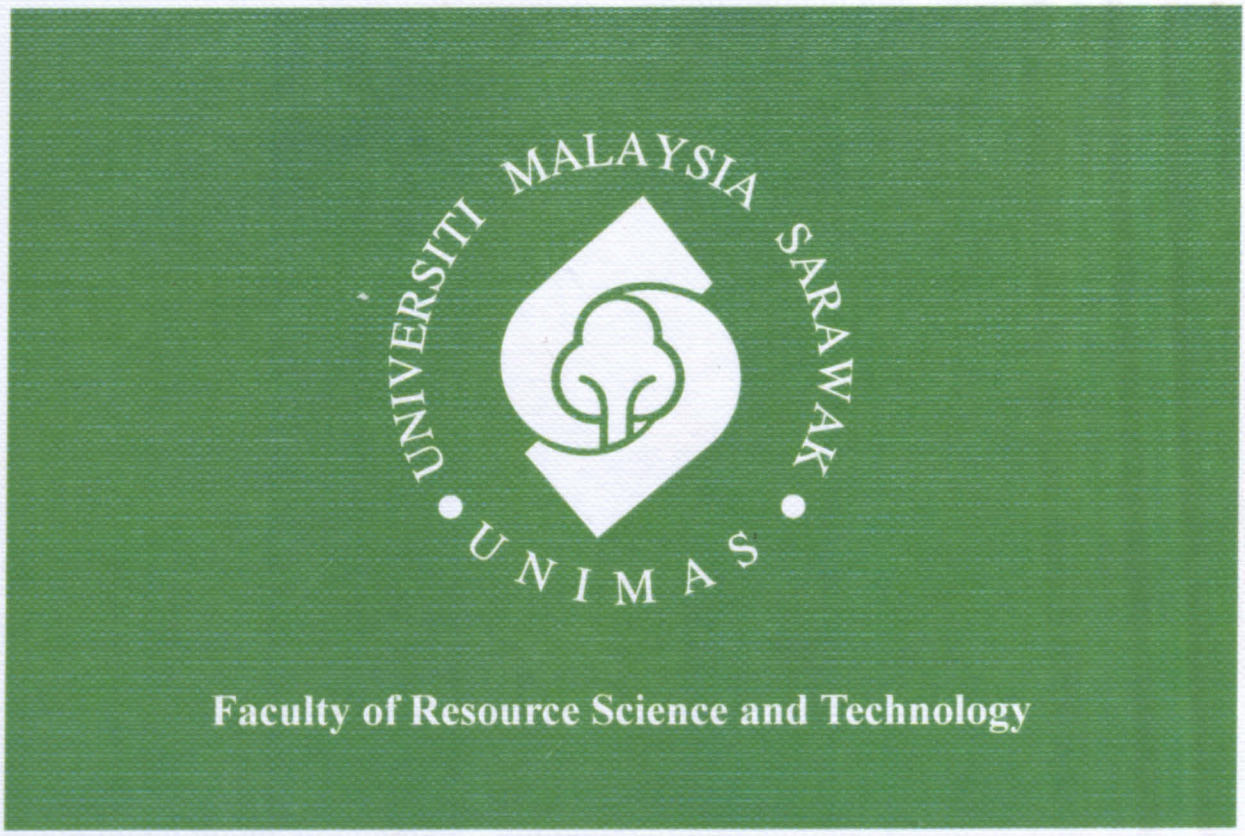


Tissue Culture of *Plukenetia volubilis* L.

Farra Amirah Binti Rusli

(54597)

Bachelor of Science with Honours
(Resource Biotechnology)
2018

Tissue Culture of *Plukenetia volubilis* L.

Farra Amirah Binti Rusli (54597)

The dissertation is submitted in partial fulfillment of requirement for degree of Bachelor Science with Honours in Resources Biotechnology

Faculty of Resource Science and Technology

Universiti Malaysia Sarawak

2017/2018

UNIVERSITI MALAYSIA SARAWAK

Grade: _____

Please tick (√)
Final Year Project Report ☑
Masters ☐
PhD ☐

DECLARATION OF ORIGINAL WORK

This declaration is made on the 7 day of JUNE year 2018

Student's Declaration:

I FARRA AMIRAH BINTI RUSLI, 54597, FRST

(PLEASE INDICATE NAME, MATRIC NO. AND FACULTY) hereby declare that the work entitled, TISSUE CULTURE OF PLUKENETIA VOLUBILIS L. is my original work. I have not copied from any other students' work or from any other sources with the exception where due reference or acknowledgement is made explicitly in the text, nor has any part of the work been written for me by another person.

