OPTIMIZATION OF *IN VITRO* TISSUE CULTURE OF *Acmella uliginosa* USING DIFFERENT CONCENTRATIONS OF INDOLE-3-ACETIC ACID (IAA)

by

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A thesis submitted in fulfillment of the requirement for degree of Bachelor of Applied Science (Natural Resources Science) with Honors

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DECLARATION

I declare that this thesis entitled "Optimization of *In Vitro* Tissue Culture of *Acmella uliginosa* using different concentrations of Indole-3-Acetic Acid (IAA)" is the result of my own research except as cited in the references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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APPROVAL

"I/ We hereby declare that I/ we have read this thesis and in our opinion this thesis is sufficient in terms of scope and quality for the award of the degree of Bachelor of Applied Science (Natural Resources Science) with Honors"

Signature	:	……....
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Date	:	……..
Signature	:	……....
Name of Supervisor II	:	……..
Date	:	……..

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Optimization of *In Vitro* Micropropagation of *Acmella uliginosa* Using Different Concentrations of Indole-3-Acetic Acid (IAA)

ABSTRACT

Acmella uliginosa or "subang nenek" is the medicinal plant broadly distributed in the tropic and sub-tropic especially in West Indies, Venezuela, Brazil, Africa, Indonesia, and Malaysia. The study was carried out to observe the effect of various IAA hormones towards the root induction from the seeds explant and to establish the in vitro micropropagation technique for *Acmella uliginosa* due to previous studies was more focusing on other species such as *Acmella oleracea*. Six different concentration of IAA hormone: 0.1 mg/L, 0.2 mg/L, 0.3 mg/L, 0.4 mg/L, 0.5 mg/L and 0.0 mg/L (control) were used in this study to observe the impact of different concentration of hormone towards rooting induction in *in vitro A. uliginosa* seedlings. The seeds were inoculated in the MS medium containing six different concentrations. After 30 days, the length of the roots generated and the mean of the root length were evaluated. Observation shows that there is a significant effect caused by the IAA hormone on the growth of root. The highest number of root regenerated from seed explant was 2.667±0.260 and it was observed in MS medium containing 0.5 mg/L of IAA. Root length, number of root and rate of root to induce was analyzed using One Way Analysis of Variance (ANOVA). Result of this study revealed, among six type of concentration, the highest response for root induction were observed at 0.5 mg/L IAA. It was observed that at 0.5 mg/L the mean length of root was 2.667±0.260 cm and mean number of root produced was 5.000±0.516 respectively. The time taken for the root to induce is 2±0.000 days. It is proved that 0.5 mg/L IAA is the optimum concentration for root to induce compared to the other IAA concentration.

Pengoptimuman *In Vitro* Mikropropagasi *Acmella uliginosa* Menggunakan

Kepekatan Indole-3-Acetik Asid (IAA) yang Berbeza

ABSTRAK

Acmella uliginosa atau "subang nenek" adalah pokok herba yang menyebar secara meluas di kawasan tropika dan sub-tropika terutama di Barat India, Venezuela, Brazil, Afrika, Indonesia dan Malaysia. Kajian ini dilakukan untuk menentukan kesan hormon IAA yang berbeza terhadap pertumbuhan akar daripada biji dan untuk mengukuhkan teknik *in vitro* mikropropagasi untuk *Acmella uliginosa* disebabkan oleh kajian seblum in hanya menumpukan terhadap *in vitro* mikropropagasi terhadap pokok lain seperti *Acmella oleracea*. Enam kepekatan berbeza hormon IAA : 0.1 mg/L, 0.2 mg/L, 0.3 mg/L, 0.4 mg/L, 0.5 mg/L and 0.0 mg/L (kawalan) digunakan di dalam kajian ini untuk mengkaji kesan kepekatan terhadap pertumbuhan akar *A. uliginosa*. Biji telah diletakkan di dalam MS media yang mengandungi enam kepekatan IAA hormon yang berbeza. Selepas 30 hari, panjang akar dan purata kepanjangan akar telah dinilai. Pemerhatian menunjukkan kepekatan yang signifikan memberikan kesan yang cepat terhadap pertumbuhan akar. Biji di dalam MS media yang mempunyai kepekatan IAA 0.5mg/L menunjukkan bacaan tertinggi iaitu 2.667±0.260. Panjang akar, bilangan akar dan purata hari untuk pertumbuhan akar telah dianalisis menggunakan ANOVA. Keputusan kajian ini menunjukkan di antara enam kepekatan yang digunakan, 0.5mg/L IAA memberikan kesan yang terbaik terhadap pertumbuhan akar. Analisis menunjukkan pada kepekatan 0.5 mg/L mean panjang akar adalah 2.667±0.260 cm dan mean bilangan akar yang dihasilkan adalah dan 5.000±0.516. Masa yang diambil untuk pertumbuhan akar ialah 2±0.000 hari. Daripada kajian ini, terbukti 0.5mg/L adalah kepekatan yang paling optimum untuk pertumbuhan akar berbanding dengan kepekatan hormone IAA yang lain.

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LIST OF ABBREVIATIONS

MS	Murashige & Skoog
IAA	Indole-3-Acetic Acid
NaOH	Sodium Hydrochloride
HCl	Hydrochloric Acid
g/L	gram per Liter
mg/L	milligram per Liter
v/v	volume per volume
mm	Millimeter
cm	Centimeter
m	Meter
µm	Micro millimeter
TDZ	Thidiazuron
GC-MS	Gas chromatography-mass spectrometry
FCA	Freund's Complete Adjuvant
ANOVA	One-Way Analysis Of Variance
PGR	Plant Growth Regulator
SD	Standard Deviation

LIST OF SYMBOLS

%	Percentage
° C	Degree Celsius
<	Less Than

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Through many decades, medicinal plants have played a crucial role in maintaining the world health care system and act as life-saving drugs and have a nutraceutical value. In order to prevent the plant is continually exist, micropropagation is one of the best technique and it has been widely used for mass propagation especially for medicinal plant that have commercial value (Sharma *et al*., 2010).

