Faculty of Resource Science and Technology

Establishment of Axenic Culture and Callus Induction of *Hibiscus sabdariffa* Linn. (Roselle) by using leaf explants.

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Bachelor of Science with Honours
(Plant Resource Science and Management)
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Establishment of Axenic Culture and Callus Induction of *Hibiscus sabdariffa* Linn. (Roselle) by using leaf explants.

Steffie Philip
(49095)

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

(Plant Resource Science and Management)

Plant Resource Science and Technology

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2017

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Final Year Project Report ☑

Masters ☐

PhD ☐

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DECLARATION

I declare that this thesis entitles "Establishment of Axenic Culture and Callus Induction of *Hibiscus sabdariffa* Linn. (Roselle)" by using leaf explants is the best 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.

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ABSTRACT

Establishment of Axenic Culture and Callus Induction of *Hibiscus sabdariffa* Linn. (Roselle) by using leaf explants.

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ABSTRACT

Roselle (*Hibiscus sabdariffa* Linn.) belongs to the family of Malvaceae which is originally from Africa. It is known as Roselle and native to Malaysia. It is an annual crop and successful cultivated in Malaysia and Sudan for commercial and economic purposes. Propagation by vegetative part of Roselle encounter problem by lowering the rate of growth and easily attacked by diseases or pathogen. In this study, establishment of axenic culture and callus induction was made to improve the protocol of sterilization and propagation by using the leaf explant. Surface sterilization step was started by put the leaf explant under running tap water for 30 minutes. Three different concentrations of Clorox (10%, 15%, 20%) were used with three different time exposures (5, 10, 15 minutes). The best protocol for surface sterilization was treatment 7 (20% of Clorox® and 5 minutes time exposures) with 83.33± 9.55 % percentage of axenic leaf explant after 2 weeks incubation. For callus induction, 6-Benzylaminopurine (BAP) and 4-Amino-3,5,6-trichloropicolinic acid (Picloram) were tested with three different concentration, 1 mg/L, 3 mg/l and 5 mg/L. The leaf explants was incubated for 6 weeks. The leaf explants started to grow callus in week 1 and the callus has white and compact structured. The best for treatment producing high percentage of callus was treatment 3 with 56.67 ± 14.1 %. 3 mg/L of Picloram was the most effective protocol for callus induction. For recommendations, selected PGR can be combining with other PGR to enhance the effectiveness of the PGR itself. Less chemical can be used to reduce the injury of tissues during surface sterilization.

Keywords: *Hibiscus sabdariffa*, Surface sterilization, Callus induction, BAP, Picloram

ABSTRAK

Roselle (Hibiscus sabdariffa Linn) adalah kepunyaan kepada keluarga Malavaceae dan berasal dari Afrika. Ia juga dikenali sebagai Roselle dan dikenali di Malaysia. Ia diklasifikasikan sebagai tanaman tahunan dan berjaya ditanam di Malaysia serta di Sudan unuk tujuan komersial dan ekonomi. Propagasi oleh tanaman keratan oleh Roselle menyebabkan banyak masalah dengan merendahkan kadar pertumbuhan dan mempercepatkan serangan penyakit. Dalam kajian ini, penubuhan kultur bebas dari pencemaran dan induksi kalus telah dilakukan untuk memperbaiki protocol pensterilan dan propagasi dengan menggunakan eksplant daun. Penstrilan di mulakan dengan meletakkan eksplan daun di bawah curahan air paip dalam masa 30 minit. 3 jenis kepekatan Clorox (10,15,20%) telah digunakam dengan 3 jenis masa (5, 10, 15 minit). Protokol yang terbaik untuk pensterilan permukaan adalah perawatan dengan 20% Clorox dan 5 minit durasi masa dan menghasilkan 83.33± 9.55% peratusan ekplan daun bebas dari kotoran selepas 2 minggu. Untuk induksi kalus, ia dikutur dalam masa 6 minggu. Eksplan daun mula tumbuh kalus dalam minggu pertama. Peratusan terbaik bagi pengasilan peratus kalus yang tumbuh adalah kaedah 3 dengan 56.6 ± 14.1%. 3mg/L Picloram adalah kepekatan yang terbaik untuk protokol yang berkesan dalam menghasilkan induksi kalus. Untuk cadangan, hormon yang dipilih boleh di gabungkan dengan hormone yang lain untuk meningkatakan kebekesanan hormone tersebut. Kurangkan pergunaan kimia dalam penstrilan permukaan untuk mengelakkan kecederaan tisu.

