Establishment Of Callus Culture Of Purple Amaranthus (*Amaranthus cruentus*)

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A proposal submitted in fulfillment of the requirements for the degree of Bachelor of Applied Science (Agrotechnology) with Honours

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DECLARATION

I hereby declare that the work embodied in this report is the result of the original research and has not been submitted for a higher degree to any universities or institutions.

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Establishment of Callus Culture Of Purple Amaranth (*Amaranthus cruentus*)

ABSTRACT

Food colourant is widely used in food industry in order to enhance the product aesthetics. Natural food colourant is safer and healthier to be consumed compared to synthetic colourant that is used extensively nowadays. Natural food colourant can be obtained from plant source. Therefore, purple amaranth or *Amaranthus cruentus* can be used for anthocyanin extraction for producing natural food colourant. The purpose of this study is to establish aseptic seedlings of purple amaranth and to induce callus from different part of explant taken from the aseptic seedlings using different plant growth regulators. Seedlings were cultured aseptically on MS basal media. After the seedlings has grown bigger, explant stem and shoot has been taken to be subcultured on different plant growth regulator at three different concentrations. The explant was subcultured on 1 mg, 2 mg and 3 mg of NAA, 2,4-D and picloram. Seedlings that has been subcultured on MS basal media without the addition of the growth hormone germinated. Explant subculture on NAA media did not produce any callus similar to the explant subcultured on picloram. Explant subculture on 2,4-D media produced callus at concentration of 1 mg. Among the three different PGR used, 2,4-D showed better growth performance. This study of establishment of tissue culture techniques for *A.cruentus* will be able to produce callus that can be used for anthocyanin production as natural food colourant.

Keywords: *Amaranthus cruentus*, purple amaranth, callus induction, NAA, 2,4-D, Picloram, anthocyanin

Penubuhan Kultur Kalus daripada Amaranth ungu (Amaranthus cruentus)

ABSTRAK

Pewarna makanan telah digunakan secara meluas dalam industri pemakanan untuk meningkatkan estetika produk. Pewarna makanan semulajadi adalah lebih selamat dan sihat untuk dikonsumi berbanding pewarna makanan sintetik yang telah digunakan secara meluas semenjak kebelakangan ini. Pewarna makanan semulajadi boleh didapati daripada sumber tumbuhan. Bayam ungu atau *Amaranthus cruentus* boleh digunakan untuk mengekstrak anthocyanin untuk menghasilkan pewarna makanan semulajadi. Tujuan kajian ini dijalankan adalah untuk menghidupkan biji benih amaranth ungu secara aseptic dan juga untuk menginduksi kalus daripada pokok berlainan bahagian yang telah dikultur secara aseptik ke dalam media berlainan hormon. Anak benih aseptik telah dikulturkan ke dalam MS media tanpa hormon. Setelah pokok membesar, bahagian pokok seperti batang dan daun telah diambil untuk di subkulturkan di dalam hormon tumbuhan yang berbeza pada tiga kepekatan yang berbeza. Bahagian pokok di subkulturkan dalam 1 mg, 2 mg dan 3 mg NAA, 2,4-D dan pikloram. Biji benih yang telah dikultur pada MS basal media telah bercambah. Bahagian tumbuhan pada media NAA tidak menghasilkan kalus sama seperti bahagian tumbuhan yang di subkultur pada media pikloram. Antara tiga jenis hormone tumbuhan yang digunakan, hormon 2,4-D menunjukkan prestasi pertumbuhan yang paling baik. Kajian pertumbuhan menggunakan teknik kultur tisu dapat menghasilkan kalus dimana ia boleh digunakan untuk menghasilkan anthocyanin sebagai pewarna makanan semulajadi.

Kata kunci: *Amaranthus cruentus*, amaranth ungu, induksi kalus, NAA, 2,4-D, Pikloram, anthocyanin

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LIST OF ABBREVIATION

UV	Ultraviolet
DNA	Deoxyribonucleic acid
Cm	Centimetre
Mg	Miligram
g/l	Gram per litre
MS	Murashige and Skoog
MT	Murashige and Tucker
PGR	Plant growth regulator
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
TDZ	Thidiazuron
BAP	6-Benzyl Amino purine
NAA	1-Naphthaleneacetic acid
2,4-D	2,4-Dinitrophenylhydrazine
NaOH	Sodium hydroxide
HCL	Hydrochloric acid
dH_2O	Distilled water
ml	Millilitre
g	Gram
mg/l	Milligram per litre

LIST OF SYMBOLS

Symbols	
%	Percentage
°C	Degree celcius
1x	One time
Psi	Pounds per square inch

CHAPTER 1

INTRODUCTION

1.1 Research Background

Natural pigments that gives colour to fruits and vegetables like pink, magenta, red, purple and dark blue is called anthocyanin. This anthocyanin have antioxidant ability which it shows many health benefits. The anthocyanin that present in plant also may help preventing the plant from any damage that cause by UV radiation. Other than can be extracted as food colourant, this anthocyanin also can be function in pharmaceutical component and also as functional foods.

Anthocyanin has their own benefits as it has antioxidant properties. One of benefits obtained from anthocyanin is it help in protection against cardiovascular disease or heart disease. Serving food that contain anthocyanin may help protect from high blood pressure and also arteriosclerosis. Death risk also may be reduced by consuming anthocyanin food which the dead is causal from heart disease or coronary artery disease. Other than that, another function of anthocyanin is it can help improving immune function in human. Bioflavonoid content in anthocyanin may protect damaging in DNA peroxidation of lipid occur.

Other than contain bioflavonoid, anthocyanin also is said to have anti-inflammatory properties and it also may reduce estrogenic activity by supporting

hormonal balance. Anthocyanin is said to have the ability of cancer protection. Due to its properties like antioxidant, anti-carcinogenic and anti-inflammatory, risk of various type of cancer can be reduced. Some studies or research has been done using in vivo or in vitro method in human and animal by using anthocyanin which the studies indicate the anthocyanin ability to fight cancer naturally by blocking cell proliferation and inhibit tumour formation by interfering with carcinogenesis process.

Apart from that, vision and eye health can be enhanced which it is one of anthocyanin benefits. It protect the eye from radical damage and night vision can be enhanced. Cognitive function also can be improved as anthocyanin may protect memory, neural function and coordination. In one of research, it shows that anthocyanin obtain from purple sweet potato inhibit peroxidation of lipid in brain tissue. Lastly, another benefits obtain from anthocyanin is it can improved performance and recovery of exercise. This is occur when anthocyanin are able to help by lowering negative effect of excessive oxygen, exhaustion and also accumulation of radicle while doing physical activities. (Jillian Levy, 2018).

However, the anthocyanin supply is very difficult and limited as the plant source is limited. The causal of limited of plant sources maybe caused by disease and that attack plant growth, variation of climate, seasonal plant sources, and need long time to cultivate the plant. So, the solution that can be used to produce anthocyanin from plant sources is by using plant cell cultures or known as tissue culture. From in vitro culture of many plant such as *Ipomoea Batatas, Fragaria Ananassa, Catharanthus Roseus, Melastoma Malabatrichum* and many else anthocyanin has already been obtained. Fruit and vegetables that are brightly in colours normally contain antioxidant. Usually, anthocyanin that has antioxidant contain in food that is purple in colour. *Amaranthus Cruentus*, is one of purple colour vegetable that contain anthocyanin which it can be produced using in

vitro method. *Amaranthus Cruentus* is one of vegetable from the Amaranthaceae family from Amaranthus genera. (Chaudhary & Mukhopadhyay, 2012).

According to Rahman & Gulshana, (2015), there were about 65 genera and 900 species of amaranthaceae plant different from Eshete, Asfaw, & Kelbessa, (2016) , which according to them, there were 70 genera with 1000 species of amaranthaceae. Amaranthaceae is a plant that has been distributed in tropical area also including temperate region. There were more than 50 species from 15 genera of amaranthaceae with their common names Alternanthera, Amaranthus, Iresine, Celosia and also Gomphrena. In Amaranthaceae family, due to it multi-coloured foliage and their beautiful inflorescence, this plant family also known as ornamental plant. Ornamental plant that from this family are *Amaranthus Candatus, A. tricolor, A. salixifolius, A.Celosia Cristata,* Iresene, Globosa and also Herbtsii. Other than ornamental plant, this family also is one of edible plant. Among edible plant that exist in Amaranthaceae family are *A. Blitum. A. Caudatus, A. Hybridus. A. Spinosus, A. Tricolor* and also *A. Viridi.* (Rahman & Gulshana, 2015).

Most of Amaranthaceae family members is annual or perennial herbs. This plant is a small tree which sometimes it can be a climbing tree. Plant from this family are able adapt in salty soils and also in arid environments. Their leaves are simple and alternate. This plant is originate in Central Asia which is Steppes. Most of the Amaranthaceae is originated from America which American people used to make drinks and flour from species of this family. Other than making drinks or flour, American people also used it for ritual as it contain the alkaloid. Alkaloid normally contain oxygen and nitrogen and also have an important physiological properties. In plant, the alkaloid function as stimulator or regulator to plant like hormone. (Eshete et al., 2016).

There were about 20 genera of Amaranthaceae that has been known in Ethiopia. Among genus that can be found in Amaranthaceae are Celosia, Digera, Pupalia, Alternanthera, Gomphrena and Lopriorea. There are many more genera that can be found under Amaranthaceae family. For the Celosia, it is a genus with annual and perennial herbs. This plant, the below part sometimes are woody and erect downwards. The leaves are alternate leaves. There are 5 species that can be obtained under genera of Celosia. For Digera genus, it is an annual herbs with leaves that are alternate and entire leaves. There are 2 sub-species that are found under Digera genus. *Digera Muricata* is one species that found under Digera and it has 2 varieties which are variety Muricata and variety Trinervis.

Other than that, Pupalia which is herbs or sub-shrubs under this genera has opposite or entire leaves. In tropic area, there are 4 species found from West Africa to Malaysia and Philipines which are *Pupalia Micrantha, Pupalia Grandiflora* and *Pupalia Lappacea* while the other one species is extending to subtropic. Alternanthera genera is a large genus with species of 200 compared to the other one with large genus which is Gomphrena where it has 100 species under this genus. Herbs is included in this genus with entire and opposite leaves. This genera was mostly obtained in tropical America but there is several in Australia. Other than tropical America and Australia, they also found in Ethiopia with 2 species which are *Gomphrena Celosioides* and *Gomphrena Globosa.* This genus was used to introduce as weed but later has been cultivated as an ornamental plant. Next, under this genera, Lepriorea is a genus with herb that are perennial and low sub-shrub. It has opposite leaves and branches. All part of plant from this genus is glabrous except for the inflorescence. (Eshete et al., 2016).