7 JUNE 2018
Date submitted

FARRA AMIRAH BINTI RUSLI (54597)

Supervisor's Declaration:

I, AP DR MOHD HASNAIN BIN MD HUSSAIN (SUPERVISOR'S NAME), hereby certify that the work entitled, TISSUE CULTURE OF PLUKENETIA VOLUBILIS L. (TITLE) was prepared by the aforementioned or above mentioned student, and was submitted to the "FACULTY" as a * partial/full fulfillment for the conferment of BACHELOR OF SCIENCE WITH HONOURS (RESOURCE BIOTECHNOLOGY) (PLEASE INDICATE THE DEGREE TITLE), and the aforementioned work, to the best of my knowledge, is the said student's work

Received for examination by: _____ Date: 6 JUNE 2018
(Name of the supervisor)
Profesor Madya Dr. Mohd Hasnain Md Hussain

Jabatan Biologi Molekul
Fakulti Sains dan Teknologi Sumber
UNIVERSITI MALAYSIA SARAWAK
94300 Kota Samarahan

I declare this Project/Thesis is classified as (Please tick (√)):

☐ **CONFIDENTIAL** (Contains confidential information under the Official Secret Act 1972)*
☐ **RESTRICTED** (Contains restricted information as specified by the organisation where research was done)*
☑ **OPEN ACCESS**

I declare this Project/Thesis is to be submitted to the Centre for Academic Information Services and uploaded into UNIMAS Institutional Repository (UNIMAS IR) (Please tick (√)):

☑ **YES**
☐ **NO**

Validation of Project/Thesis

I hereby duly affirmed with free consent and willingness declared that this said Project/Thesis shall be placed officially in the Centre for Academic Information Services with the abide interest and rights as follows:

- This Project/Thesis is the sole legal property of Universiti Malaysia Sarawak (UNIMAS).
- The Centre for Academic Information Services has the lawful right to make copies of the Project/Thesis for academic and research purposes only and not for other purposes.
- The Centre for Academic Information Services has the lawful right to digitize the content to be uploaded into Local Content Database.
- The Centre for Academic Information Services has the lawful right to make copies of the Project/Thesis if required for use by other parties for academic purposes or by other Higher Learning Institutes.
- No dispute or any claim shall arise from the student himself / herself neither a third party on this Project/Thesis once it becomes the sole property of UNIMAS.
- This Project/Thesis or any material, data and information related to it shall not be distributed, published or disclosed to any party by the student himself/herself without first obtaining approval from UNIMAS.

Student's signature _____ (Date)

Supervisor's signature _____ (Date)

Current Address:
FAKULTI SAINS DAN TEKNOLOGI SUMBER, UNIVERSITI MALAYSIA SARAWAK,
94300 KOTA SAMARAHAN, SARAWAK

Notes: * If the Project/Thesis is **CONFIDENTIAL** or **RESTRICTED**, please attach together as annexure a letter from the organisation with the date of restriction indicated, and the reasons for the confidentiality and restriction.

[The instrument was prepared by The Centre for Academic Information Services]

ACKNOWLEDGEMENTS

I would like to give my deepest thanks to my supervisor, Associate Professor Dr. Mohd Hasnain Bin Md. Hussain for the assistance and guide for along the way through my project and thesis.

I would like to show gratitude to Post-Graduate at UNIMAS under Associate Prof. Dr. Mohd Hasnain Bin Md. Hussain. They always helped me get results of better quality for my project. I would like to thank my fellow final year student under supervision of Associate Prof. Dr. Mohd Hasnain Bin Md. Hussain for their feedback, cooperation and of course friendship along the final year project been conducted.

Nevertheless, I would like to thank my friends for accepting nothing less than excellence from me and always give support during the semester. Last but not the least, I would like to thank to my family especially my parents and to my brothers for supporting me spiritually throughout writing this thesis and my life in general.

Tissue Culture of *Plukenetia volubilis* L.