Kata Kunci: Hibiscus sabdariffa, Penstrilan permukaan, Induksi kalus, BAP, Picloram

TABLE OF CONTENT

LIST OF ABBREVIATIONS

BAP	6-Benzylaminopurine
BA	Benzyladenine
CRD	Completely Randomized Design
MS	Murashige and Skoog (1962)
PGR	Plant growth regulator
PPM	Plant Preservative Mixture
TET	Tetracycline
UNIMAS	Universiti Malaysia Sarawak
PICLORAM	4-Amino-3,5,6-trichloropicolinic acid

LIST OF TABLES AND FIGURES

Tables Page

Figures

LIST OF APPENDICES

1.0 Introduction

1.1 Research background

Hibiscus sabdariffa Linn. Var *sabdariffa* popularly known as roselle and very native to Asia especially Malaysia. *H. Sabdariffa* is an annual shrub, herbaceous shrub, cylindrical red or green stalk and can grow up to 3 m in height (Morton, 1987). According to Daudu *et al*. (2005), there is variety of language in pronouncing *H. sabdariffa* for its vernacular name such as *manipura* (Sanskrit), *gongura* (Hindi), *Sheria* (Oriya), *karkade or carcade* (African). Leaves are compound altenate and deeply 5 to 7 lobed. The flowers are yellow with a rose eye and calyx with bright red and edible especially for making jam or healthy juice (Ismail *et al*., 2009).

According to Ismail *et al*. (2009), Hibiscus is classified as common flowering plant grown in worldwide and reported more than 300 species have been found. There are 2 main types of types of variety of roselle of *H. sabdariffa* species, *H. sabdariffa* Var. *altissima* Wester and *H. sabdariffa* Var. *sabdariffa* (Morton, 1987). He reported that *H. sabdariffa* Var. *altissima* Wester are most economically important and cultivated for fibre source in India.

Hibiscus species are conventionally propagated from stem but currently it can be propagated from seed in bulk number. Vegetative propagation of cutting is a detached piece from plant and re-growing of the part or tissue in different medium. Vegetative propagation helps in generate clones in easy ways and reduce the length of juvenile method. Roselle seeds can be raised in nursery beds by controlling nutrient and temperature. In addition method of vegetative propagation for seed also can be practiced in micro-propagation by plant tissue culture and this application are important for biotechnologies tool especially for economic and research purposes (Govinden-Soulange *et al*, 2009).

The benefits and importance of *H. sabdariffa* are widely applied in worldwide. According to Quezon (2005), it classified as versatile plant same as coconut tree. Similar as coconut tree, every part of the plant have its own benefits and producing high economic value. Calyces are the most exploited part of *H. sabdarffa*. It been used as juice or tea, jams and herbal drink (Ismail et al., 2009, as cited in Tsai *et al*. 2002). *H. sabdariffa* produced coast bast fibre which commonly used for making ropes and sacks (Fathima & Balasusbramanian, 2006). In addition, *H. sabdariffa* also applied in traditional treatments such as to cure hypertension, heart problem and digestive problem. Other uses of *H.sabdariffa* are chickens food, mycological staining and paper pulp.

H.sabdariffa use genetic engineering methods to develop rapid development of new cultivars with desired traits. According to Gomez-Leyva *et al*. (2008), in *vitro* regeneration system of *H.sabdariffa* is currently available which were based on meristem tissue. In 2010, somatic embryogenesis for *H. sabdariffa* is reported. The genetic improvement of *H. sabdariffa* are from the genetically transformation plant by practicing out dynamic protocol of regeneration of *H. sabdariffa* through somatic embryogenesis (Sie *et al*., 2010)

From previous study from Govinden- Soulage *et al.* (2009), vegetative propagation and regeneration of tissue culture through Indirect and direct organogenesis are carried out. Sie *et al*. (2010) reported also callus induction and somatic embryogenesis also worked in this species and tested different type of explant, different concentration of sucrose used and manipulated PGR to achieve their objective.