Amaranthus from the Amaranthaceae family genus has annual plant more rarely compared to perennial herbs. It can be monoecious or diecious plant. In Euthopia, there were about 11 species that has been recorded which many of the species in this genus

found in that country are edible and some are cultivated for their leaves. This genus that are edible include *A. Caudatus* and *A. Hybridus* which it subspecies is *A. Cruentus*. Other than that, other edible species incude *A. Spinosus* and *A. Dubius* which both of this species occur together, *A.Tricolor* and a few species more. Amaranth grain are very small and light in colour with a good light taste or mild flavour.

The anthocyanin of the plant belong to amaranthus family can be produced by using plant tissue culture technique which callus can be produced and the extract to obtained the anthocyanin. Plant tissue culture is an art or a science in plant cells growth which this technique must be done in controlled environment and sterile growth condition and medium. Part of plants that can be used to culture on MS media is plant tissue, leaves, stem, single cells and roots. Those part of plant can be used to generate a new plant on media that is supplied with nutrient that required to grow a plant and plant hormone also may be added. (Ikenganyia, Anikwe, Omeje, 2017).

In tissue culture, hygiene is important which is necessary in media preparation and also during culture. The hygiene and sterilization is crucial to avoid contamination of plant occur. There are few reason or sources of contamination that occur in plant tissue culture. Culture media can be one of the contamination sources. Media that used to for plant culture not only support plant growth but it also able to support growth of other organism likes bacteria and fungi which can cause plant death. Before cleaning the contaminated culture, autoclave is needed to avoid the contamination being spread to other plant or culture instrument. Environment is other sources of contamination which the environment include air, temperature, radiation, moisture and many other. During culture the used of laminar flow hood is to avoid contamination due to constituent of air.

Other than that, other sources of contamination is the use of plant materials. The surfaces of plant may carry significant amount of microorganism. The microorganism that carried by the plant surface can be removed by doing surface sterilization before culture the plant on media. Culture media containers or instrument is one of sources for contamination. Equipment used during culture of plant need to be autoclaved to remove unwanted organism. Management practices like plant handling can be one of contamination sources. All possible of contamination sources has been identified. There are few steps of sterilization that can be used which are chemical method which use chemical substances to sterilize and ultra-filtration method which this method is suitable for unstable media component at high temperature. Other than that, other method of sterilization are dry heat method and wet heat method. Dry heat method oven may be used while wet heat method like autoclaved using steam under pressure. (Ikenganyia, Anikwe &Omeje, 2017).

There are three types of culture which are organ culture, culture of unorganised cells and also culture of single cell origin. Callus culture is a culture under culture of unorganised cells. Callus is formed when there are multiplications of plant cells in disorganised way which the callus is a tissue that is coherent and amorphous. Callus often occur on the part by wounding of an intact plant with the insect or microorganisms presence as result of stress. Initiation of callus can done using in vitro method by placing a small pieces of a whole plant under sterile condition. Establishment of callus culture can be from gymnosperm, mosses, ferns and also thallophytes despite most of experiments that has been conducted use tissue of a higher plant. Primary callus is callus that formed from the original explant while callus that initiated from tissue pieces deserted from primary callus is called secondary callus. (George, Hall, & Klerk, 2008).

1.2 Problem Statement

In order to enhance the aesthetics of a product and also its novelty and festivity of a product, food colourant are one of common additive that is widely used in production of food and beverage. Other than that, colour also important in perceiving flavour of food product. There are 2 types of food colourant which are natural and artificial or synthetic food colourant. The artificial food colourant is make by using chemical substances for food appearance enhancement. Commonly, in food industry, artificial food colourant has been used as it is easy to get. However, synthetic colour is more harmful to be used especially to human health. In children, the artificial food colourant may cause hyperactivity in sensitive children. Other than that, the use of food colourant may also cause cancer and some of it cause allergic.

Therefore, natural food colourant is needed to minimize the use of synthetic food colourant. The natural food colourant or known as anthocyanin can be obtained from fruit and vegetable which it is not harmful to human health. Hence, this project is being design to see whether purple colour of callus from purple amaranth are able to produce by using tissue culture method. The purple colour callus can be used to extract anthocyanin or food colourant.

1.3 Objective

1. To establish aseptic seedlings of *Amaranthus cruentus*
2. To induce callus from *Amaranthus Cruentus* from stem and shoot from aseptic seedlings using different plant growth regulators

1.4 Hypothesis

Null Hypothesis

1. Callus from stem and shoot of *Amaranthus Cruentus* from aseptic seedling could not be induced by using different plant growth regulators.

Alternate hypothesis

1. Callus from stem and shoot of *Amaranthus Cruentus* from aseptic seedling could be induced by using different plant growth regulators.

1.5 Scope of Study

The present study is done using seed of purple amaranth or known as *Amaranthus cruentus.* This explant was cultured on MS basal media for germination. After that, the plant growth need to be subcultured on media contain auxin at different concentration and different type of auxin.

1.6 Significance of Study

The callus induced in this research can potentially be used for anthocyanin production which are edible food colourant, as the natural food colourant can be obtained from plant source and has many benefits compared to synthetic food colourant. Purple amaranth is one of vegetable that has potential to produce anthocyanin.

CHAPTER 2

LITERATURE REVIEW

2.1 *Amaranthus Cruentus* (Purple Bayam) Plant

Most species belong to Amaranth genera are edible which it produce cereal like grain in quite large quantity. Amaranth plant is one of easy grow plant and rich in nutrient content. It can used to fight against hunger and malnutrition as it known as pseudo cereal that is underutilized. Amaranthus main species are *Amaranthus caudatus*, *Amaranthus cruentus* and *Amaranthus hypochondriacus* has been grown in West Africa about a very long time ago as a food crop as the Amaranth seed contain dietary fibre, iron and calcium in quite large amount and also contain few important nutrient. Other than contain dietary fibre, the seed also is said to have high amount of methionine, lysine and cysteine which can bind with amino acid producing sources that are excellent with high quality and balanced protein. The balanced protein were more complete better than protein that is found in other most grains. *Amaranthus caudatus* leaves are just like other amaranth which it can eaten or can also be boiled. (Jillian Levy, 2018).

Amaranthus cruentus is one of leafy vegetables that is very popular in many countries. Even though this vegetables is originated from South America, this vegetable also is has been widely cultivated in Nigeria and other West African countries. Due to it early maturation, this vegetables are widely cultivated other than their ability to survive in mixture with other types of crop. Therefore, this crop is one of the most preferable

crop for farmer to cultivate as this crop give income fastest while farmer still wait for other crops to be harvested.

This crop is cultivated in many area by many people including in rural area, urban area, and also being planted by many farmer. 40 percent form this crop yield collection is eaten by farmer while the rest has been marketed.

As this plant is easy to cultivate and grow, this crop has become an important commodity of economic activity in market for rural women. According to (FAO 2007) from article Law-ogbomo & Ajayi, (2009) the production for this crop in US is about 77.27 tan per hectare while for world average the production is about 14.27 tan per hectare. This crop has higher planting density. Growth and yield of this crop can be improved by applying fertilizer or organic fertilizer which by applying fertilizer is the best way to improve crop growth which it is fast easy way.

Figure 2.1 : *Amaranthus cruentus* flower

Source: https://alchetron.com/Amaranthus-cruentus

Figure 2.2: *Amaranthus cruentus* leaves which is edible

Source: https://alchetron.com/Amaranthus-cruentus

Amaranthus cruentus is a vegetable crop or flowering plant that produce good and nutritious grain of amaranth which has been cultivated as sources of grain as it is easy and fast growing crop. It is originated from *Amaranthus hybridus* which both of this crop share the same morphological features. This plant can grow until 2m in height. A. cruentus is an annual herb with dark pink flowers on this plant. This crop is suitable to be planted on light clay and sandy loam soil that is rich with organic matter with suitable pH of 5.5 to 6.5 which means not too acidic and not too alkaline.

Other than that, to plant this purple amaranth, good drainage is required to ensure good growth. Suitable bed is needed with good measurement and suitable planting distance to ensure this plant grow in good condition. This purple amaranth is plant by sowing seeds on bed. Some of farmer preferred to sow in seed tray and then transplant on the bed. Watering is an important care and crucial for this crop after planting to ensure this plant grow healthily. Normally, the seeds will start to germinate after 2 or 3 days after sowing the seed. About 1 to 3 kg of amaranth seed is required for 1 hectare of land.

Other than watering, another care needed for this plant is weeding to ensure this plant get enough nutrient without need to be compete with other weed grow near them. Weeding must be done carefully to avoid affecting it roots. This purple amaranth can be harvested after 3 to 4 weeks after sowing. This crop can be harvested when it grow up about 20 to 25 cm. This crop can be harvested by cutting it or pull out from the ground. Research has been done around Malacca and Johore which it shows that the yield of this crop is approximately around 10 000 to 20 000 kg per hectare with frequency of 8 to 10 times per year. Amaranth seeds contain about 13 % to 15 % of protein which is the highest among any grain. Other than contain high protein, its seed also contain fibre, potassium, iron, calcium phosphorus, zinc and also contain vitamin A and C. (Hosnan, 2013; Lawal, 2017).

2.1.2 Classification Of *Amaranthus Cruentus*

Amaranthus cruentus is a crop that other than produce nutritious staple grain, it also known as flowering plant which is normally cultivated for it grain source. This amaranth also has many other name. In Mexico, this crop is known as hautli while in English this crop has various name which are blood amaranth, purple amaranth, red amaranth, prince's feather and also Mexican grain amaranth. In Malaysia and Indonesia, it is known as bayam merah atau bayam ungu. Other than that, in Maharashtra, this purple amaranths is called as shravani math rajgira. Usually amaranth is green in colour but for has colour of purple variant which this purple amaranth used to grow for inca rituals. (anon, 2018)

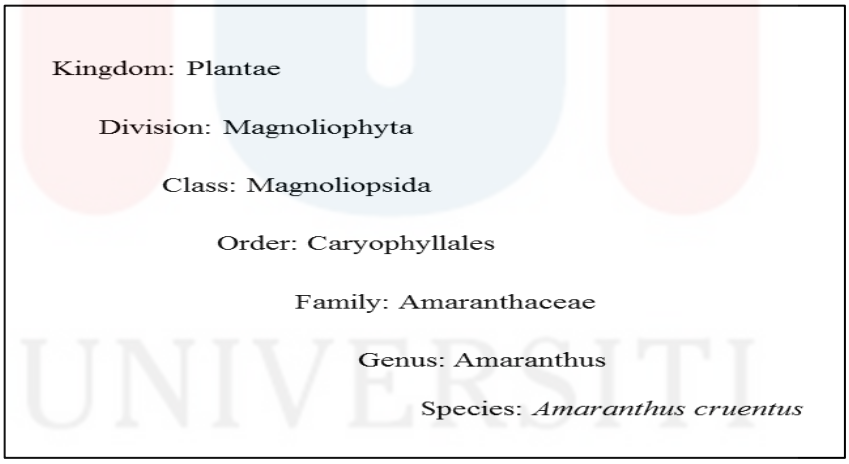


Figure 2.3 Plant Taxonomy of *Amaranthus cruentus*

The plant taxonomy or classification is as shown in figure 2.3. (USDA, n.d.).This plant is from kingdom Plantae and from Amaranthaceae family. It is belong to genus Amaranthus and this plant scientific name is *Amaranthus cruentus.*

2.1.3 Pests And Disease Problem In Purple Amaranth

Amaranth also have their disease and pest problem. For *Amaranthus cruentus*, common pest that attack this plant is aphids. Aphids cause damage to plant by sap sacking from the leaves of the plant. This will cause wilting to the plant leaves. Fatal plant disease that is normally cause by aphids is known as Aster yellows which its sometime affect this purple amaranth. By controlling or reducing aphids attack, the disease can be controlled. In order to reduce the aphids attack, there are 2 ways can be used which the first way is by spraying the plant with water stream or another way is by cover up the leaves on both upper and down part using insecticidal soap or insecticidal oil. (Christensen, 2018).