Farra Amirah Binti Rusli

Resources Biotechnology
Programme Faculty of Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

Plukenetia volubilis (Linn.) is a Peruvian plant in the *Euphorbiaciae* family. The present study investigate on the sterilization of *P. volubilis* leaves and stems. Various levels of bleach with exposure to ethanol were used to surface sterilized the leaves and stems. Surface sterilization using 20% of chlorox concentration for 1-5 minutes with exposure to ethanol had been found effective in controlling the contamination of stem. Calluses were form from sterilize plant stem that gained from embryo of *P. volubilis* that had been introduced on different basal media to study the effect of different medium (MS and WPM medium). The callus were induced by NAA and BAP. Greenish with light yellowish granulated calluses were successfully induced by the NAA with concentration of 0.5 and BAP of 0.5. Further research on the above-mentioned factors for *P. volubilis* should be conducted due to its high potential in medical application and commercialized products.

Key words: Sterilization, leaf explants, stem explant, embryo, callus induction

ABSTRAK

Plukenetia volubilis (Linn.) adalah tumbuhan berasal dari Peru dalam keluarga Euphorbiaciae. Kajian ini melihat pada pensterilan daun dan batang P. volubilis. Pelbagai peringkat peluntur dengan pendedahan kepada etanol digunakan untuk permukaan daun dan batang disterilkan. Pensterilan permukaan menggunakan 20% kepekatan kloroks selama 1-5 minit dengan pendedahan kepada etanol didapati berkesan dalam mengawal pencemaran batang pokok. Kalus di bentuk menggunakan batang tumbuhan yang steril yang diperolehi daripada embrio P. Volubilis. Embrio telah diperkenalkan di media basal yang berbeza untuk mengkaji kesan medium yang berbeza kepada pertumbuhan tumbuhan (medium MS dan WPM). Kalus diinduksi oleh NAA dan BAP. Kalus berwarna hijau serta kekuningan cair telah berjaya diinduksi oleh NAA dengan kepekatan 0.5 dan BAP dengan kepekatan 0.5. Kajian lanjut mengenai faktor-faktor yang disebutkan di atas untuk P. volubilis perlu dijalankan kerana potensi tinggi dalam aplikasi perubatan dan produk-produk komersil.

***Kata kunci**: Pensterilan, eksplan daun, eksplan batang, embrio, induksi kalus*

Table of Content

List of Tables

List of Figures

List of Abbreviations

BAP - Benzyl adenine

EtOH - Ethyl alcohol

MS - Murashige & Skoog Medium

NAA - Naphthaleneacetic acid

pH - Potential hydrogen

WPM - Woody Plant Medium

CHAPTER 1: INTRODUCTION

Sacha Inchi (*Plukenetia volubilis* L.) is a Peruvian plant. It is located and can be found in the Amazon rain forest or on the east slope of the lower Andes. The indigenous plant thrives in warm climates, up to elevations of 1,700 meters, as long as it has a consistent source of water and decent drainage. Acidic soils and sedimentary flats near rivers are the best area for Sacha Inchi to grow. Sacha inchi is an eye-catcher plant at two meters high, with a delicate white flowers, a split leaves and star-shaped green fruits produced for every nearly year-round. In the star-shaped fruit, it will form a dark brown seeds which look similar to nuts once it ripen. These almond-like seeds are called Sacha peanuts, Inca peanuts or mountain peanuts (Briggs, 2015).

Sacha peanuts contain the amino acid tryptophan, which is known to improve your mood. After the fruit has been de-seeded it is then used in the production of flour, bread and protein powder. The seed is dark brown and oval, rich in proteins, vitamin E, and especially in polyunsaturated oils omegas 3, 6 and 9. This culture has a great agro-technological potential and is currently widely explored by Peruvian industries in the production of its oil, possessing nutraceutical properties and wide use in the cosmetic industry. The high omega 3 content in the oil, described by Guillén *et al.* (2003). The oil was responsible for the reduction of triglyceride levels (Huamán *et al.*, 2008). Since sacha inchi was not originally from Malaysia, and it rich of omega 3, so sacha inchi demand increase in Malaysia due to its benefits. Based on Berita Harian reporter said, sacha inchi was propose by Pihak Berkuasa Pekebun Kecil Perusahaan Getah (RISDA), as one of the new plant that can be introduce in Malaysia agriculture (Zahari, 2017). The chairman of RISDA, Datuk Zahidi Zainul Abidin, RISDA provise RM300,000 for the Sacha Inchi plantation. This project will start from peninsular Malaysia.