1.2 Problem statement

From previous study (Govinden *et al*., 2009) some study had been made in establishment of axenic culture. However, the research need to be continue in order to achieve better achievement in minimize less contamination and survive culture. Due to high economic

value in medicine, food, beverages and ornamental, (Morton, 1987). In order to produce good mother plant, contamination free cultures need to be done such as through tissue culture. Apart from that, there are less study and information about *H. sabdariffa* especially to propagate it in large number in shorter time. Therefore, by establish protocol of axenic culture and induce callus by using leaf explant of *H. sabdariffa* to further improve discovery about this species in tissue culture approach.

1.3 Objective

The main objectives of this study are to establish the axenic culture of leaf explant of *H. sabdariffa* and to induce callus from leaf explant oh *H. sabdariffa* by using Picloram and BAP.

2.0 Literature review

2.1 Origin, distribution and ecology of *Hibiscus sabdariffa*

H. sabdariffa is originally from Africa and been planted about 6 years ago in Sudan. It is an annual crop or shrub and successfully cultivated in Malaysia, India and Sudan for commercial and economic purposes (Puro *et al*., 2014). In Sudan, it became the major producer of *H. sabdariffa* and widely grown for major export (McClintock & Tahir, 2004). Mohamed *et al*. (2012) reported that *H. sabdariffa* requires 130-250 mm rainfall in few months of growing phase even though its influence the standard of the yield. The optimum temperature for good growing of *H. sabdariffa* is 25 ° C. *H. sabdariffa* classified as short day plant ad requires 10 to 12 hours for flowering. Normally, this plant prefer field setting with complete exposition of sunlight. According to Ansari *et al*. (2013), *H. sabdariffa* well tolerates in floods and heavy winds especially in tropical and subtropical climate.

2.2 Classification and description of *H. sabdariffa*

H. sabdariffa belongs to family Malvaceae which is originally from Africa. The leaves are divided into 3 to 5 lobbed with long up to 15 cm and arranged alternately. Leaves are dark green in colour. The flowers are pink to yellow colour with 8 to 15 cm in diameter and have a red dot at the centre. The mature calyces are fleshy red and edible. The morphological characteristics of *H. sabdariffa* are illustrated in Figure 1. According to Qi *et al* (2005), the flowers have both male and female with short peduncles. The calyces take about six months to mature. The *H. sabdariffa* can grow up to 3m depending on location and season of sowing (Gomez-Leyva *et al*, 2005). In other country, *H. sabdariffa* are locally known by different vernacular name in variety of languages such as *Mesta* or *Meshta, sorrel, Rosela , Karkade , cranberry* and *krajeab* (Daudu *et al.,* 2005).