Plant tissue culture is a method used to propagate plant under a good and sterile condition to produce the clone of the plant (Hussain *et al*., 2013). According to Thangavel and Ebbie (2014) and Pandey *et al*. (2014), most of the medicinal plant are being threatened its existence by the ever changing of its habitat ecology, disability to produce viable seeds or the seeds that has been produced is too slow to grow due to imbibition and the vast production of free-disease plant remains a major problem. Thus, plant micropropagation technique provide a convincing alternative method for producing true to type, rapid and mass multiplication of medicinal plants in disease-free condition.

Genus *Acmella* belongs to the family of Asteraceae which consist of 30 species including *Acmella uliginosa* (Chung *et al*., 2007) *A. uliginosa* is a herbaceous and annual plant and sometime perennial herb. Stems solitary or several from base, erect to

ascending or occasionally decumbent, green to purple, glabrous to moderately pilose is a flowering plant that are broadly spread in the tropical and sub-tropic especially in West Indies, Venezuela, Brazil, Africa, Indonesia, and Malaysia (Pandey *et al.*, 2014).

1.2 Problem Statement

A. uliginosa is an important plant in the medicinal field that are broadly distributed in the tropic and sub-tropic especially in West Indies, Venezuela, Brazil, Africa, Indonesia, and Malaysia but the previous study focus more on the other species such as *Acmella oleracea*.

1.3 Objective of Study

1) To established optimum *in vitro* propagation for *A. uliginosa*.
2) To determine the effect of various Indole-3-Acetic Acid (IAA) concentration on the growth of *A. uliginosa*.

1.4 Scope of Study

The sampling of *A. uliginosa* plants was focused around Universiti Malaysia Kelantan, Jeli Campus, Kelantan. This study was focusing on the establishment of *in vitro A. uliginosa* which is one of the medicinal values that have been used widely in the medicinal industry. The collected fresh sample was used to establish the *in vitro* micropropagation of *A. uliginosa.* This study also was done to determine the effect of hormone on the growth of this species *in vitro*.

1.5 Significance of Study

The established *in vitro A. uliginosa* gave benefit to the future downstream researches related to the pharmacological and medicinal industry. Firstly, this study was conducted to determine the ideal concentration of growth hormone (IAA) to obtain the optimum growth of *A. uliginosa.* At the end of the study, optimization of *in vitro* micropropagation of *A. uliginosa* was established.

CHAPTER 2

LITERATURE REVIEW

2.1 Asteraceae Family

A. uliginosa is a member of the Asterales order which is known for herbaceous plant (Table 2.1). Asteraceae or more commonly known as aster or sunflower is one of the largest angiosperm family with more than 1620 genus and 23600 species of herbaceous plants, shrub and trees distributed throughout the world. Figure 2.2 shows the distribution of *A. uliginosa* around the world.

Figure 2.1: World Map shows the distribution of *A. uliginosa* (the areas are in the red region).

(Source: Retrieved April 8, 2018 from https://www.cabi.org/isc/datasheet/120415)

Table 2.1: Taxonomy of Asteraceae Family (NRCS)

Kingdom	Plantae
Class	Magnoliopsida
Order	Asterales
Family	Asteraceae

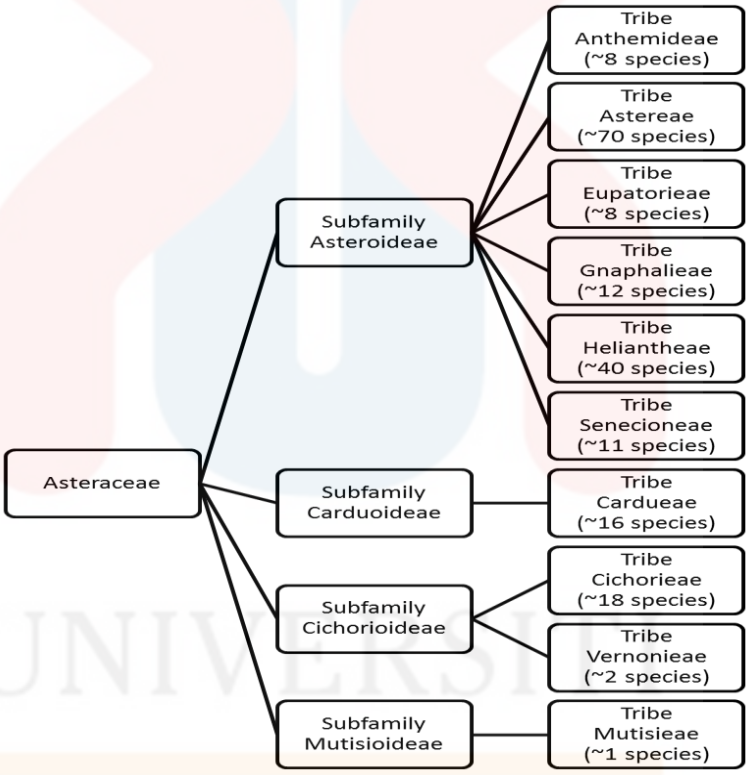

Figure 2.2: Classification of Asteraceae Family

2.2 Botanical of *Acmella uliginosa*

A. uliginosa or also known as marsh para cress and it belong to the Asteraceae family. *A. uliginos*a (Asteraceae) is a flowering plant that are broadly distributed in the tropic and sub-tropic especially in West Indies, Venezuela, Brazil, Africa, Indonesia, and Malaysia (Pandey *et al*., 2014) It is usually used by local Malay people to cure the toothache, mouth ulcer, sore throat and stomach ache. The antinociceptive property has been identified in the methalonic flowers extract (Khatun *et al*., 2015). In Benin, Porto Novo, Africa they usually use the leaf as the vegetable in the rural area and it has been domesticated. The leaf itself also can produce a good dewormer and antibiotic (Dansi *et al*., 2008). *A. uliginosa* is an involucral bract with 5.6 uniserate, achenese glabrous with margin ciliate. The habitat of these species are typically in a field, waste ground, damp location in fallows and semi aquatic prairies and it is categorized as an invasive species in New Caledonia and Fiji

2.3 Traditional use of *Acmella uliginosa.*

A. uliginosa can be cooked such as making sauce, hot peppery flavor or eaten raw as vegetable. It also can act as milk booster for lactating mother. It is known as herbaceous plant that are related to the mouth ulcer, toothache, sore throat and stomach ache *(*Ong *et al*., 2011). The leaf itself also can produce a good dewormer and antibiotic (Dansi *et al*., 2008).