The absence of reliable methods of asexual propagation of the sacha inchi has limited its planting and its use at an industrial level (Ruiz & Mesén, 2010), since it is not possible to clone mother plants by micropropagation process to help in increasing the quantity and quality of this plant, it lead to this study to fulfil the high demand of *P. volubilis* plant in Malaysia. This plant also had lack of studies. From this experiment, by using in vitro propagation, it can try to find ways to decrease the non-uniform planting and get a preliminary data of the research. The objectives of this research study are to evaluate a proper method for *P.volubilis* explant from the outside sterilization method, to evaluate the effect of different basal medium for Sacha Inchi to grow and to evaluate the effect of different concentration of hormone used for callus induction. Since there are less studies of sterilization on *P. volubilis* explant, the tissue culture part of callus induction and usage of different basal media for growing the plant might can help the future study of *P. volubilis* for other researchers to do tissue culture using this plant.

CHAPTER 2: LITERATURE REVIEW

2.1 Sacha Inchi (*Plukenetia volubilis* Linneo)

Known by the Incas for thousands of years, the creeper *Plukenetia volubilis* Linneo, or more commonly called Sacha inchi, is a *Euphorbiaceae* native to the Amazon jungle, with a center of origin in Peru, Colombia, Venezuela and Brazil. The seed is dark brown and oval, rich in proteins, vitamin E, and especially in polyunsaturated oils. (Bordignon *et al.*, 2012). *P.volubilis* is a biodiversity hotspot and is plenty in tropical plant resources. (Cao *et al.*, 2006).

Sacha inchi, also known as the Inca peanut, is the seed of a plant that grows in the highlands of Peru. Despite being a fairly recent discovery in the health community of the United States, sacha inchi has been cultivated and used as a food source for 3,000 years in the Amazon rainforest. The fruit that these seeds grow in is inedible, but when lightly roasted with low heat the seeds take on a crisp nutty flavor. The oleaginous seed obtained from the star-shaped fruit of *Plukenetia volubilis* L. characterized by its content of omega fatty acids.

2.2 Classification of *Plukenetia volubilis*

Plukenetia volubilis also have their classification like the other plants. Below are the list of information based on the classification of *P. volubilis* (Mc-Bride, 1951):-

Kingdom	**Plantae**
Division	**Angiospermae**
Class	**Dycotiledonea**
Order	**Geraniales**
Family	**Euphorbiaceae**
Genus	***Plukenetia***
Species	***Volubilis Linneo***
Scientific name	***Plukenetia volubilis L.***
Common name	**Sacha Inchi**

Table 2.1: Classification of *P. volubilis*

2.3 Sterilization technique

Sterilization or aseptic technique are the way to prevent from any contamination or the exclusion of any microorganisms while doing experimental procedures. Using active grow and healthy part of plant use in tissue culture can be disrupt when plant part get stress from any of disease and insect and sterilization method can be used for these plant can be used for tissue culture. Aseptic technique are absolutely can produce a contamination-free plant for the establishment of tissue, plant cell and organ structure (Misra *et al.*, 2012). The common contaminant are fungus, bacteria and insect.

2.4 Micropropagation

According to Royal Horticultural Society (2017), tissue culture is the cultivation of plant and cells, tissues, or organs on specially formulated nutrient media. Micropropagation has become an important part of the commercial propagation of many plants because of its advantages as a multiplication system (Iliev *et al.*, 2010). Under the right conditions, an entire plant can be regenerated from a single cell. Application for Sacha Inchi propagation improvement which will include the micropropagation via the shoot and the regeneration from callus cultures. Different types and concentrations of plant growth regulators were used to induce adventitious shoot regeneration. (Mandal & Laxminarayana, 2012).

2.5 Hormone

Plant hormone that usually been use for plantation are auxin and cytokinin. Auxins and cytokinins, as growth regulators, have basic roles to play in plant tissue culture. Often, they are used together but with different concentration rations which subsequently determine the type of culture regenerated. A high cytokinin to auxin ratio supports formation of shoots, whereas a high auxin to cytokinin ratio favors formation of roots. (Machuki, 2013). Auxins play a role as a promoter to cell division and growth in explants and support callus induction hence growth. The pattern of auxin distribution within the plant is a key factor for elongation during growth of the stem and root, its reaction to its environment, and specifically for development of plant organs such as leaves or flowers (Friml, 2003). The mostly used type of auxin for tissue culture is called 2, 4-Dichlorophenoxyacetic acid (2, 4-D). While, for cytokinins, it support cell division. The two main types of cytokinins used in tissue culture are benzylaminopurine (BAP) and kinetine. Cytokinin acts to regulate auxin distribution in the root apical meristem. (Zhang *et al.*, 2013).

