important source of medicinal plant. It has been used to provide treatments for chronic disease patients who had typically developed their blunt taste sensation due to various treatments (Peregrin, 2009). Another issues concerning health benefits were an extravagantly consumption of sugar that may impose several negative effects which were hazardous to human health. Many artificial sweeteners such as aspartame had been used widely as it can alter our sweet desire without undesirable calories on blood sugar levels and health. Nevertheless, the circumstances after consumption of aspartame gave an unpleasant side effect. However, miracle berry eliminates the possibilities of having any side effects after consumption (Levin, 2012).

This has been proven by Foods and Drugs Administration (FDA) in 1970's that miraculin compound were certified to be legally used as sugar substitute subsequently having examined for safety and efficacy. This had been further supported by Dr.Linda Barthoshuk in the 1960's in an experiment conducted for Smell and Taste in University of Florida Center that no health risk were related to the consumption of miracle berry fruit. Hence, it has been commercialized as substitute for food additives as sweetener due to the facts that only little amount were utilised to make the food taste sweet as compared to the excessive used of food additives (Bartoshuk, 1974). In short, this fruit had been used as a medicinal plant which is believed to aid in diabetic patients and also as a source of daily products in food manufacturing as food sweeteners. Due to its increasing economically medicinal values and great commercial potential of miraculin, there has been a high growing demand for the production of a large scale yield of *Synsepalum dulcificum*.

However, raising the plants from seed posed several problems. As stated by Ogunsula and Ilori (2008), the seed are recalcitrant and (Chen *et al*., 2012) this plants are "difficult to root" species thus production of large scale yield had been an obstacle. Following a great commercial potential of miraculin, there is a need to develop an effective way to yield more miracle berries. Therefore, there is a need to investigate other effective methods that can contribute to the regeneration of this species. Relatively little information were documented about the growth and development of *Synsepalum dulcificum* in tissue culture practices. Several successes have been reported in regenerating plants through propagation by stem cutting method by (Chen *et al*., 2012) and *in vitro* propagation through embryo and nodal cultures by (Ogunsula and Ilori, 2008). The recognition of the planting method had to be devised to search out an alternative approach in regenerating miracle berry plants other than conventional method of propagating by stem. Hence, an alternative approached

by using tissue culture method on leaves part as source of explants had been used in this study. It is worth seeking of developing this technology practices (tissue culture) to aid in future studies on this plant with potential application to its propagation for its recognition potential in nutritional and medicinal purposes.

According to Razdan (2003), tissue culture techniques have highly been used to assist the traditional techniques for plant improvement. Anchoring from this method, a number of plants can be regenerated or produced in vast amount in a short period of time. So far, there have been no report and research results documented regarding the regeneration of Miracle berry plants through leaf explants by tissue culture methods. Hence, the need to investigate the regeneration of Miracle berry plant has been the centre of this research study. This research study emphasized on the callus induction of Miracle berry plant through leaf explants and seed culture by tissue culture methods. According to Ogunsula and Illori (2008), Miracle berry plant are recalcitrant and is rather difficult for this plant to be propagated by seeds or through cutting as the seeds desiccated in a rapid rate after being harvest. Therefore, it is essential to look for alternatives ways to regenerate this plant other than propagating methods. Hence, the present objectives of this study are:

1. To establish an axenic culture of *Synsepalum dulcificum* explants.
2. To determine the effect of different Plant Growth Regulators on callus formation from leaf explants and seed culture.

2.0 LITERATURE REVIEW

2.1 The Botanical Description of *Synsepalum dulcificum*

The taxonomic classification and nomenclature of *Synsepalum dulcificum* are as follow:

Division: Magnoliophyta

Class : Magnoliopsida

Order : Ericales

Family : Sapotaceae

Genus : *Synsepalum*

Species : *dulcificum*

Scientific name: *Synsepalum dulcificum*

Synsepalum dulcificum is a small, evergreen shrub grows very slowly to a height of 4 to 6 feet in container, and 10 to 15 feet in natural habitat. The growing plant eventual size depends on where the plant is being grown. A 10 years old plant might be easily only 4 to 5 feet tall. It forms an oval to pyramidal shaped bush or small tree.

The leaves are dark green, glossy and glabrous. The foliage is elliptical in shape, with a cuneate base, entire leaf margin and an obtuse apice. The leaves are 5-10 cm long and 2-3 cm wide. The flowers are quite small and points downwards. It measures approximately 1 cm from the brown base to the creamy- white coloured pedal tips.