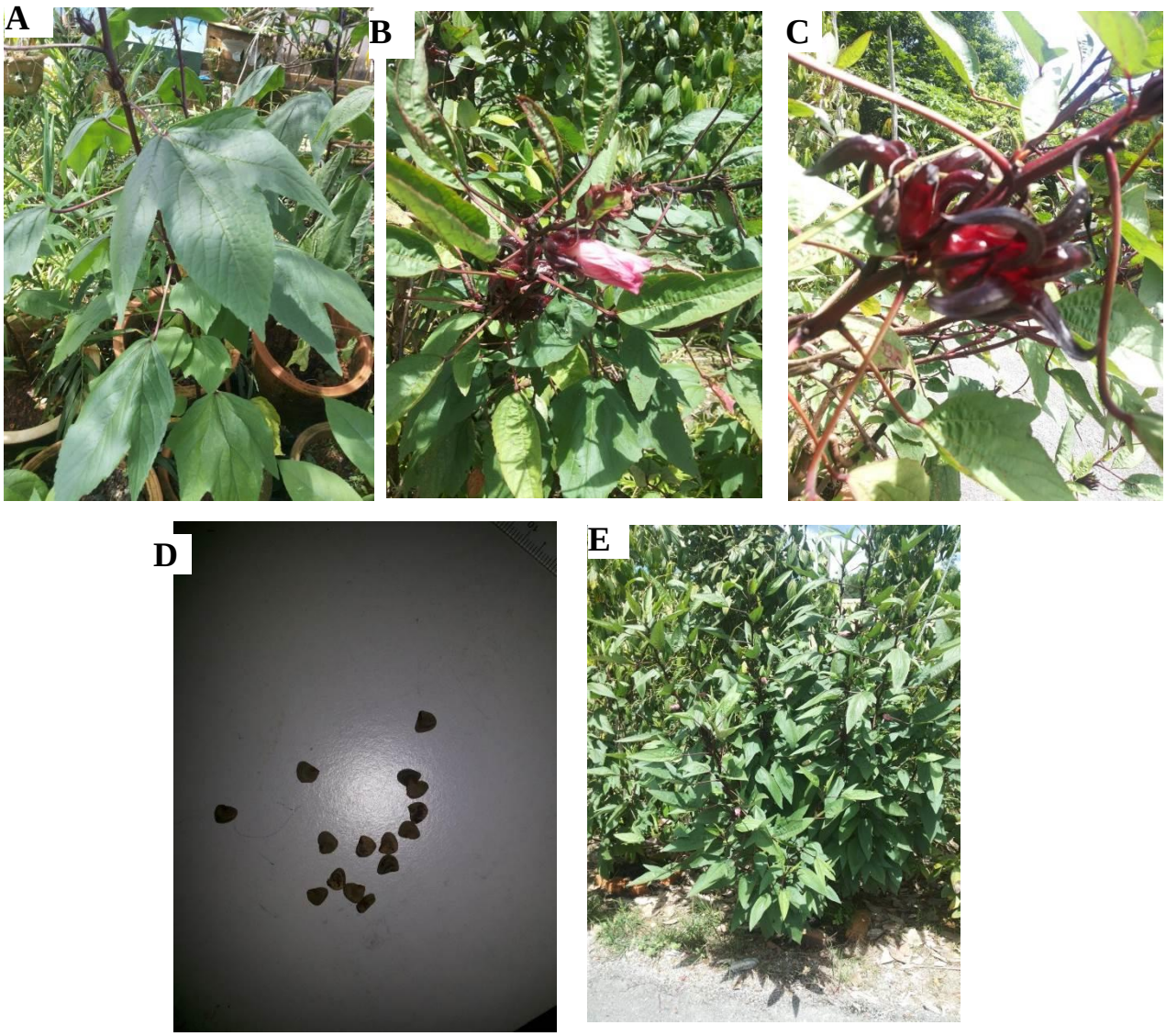

Figure 1. Morphological characteristics of *H. sabdariffa.* Taken from Kampung Lanchang, Serian.

A)Leaves; B)Pink flower; C) Red calyces; D) Dark brown seeds; E) 1 year old tree of *H. sabdariffa*

2.3 Importance of *Hibiscus sabdariffa*

2.3.1 Food and beverages

According to Quenzon (2005), *H. sabdariffa* classified as flexible or versatile plant which every part of the plant including seed, fruits, calyx and leaves are used in various food and beverages. The seed of *H. sabdariffa* can be eaten roasted as snacks and the seed oil used in making soap and cosmetics (Ansari *et al*., 2013). Apart from that, seed also been used in coffee substitute which is high in protein (Qi *et al*., 2005). Nyam *et al*. (2012) tested that

H. sabdariffa seed used in bakery ingredients by replacing the cookie flour with *H. sabdariffa* seed powder. They found that different percentage of seed powder can influence the aroma, appearance and crunchiest of cookie itself.

Calyces are the most exploited part of this plant because it's been used for tea, jams, jelly, wine and syrup (Morton, 1987). According to Ismail *et al*. (2009), calyces contain foremost of vitamin C and very satisfactory to make healthy juice and they also reported that *H. sabdariffa* contains triple of vitamin C compares than blackcurrant and citrus. Leaves of *H. sabdariffa* are can be eaten as salads or cooked with other vegetables. Besides that, it also added as seasoning especially for curies (Ansari *et al.*, 2013).

2.3.2 Paper industry and fibre production

In United States and Asian countries, the bast fibre and the stem of *H. sabdariffa* are used in paper manufacture (McClintock & El-Tahir, 2004). *H. sabdariffa* also used in production of coast bast fibre which normally used in manufacture of ropes and sacks (Fathima & Balausbramanians, 2006). Besides that, production of fibre coast also important for India especially for economic purposes (Morton, 1987).