2.4 Callus

Callus was famously known cell growing and accumulation of callose associated with wounding. (Ikeuchi, Mapes & Iwase, 2013). Production of callus can be from a single differentiated cell, and many callus found are totipotent cell and callus can be used to form new whole plant. (Steward *et al., 1958*; Nagata and Takebe, 1971). The other ways to form new plant, callus cells can undergo somatic embryogenesis which embryos generated from adult somatic cells (Steward *et al.*, 1958). Thus, at least some forms of callus formation are thought to involve cell dedifferentiation. However, there are many different type of callus based on their macroscopic characteristics, such as friable, compct callus, has rooty, has shoot, and embryogenic callus. (Zimmerman, 1993; Frank *et al.*, 2000). Therefore, the term callus includes cells with various degrees of differentiation.

CHAPTER 3: MATERIALS AND METHODS

The material used for this study are Sacha Inchi seed (gained from Kampung Tebedu Mawang, Serian Sarawak), Murashige & Skoog Medium, MS (Duchefa Biochemie, The Netherlands), Woody Plant Medium, WPM (Duchefa Biochemie, The Netherlands), Naphthaleneacetic acid, NAA and benzyl adenine, BAP (Duchefa Biochemie, The Netherlands), Tween 20 (Merck Schuchardt, Germany), Sucrose (Fisher Scientific UK, UK), Vertical Laminar Flow Cabinet (ESCO, US), Autoclave (Astell Scientific Ltd, UK).

3.1 Sample collection

Sacha Inchi plant was taken from Kampung Tebedu Mawang, Serian Sarawak. The samples putted in appropriate bags and then the sample keep in refrigerator to keep it cool and moist to avoid sample withered before the day of experiment.

3.2 Media preparation

For media preparation for Sacha Inchi in-vitro propagation, MS (Murashige & Skoog, 1962) medium and Woody Plant Medium, WPM (Lloyd & McCown, 1980) used for the experiment. For 100ml of MS medium, about 3g sucrose, 0.44g of MS powder and 100ml of distilled water mixed together. While for WPM, about 3g sucrose, 0.246g of WPM powder and 100ml of distilled water mixed together. The suitable pH for both medium for in-vitro studies, around ±5.7 pH are recommended. For 100ml of medium, can be used to prepare around five plate of media in a petri dish for tissue culture.

3.3 Sterilization of explant (leaves)

Sacha Inchi leaves used in this study to test which concentration of bleaches suitable for sterilize the explant. Young leaves sterilized with 70% EtOH and then sterilized with 2%, 10% and 15% Clorox which mixed with Tween 20 about one drop (20µl) for about 5, 10 and 20 minutes for every each of the leaves that sterilized with different Clorox concentration to avoid contamination (Daud *et al.*, 2012). About nine plate of MS media prepared and each plate had been replicated.

3.4 Sterilization of explant (stem)

Sacha Inchi stems used in this study to test which concentration of bleaches suitable for sterilize the explant. Young stems had been washed under running water, and then brushed with diluted deepole detergent. After that, the explant stems sterilized with 70% EtOH and then sterilized with 2%, 10%, 15% and 20% Clorox which mixed with Tween 20 about one drop (20µl) for about 1, 5, 10 and 15 minutes for every each of the stem that sterilized with different Clorox concentration to avoid contamination (Daud *et al.*, 2012). About nine plate of MS media prepared and each plate had been replicated.

3.5 Preparation of explant (leaves and stem)

After surface sterilization, explants leaf/stem were prepared by aseptically removing the entire unwanted part of plant. The resulting leaf strips were cut into small squares about 5-8 mm, while, for the stem part, the stem part were cut into small size around 5 mm. Leaf, were placed onto the MS medium (Murashige & Skoog, 1962) supplemented with 30g/L sucrose and gelled with 2.5g/L gelrite without hormone supplements. The leaf explants were inoculated into petri dishes containing 20 ml of MS medium. All steps were carried out in the laminar flow cabinet. Cultures were provided with light. The explants in the Petri dishes were observed every day. The number of clean and alive explants, as an example those plants which were without bacterial or fungal contamination and still green and growing, was recorded after seven day.