2.4 Medicinal Properties of *Acmella Uliginosa.*

According to Rathore *et al*. (2007), *A. uliginosa* shows local sedating properties that usually used by local people to reduce the pain. Because of the studies on the other different potential anti-arthritic plants such as *Nyctanthes arbortristis* and *Aristolochia bracteata, A. uliginosa* has been considered to have a good inflammation inhibitory active values which can be considerably brought a changes in parameters toward normalcy in disease models.

2.4.1 Curing Potential of *Acmella Uliginosa* in FCA-Induced Arthritis

Freund's complete adjuvant (FCA)-induced model in rats usually is the best way of simulating a human disease condition. The technique is already been used as a model of chronic inflammation in rats and it is proved that it was a considerable relevance for the study of pathophysiology and pharmacological control of inflammatory processes. The crude extract of *A. uliginosa* can reduce the swelling of the paw in in FCA-induced arthritic rats when fed orally and because the swelling of the paws is the sign of the inflammation. The group treated by the aqueous extract of *Aloe vera* gel and *A. uliginosa* that have been proportionally combined showing the best result in increasing the serum protein and blood hemoglobin level (Balasubramanian *et al*., 2005). The level of albumin also decrease along with the arthritic severity (Ekambaram *et al.*, 2010).

2.5 Bioactive Constituents of Methanol Fraction of *A. uliginosa* Leaves

Varieties of compounds in the methanol lead extract of *A. uliginosa* have been identified by Gas chromatography–mass spectrometry (GC-MS) analysis. Through this identification, the ethnomedical relevance of this plant has been confirmed in the use of management and treatment of diseases. The effectiveness of this plant has been confirmed by the local dwellers and it is probably due to single effects as interplay of phytocompounds present in the plant. However, the definite mode of actions of the extract's oil has not been established but the mechanism suggested here in the study cannot be discarded (Ogunwande *et al*., 2010). This observation is in line with the report of Balamurugan *et al*. (2012). The GS-MS Chromatogram of *A .uliginosa* methanol leaves extract detected six peaks and this signified six compounds. The results revealed that the following compounds hexadecanoic acid (8.68%), hepta-9, 10, 11-trienoic acid (19.36%), octadecenoic acid (8.14%), 5-hydroxylmethyl heptadecane (14.02%), docosane aldehyde (41.72%) and 1-thoxyoctadecane (8.08%) were found as the major compounds in the methanol extract of leaves of *A. uliginosa* plant. These reports indicate that the economic value of this particular plant species is high owing to the medical property found. The high medicinal values stresses the plant must be grown in a sustainable environment as the habitat is facing rapid loss due to deforestation.

2.6 Developing *In Vitro* Micropropagation of *A. uliginosa*

Nowadays, due to increase demand of the medicinal plant in pharmacological industry, plant tissue culture offers as an alternative method to propagate the plant in short time

with homogenous growth and secondary metabolite production useful in pharmacological studies.

According to Sethi and Sharma (2011), plant tissue culture can be divided into different type of culture such as callus culture, meristem culture and internodal segment culture. For callus culture, it involves the growth of the callus that composed of differentiated and non-differentiated cell and then was followed by the procedures that will induce the organ differentiation. Furthermore, the culture usually sustained on a gel medium that composed with agar and a mixture of macronutrient and micronutrients depending on the type of the cell.

Meristem tissue culture is one of technique used for the viruses' elimination that is related to the pathogens from a number of vegetative propagated plants. In 1952, Morel and Martin developed the technique of meristem culture for *in vivo* initiation of Dahlia. Shoots of all gymnosperm and angiosperm grow by virtue of their apical meristem and then the meristem was isolated and the MS basal salt has been used for meristem culture.

According to (Sharma *et al*., 2010) various concentration of thidiazuron (TDZ) were used to observe the development of nodal explants. The plant shows an increase in size of leaves and then the present of the nodes and followed by the induction of shoot buds when TDZ was used with basal medium. When the TDZ is at lower concentration, shoot buds produced directly from node without callus. The plant shows an increasing in the leaves enlargement when the nodal segments were cultured on to medium containing 0.5 μM TDZ and the size is twice to their original size within 4 - 5 days of culture

followed by shoot bud induction within 15 - 20 days study regeneration potential of nodal explants.

2.7 Explant Preparation for *In Vitro* Culture

2.7.1 Explant Type

Different types of explant can be used in micropropagation of *A. uliginosa* such as internodal segment, leaves and seed.

2.7.2 Surface Sterilization of Explants

The explants were sterilized by the immersion in the 70% of ethanol for 2 minutes. After that the surface were sterilized using 15% of Clorox for 20 minutes in the laminar air flow followed by rinsed with double distilled water. The seeds were dried on the sterile filter paper.

2.8 Growth Media Composition

The composition in the culture media is one of the most important things in governing the growth of the plant. The culture media basically made up of macronutrients, micronutrients, vitamins, amino acid or other nitrogen supplement, sucrose, other undefined organic supplements, agar and growth regulators.