2.3.3 Mycological staining and dye

Based from previous study from Abu Bakar *et al.* (2012), microscopic identification of fungal mycelial structures is stained by the extraction of natural dye from the calyces of *H. sabdariffa* for the single staining. Red dyes from calyces are used to compare performance of the synthetic dye such as lacto-phenol and methylene blue stain in identification of fungal mycelial. There are some fungal that involved such as *Penicillium ctrinum, Cladosporium* spp and *Mucor* spp. Abu Bakar *et al.* (2008) concluded that the performance of red dye from calyces is both the same from the synthetic dye. This shows that the red dye from calyces can be an alternative source for natural or low cost dye for fungal staining.

Furthermore, red calyces of *H. sabdariffa* can be used as dye and produce different colour such as red, yellow and blue. This can reduce the applications of hazardous effect especially on human and surrounding. According to Ismail *et al.* (2008), it also been used in West India as colour ingredient in alcoholic beverage.

2.3.4. Other uses

The seeds can be used to feed chicken and the residue of the oil extraction is used for cattle when the amount is large (Morton, 1987). In addition, in Europe the red stalk with ripe red flower use widely in ornament especially in garden.

2.4 Medicinal value

H. sabdariffa are used widely in India and Africa to works as traditional medicine especially for folk. According to Mohamed *et al.* (2012), this plant abundantly in anthocyanin and protocatehuic acid. Anthocyanin is a water-soluble pigment that important for the colour of calyces. According to Wahabi *et al.* (2012), anthocyanin and proanthocyanin are compound can work well for lowering blood pressure. Calyces are the most exploited part for *H. sabdariffa* because it accommodates high of vitamin C (Amin *et al.*, 2008). The mature calyces are classified as diuretic and tonic which can lower the viscosity of the blood and to encourage intestinal peristalsis (Morton, 1987). Dried calyces contain high amount of ascorbic acid, flavonoid gospetine and sabdaretine that responsible to increase urination. The leaves can treat colds, toothache, urinary tract infection, and ulcers. Besides that, citric acid in *H. sabdariffa* used as cooling herb especially for sunburn skin (Morton, 1987). Scurvy can be treated by leaves and mature roots.

Based on studies from Chun-Tang *et al.* (2015), leaf extracts of *H. sabdariffa* can prevent Human Prostate Cancer cell Invasion. This study conclude that leaf extract from this plants can inhibit the growth of Prostate tumour and classified as anticancer agent that can naturally uses as medicine plant.

Additionally, *H. sabdariffa* seed also a source of vegetable oil that is low in cholesterol but good source of lipid-Soluble antioxidant and high amount of protein compared to *Passiflora edulis* (passion fruit) (Ismail *et al.,* 2008). Furthermore, lotion made from leaf extracts of *H. sabdariffa* can treat sores and wounds. In domestic market, dried flower highly exported for making herbs tea which reduces cold, cleaning mucous and blocked nose, inhibit kidney failure and reduce fever (Mahadevan *et al.*, 2009).

2.5 Tissue culture of *H. sabdariffa*

Plant tissue culture also known as micro propagation technique that applied in propagation of huge number of plantlets in aseptic surrounding. There are plenty of choices of medium used as medium in tissue culture such as Murashige and Skoog (1962), Gamborg (B5), Nitch Media White and LS media. Generally in tissue culture, Murashige and Skoog (1962) are usually used especially for growing callus and generating different plant organs. In this study, MS stock solution will be used in establishment of axenic culture and direct shoot organogenesis.

According to Govinden- Soulange *et al*. (2009), regeneration of *H.sabdariffa* by tissue culture are more achieved by using nodal explant. Murashige and Skoog (1962) are supplemented with different concentration of BAP and Kinetin. Govinden-Soulange *et al*. (2009), shown that *H. sabdariffa* can be propagated by vegetative method and tissue culture. In previous study from Sie *et al.* (2010) stated that *H. sabdariffa* can go through of callus induction and somatic embryo initiation. There are 2 genotypes involve in this study, *H. sabdariffa* Var. sabdariffa and *Hibiscus sabdariffa* Var. altissimma. Sie *et al.* (2010) conducted this study to show how explant type, concentration of sugar and PGR can influence the performance of culture based on 2 different selected. Gomez-Leyva *et al.* (2008) also conducted a research by using shoot apex and stated that these studies are the first report of inductive effect of BA on production of multiple plants from shoot apex.