3.6 Seeds preparation for embryo planting

For media preparation for Sacha Inchi in-vitro propagation, MS (Murashige & Skoog, 1962) medium and Woody Plant Medium, WPM (Lloyd & McCown, 1980) had been used for the determination of the differences of using different medium. For the seeds, the seeds coat were broke for short terms of plant to germinate. After that, the seeds had been sterilized with 70% EtOH and then soaked with sterilize distilled water for five hours. Then, sterilize the same seeds with 20% Clorox mixed with Tween 20 about one drop (20µl) for about 20 minutes to avoid contamination (Patthanajuck & Bunnag, 2017). After sterilization, the seeds were dissected to access the embryo explant. Sacha Inchi embryo were introduced to the MS and WPM medium, under dark condition for about 1 week to induce shoot formation.

Figure 3.1: Seed ready to be dissected

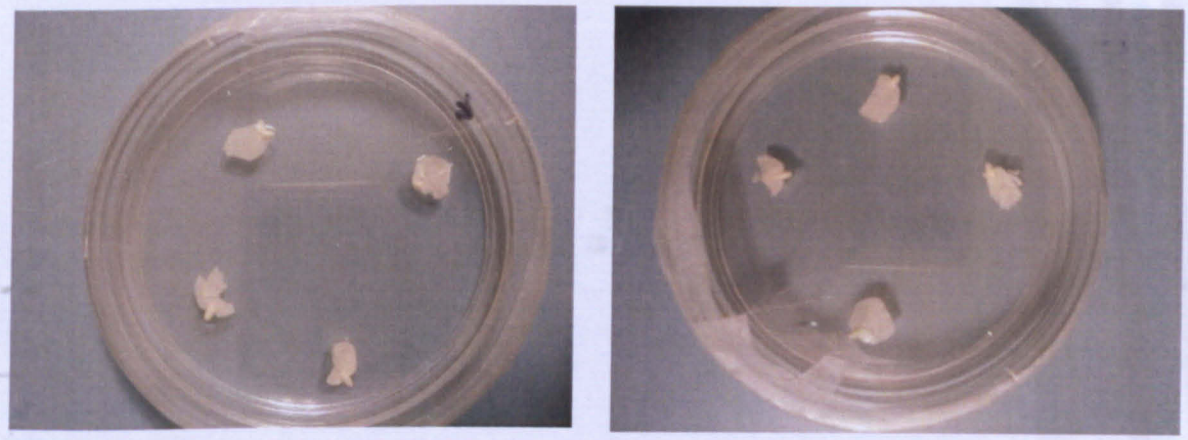


Figure 3.2: Embryo introduced into basal media

3.7 Dissection of stems from sterilized explant for callus induction

After the elongation of embryo form, the plant were put under light condition for two to three days. About five plate of embryo on MS media and five plate of embryo on WPM media prepared to differentiate the effect of different media used for embryo elongation. When green plantlets form, apical shoot, middle part and the root part were cut. The middle part (stem) become as the explant for callus induction. The explant transferred to different testing media with different concentration of hormone. MS media used for the hormone concentration (auxin and cytokinin) determination. The hormone concentration were controlled with concentration of 0, 0.1, 0.5, 1.0, 2.0 mg/L for each hormone. About 25 sample for the hormone determination part had been prepared. Each of the sample plate was replicated.