The flowers only partially open, revealing the stigma and pistil, a notable characteristic of the Sapotaceae family. As the flower ages and the ovary ripens, the petals turn a dark red and open up more, revealing the inside workings.

The fruit is the most miraculous part of this plant. Fruits are bright red, 2-3 cm long and ellipsoidal in shaped. The fruit itself is not particularly flavourful, however, once eaten; anything bitter or sour will become sweet taste and the effect may last for about thirty minutes to a few hours, depending on the conditions in which the plant itself was grown. The colour of the miracle fruit plant does not vary much throughout its lifespan. Its bark is a light grey to medium brown. The foliage is a deep green like many tropical. Flowers are creamy-white with brown bases. The miracle fruit itself is bright red or scarlet in colour (Pascoe, 2011).

It has an inconspicuous brown-and-white half inch flowers followed by bright scarlet, 1 inch football-shaped fruit, sweet and pleasant tasting. The plant starts fruiting when only 1 feet tall. It produces fruit practically year around. In native habitat, two large crops are available yearly, each after a rainy season. The mature bushes usually have a few fruits hanging around all years. It will usually takes about 2 to 3 years for seed to fruit whereas from flower to fruit usually results in 30 to 45 days (Anon, 2014). *Synsepalum dulcificum* or known as Miracle Berry is an important source of medicinal plants that is widely used worldwide. According to Ogunsula and Illori (2008), its habitat lies within evergreen tropical area which has a shrub plant habit. It has a distinctive characteristic that can altered or changed all sour foods into sweet due to the presence of Miraculin (a type of glycoprotein) that binds to our sweet receptors and changes its configuration on our tongues. Production of large scale yield of miracle berry has been hindrances since the plant are difficult to root species and the seeds are recalcitrant.

2.2 History of *Synsepalum dulcificum* Daniel

Miracle berry plants were originated in West Africa and it has been widely used as an important source of food to native African for centuries where they relies on this miracle berry as a source of foods which barely within few basic foods, mostly were of sour taste (Anon, 2014). When these miracle berries were first tasted, the magnificent altering taste of sour foods into sweet had become a surprised. Anchoring from this finding, the West African natives had utilised this miracle berry fruit to sweeten sour palm wine (beer) which were regarded as *Pito*, and fermented maize bread which were known as *Kenkey*. Their berries were used as food taste modifier and preservative where available food were preserved for a long periods of time. Due to this unique characteristics displayed by this berry, there is a tendency for the identification of this berry. As stated by Sabel (2012), in the early of the year 1700; these miracle berries were authenticated by a French explorer Reynaud Des Marchais following the consumption by the native African. Subsequently during the mid-1880s, this berry fruit had been generalized and issued by a botanist after Dr. W.F. Daniell. According to Anon (2014), this astonishment to West African was not botanically or taxonomically identified, described and named until the middle of the 19th century as *Synsepalum dulcificum*, which is a member of the Sapotaceae family, relative of the sapodilla (*Manilkara zapota*). Much research had been conducted on miracle berries as possible alternative for sugar substitute. As such, *Synsepalum dulcificum* plants were introduced to America by David Fairchild during the dawn of 20th century. Bill Whitman was the first to grow the plant successfully in the United States of America. He had a seedling all of 7" tall and a cutting of 4" bearing fruit upon the young twigs.

2.3 Habitat and Distribution of *Synsepalum dulcificum*

As stated by Akpla and Fandohan (2014), the current geographical range habitat of *Synsepalum dulcificum* lies on southern Benin below 7˚ north as shown in Figure 1. The Republic of Benin (6-12 ℃N; 0.40-3 ℃E) is a part of West African country located within the Dahomey Gap as shown in Figure 2. Geographically, Benin had a coverage area of 112,622 km ²of which 22.7 % of the area is legally protected. Much information regarding its distribution had been retrieved. Likewise, this species had been distributed to United States of America (USA) in the year 1919 by Fairchild (1931). In fact, it has been successfully grown by Bill Whitman in the 19th century. This miracle berry had been widely produced and cultivated in Taiwan for large production (Fooladi, 2012). Furthermore, Kurihara and Beidler (1968) stated that these miracle berries were also encountered in Oban Hills in Cross River National Park and were also available in Japan (Madison, 2008). Therefore, this remarkably proven that their distributions in Asia region were significant. Nonetheless, the present study of this species did not yield any documentation on how it has been distributed to Malaysia. In brief, this species were believed to have distributed across a number of regions.