2.8.1 Agar

Agar is the most widely used as a gelling agent in preparing semisolid and solid for plant tissue culture media. The advantages using the agar is when it is mixed with the water, the gel that formed will melt approximately at 60°C - 100°C and then solidifies at 45°C approximately, thus make the agar are stable at all incubation temperatures. The agar also does not react with media constituents and does not being digested by the plant enzymes. The concentration and the brand type of agar used influenced the firmness of an agar gel and the pH of the medium. The agar concentrations commonly used in plant cell culture media range between 0.5 and 1.0%; these concentrations give a firm gel at the pH's typical of plant cell culture media (Abobkar *et al.,* 2012)

2.8.2 Plant Growth Regulator (PGR)

Plant growth regulators or also called as plant hormones are the chemicals that will influence the cell, tissues and organs differentiation and growth. Currently, there are five recognized groups of plant hormone that are auxin, gibberellins, cytokinins, abscisic acid (ABA) and ethylene. Ethylene is not commonly used in tissue culture due its properties where it is commonly involved in abscission and flower senescence in plants. Auxin responsible to stimulate the cell elongation such as initiation of the root, apical dominance and vascular differentiation. Synthetic auxin that used in the tissue culture is Indole-3-Acetic Acid (IAA). Cytokinin is responsible to stimulate the cell division and shoot bud induction formation in tissue culture. Synthetic cytokinin used in tissue culture is BAP.

2.8.3 Carbohydrate

Carbohydrate is commonly added into culture medium as an energy source and an osmotic agent. Sucrose 30% is commonly used as a carbon source for *in vitro* propagation of plant.

CHAPTER 3

MATERIALS AND METHODS

3.1 Study Area

3.1.1 Universiti Malaysia Kelantan, Jeli Campus, Kelantan

Universiti Malaysia Kelantan, Jeli Campus, Kelantan is located in Jeli, Kelantan which is adjacent to the Perak, Malaysia and Thailand. The study was done around UMK, Jeli Campus. Figure 3.1 shows the maps of the study area.

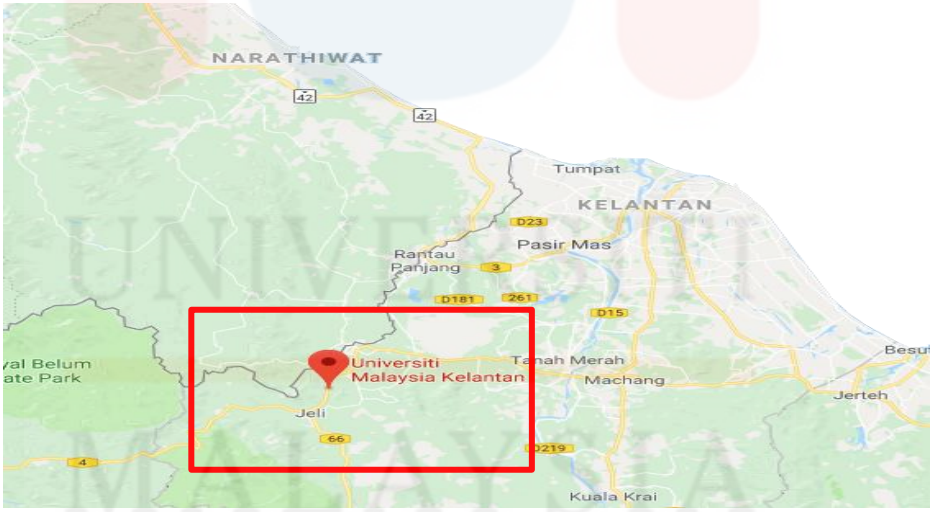

Figure 3.1: Maps Universiti Malaysia Kelantan, Jeli Campus

3.2 Materials

Beakers, glass rod, volumetric flask, conical flask, culture test tube, Erlenmeyer flask, measuring cylinders, petri dishes, short jam jars, tall jam jar bottles will be washed using soap and rinsed with distilled water and to sterilize the instruments and materials that was used for the culture work such as scalpel, forceps, spatula, disposable blades and scissors autoclave (TOMY SX-500) will be used. All the sterilized items were dried in oven at 50 ºC. Oven, autoclave (TOMY SX-500), laminar flow hood, magnetic stirrer hot plate and weighing machine are the laboratory apparatus that will be used in this study.

The chemicals that will be used to prepare macronutrients (Stock A) are ammonium nitrate (HmbG chemicals), potassium nitrate (Bendosen), calcium chloride dehydrate (Bendosen), potassium dihydrogen phosphate (HmbG chemicals) and magnesium sulphate heptahydrate (Bendosen). Sodium molybdate dehydrate (HmbG chemicals), copper sulphate-5-hydrate (Bendosen), cobalt chloride hexahydrate (Bendosen), manganese sulphate (Bendosen), zinc sulphate heptahydrate (Bendosen), boric acid (QRëC) and potassium iodide (Bendosen) will be used to prepare micronutrients (Stock C). The vitamin stock solution will be prepared using thiamine hydrochloride (Duchefa Biochemie), glycine (HmbG chemicals), nicotinic acid (Duchefa Biochemie) and pyridine (Duchefa Biochemie). Ethylenediaminetetraacetic (QRëC) is a sodium iron which is will be used to prepare iron stock solution. Myo stock solution will be prepared using myo-inositol (Duchefa Biochemie).

3.3 Methods

3.3.1 *Acmella uliginosa* Sampling

The sampling of *A. uliginosa* is done around the Universiti Malaysia Kelantan Jeli Campus area. The method used for sampling is random sampling. *A. uliginosa* that can be spotted along the river and watered area was photographed and collected. Their natural habitat also had been recorded. Figure 3.2 shows *A. uliginosa* found growing wildly at Universiti Malaysia Kelantan, Jeli Campus. The sample identification was confirmed by referring to an article entitled "New variety of *A. uliginosa* (Asteraceae) from Kerala, India" wrote by Reshmi in 2016.

Figure 3.2: *A. uliginosa* at Universiti Malaysia Kelantan, Jeli Campus.

3.3.2 Identification of the Plant Sample

A. uliginosa can be identified with leaves lanceolate to narrowly ovate or sometimes ovate and it is based on attenuate where; heads 6-8mm tall, 4-6 mm in diameter,

phyllaries 5-6, uniserate and ferally naturalized plants (Chung *et al*., 2007) Figure 3.3 shows the flowers and seeds of *A. uliginosa*.