Other than that, *Amaranthus cruentus* also subject to disease like leaf spot, rust and also root rots. Humid weather possibly aggravate the pest and disease problem. Thus, to reduce this problem, few things can be done like planting this amaranth in well-drained soil or treat the disease with fungicide. Besides that, suitable spacing for the plant is important during sowing to ensure air can freely circulate around the plants. Other care is leaves that has been attacked by disease must be removed and debris on the ground must be picked. Fungicide spraying is one of the method to reduce pest and disease. For plant that has been seriously infected, it must be destroyed to avoid the disease infect other plant. (Christensen, 2018).

2.1.4 Nutritional Properties Of Purple Amaranth

In *Amaranthus cruentus*, there are few nutritional value which is good for human health. It contain about 2.05 mg / 100 g of calcium which the function of calcium is for blood clotting, for strengthen the bone, contraction and relaxation of muscle and also help to absorb vitamin. Other than calcium, *Amaranthus Cruentus* also contain potassium and magnesium of 2.53 mg / 100 g. Their function is help to control contraction of skeletal muscle and also transmit nerve impulse. Other than that, blood pressure also can be decrease when consume vegetables that contain both magnesium and potassium. Iron also can be found in purple amaranth. In the formation of haemoglobin, iron is needed. *Amaranthus cruentus* contain high amount of iron which needed for haemoglobin. Thus, it is recommended for anaemic recovery to consume vegetable that contain iron. (Mensah, Okoli, Eifediyi, & Ohaju-Obodo, 2008).

Besides that, this plant also contain ascorbic acid in quite large amount. Ascorbic acid is an antioxidant which can help in body protection against various disease especially cancer. It also may protect from degenerative disease like diabetes mellitus type 2 or arthritis disease. Ascorbic also function to help in strengthen the immune system. Other than ascorbic acid, iron, calcium, potassium and magnesium, this plant also has other nutritive value which they also contain alkaloid, inulin, saponin and tannin. There are 97 % of usage of *Amaranthus cruentus* in Nigeria as this plant has many benefits and contain many good nutritive value. (Mensah et al., 2008).

2.1.5 Use Of Purple Amaranth

Amaranthus cruentus has been commonly eaten as vegetables by many people all over countries. This plant has been grown commercially in South Africa and being selling in supermarket in form of canning. Other than being eaten as vegetable, this plant can be used as ornamental plant. This purple amaranth also has been used as for fodder, and making food dye by extracting their colour. This plant has been prepare for potash by burn the dried plants in Renin Republic.

This species has several benefits for health and also medicinal properties and medicinal use. Amaranthaceae species can be used to prevent children grow retarded. It also can help in prevention of premature old age. Constipation also can be treat by consuming this vegetables as it contain many nutritive value. Other than that, this vegetable can help in treating kidney complains and anaemia. Water extraction of this plant can be used to treat limb pains as expellant of tapeworm, dressing wound and treat tumour. The roots of this plant can be mixed with honey and used for infant laxative.

Other than being used commercially and used for their medicinal properties, this plant also can be used industrially. Amaranthus cruentus also has function in computer industry, which it is use as lubricants as cited in Alegbejo, (2013). Apart from that, this plant also can be used in cosmetics and also health food. (Alegbejo, 2013).

2.2 Plant Tissue Culture

Plant tissue culture on artificial media was first commercialize happen in growth and germination of orchid in 1920. After a good artificial media created in 1962 which is known as Murashige and Skoog medium, plant tissue culture has been commercially took off. Plant tissue culture is a fundamental of science that help in understanding the growth and development of a plant. The first attempt using tissue culture technique is done by a botanist from German which known as Gottlieb Haberlant in 1902. He selected a cell from a single plant and the isolate it for culture purpose on knop's salt solution with glucose but he has failed the experiment because the culture technique use by him does not use plant hormone which is necessary for plant growth and cell division. (Dagla, 2012).

Plant tissue culture is a production of a new complete plant or growing and multiplication of plant by using plant part such as cells, tissue and organ using artificial medium and addition of plant hormone in the medium under sterile technique and condition to support plant growth. Aseptic control environment is needed for doing tissue culture. Plant part like stems, root, shoot and nodal explant can be used in doing tissue culture technique. Clean environment is needed with suitable temperature, light, humidity and proper ventilation according to plant types and requirement. Plant tissue culture is widely used for multiplication of plant in wide-scale used. There are many benefits and used in tissue culture especially for elimination of virus, genetically manipulation and plant improvement. There are favourable condition that required to do tissue culture which are suitable medium for plant growth that contain nutrient supply for the cell to growth into a plant is needed and selection of appropriate tissue part is required. (Daud, 2017).

2.2.1 In Vitro Propagation

Tissue culture method is a method that can be associated with plant tissue maintenance using in vitro techniques or condition. In vitro technique is a technique where the process is carried out outside of plant part which is normally carried in glass container or in a closed test tubes. In Latin, in vitro is define as inside a glass. The plant tissue that has been isolated is left inside a suitable culture container such as petri dish, glass jar or test tube. The container containing tissue left inside must contain suitable medium to give nutrient to plant. Tissue culture techniques also always recognised as in vitro propagation which sometime this techniques also known as micro propagation techniques. Culture is known as planting a plant tissue into an artificial media under aseptic techniques which mean sterile condition and free from microorganism while micro is one of technique involving plant cell that is breed in a small places. (Daud , 2017).

According to Ravi, (2015), recent years, technique planting of plant using in vitro propagation is increasing and also this technique is one of conserving of plant that grow vegetatively. For maintaining or conserving germplasm under in vitro method, clonal propagation is it prerequisite. For almost more than three decade, plant propagation using in vitro method in culture tissue has been successfully used for cloning plant and produced plant in large quantity.

In vitro propagation technique involve a process of small plant tissue or known as explant being separated from plant organ and then culture in sterile medium and then a new plant is produced. The new plant that has been produced has same characteristic and nature to the mother plant. (Daud, 2017).

In preparing in vitro propagation, good plant material must be selected and identify suitable plant material that would like to be grown on media. There are two types of culture tissue which are organized and unorganized cell. For the organized cell, there are shoot culture, meristem culture, embryo culture and node culture while for unorganized culture, there are callus culture, suspension culture, protoplast culture and anther culture. Shoot culture is a growing of shoot tips, buds of the plant. Meristem culture is a growth of shoot apex to give single shoot while node culture is growth of bud producing single shoot. Callus culture is growth of cell masses that arises from unorganized growth of small plant. Growth of plant cell on a liquid medium from clumps of small cell is called suspension culture while protoplast culture is taking a part from isolation of plant cell without their cell wall. Anther culture is a technique where anther that is complete contain pollen microspores that is immature. (George et al., 2008).

2.2.2 Advantages And Application Of In Vitro Propagation

Planting plant vegetatively or through conventional method is not enough satisfactory. Micro propagation is one of alternative method in planting plant. There is a lot of benefit or advantage can be obtained by using tissue culture method or known as micro propagation.

According to Shahzad et al., (2017), by using tissue culture or micro propagation technique, a true to type plant can be produced easily and the plant is free from disease. Before start culturing the plant, disease will be eliminated first such as elimination of bacteria, fungus and virus by aseptic technique. Uniformity of plant production in higher degree can be produced using tissue culture methods. True-type plants will be produce

and possibility of market demand can be fulfil. Using tissue culture also does not need or required a large and big area to produce plant as this technique required only small space. Besides that, the necessary of time and cost in production can be reducing.

By using in vitro propagation method only taking small piece of plant can produce a large number of new plant. The benefit is number of explant required to produce new plant is very small number and limited. Plant also can multiply rapidly by using tissue culture. Plant can be produced in large scale. In the beginning this technique is apply to plant that is hard to produce sexually. This phenomenon has been continually being used as many plant can be produced in one time. Lastly, a quality plant can be produced as this plant is a disease free plant production. It has ability to withstand an attack from pathogen and pests. Thus, good quality of plant can be produced.

The use of tissue culture in the field of agriculture allows botanists to introduce this method in plant sciences. This method can be applied in plant breeding which a plant that free from viruses can be raised up and grow. In plant breeding also, plant that is homozygous can be obtained by diploidization in single plant generation. Other than plant breeding, this method also can be applied to industrial production of nature of plants. Lastly, germplasm conservation also can be applied using tissue culture method as the germplasm conservation can regenerate whole plant successfully. (Dagla, 2012).

2.3 Plant Growth Media

Once an explant being isolated from its original plant, the explant will no longer able to receive any hormones or nutrient that provided to it before this from its plant. So, nutrient is needed by the plant and must be provided to it in order to ensure in vitro growth of the plant. Medium culture is a basic of plant nutrient composition that is required for plant growth. There are many media composition that has been created. Among media that has been created are Murashige and Skoog medium (MS medium), Murashige and Tucker medium (MT medium), N6, B5 and many other medium for plant culture. Most of medium that commonly being used is MS medium because it is suitable for all plant type and also for plant cell. This MS medium consists of high concentration and quantity of mineral salt. A media should consist of an important component in order for ensure plant growth and developed healthily. Essential nutrient and main component is needed in a medium. (Daud, 2017).

The developmental and growth of plant is different according to their nutritive requirement that is needed by each plant. Media that contain micronutrient, macronutrient, amino acid, vitamin, carbon source, supplement of organic material is the best and ideal media for culture of plant cell. Inorganic salts also important in plant cell media culture. Medium that is going to be selected in plant culture depends based on species of plant. Toshio Murashigue and Kolke Skoog were the first inventor of Murashige and Skoog medium. This media are created to support culture of Tobacco from callus and shoot regeneration and also explant plantlets. (Trivedi & Dahryn Trivedi, Gopal Nayak, Khemraj Bairwa, 2015).