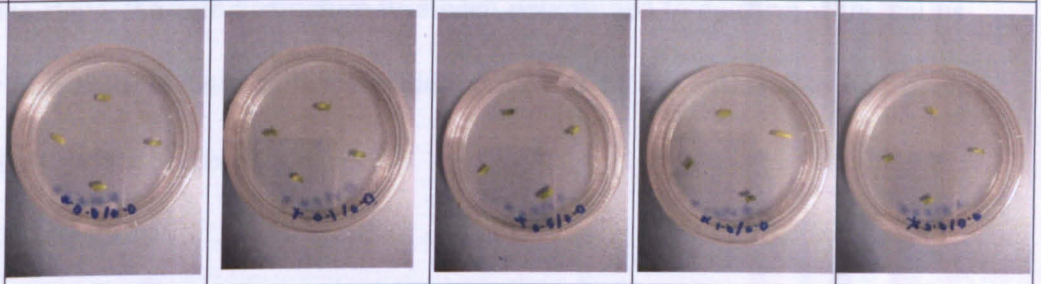
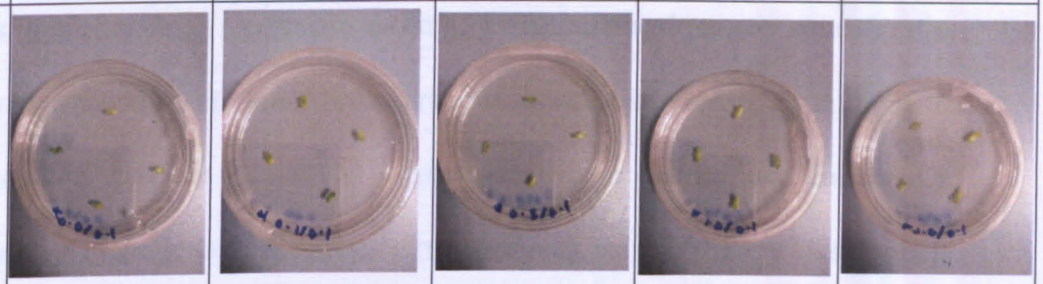
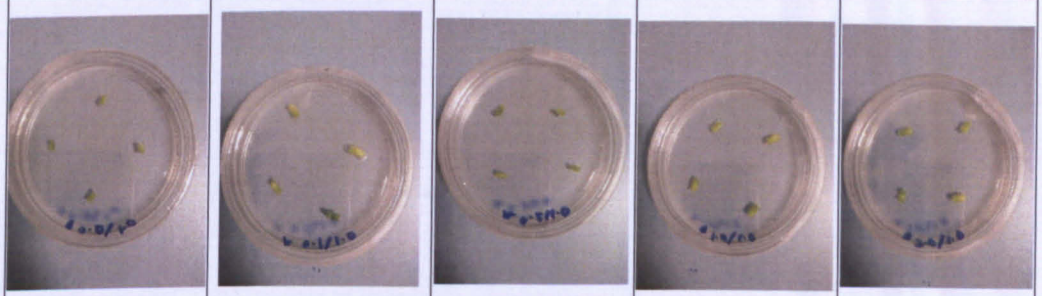
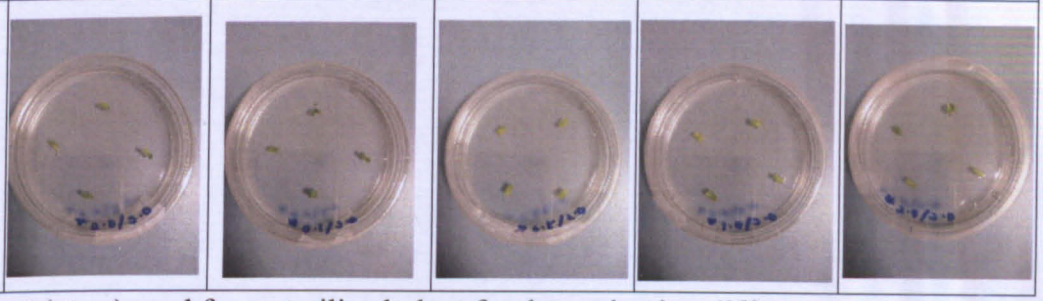
		Auxin (NAA)				
		0.0	0.1	0.5	1.0	2.0
Cytokinin (BAP)	0.0					
	0.1					
	0.5					
	1.0					
	2.0					

Table 3.1: The explant (stem) used from sterilized plant for determination different concentration of hormone NAA and BAP

3.8 Observation of callus under microscope

Callus were observed under stereomicroscope for determination of the structure and color of the callus. During this steps, the callus structure was been determined to confirm if the callus are embryogenic or non-embryogenic callus. Besides, the color of the callus also had been observed (green, yellow, white and brown in color).

3.9 Data Collection

Data collection of sample after being sterilize to determine which of the detergent concentration reduce or make a better aseptic technique from contamination. Moreover, the results of the better media between MS medium and WPM medium for tissue culture also collected to differentiate the effect of plant growth if tissue culture done by using different basal media. The length of stems collected and calculated by using mean and variance formula. Besides, the callus induction with different concentration of hormone (NAA and BAP) results also being collected to differentiate which of the hormone concentration of auxin and cytokinin give a better result of callus induction.

CHAPTER 4: RESULTS

4.1 Sterilization of leaves

Sacha inchi plant was treated with different concentration of bleach for the sterilization of the leaves surface. However, the effectiveness of treatment should not harm the explant. Based on Table 4.1. After sterilization of *Plukenetia volubilis* leaves, the contamination rate after sterilize using 2% bleach are 90% for five minute, 40% for 10 minutes and 100% for 20 minutes. Moreover, for leaves that sterilize using 10% bleach rate of contamination are 100% for five minute, 30% for 10 minutes and 100% for 20 minutes. While for 15% bleach that used for leaves sterilization, the contamination rate are 40% for five minute, 50% for 10 minutes, 100% for 20 minutes.