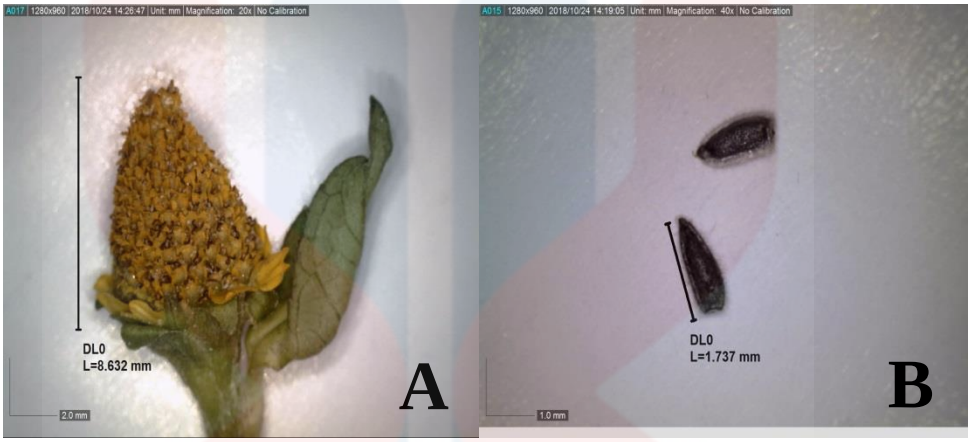

Figure 3.3: Flowers and Seeds of *A. uliginosa* were taken using digital microscope under 20 x and 40 x magnification in Tissue Culture Laboratory, UMK Jeli Campus

3.3.3 Preparation of Murashige and Skoog (MS) Medium

The Murashige and Skoog (MS) medium were prepared following protocol by Murashige and Skoog (1962). Micropagation of *A. uliginosa* requires basal media with micronutrients, macronutrients, 7.8g/L iron source, 30g/L sucrose and organic supplement formulation. The pH was adjusted to 5.7 -5.8 by using 0.1 NaCl and 0.1 HCl. The culture bottle were filled with the molten MS medium approximately 20mL for each and the bottles were labeled with the date and name and covered by the plastic lids. The sterile MS media that has been autoclaved 121°C temperature at 1.5 atm pressure was left to solidify and stored in the clean cabinet in the Tissue Culture Laboratory Universiti Malaysia Kelantan, Jeli Campus.

3.3.4 Optimization of Surface Sterilization of *Acmella uliginosa*

A. uliginosa seeds were obtained from the matured flower. Seeds of *A. uliginosa* were then washed with the soap water and under running tap water for 20 minutes. Then, seeds' surface was sterilized by the immersing in the 70% of ethanol for 2 minutes. After that, the surface were again sterilized using 15% of Clorox® for 20 minutes in the laminar air flow followed by rinsed with double distilled water. The seeds were dried by bolting on the sterile filter paper (Inoka & Dahanayake, 2015)

3.3.5 *In Vitro* Culture Establishment of *Acmella uliginosa*

After the surface sterilization, the seed coats of *A. uliginosa* were removed using the sterile scalpers and blade (Inoka & Dahanayake, 2015). Three seeds then were inoculated in each of the culture bottles containing six different concentrations; 0.0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/L of IAA hormones. All culture were maintained in the growth chamber at 25± 2 ºC under 16 hours light photoperiod provided by white fluorescent tubes (Thingbaijam *et al*., 2014). The cultures was maintained by regular subcultures at 4 weeks intervals on fresh MS medium (Behera & Sahoo, 2009). The data about the root induction of the seedling have been recorded.

3.3.6 Statistical Analysis

The data that has been obtained from 3.3.5 on the effect of the IAA on the growth of *A. uliginosa* was recorded and analyzed using a One-Way Analysis of variance (ANOVA). The means was compared by Tukey test at 5% probability level.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 *In Vitro* Micropropagation of *Acmella uliginosa*

4.1.1 Establishment of Aseptic Explants of *A. uliginosa* Using Internodal Segment

As the internodal segment were used as the explant, establishment of the aseptic explant were more challenging due to its hairy wood. Three types of surface sterilization treatments were applied to the *A. uliginosa* explants. For the first treatment, the explants were treated with 15% (v/v) Clorox® for 10 minutes and 20% ethanol for 20 minutes. The explants were washed thoroughly with sterile distilled water before and after sterilizing with ethanol.

Thirty explants were treated with second treatment by which the explants were immersed with 50% (v/v) Clorox® and 70% ethanol for 3 minutes consecutively. The explants were rinsed with sterile distilled water after each sterilization of Clorox® and ethanol. In the third treatment, the internodal segments were dipped in 100% of Clorox®. The internodal segments were washed three times thoroughly with sterile distilled water. Then, the pieces were immersed in 95% (v/v) ethanol for 15 seconds. After that, they were rinsed three times with sterile distilled water.

According to Sarmila (2018) the most efficient way to reduce the contamination of the explant is to surface sterilized with 95% ethanol and followed with 100% (v/v)

Clorox® solution but still cannot resolve the contamination of the internodal segment for *A. uliginosa*. It is because woody or field-grown plants are sometimes difficult to disinfect. Figure 4.1 shows the contaminations occur in the internodal segments culture.

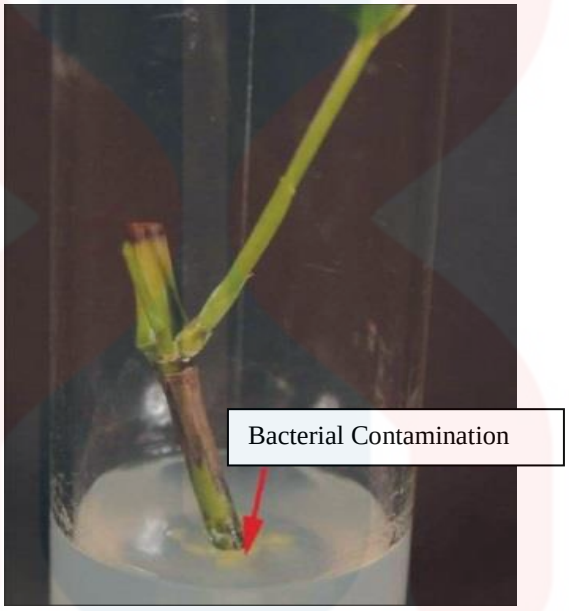

Figure 4.1: Four-week old internodal segment culture in control MS media. The arrow shows the bacterial contamination around the internodal segment.