Macronutrient that is used in media is consist of nitrogen, phosphorus, postassium, calcium, magnesium, sulfur and ferum while micronutrient consist of manganese, copper, zinc, boron, chlorine, cobalt and molybdenum. Macronutrient needed in large quantity by plant as it is a main nutrient needed. Micronutrient is second important for plant after macronutrient as this nutrient important for process of metabolism and plant physiology processes. Other than macronutrient and micronutrient, vitamin also needed for plant growth through in vitro culture as vitamin is used to promote plant growth and development. Few vitamins can be used in media where some of it are vitamin B1, vitamin B6 and Myo-inosotol. Agar is one of other component need in preparing media as the agar help in solidifying the agent which the purpose is to support plant growth. Agar is made up of polysaccharide that being obtained from several species of algae and seaweed. There are several substances that can replaced used of agar which are gelrite, alginate and gelatine. Quantity of agar used must be in correct amount to ensure plant growth in good condition and agar constituent must be suitable to plant growth in it. Normally, the correct and suitable amount of agar is 8g/L for good growth development. Media also must contain plant hormone or plant growth regulator to support plant growth. (Daud, 2017)

Plant hormone is one of factor determining successful of plant tissue culture. There are five commonly recognized of plant hormones which known as ethylene, auxin, abcissic acid, cytokinin and gibberellin. This 5 substances is the most important in plant growth as they regulate the growth and developing of plant tissue culture and organs. This hormones must not be ignored in tissue culture as they have regulatory-roles.

Plant hormones has been synthesized playing hidden role in growth and development of plant and is said to be active. Plant growth regulator is a compound that is synthetic and they act like a natural plant hormones. Many of plant growth regulator has discovered endogenous hormones equivalently and their biological activity. In few cases, the balancing of plant hormones that being used to treat a plants, may be affected by plant growth regulator. (Rademacher, 2015)

Plant growth regulator (PGR) can be combined with another two or three different PGR which is required by plant and they either can be used simultaneously on the growth media or used separately in sequence. Among plant hormone that has been used extensively in micro propagation or culture tissue techniques are auxin, cytokinin, gibberellin, ethylene and abcissic acid. The need of plant hormone for in vitro propagation of culture tissue is not too much which it is needed in small amount to support the development of plant. Each of the hormone has their own function in supporting plant growth. The hormone has its biochemical and physiology properties once added to medium culture. (Rademacher, 2015).

Auxin has vital function through in vitro culture where the auxin give response towards callus induction, root development and suspension of cell. Cytokinin hormone in plant pays role in development of cell culture and growth of organ. Cytokinin also helps in cell division stimulation and proliferation of shoot. It also prevent the growth of root in plants. Other than ctyokinin and auxin, gibberellin is widely used for cell differentiation and cell multiplication. Gibberellin also act to prevent root and shoot formation. Among all of the hormone that needed for tissue culture medium, auxin and cytokinin is the most important in plant growth. Auxin hormones that used in tissue culture helps to stimulate cell or organ suspension. Other than that, it help in stimulating growth of callus and root. Cyokinin roles is to stimulate shoot growth and formation. It also helps in cell division

induction. Cytokinin important in determining cell division and morphogenesis of plant in tissue culture.

Most commonly auxin that have been widely used in tissue culture field is Indole-3-acetic-acid or known as IAA. Other auxin that used is IBA which stand for Indole-3-Butyric acid. There are synthetically produced auxin that normally being used in plant tissue culture are 2, 4-di-chlorophenoxyaceticacid and 1-naphthaleineacetic acid or NAA. For cytokinin, the most commonly used in plant tissue culture are zeatin, N-Isopentenylaminopurine (2iP), BAP which stand for 6-Benzylaminopurine and lastly kinetin, K. For gibberellin hormone, there is GA3, Gibberillic acid that can be used in tissue culture. Among hormone used in doing tissue culture is synthetic hormones such as BAP, Indole-3-Butyric acid and TDZ which is known as Thidiazuron. Hormones responses and activity depend on type and concentration of hormone use and also depends on plant physiology and genotype.

In medium contain more auxin, the root development is good while medium has higher cytokinin compared to auxin, shoot formation normally occur. For shoot induction growth, BAP is the most suitable hormone compared to kinetin. TDZ hormone may be added to BAP as this hormones can help in increasing of shoot proliferation for some species of plant. The shoot is from explant culture like leaf petiole, peduncle and many else. By choosing suitable and good hormones, production of plantlets can be complete and perfect. Combination of 0.5-2.0 Mg/L NAA hormones with 0.5-2.0 Mg/L BAP can be added to MS medium with suitable optimum concentration able to produce adventure shoot induction. (Daud, 2017)

2.4 Explant Selection And Preparation

Explant is a plant part like cell or organ of a plant that used to do in vitro culture. There are many plant part can be used as explant for doing tissue culture. Based on article Emeritus, (2013), there are several factors that influence the explant selection. First of all, size and location of explant used must be known. Selecting smaller size of explant will cause the process of culturing harder than choosing bigger size. This is because by choosing small size, additional component must be added to the medium. Larger explant is a good choice as it contain more nutrient in it. Besides, larger explant contain plant growth regulator to sustain culture. Plant also will have different hormonal balance. Second factor that need consideration is source plant quality. Healthy plant is a good choices to obtain explant. Before choosing explant, make sure their mother plant is free from disease and virus, do not get water stress and get enough of nutrient. This is to ensure, once the explant has been grown on nutrient media, the plant can grow healthily without any contamination occur in the culture. Age of the explant chosen also is one of consideration in selecting the explant. Year season have contribute effect in response to the culture of the plant and contamination. The age of plant can affect or influencing the development of callus. Young explant tissue is better to be chosen compared to old tissue as the young tissue have better respond to in vitro treatment. Genotype of plant also is one of contribution factor to choose explant. Some of genotype of plant will not give any response towards cell culture. Identification of which plant genotype that will give respond after culture is important thing. (Emeritus, 2013).

Explant that will be used on artificial media or nutrient media must be sterile. Those explant can be sterilized by using bleach agent before culture it. Choosing suitable plant part as explant and its preparation with medium culture is important factor in that contribute to successful of in vitro propagation.

Explant must be free from microorganism and in sterile condition to reduce or avoid contamination from occur. Cleaning process of explant also must be done carefully and perfectly to ensure no side effect to the explant condition. In term of explant growth, for shoot explant, the shoot can be taken from leaf with 5, 6 or 7 position of the plant.

For the explant of nodes or nodal explant, distal cutting is from upper side while the bottom cutting is known as proximal. This knowledge is important for the explant position in medium culture. Sometimes, for the nodal or leaf explant, the plant part cutting must be placed inside a medium culture in vertical position with the medium. This condition can create a fragmented area that touches the media. Through this method, the cutting part can obtained nutrient easily and the plant growth regulator can permeated easily into the plant. Contamination of the explant must be avoided by doing the culture carefully. (Daud, 2017).

2.5 Plant Callus Culture

Callus is a word originated from Latin word which is Callum that has meaning of hard. Unorganized cell masses could be generated by plant such as callus or tumours in responsible to stresses such as wounding, or pathogen infections. Callus is a tissue mass that is unorganized that growing in solid substrate. It comes from plant naturally in response to wounding, graft union or infestations. Callus can be multiplied and then clone

into a new whole plant. Callus has been referred by plant biology before as massive cell growth and callose accumulated associated with wounding.

Callus also undergoes somatic embryogenesis under certain condition which embryogenesis is a process which embryos are generated from adult somatic cells.

Other than that, callus tissue also gives meaning of unorganized proliferative mass of cells that is produced from plant cell that has been isolated or tissue or organs that grown aseptically on artificial media under controlled condition and environment. Callus culture has potential to develop normal root, shoots or embryoid to form a plant. Formation of callus is due to infection of microorganisms from wounds cause by endogenous growth hormone stimulation which are auxins and cytokinins.

There were few factors that affecting callus culture which are plant growth regulator used to induce callus, source and genotype of explant use in the culture, medium composition, temperature of surrounding and also plant condition of its physiological and growth. Callus has it importance for growing it which are by using callus, a whole plant can be developed or regenerated through nutrient and hormone manipulation. This also known as morphogenesis or plant regeneration. Callus also can be used to culture in liquid media which known as suspension culture. (Ikeuchi, Sugimoto, Iwase, 2013).

2.6 Previous Study of Amaranthus Plant Using Tissue Culture

Previous study has been done by M.Biswas, S.Das, & S.Dey, (2013), by using *Amaranthus tricolor*. The seed of this plant was inoculated on MS media that containing phytagel for germination. After 1 month the seed has been grown in vitro, the seedling has been used as explant for initiation of callus by using it few part like leaf, stem and root. The MS media that used for subculture this explant has been supplemented with 0.5 mg/l and 1.0 mg/l of 2,4-D for initiation of callus. Other than that, in this research, combination of media also has been done by combining NAA hormone with BAP hormones. As the result callus could be induced.

Other research also has been done by Bernardo, Aspuria, Yebron, (2017),which in this research *Amaranthus spinosus* and *Amaranthus tricolor* has been used as explant. Seed of this explant has been germinated using in vitro method. Once the seedling has grown, the hypocotyl explant of the grown seedling has been taken and being culture on MS media containing BAP at concentration of 0.5 mg/l and 1.0 mg/l. Other hormone used were 1.0 mg/l, 5.0 mg/l and 10.0 mg/l of NAA and 2,4-D at 0.5 mg/l, 1.0 mg/l and 2.0 mg/l. As the result, seed that has been sterilized for longer time give the higher contamination rate which at 90.55 % of contamination while seed being treated for 10 min. However, callus has successfully been induced in this study.

Other study using *Amaranthus cruentus* seed being culture on MS media under aseptic condition has used hypocotyl as explant were placed on media containing 2,4-D, NAA and BAP hormone at different concentration. After 4 week callus still cannot be obtained. However, callus obtained on BAP supplemented media was colourless and friable. (Kitti. et al, 2017).

CHAPTER 3

MATERIALS AND METHODS

List Of Materials and Chemicals

Schott bottles, measuring cylinder, balancer, spatula, jug, pipette, hot plate magnetic stirrer, micropipette, aluminium foil, distilled water, conical flask, dropper, pH meter, glass jar, microwave oven, balancer, wrapper, sterile forceps, spirit lamp, conical flask, spray bottle, knife, autoclave machine, laminar flow hood, paper towel, test tube, 1.0 M NaOH, 1.0 M HCL, Iron, vitamin, Macronutrient, Micronutrient, gelrite, sucrose, ethanol, tween 20, Clorox, NAA hormone, 2,4-D hormone, Picloram hormone, Amaranth seed.

3.1 Preparation of Stock Solution

4 sets of stock solution has been made based on groups of nutrients required for plant tissue culture which are macronutrient, micronutrient, iron and vitamins. A required amount of chemical need to be weighed according to the calculation provided in the table. The stock of the nutrients has to dissolve in appropriate quantity of distilled water. After the solubilisation complete, the final volume has been made up. The solution was transferred into schott bottle and then label the bottle with type of stock solution, media type, date preparing the solution, instruction on how to use and followed by name of the owner. Then, the solution was stored at 4°c for MS medium preparation. Table 3.1 shows list of chemical substances that needed to prepare stock solution with the volume needed for each stock.