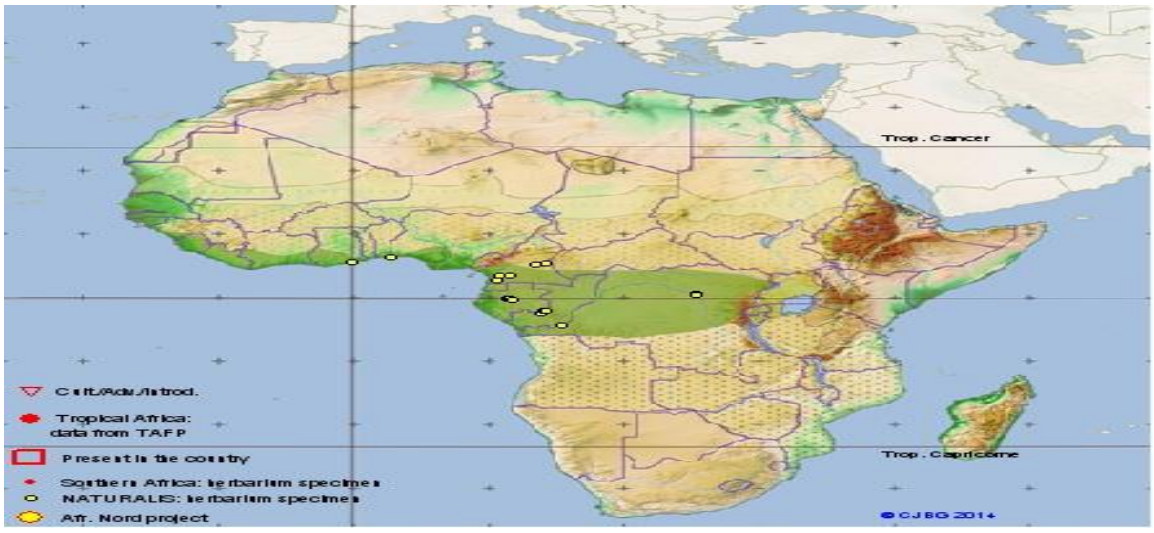

Figure 1: Geogrphical range habitat of *Synseplaum dulcificum* in Tropical West Africa Region (Adapted from Fleurs, 1997).

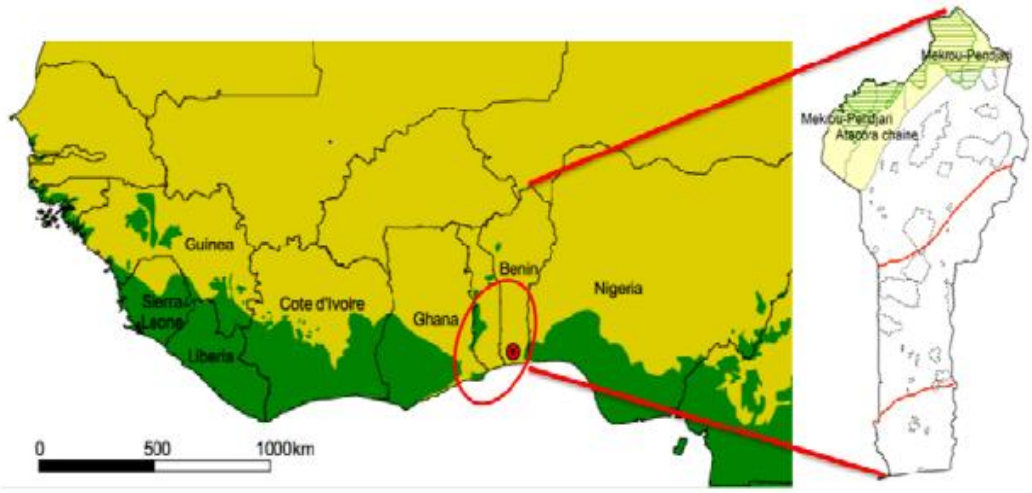

Figure 2: Map showing Benin within the Dahomey gap encircled (Adapted from Olson *et al*., 2001).