Due to that, the explant part has been changed from internodal segment to the seeds and it shows a satisfactory growth with a proper surface sterilization. In sunflower family, hypocotyl and cotyledon explants are good regeneration material that shows better regenerative behavior when kept in the culture (Ozyigit, Gozukirmizi & Semiz, 2007). The *A. uliginosa* seeds were grayish-green and encase in tear-dropped black shells. The size of the seeds can reach 1mm until 2mm only. Figure 4.2 shows the seed of *A. uliginosa.*

Figure 4.2: Seeds of *A. uliginosa* was taken using digital microscope under 40 x magnifications in Tissue Culture Laboratory, UMK Jeli Campus

4.1.2 Establishment of Aseptic Explants of *Acmella uliginosa* Using Seed

Surface sterilization protocol was done for the seeds obtained from the flower *A. uliginosa.* The percentage of survivability for all explants after seed surface sterilization were 100% as the explants remained grayish-green in color after five days of culture and some of the culture were observed to have root induction and shoot proliferation. The use of seed as explants is because the seed is simply germinated under in vitro conditions and to reduce the chances of contamination which is very high if we take other plant parts as explants. Some plants like *A. uliginosa* and orchid have very small seeds and the seeds are more reliably grown from seed in sterile culture. Figure 4.1 shows the explants that have produced the longest root and tallest shoot.

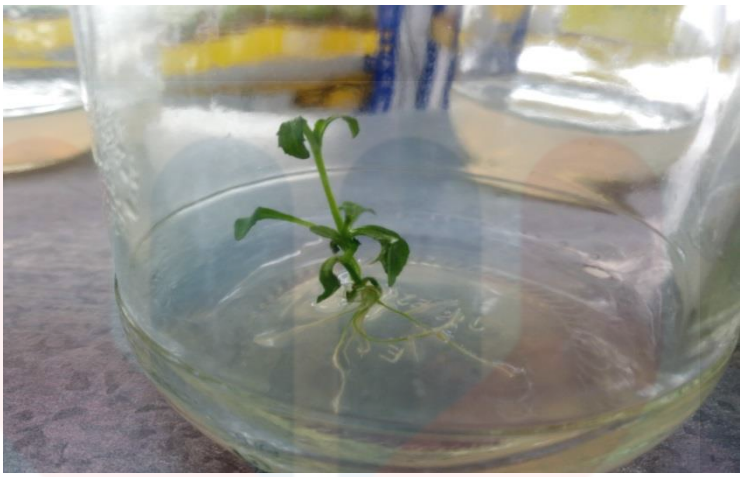

Figure 4.3: Four-weeks old *A. uliginosa* produced from seeds cultured in MS media containing 0.5 mg/L IAA hormone in the growth chamber at 25± 2 ºC under 16 hours light photoperiod provided by white fluorescent tubes

After a few weeks**,** contamination was observed in several cultures. The bacteria and fungus seem to cover the explant and media**.** It was turned into cotton-like structure in 'fuzzy' patches with rapid spreading surrounding the explants on the agar was observed (Figure 4.2).

Figure 4.3: Contamination Four-week old *A. uliginosa* plantlets produced from the seed cultured in MS media containing 0.2 mg/L IAA hormone

The color of the fungus normally was different depending on the species and in this study shows that the fungus turned into yellow brownish. The contamination occurs possibly due to the activity in the growth room. (Ugenthira, 2014)

4.1.3 Effect of Different Concentrations of IAA Hormone on Root Induction

MS basal medium containing IAA hormone affected the growth of the roots of *A. uliginosa.* The highest mean number of root (5.330 ± 0.516) of *A. uliginosa* per explant and mean of the root length (2.667 ± 0.260) were observed on 0.5 mg/L. The lowest mean number which no growth of the root was observed in control medium which is no hormone was being put. *A. uliginosa* had the highest root induction at 0.5 mg/L of IAA. It is shows that the root is thicker and denser in the MS medium containing 0.5 mg/l IAA rather than hormone free MS medium. There was a significance study that shows why there is no root induction, it was mainly because of the low in endogenous phytohormone level in the explants and the absence of plant growth regulators supplied to the medium (Jimenez, 2005). Plant growth hormone is important in the induction of root from the seed explants (Banerjee *et al*., 2007). Figure 4.5 shows the root induction from seed in various concentration of IAA hormone; 0.0, 0.1, 0.2, 0.3, 0.4, and 0.5 mg/L.