Table 3.1 List of chemical substances for stock preparation

A. Micronutrient	1X (G/L)	50X (G/500mL)
NH_4NO_3	1.65	41.25g
KNO_3	1.90	47.50g
$CaCl_2.2H_2O$	0.44	11.00g
$MgSO_4.7H_2O$	0.37	9.25g
KH_2PO_4	0.17	1.7g
B. Macronutrient	**1X (G/L)**	**1000X (G/500mL)**
$MnSO_4.4H_2O$	0.02230	11.15g
$ZnSO_4.7H_2O$	0.00860	4.30g
H_3BO_3	0.00620	3.10g
KI	0.00083	0.415g
$NaMoO_4.2H_2O$	0.00025	0.125g
$CuSO_4.5H_2O$	0.000025	0.0125g
$CoCl_2.6H_2O$	0.000025	0.0125g
C. Vitamins	**1X (G/L)**	**500X (G/500mL)**
Myo–inositol	0.1	25g
Glycine	0.002	0.5g
Thiamine-HCL	0.001	0.25g
Nicotinic acid	0.0005	0.125g
Pyridoxine-HCL	0.0125	0.125g
D. Ferum source	**1X (G/L)**	**200X (G/500mL)**
$FeSO_4.7H_2O$	0.0278	2.78g
$Na_2EDTA.2H_2O$	0.0373	3.73g

3.2 Preparation Of Murashige and Skoog (MS) Media

About 300 ml of distilled water was added into a 1-liter conical flask. Then stir bar was placed into it to make the solution stirred while prepared it. 30 g/L sucrose was added then add the previous stock solution that has been prepared accordingly into a conical flask. Bring the volume near to 400 ml by adding distilled water to the solution. pH has been checked by using pH meter and adjust pH by adding NaOH or HCl until the pH reach about 5.8. Addition of 8g agar has been done into the solution and being dissolved by using microwave oven. After all component has dissolved, the solution was transferred into glass jar. The cap of the vessel containing the medium must be kept loosely while sterilizing. The jar of the medium, sterilized using an autoclave for about 15 minutes at 121°C at 15 psi. Let it cool and then it has been stored in media room before culture. The media solution prepared is MS basal media and media contain hormone must be supplied with plant growth regulator at different concentration like 1 mg, 2 mg and 3 mg of NAA, 2,4-D and picloram.

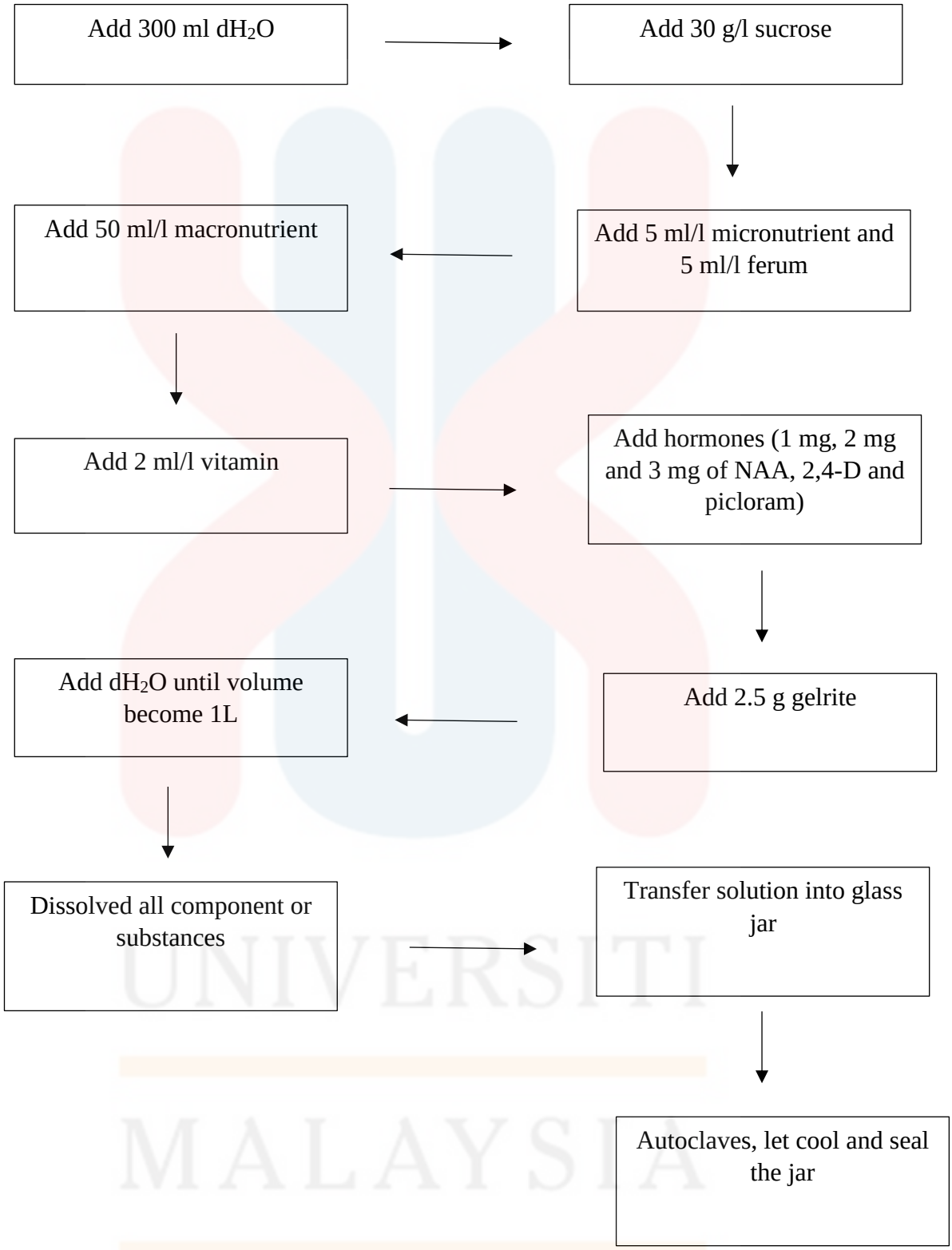


Figure 3.2 Flowchart for MS media preparation

3.3 Sterilization Of Amaranth Seed

Firstly, purple amaranth seed was taken and then the seed has been washed by mix water with 3 drops of tween 20. After that, the seed has been rinsed by using tap water. Set up laminar flow hood by spraying ethanol, set up UV light and blowing it. The sterile seed was brought into laminar flow hood. Then, inside the laminar flow, the seed was washed by using 95 % ethanol about 1-2 minutes and then rinsed using distilled water. After that, wash seed using Clorox. Rinse the seed using distilled water 3 times. The seed ready to be cultured on MS basal media. The seed has been scattered about 5-6 seed in one jar. Sealed the jar using parafilm. Transfer the jar into growth room and placed in continuous light.

3.4 Amaranth Subculture Taken From Aseptic Seedlings

Once the seed has been germinated and the plant grow bigger, then it need to be subculture on media contain different hormone with different concentration for obtaining callus. The explant was brought into laminar flow, and being transferred into media contain plant growth regulator. Shoot and stem was taken as explant and being cut into small part then placed it onto media contain hormone of 1 mg, 2 mg and 3 mg of NAA, 2,4-D and pichloram. Each hormone was done about 4 jar which 2 jar has been cultured using stem as explant and another 2 jar used shoot as explant. Then callus growth has been observed if there were any formation of callus.

3.5 Data Observation And Analysis

Growth of the cultured seed has been observed. The result of the seed germination obtained is recorded. The observation for seed germination has been made based on how many times culture has been done, jar produced per culture, seed germination rate and also average leaves number.

Then data subculture of the explant taken from aseptic seedlings then being placed on different hormone at different concentration also has been recorded. Table 3.5 shows experimental design for amaranth subculture. The data obtained has been analysed by using one way analysis of variance (ANOVA) and Tukey's test through SPSS software.

Table 3.5 Experimental design of explant on different type of hormone at different concentration

Source of planting material	**Treatment**		
	NAA	**2,4-D**	**Picloram**
Shoot	1 mg/L	1 mg/L	1 mg/L
	2 mg/L	2 mg/L	2 mg/L
	3 mg/L	3 mg/L	3 mg/L
Stem	1 mg/L	1 mg/L	1 mg/L
	2 mg/L	2 mg/L	2 mg/L
	3 mg/L	3 mg/L	3 mg/L

CHAPTER 4

RESULTS & DISCUSSION

This experiment has been done by culturing seed of *Amaranthus cruentus* or known as purple amaranth on MS basal media which means media without presence of any hormone or plant growth regulator. Then, the seed was subculture on media containing different plant growth regulator at different concentration. All hormone used were auxin hormone which are 1-Naphthaleneacetic acid, 2,4-dinitrophenylhydrazine and picloram. This research is about the production of callus that need to be obtained from this culture. Edible food colourant can be produced by using the callus obtained from purple amaranth. There are few factors that may affect the production of callus while conducting this research which are sterilization of seed, equipment used sterilization, quality of seed, hormones used for obtaining callus, right concentration of plant growth regulator and also growth room condition with adequate amount of light presence and suitable temperature.

Ms media has been used in this research because it has balanced composition of nutrient compared to other type of media. In Ms media, it contain macronutrient, micronutrient, vitamins and iron sources which is complete component required for plant to grow and also highly suitable for most plant species. This type of media has been used extensively in tissue culture. Therefore, this media is selected for germinating and

growing the purple amaranth. For seed germination, MS basal media is used as it is favourable for seed germination.