Bleach concentration (%)	**Time for soak leave in bleach (minute, m)**		
	5	**10**	**20**
2	90%	40%	100%
10	100%	30%	100%
15	40%	50%	100%

Table 4.1: Contamination rate of explant (leaves)

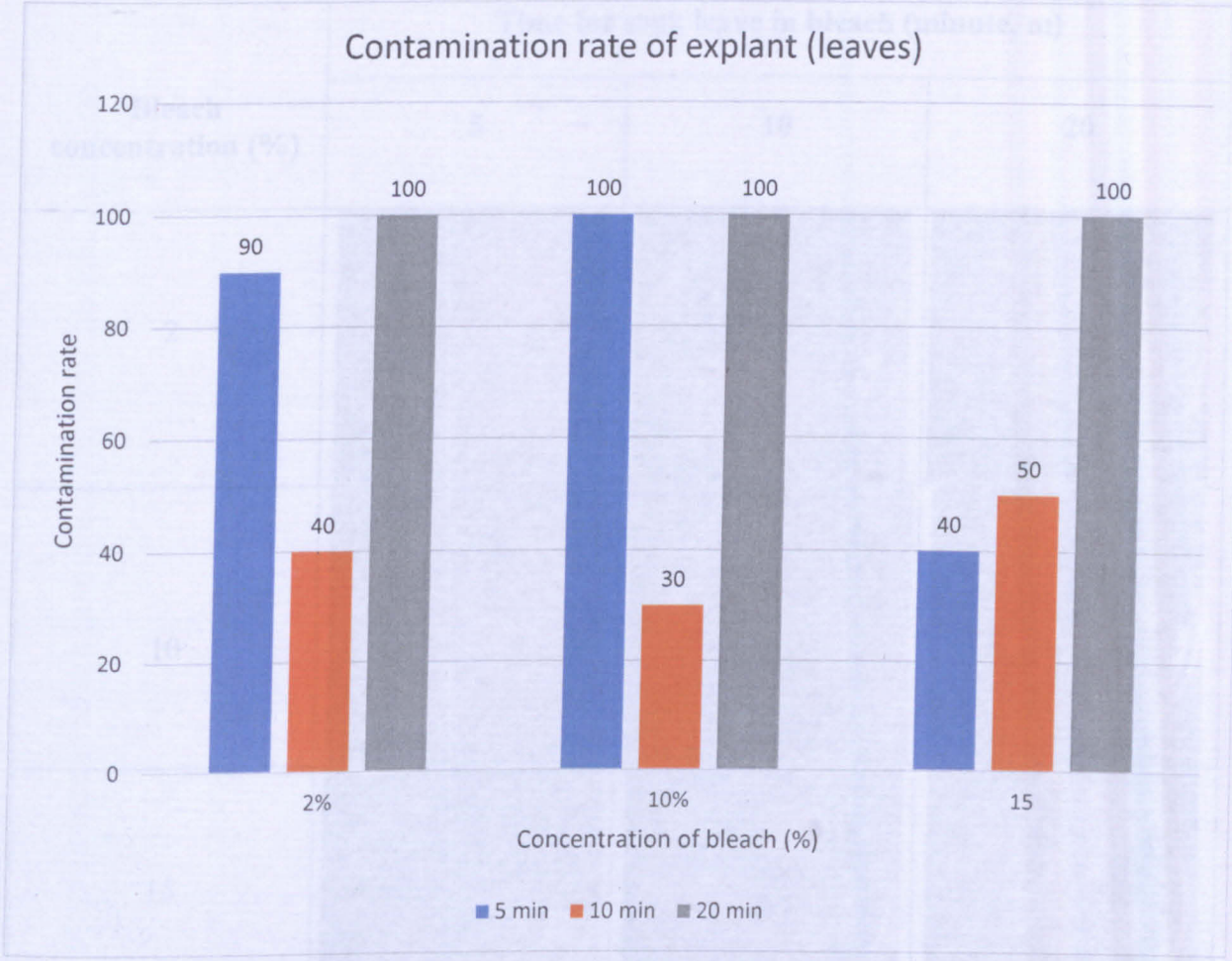


Figure 4.1: Bar chart of contamination rate after sterilizing explant (leaves)

Figure 4.1 shows the rate of contamination by using bar chart of contamination rate against concentration of bleach used for sterilization of explant.