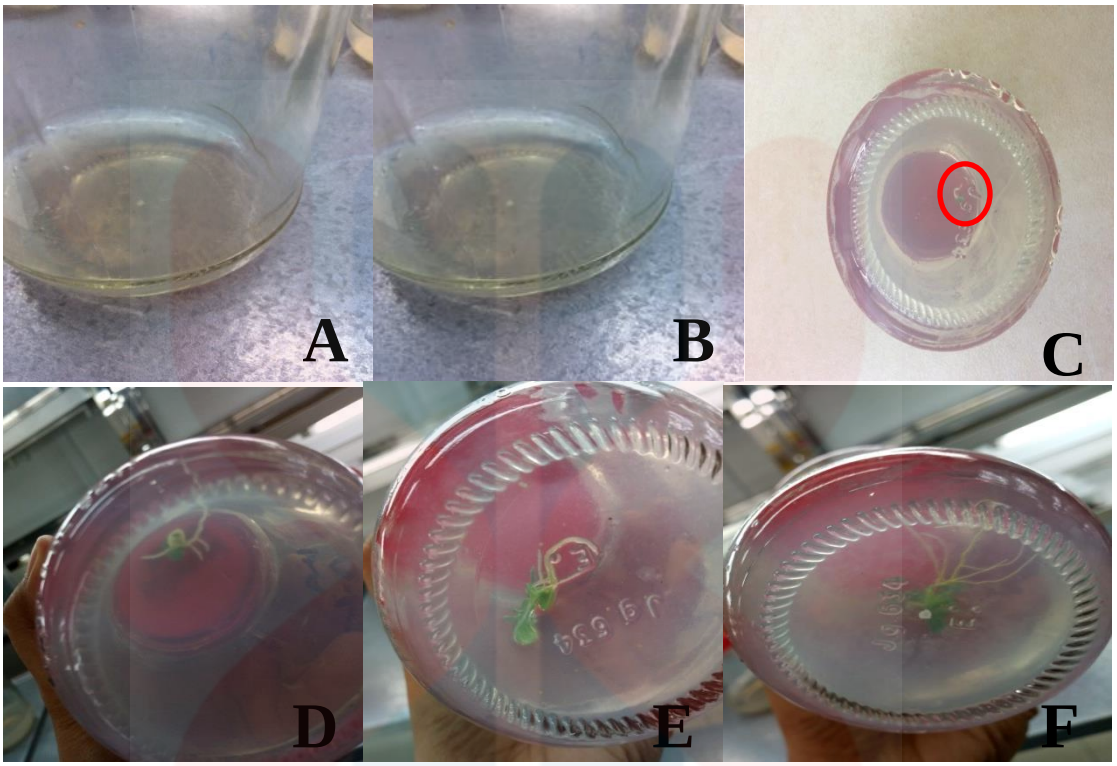

Figure 4.4: Root induction for *A. uliginosa* based on IAA hormone concentration where, A: 0.0, B: 0.1 mg/L,

C: 0.2 mg/L, D: 0.3 mg/L, E: 0.4 mg/L, F: 0.5mg/L

4.2 Various Hormone Concentration Factors Affecting Root Induction

4.2.1 Various Hormone Concentration Factors Affecting Root Length

The effect of various concentration hormones (0 – 0.5mg/L) on the root induction was tested by measuring the length of the root after 30 days. The mean length of the root was presented in the table 4.1. Based on the table 4.1, the result indicated that the root induction could be highly induced on MS medium containing 0.5 mg/L IAA hormone within 30 days. The MS media supplemented with 0.5 mg/L hormone gave higher

growth than MS media supplemented with 0.0 mg/L hormone. This result can be supported with the research reported by Inoka and Dahanayake (2015). The mean root was 2.667 ± 0.260 when using 0.5 mg/L compared to 0.000 ± 0.000 when using 0.0 mg/L hormone.

Table 4.1: Effect of various concentration of IAA hormone (0 – 0.5mg/L) on root induction (cm) from seed explants of *A. uliginosa* (mean root values followed by the same letters indicating they are not significantly different; HSD, $p < 0.05$)

Concentration of IAA Hormone (mg/L)	**Mean Length of the Root (cm)**
0	$0.000 \pm 0.000_{d}$
0.1	$0.000 \pm 0.000_{d}$
0.2	$0.908 \pm 0.058_{a}$
0.3	$1.275 \pm 0.186_{b}$
0.4	$2.217 \pm 0.282_{c}$
0.5	$2.667 \pm 0.260_{e}$

- Mean ± Standard Deviation (SD) of 6 replicates per treatment for all root induction studies
- At 30 days after root induction, mean root length was recorded as 1.178 ± 1.039

4.2.2 Various Hormone Concentration Factors Affecting Number of Root Produced

The seed explants cultured on MS medium supplemented with 0.5 mg/L produced the highest amount of root (5.330 ± 0.516) meanwhile MS medium supplemented with 0.0 and 0.1 shows no root induction within 30 days. This was significantly higher than the amount of root induced on MS medium contained 0, 0.1, 0.2, 0.3 and 0.4 mg/L respectively. This shows that the higher amount of hormone concentration, the higher

the number of root produced. Table 4.2 shows the result of various hormone concentrations affecting number of root produced.

Table 4.2: Effect of various concentration of IAA hormone (0 – 0.5mg/L) on number of root produced from seed explants of *A. uliginosa* (mean root values followed by the same letters indicating they are not significantly different; HSD, $p < 0.05$)

Concentration of IAA hormone (mg/L)	**Mean number of the root produced**
0	$0.000 \pm 0.000_{d}$
0.1	$0.000 \pm 0.000_{d}$
0.2	$2.000 \pm 0.516_{a}$
0.3	$3.000 \pm 0.632_{b}$
0.4	$4.000 \pm 0.516_{c}$
0.5	$5.000 \pm 0.516_{e}$

- Mean ± Standard Deviation (SD) of 6 replicates per treatment for all root induction studies
- After 30 days of cultured , mean root length was recorded as 2.390 ± 1.990

4.2.3 Various Hormone Concentration Factors Affecting Rate of Root Produced

From the observation, the rate of root induction from single seed explants cultured, were not significantly different between MS medium containing 0.4 and 0.5 mg/L. The rates of root induction for both concentrations were 3.000 ± 0.000 and 2.000± 0.000 days. The lowest mean for the rate of root induction (0.0 cm) was observed in 0.0 and 0.1 mg/L IAA. Rate of root produced depends on the concentration of IAA hormone, this can be proved by the table 4.3 where the higher the concentration of hormone, the rate of root induction is faster. However, future studies could be conducted to investigate effect of

wider range of IAA concentration on root induction which could not be accomplished in this study due to time constraint.