4.1 Seed Germination on MS Basal Media

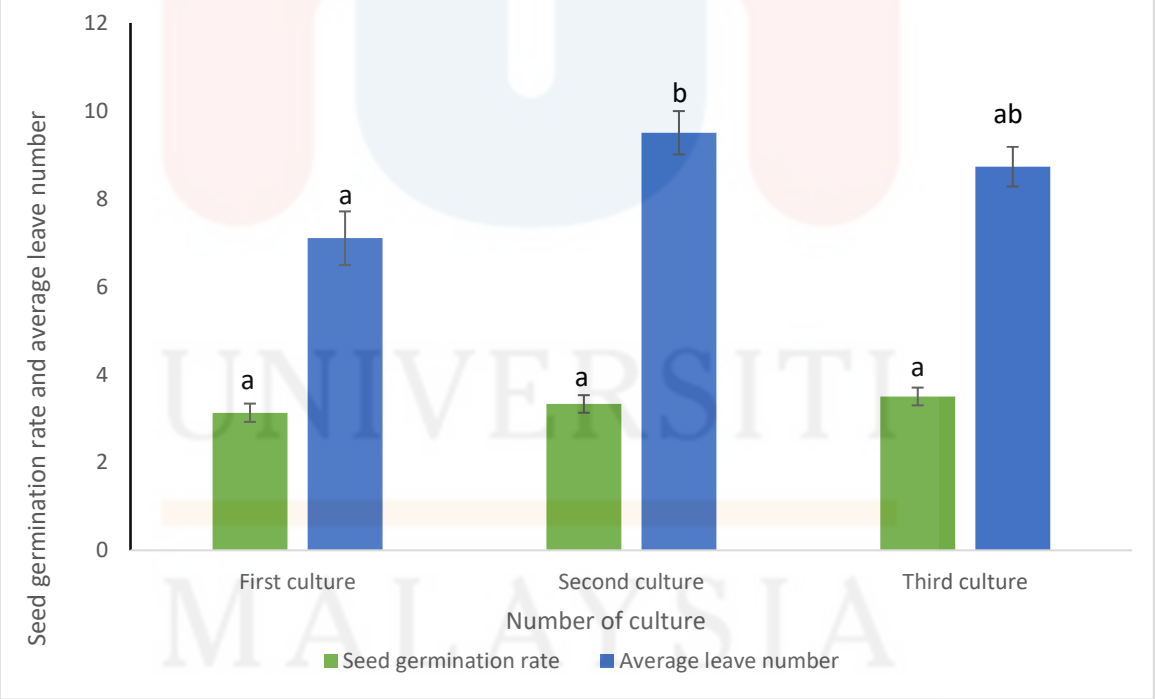
Surface sterilization of seed is important to ensure the seed is completely sterile before culture on media to avoid contamination. Seed that has not been sterilized properly can cause contamination of bacteria, thus, percentage of contamination can be increase. Normally, for sterilization process the common sterilizing agent used are sodium hypochlorite, mercuric chloride, alcohol, flugal and nystatin. For this research, sterilizing agent that has been used were tween 20, sodium hypochlorite and also ethanol. Bleach is normal sodium hypochlorite used in sterilizing. The purple amaranth seed was washed using Clorox. Sodium hypochlorite is one of sterilizing agent that is easy to get at lower cost and effective in killing bacteria and microorganism. It has wide spectrum in bacterial killing. However, the rate of application should be consider to avoid over sterilizing and causing harm to explant or seedlings.

Other powerful sterilizing agent used is ethanol but it is phytotoxic. Normally, explant were exposed to ethanol only for a short period like seconds or minutes depends on the concentration used. Therefore, this seed was immersed in ethanol only for 1 to 2 minutes to avoid toxicity occur to seed. Other than that, tween 20 or known as polysorbate 20 has been used before sterilize using ethanol and sodium hypochlorite. Tween 20 act as surfactant that can enhance the sterilization effectiveness.

The data of seed germination has been observed and has been analysed by using the SPSS software to check it significant value and also it means.

Table 4.1 Seed Germination and Average Leaves Number from Aseptic Seedlings

Batch number	Seed germination rate	Average leave number
First batch	3.13 ± 0.21	7.10 ± 0.61
Second batch	3.33 ± 0.20	9.50 ± 0.49
Third batch	3.50 ± 0.20	8.73 ± 0.45



*The similar letter indicates that the result are not significantly different while the different letter indicates the result is significantly different. Uses Harmonic Mean Sample Size = 30.000

Figure 4.1 Seed Germination and Average Leaves Number From Aseptic Seedlings

Three batches has been made for seed cultured on MS basal media which each batch has different method of sterilization to improve the seed germination rate. For first batch, the concentration of sodium hypochlorite is high and the time for ethanol sterilization is longer compared to second and third batch which the concentration of sodium hypochlorite is reduced and time taken for seed wash in ethanol is shorter.

Table 4.1 and figure 4.1 shows the result of seed germination and average leaves number from aseptic seedlings that has been cultured on MS basal media. After culture on MS media, the seed was observed. Based on the observation, seed germinated about 2 weeks, then the seed started to grow.

From table 4.1, mean and standard deviation for seed germination for the three culture has been analysed. Third culture has the highest value for seed germination rate which are 3.50 followed by second culture with value of 3.33 and then the first culture with value of 3.13 . Significance value for seed germination is 0.45 and the significance value for average leaves number is 0.006. The significance value for seed germination is exceeding 0.05 which means the seed germination rate is not significance to in-vitro culture of the aseptic seedling of the purple amaranth. Significance value for average leaves number is less than 0.05 which means it gives significance towards establishment of aseptic seedlings of purple amaranth. Based on the results shows in figure 4.1, there are only one culture that shows 2 subset which subsets ab on third culture for average leaves number. Other than that, for average leaves number, it only shows subset a for first culture and subset b for second culture. For seed germination rate, there were only one subset obtained. The similar subset shows that the seed germination rate is not significance.

Other than analyse data for seed germination and average leaves number, result for contamination and survival percentage also has been recorded. It is shown based on the table 4.1.2 and figure 4.1.2.

Table 4.1.2 Percentage of contamination and survival for aseptic seedlings

Number of Culture	Percentage Contamination (%)	Percentage Survival (%)
First Culture	60	40
Second Culture	31	69
Third Culture	27	73

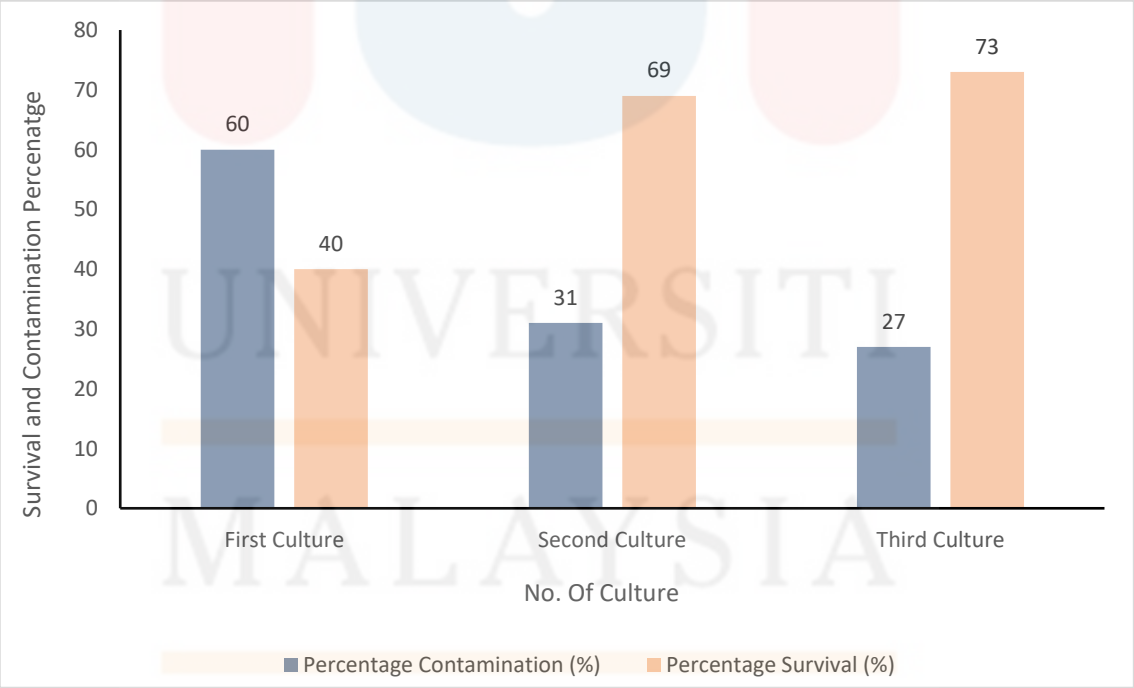


Figure 4.1.2: Percentage of contamination for three time culture

Figure 4.1.2 and table 4.1.2 shows the percentage of contamination that occur for each culture. The highest contamination percentage was recorded in the first culture which 60 percent of the culture has contaminated and the survival percentage of 40 percent. The percentage of contamination has been reduced for the second and third time doing the culture.

The contamination percentage during second culture has decreased to 31 percent of contamination. For the third culture, the contamination percentage has decreased a bit from the second culture with 27 percent of contamination. Sterilization method is important during culture to avoid contamination. Therefore, in order to reduce the percentage of contamination, sterilization must be done properly.

Other than result obtained from ANOVA analysis as present in table 4.1, and result of contamination from table 4.1.2, this seed germination result also is shown in figure 4.1.3 which this figure shows the seed condition after culture on MS media after 2 weeks.

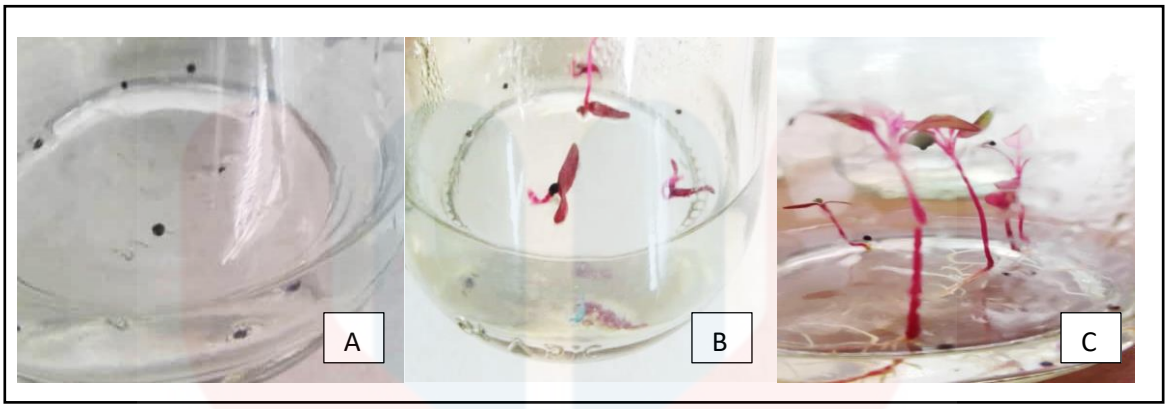


Figure 4.1.3 Effect of seed germination on MS basal media after 1 month of culture.

Figure 4.1.3 shows that seed condition after 1 month being culture on MS media with zero hormone. The result shows the condition that occurred on the three culture of the seed. Some of the seed does not germinated at all as can be seen from figure 4.1.3 (A). The seed still did not germinate even after a month cultured on MS media.

In the figure 4.1.3 (B), the seed start to germinate after 2 weeks and grow after that but after a month, the condition of the plant is not in good condition. As can be seen from the picture from figure 4.1.3 (B), the seedling germinated was stunted with leaves fall downward. However, the seed in figure 4.1.3 (B), only occurred for the first culture and did not occur on second or third culture.

In figure 4.1.3 (C), the seed germinated and it grow well until produce leaf and healthy root. The leaf and the stem can be used for subculture in media supplied with plant growth regulator.

Germination of seed was a critical and important part for successful growth of a crop. Environment is one of factor that influence the seed germination which the environment factor include suitable growth room, temperature, light and moisture.

However, if all the condition is in favourable condition, the seed still will fail to germinate if the seed shows it dormancy. The seed viability can be seen through the ability of seeds to germinate well. Seed dormancy distributes seed germination in time. To reduce or to overcome seed dormancy, there were few factors that need to be considered. One of the factor need to be considered in order to promote germination of seed by using proper salt solution. Other than that, seed that shows it dormancy can be germinated by stimulation of gibberellin, ethylene and cytokinin. This can be supported by previous research (Tiryaki, Korkmaz, Nas, and Ozbay, 2005).