There are different concentrations of BA, thidiazuron and m-*topolian* was supplemented in MS medium for shoot production.

2.6 Direct shoot organogenesis

PGR are divided into 5 major groups such as auxin, cytokinin, gibberellins, abscisic acid and ethylene. This entire major group have their own roles especially in production of callus and roots. Normally, it stimulated cell, elongation and division in cambium tissue (Gaba, 2005). Cytokinin works in cell division and stimulates growth of shoots of *in vitro*. (Beyl, 2005). According to Beyl (2005), BA has stronger activity compare that zeatin.

According to Malik *et al*. (1993), cytokinin or cytokinin-like substance can be one of the principal in procedure of direct germination of seeds on continuous exposure of seedling in same medium. In multiple shoot regeneration in *H. sabdariffa* by Gomez-Leyva and Gonzalez (2008), the shoot tips were cut after 10 day of germination with help of 4.48 µM of BA. The shoot productions are obtained from shoot tips of the seedling and supplement at various type of PGR such as BA, TDZ and *m*-topolin. Gomez-Leyva and Gonzalez (2008) reported that BA has inductive effect on production of multiple plants from shoot apex of roselle and proved that regeneration of this plant without help of rooting medium.

According to Govinden-Soulange *et al*. (2005), direct organogenesis of *H. sabdariffa* are invented from nodal segment from *in vitro* germinated seedling. BAP and kinetin are used in that experiment and showed that kinetin show the highest number of shoot formation compares to BAP. But, Govinden-Soulange *et al.* (2009) also reported that single node explants can also be applied through *in vitro* even in very low level or in absence of cytokinin.

2.7 Contamination in Tissue culture

Contamination is a biggest problem in establishment of axenic culture. The existence of microbial contaminant can highly affect the performance of explant used in tissue culture. According to Kane (2005), the successfulness of establishment of aseptic cultures of meristem explant depend on explatation time, position of the explant on the stem, explant on the stem, explant size and polyphenol oxidation.

Surface sterilization was less effective in control group of contamination. The use of fungicide such as benomyl is not being encouraged in some researchers. Contamination in plant tissue by different sources can reduce the productivity and postponed the effectiveness of culture. Plant Preservative Mixture (PPM) is works in prevention growth of microorganism and can be applied up to 4ml/L (Colgecen *et al.*,2011). According to Colgecen *et al*. (2011), PPM can be used depending on plant species with no negative effect on plant growth. In addition, medium incorporated with PPM and tetracycline (TET) help inhibit microbial grow in culture media.

Sodium Hypochlorite usually used in laundry bleach and commercial bleach called as Clorox™ (5.25 % active ingredient). It most frequent choice in tissue culture method. Ethanol is one of powerful sterilized agent and been exposed towards explant for only seconds or minutes.

Apart from that, there a few sterilizing agent used widely in tissue culture such as mercuric chloride, with or without combination of Sodium hypochlorite (Jan *et al.,* 2013). This sterilizing agent can be observed effective in strawberry explant with mercuric chloride (0.1%) with 4 min time exposure.

2.8 Effect of Picloram in tissue culture.

Picloram is one part of PGR which act as auxin. According to Benlioghu *et a.l* (2015), Picloram can produce callus up to 90% with combination of 2,4-D in different in 3 different types of rice genotypes. The callus induction was from embryos of 3 different genotypes after 14 day. Apart from that, effect of picloram also seen on somatic embryogenesis of *Phyla nodiflora* (L.) Greene. The plant regeneration was via somatic embryogenesis in suspension culture derived from leaf and stem explants (Ahmed *et al*., 2011).

Furthermore, the best calli with healthy morphology ad growth was observed from leaf explant of *Ficus deltoidea* Jack (Kiong *et al*., 2007). Kiong *et al*. (2007) concluded that MS media supplemented with 1 mg l^{-1} Picloram gave the best growth performance growth of calli.