Table 4.3: Effect of various concentration of IAA hormone (0 – 0.5mg/L) on time taken for root to produce (days) from seed explants of *A. uliginosa* (mean root values followed by the same letters indicating they are not significantly different; HSD, $p < 0.05$)

Concentration of IAA hormone (mg/L)	Time Taken for Root to Produce (days)
0.00	No growth
0.10	No growth
0.20	$19.000 \pm 0.139_a$
0.30	$7.000 \pm 0.117_b$
0.40	$3.000 \pm 0.000_c$
0.50	$2.000 \pm 0.000_c$

- Mean ± Standard Deviation (SD) of 6 replicates per treatment for all root induction studies

4.2.4 Various Hormone Concentration Factors Affecting Rate of Root and Shoot Proliferation

Shoot proliferation was observed on MS medium supplemented with (0.2, 0.3, 0.4 and 0.5) mg/L IAA hormone but there is no growth in 0.0 and 0.1 mg/L. The highest mean number of shoot proliferation was produced in MS medium supplemented with 0.5 mg/L IAA hormone (table 4.4). According to the Ozyigit *et al* (2005, 2007), it was recorded that the most suitable condition for shoot induction of sunflower was found to occur on MS media supplemented with 0.5 mg/L hormone. It is proved that the hormone concentration at 0.5mg/L was the best condition in order to have faster root induction and shoot proliferation which is it only takes two days for the root to induce. It is because the auxin is particularly involved in the culture medium to stimulate callus

production and cell growth, to initiate shoot, particularly root and promote somatic embryogenesis (Taji *et al.*, 1995)

Table 4.4: Effect of various concentration of IAA hormone (0 – 0.5mg/L) on rate of root induction (cm) and shoot induction from seed explants of *A. uliginosa* (mean root values followed by the same letters indicating they are not significantly different; HSD, $p< 0.05$)

Concentration of IAA hormone (mg/L)	**Rate of Root Induction**	**Shoot Proliferation**
0.00	No growth	-
0.10	No growth	-
0.20	$19 \pm 0.139_a$	+
0.30	$7 \pm 0.117_b$	+
0.40	$3 \pm 0.000_c$	++
0.50	$2 \pm 0.000_c$	+++

- Mean ± Standard Deviation (SD) of 6 replicates per treatment for all root induction studies
- Shoot was quantified as less than 5mm, between 5mm and 10mm and greater than 10mm, as indicated by +, ++, +++ respectively.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

According to the result, although three surface sterilization methods were applied to internodal segments using Sarmila (2018) method, there is no growth in the culture and the explant easily to contaminate due the reason of field-grown plant are sometimes difficult to disinfect. Due to that reason the explant were changed into seed explant and the surface sterilization using Inoka & Dahanayake (2015) and it shows the satisfactory result where there was a growth observed in the seeds culture. In short, a surface sterilization protocol for the seeds explants was established root and shoot were induced using different concentration of IAA hormone. The optimum root induction was observed in the MS medium containing 0.5 mg/L of IAA and the hormone free MS medium shows that there was no root induction. It was shows that at 0.5 mg/L the mean length of root and mean number of root produced were 2.667±0.260 and 5.000±0.516 respectively. The time taken for the root to induce in 0.5mg/L also was shorter where it only took two days to induce. Among two explants tested, seeds show the good root induction rather than the internodal segment. It was proved that the higher the concentration of hormone used, the faster the explant to grow for the conservation purposes. Thus, this can be concluded that the main objective of this study is achieved.

5.2 Recommendation

As the recommendation, in order to avoid any contamination it was important to keep the workplace sterilize and the door of the culture is always being close to avoided any pathogen that could cause the contamination towards the explant. Other than that, the culture should always being put in the optimal condition which was low in temperature but at the same time the light was provided in order for them to grow. The hardware that has been autoclave should always being put in a sterile condition to avoid any lab pathogens. In micropropagation studies, other explants such as root or leaf can be used for research purpose. Furthermore, different types of auxin and cytokinin can be used in the research to observe the growth of the plant especially for root and leaf induction. Increasing the concentration and using various auxins and cytokinins used may result in plant regeneration.

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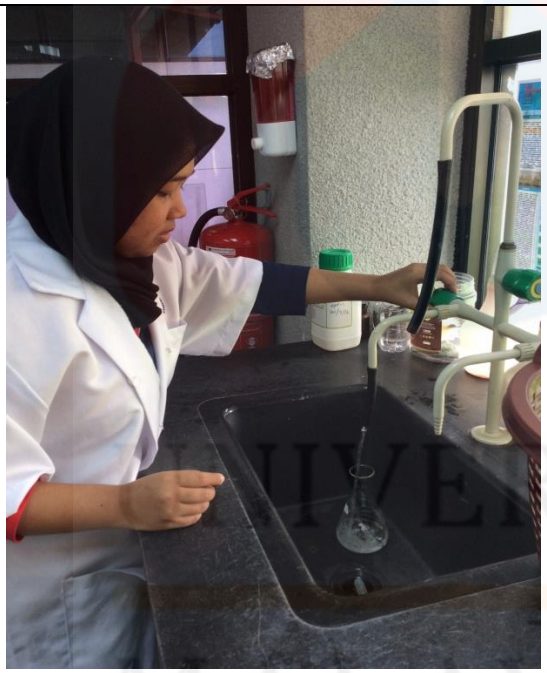
APPENDIXES A

Raw Material	Description
	*Acmella uliginosa* at UMK Jeli Campus.

APPENDIXES B

Chemicals	Description
Macronutrients	Ammonium nitrate (HmbG chemicals), potassium nitrate (Bendosen), calcium chloride dehydrate (Bendosen), potassium dihydrogen phosphate (HmbG chemicals) and magnesium sulphate heptahydrate (Bendosen).
Micronutrients	Sodium molybdate dehydrate (HmbG chemicals), copper sulphate-5-hydrate (Bendosen), cobalt chloride hexahydrate (Bendosen), manganese sulphate (Bendosen), zinc sulphate heptahydrate (Bendosen), boric acid (QRëC) and potassium iodide (Bendosen)
Vitamin	Thiamine hydrochloride (Duchefa Biochemie), glycine (HmbG chemicals), nicotinic acid (Duchefa Biochemie) and pyridine (Duchefa Biochemie).
MS Iron	Ethylenediaminetetraacetic (QRëC)
Myo-Inositol	(Duchefa Biochemie).
Plant Agar	Duchefa Biochem
Sodium Hydroxide	1M
Hydrochloride Acid	1M
Ethanol	70%, 95%
Clorox	15%
IAA Hormone	Indole-3-Acetic Acid

APPENDIXES C

Field Work/ Laboratory Work	Description
	Sampling of *Acmella uliginosa*.
	*A. uliginosa* was washed with the soap water and under running tap water for 20 minutes.

	Preparation of MS media.
	Inoculation of *Acmella uliginosa* seeds in laminar flow hood.