Other than seed dormancy, sterilization method is one of the factor that cause seed cannot germinate well. However, in tissue culture, for successful in vitro seed propagation, maintenance under aseptic condition is crucial. Ethanol that has been used as sterilizing agent is a strong and phytotoxic. For the first culture of the amaranth seed, seed has been exposed in ethanol for a longer period of time compared to second and third culture. Therefore, during the first culture, the germination rate of seed is quite slow. There was improvement in seed germination effectiveness for the third culture compared to first and second culture as seed has been exposed for short period of time in ethanol. In some research, it has been reported that alcohol are rapid bactericidal rather than bacteriostatic. Other than alcohol, sodium hypochlorite or bleach is used as sterilizing agent is this research. It is reported to be very effective against many types of bacteria.

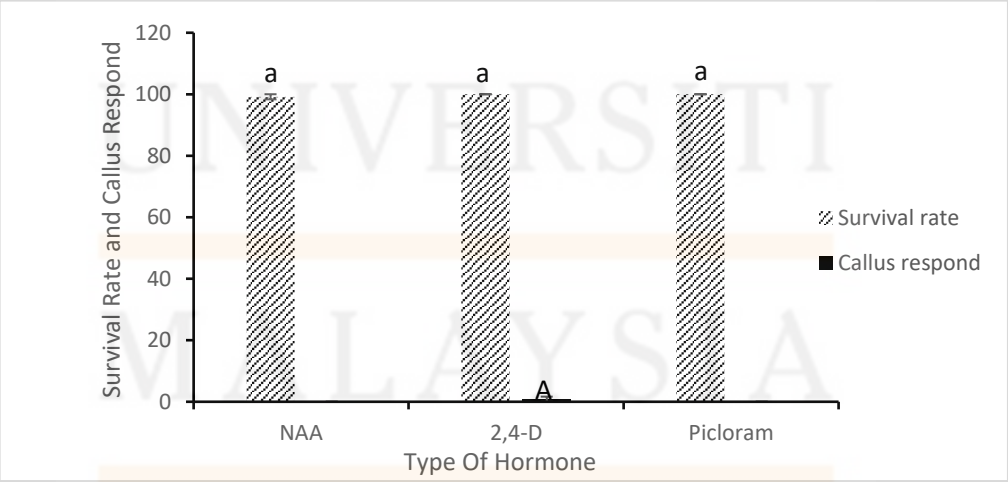
However, in this study, the usage of high concentration of sodium hypochlorite has caused negative effect in seed germination which resulting in lower germination rate. As can be seen in figure 4.1 (B) which the seed growth has been stunted but not occur on third culture as the concentration has been reduced. (Sen, Jamal, 2013).

4.2 Maintaining Subculture of Amaranth on Media Contain Hormone

After seed has germinated and grow bigger, subculture is needed to ensure the explant got enough nutrient for growth support. Therefore, subculture of the purple amaranth obtained from aseptic seedling has been done. Other reason for doing the subculture is to induce callus. The callus induction has been observed on different hormones or PGR at different concentration.

Table 4.2.1 Effect type of hormone on callus respond and survival rate

Type of hormone	Survival rate	Callus respond
NAA	99.17 ± 0.83	0.00 ± 0.00
2,4-D	100.00 ± 0.00	0.83 ± 0.83
Picloram	100.00 ± 0.00	0.00 ± 0.00



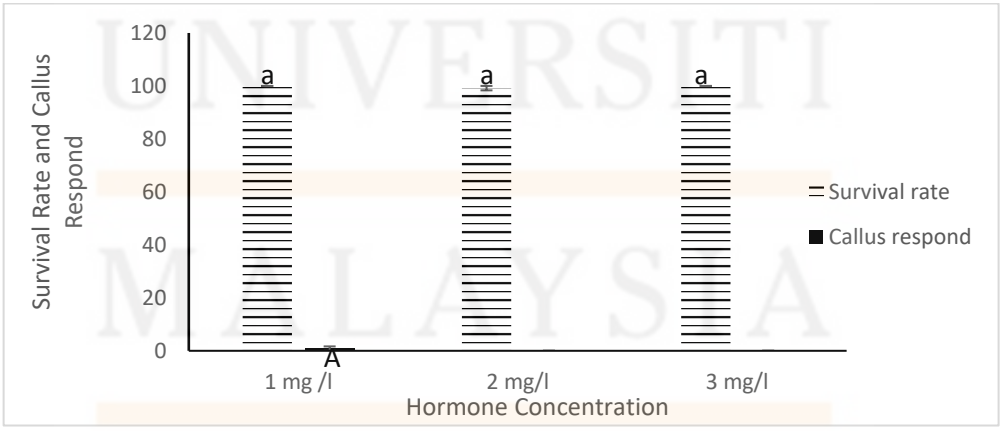
*The similar letter indicates that the result are not significantly different. Uses Harmonic Mean Sample Size = 12.000

Figure 4.2.1 Effect type of hormone on callus respond and survival rate

Based on figure 4.2.1 and table 4.2.1, it shows that the effect on type of hormone towards callus respond and also survival rate. The highest mean of survival rate obtained from hormone 2,4-D and picloram hormone which both of the mean value is 100.00 ± 0.00 respectively. NAA show less mean value compared to picloram and 2,4-D hormone which the value is 99.17. From analysis of data that has been done, the significance value for both survival rate and callus respond is 0.379 which means the significance value is exceed than 0.05. Thus, it can be conclude that the type of hormone does not gives any significance towards subculture of amaranth on media containing different hormones.

Table 4.2.2 Effect of hormone concentration on survival rate and callus respond

ormone concentration	Survival rate	Callus respond
1 mg /L	100.00 ± 0.00	0.83 ± 0.83
2 mg/L	99.17 ± 0.83	0.00 ± 0.00
3 mg/L	100.00 ± 0.00	0.00 ± 0.00



*The similar letter indicates that the result are not significantly different. Uses Harmonic Mean Sample Size = 12.000

Figure 4.2.2 Effect of hormone concentration on survival rate and callus respond

Based on table 4.2.2 and figure 4.2.2, the result shows the effect of hormone concentration towards survival rate of explant and callus respond. From the result in table 4.2.2, highest mean obtained for survival rate is at 1 mg/L and 3 mg/L concentration with mean value of 100.00 ± 0.00. The mean value for 2 mg/L shows less value than 1 mg/L and 3 mg/L with mean value of 99.17 ± 0.83.

For callus respond, the value obtained is 0.00 ± 0.00 for 2 mg/L and 3 mg/L of concentration. However, for 1 mg/L the mean value obtained for callus respond is 0.83 ± 0.83. This shows that, the callus only obtained on PGR at 1 mg/L concentration while for 2 mg/L and 3 mg/L concentration, there were no callus obtained as can be seen from figure 4.2.2.

Based on the hormone concentration results, significance value for both survival rate and callus respond is same which the value is 0.379. This value is more than 0.05 which means that the value does not give any significance towards concentration of hormone with subculture of purple amaranth.

Other than result that has been analysed by using SPSS software, figure below shows the result of plant condition on media contain different hormone at different concentration.

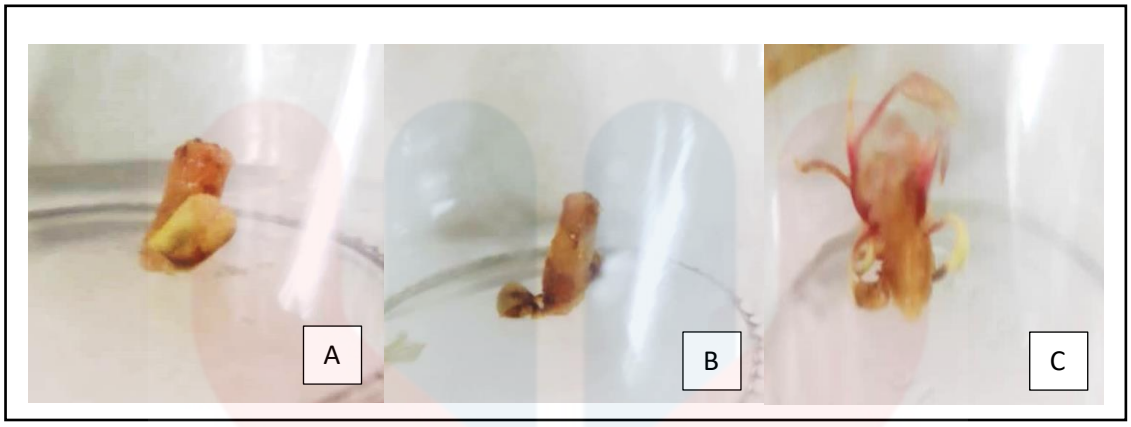


Figure 4.2.3 Explant condition after 5 weeks being subcultured on MS media+NAA (4.2.3 (A) is 1 mg/L NAA, 4.2.3 (B) is 2 mg/L NAA, 4.2.3 (C) is 3 mg/L NAA)

Based on the picture shown on figure 4.2.3, the culture on NAA media, there were no callus obtained on the three subculture. Growth performance of the explant on NAA media shows that 3 mg/L NAA has better growth compared to 2 mg/L NAA and 1 mg/l NAA while in 2 mg/L NAA is better compared to 1 mg/L NAA. This shows that the higher the concentration of NAA, the better the growth of the explant. Part of explant that has been used were stem and leaves part. As can be seen from figure 4.2.3, the leaves part on 3 mg NAA grow bigger compared to stem part that has been subculture on NAA with concentration of 1 mg/L and 2 mg/L. This shows that shoot grow better than stem on media contain NAA hormone.

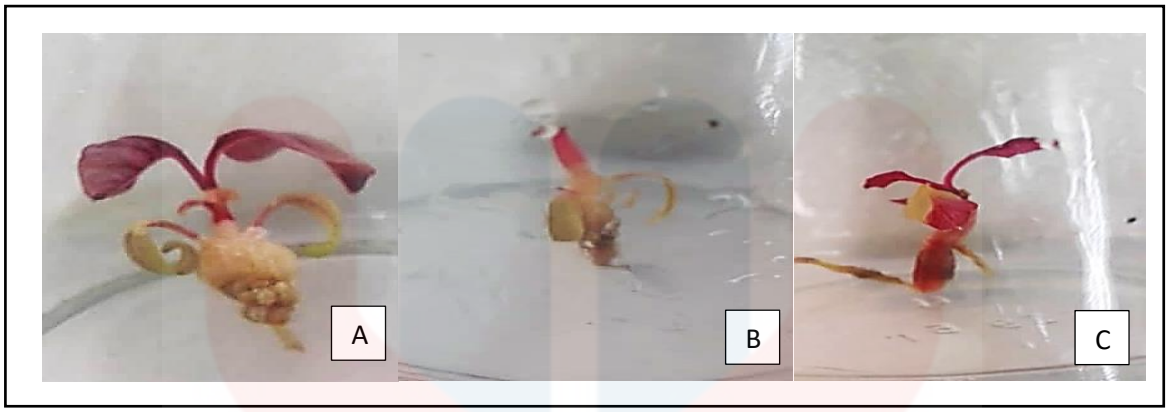


Figure 4.2.4 Explant condition after 4 weeks being subcultured on MS media+2,4-D (4.2.4 (A) is 1 mg/L 2,4-D, 4.2.4 (B) is 2 mg/L 2,4-D, 4.2.4 (C) is 3 mg/L 2,4-D)

Based on figure 4.2.4, there were no callus obtained on media contain 2,4-D hormone at different concentration. However, the growth of the explant is good on hormone 2,4-D on three subculture. On 1 mg/L 2,4-D, leaves part has been taken for subculture.