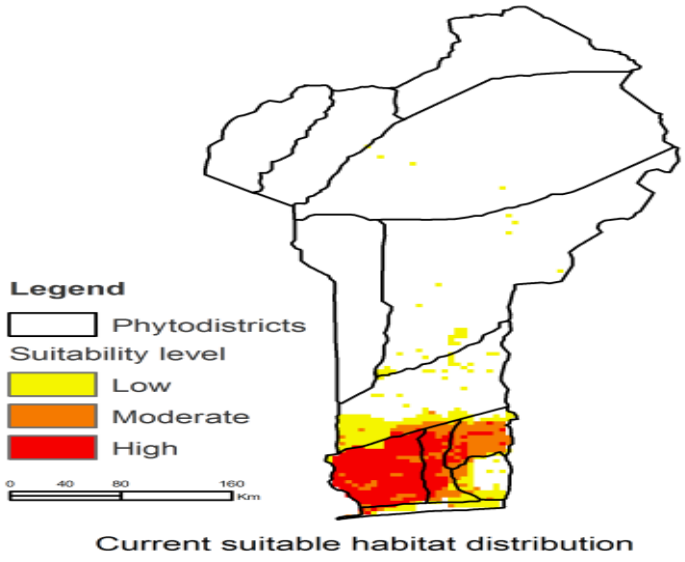

Figure 3: Map showing the current suitable habitat distribution of *Synsepalum dulcificum* (Adapted from Akpla *et al*., 2014).

Climatically, Benin is subdivided in three main regions: the semi-arid, the sub-humid dry and the sub-humid humid. According to Inglett and May (1968), miracle berry were farms growing based and around dwellings occurs in surrounding forests, and is common on the coast in Ghana. The predicted range under the current climate conditions shows that the suitable areas for *Synsepalum dulcificum* were located in the south of Benin as shown in Figure 3, which was characterized by wet subequatorial climate. The highly current

suitable areas for the species cultivation (6,510 km²) were imbedded in the West Plateau and Ouémé-valley phytodistricts. The moderately suitable habitats (3,671 km²) were mainly in the east Plateau phytodistrict while the poorly suitable areas correspond to the Coastal phytodistrict and the southern part of Pobe and Zou phytodistrict. However, globally apart from the south–eastern part of Zou phytodistrict, there were only some spots of poorly suitable habitat in the remaining parts of this phytodistrict up to the centre of Benin. Moreover, no information and records were obtained in the centre and north of the country.

According to Sabel (2012), *Synsepalum dulcificum* originates from Tropical West Africa, which had been highly commercial farmed by farmers of rare fruit in Taiwan, Jamaica, Puerto Rico, Guam, and South Florida. Its habitat lies within and grows ideally in a humid, warm environment and while resistant to varying water levels; it cannot withstand any sort of freezing. This plant was well adapted to a slightly acidic soil environment (pH 4.5-5.8) and produces edible fruits about 3 years after planting. The fruit grows in a single season ranges throughout from May to September, though berries are generated year-round. When grown from seedlings, it takes three to four years before fruiting occurs; the bush grows slowly and eventually reaches six to fifteen feet in height when fully mature (Adansi, 1970).

2.4 Chemical Constituents of Miracle Berry

The active component constituent in this miracle berry is miraculin compound (Gollner, 2008). Miraculin (MCL) is a glycoprotein molecule extracted from fruit *Synsepalum dulcificum* which is native to the African. According to Sabel (2012), miraculin is a single polypeptide chain which consists of 191 amino acids with the presence of two glycosylated polypeptides which were Asn-42 and Asn-186 linked by disulfide bond. As stated by Temussi (2006), miraculin is a macromolecule compound which has a molecular mass of 24,600 and it has been verified to be approximately 400,000 times sweeter than sucrose on same molecular mass basis. Its properties as food modifier had been remarkably recognised which were able to convert taste bud stimuli from sour taste into sweet.

According to Gimbson (n.d.), this glycoprotein molecule adheres to the sweet receptors of the tongue and it triggers the inactivation of the sour and bitter taste bud which leads the sour taste food into sweet. Upon consumption, this miraculin compound will retained on tongue bud and this configuration will triggers sweetness after tasting sour foods in tandem this effect usually lasts for one to two hours (Koizumi *et al*., 2011). The sweet receptors will recognized the sour food as sugar which taste sweet and this effect will last for at most an hour right until the saliva washes off the protein molecule. This effect is apparent when it comes in contact upon acidic pH. Miraculin compound has the ability to evoke the sweetness taste from several acid formulations which primarily includes hydrochloric acid, oxalic acid, lactic acid, formic acid, acetic acid and citric acid. The actual sweetness effect is dependent upon the rate of acidity (Kurihara and Beidler, 1968).

In addition to miraculin, other secondary metabolites chemical constituents have also been reported to be isolated from miracle berries. These include tannins and other poly-phenolic