After 4 weeks being subculture, the explant grow bigger and produces new leaves. The stem also increase its length compared to before subculture as can be seen in figure 4.2.4 (A). Callus also obtained from this hormone.

On 2 mg/L 2,4-D, as can be seen in figure 4.2.4 (B), the explant that has been used for subculture was stem part. This explant on concentration of 2 mg/L also has grown bigger from the initial size during explant. On 3 mg concentration of 2,4-D, the plant also grow bigger and healthy as can be seen in figure 4.2.4 (C). Even though there were no callus obtained from explant on 2,4-D hormone, the explant that subculture on this hormone has grown bigger healthily and there is no contamination occur after being subculture.

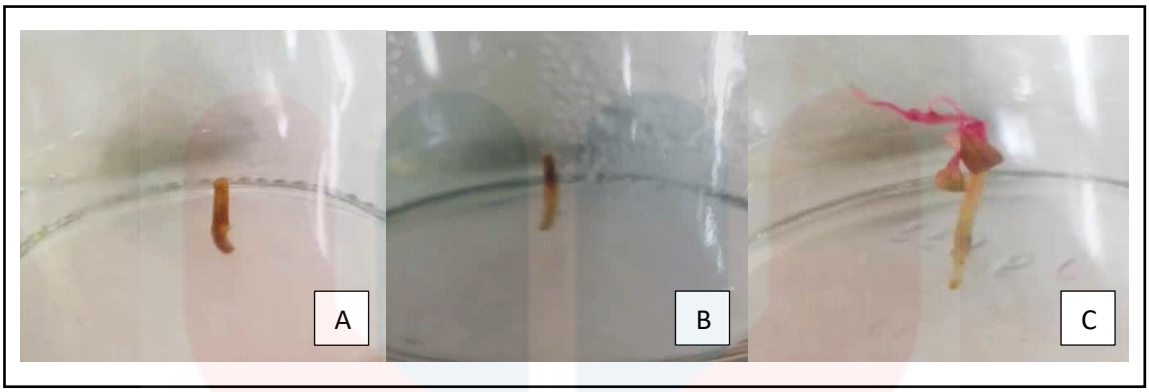


Figure 4.2.5 Explant condition after 4 weeks being subcultured on MS media+picloram (4.2.5 (A) is 1 mg/l picloram, 4.2.5 (B) is 2 mg/l picloram, 4.2.5 (C) is 3 mg/l picloram.

Based on figure 4.2.5, the results shows the explant condition after being subculture on picloram hormone. Explant on figure 4.2.5 (A) and 4.2.5 (B) growth was the same with hormone at different concentration. Explant in figure 4.2.5 (C) shows better growth compared to explant being subculture on 1 mg/L and 2 mg/L picloram. The higher concentration of picloram, the better the plant growth. There were no callus obtained from explant subculture on this hormone. However, there is no contamination recorded when the explant subculture in picloram. For 1 mg/L and 2 mg/L picloram, the part of plant used was stem. The stem does not grow well on hormone picloram with lower concentration. For 3 mg/L picloram hormone, the explant used was shoot. The shoot grow longer after being subculture. 3 mg/L picloram gives respond to shoot growth of amaranthus.

Based on the result, the effect of auxin hormone that has been used, 2,4-D is the better for purple amaranth to grow compared to NAA and picloram. NAA has the slowest grow and took longer time to grow the explant compared to 2,4-D and picloram.

The respond of the callus is very slow, therefore, further respond still in waiting. 2,4-D is good hormone for callus formation. There were previous study that shows callus induction was better in media supplemented with 2,4-D hormone. (Singh, Kandasamy, & Odhav, 2009).

Amount of concentration of NAA used in MS media is not sufficient to promote the growth of callus or formation of callus. There were study used higher concentration of NAA to obtain callus. Based on previous study, NAA concentration used were 1 mg/l, 5 mg/l and 10 mg/l. Therefore, in this research, high concentration of NAA is required to produce callus. While for 2,4-D hormone, the concentration used has enough. (Bernardo, Aspuria, Yebron, 2017).

There are few factors the callus cannot be obtained. Seed sterilization is one of the factors that affect the germination and grow of the seed, thus the explant used for subculture also affected. Contamination percentage could be reduced by increasing concentration of Clorox but seed germination percentage could be decrease and stunted.

Other than that, the seed germinated into explant causing the explant to grow unhealthy. If the unhealthy plant has been used for subculture, then the callus could not be obtained. Other than that, factors that cause callus failed to induce is the surrounding factor like temperature and adequate light. Temperature is one of factor to grow the plant.

Besides that, size and maturity of plant during subculture also cause callus cannot be obtained. Explant used should be more matured and grow up more before doing subculture

Some studies state that media that supplied with auxin alone were unable to induced callus. Combination of PGR like auxin and cytokinin was very effective in formation of callus.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

In this study it shows that sterilization method can affect the seed germination rate of the aseptic seedling establishment. It shows that, Clorox should not be used at high concentration even though the Clorox is very effective against many types of bacteria. However, the use of high concentration of sodium hypochlorite or known as Clorox has caused negative effect towards seed germination and its growth performance. Other than sodium hypochlorite, the use of alcohol or ethanol for a longer period of time also may affect the seed germination as it will cause toxicity towards the seed germination. However, the first objective of this study has been achieved which aseptic seedlings of purple amaranth (*Amaranthus cruentus*) was established successfully. Based on data analysis, the type of hormone and hormone concentration towards induction of callus are not significance since the value obtained $P \geq 0.05$, thus null hypothesis is accepted. According to this study, the most suitable hormone for induction of callus is 2,4-D hormone as on 2,4-D hormone, there were one callus obtained at concentration of 1 mg/L.

5.1 Recommendation

In the current study, limitation has occur which might be the reason of error occur in this study. The surrounding of the tissue culture lab is one of the factor that influence growth of explant through in vitro method. For plant tissue culture method, sterile condition is the most important things to ensure plant grow healthily. Hygiene and sterile condition can support plant growth as there is no contamination will occur. Therefore, in future study, the lab must be ensure cleaned from any contamination, clean and sterile to avoid plant contamination.

Other than that, further research is required for better achievement and understanding of aseptic sterilization actual mechanism as sterilization is important factor for plant growth through in vitro method.

In the future, before doing subculture on media contain hormone, explant need to be more matured and grow stronger. Size and maturity of explant is one of the factor that influences the induction of callus.

On the other hand, combination of plant growth regulators can be used in order to fasten callus production. Combination of auxin and cytokinin could be used for future research as the combination of this two hormones can give better achievement of callus induction.

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APPENDICES

Table A1 ANOVA Test for aseptic seedlings

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Seed_Germination	Between Groups	2.022	2	1.011	.817	.445
	Within Groups	107.633	87	1.237		
	Total	109.656	89			
Average_Leave_Number	Between Groups	90.156	2	45.078	5.492	.006
	Within Groups	714.067	87	8.208		
	Total	804.222	89			

Table A2 Mean and standard error for aseptic seedling

		N	Mean	Std. Deviation	Std. Error
Seed_Germination	Replication 1	30	3.13	1.137	.208
	Replication 2	30	3.33	1.093	.200
	Replication 3	30	3.50	1.106	.202
	Total	90	3.32	1.110	.117
Average_Leave_Number	Replication 1	30	7.10	3.346	.611
	Replication 2	30	9.50	2.701	.493
	Replication 3	30	8.73	2.477	.452
	Total	90	8.44	3.006	.317

Table A3 Tukey's and Duncan's test for seed germination

Seed_Germination

Replication		N	Subset for alpha = 0.05
			1
Tukey HSD[a]	Replication 1	30	3.13
	Replication 2	30	3.33
	Replication 3	30	3.50
	Sig.		.412
Duncan[a]	Replication 1	30	3.13
	Replication 2	30	3.33
	Replication 3	30	3.50
	Sig.		.233

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 30.000.

Table A4 Tukey's and Duncan's test for average leaves number

Average_Leave_Number

Replication		N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	Replication 1	30	7.10	
	Replication 3	30	8.73	8.73
	Replication 2	30		9.50
	Sig.		.075	.556
Duncan[a]	Replication 1	30	7.10	
	Replication 3	30		8.73
	Replication 2	30		9.50
	Sig.		1.000	.303

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 30.000.

Table A5 Mean and standard error for type of hormones

		N	Mean	Std. Deviation	Std. Error
Callus_respond	NAA	12	.00	.000	.000
	2,4-D	12	.83	2.887	.833
	Picloram	12	.00	.000	.000
	Total	36	.28	1.667	.278
Survival	NAA	12	99.17	2.887	.833
	2,4-D	12	100.00	.000	.000
	Picloram	12	100.00	.000	.000
	Total	36	99.72	1.667	.278

Table A6 Mean and standard error for hormone concentration

		N	Mean	Std. Deviation	Std. Error
Survival	1mg/L	12	100.00	.000	.000
	2mg/L	12	99.17	2.887	.833
	3mg/L	12	100.00	.000	.000
	Total	36	99.72	1.667	.278
Callus_respond	1mg/L	12	.83	2.887	.833
	2mg/L	12	.00	.000	.000
	3mg/L	12	.00	.000	.000
	Total	36	.28	1.667	.278

Table A7 Anova test for type of hormone

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Callus_respond	Between Groups	5.556	2	2.778	1.000	.379
	Within Groups	91.667	33	2.778		
	Total	97.222	35			
Survival	Between Groups	5.556	2	2.778	1.000	.379
	Within Groups	91.667	33	2.778		
	Total	97.222	35			

Table A8 Anova test for hormone concentration

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Survival	Between Groups	5.556	2	2.778	1.000	.379
	Within Groups	91.667	33	2.778		
	Total	97.222	35			
Callus_respond	Between Groups	5.556	2	2.778	1.000	.379
	Within Groups	91.667	33	2.778		
	Total	97.222	35			

Table A9 Tukey and Duncan test for callus respond on different type of hormone

Callus_respond

	Type_Of_Hormone	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	NAA	12	.00
	Picloram	12	.00
	2,4-D	12	.83
	Sig.		.447
Duncan[a]	NAA	12	.00
	Picloram	12	.00
	2,4-D	12	.83
	Sig.		.257

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 12.000.

Figure A1 Media prepared and stored

Figure A2 Plant culture in growth room