Establishment of Axenic Explants and Callus Culture of *Annona muricata* Linn.

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Establishment of Axenic Explants and Callus Culture of *Annona muricata* Linn.

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A thesis submitted in partial fulfillment of the requirement for the degree of Bachelor of Science with Honours

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Date : 19 May 2017

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LIST OF ABBREVIATIONS

ANOVA	-	Analysis of Variance
BAP	-	N6 – benzylaminopurine
EtOH	-	Ethyl alcohol
HCI	-	Hydrochlorix acid
HgCl2	-	Mercuric chloride
IAA	-	Indole-3-acetic acid
KOH	-	Potassium Hydroxide
mg/l	-	milligram per litre
MS Media	-	Murashige and Skoog Medium
NAA	-	I-Napthaleneacetic
NaOCl	-	Sodium hypochlorite
LSD	-	Least Significant Difference
PGRs	-	Plant Regulator Growth
2,4-D	-	2,4- dichlorophenoxy acetic acid

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Establishment of Axenic Explants and Callus Induction of *Annona muricata* Linn. Leaves

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ABSTRACT

Soursop (*Annnona muricata* L.) has been commercially exploited for its medicinal values. Large scale planting of the species requires huge number of planting stock that could not be achieved by using seeds as the seed possess some degree of dormancy. Thus, vegetative propagation by tissue culture holds great potential to produce quality planting stock. This study was designed to determine a working protocol for axenic leaf explants establishment and callus induction of *A. muricata*. For surface sterilization experiment, the leaves were cut and washed with Tepol detergent, followed by immersion in 200ml of 70% ethanol for 30 seconds, before they were soaked in different concentration of Chlorox® for different exposure times. The second experiment aimed at looking on the best 2,4-D concentration for callus induction. The results showed that 15% Chlorox® with 30 minutes exposure produced the 100% axenic explants. Secondly, leaf explants placed on MS + 4.0mg/ml 2,4-D + 1.0 mg/ml BAP and MS + 6.0 mg/ml 2,4-D + 1.0 mg/ml BAP were the most number of explants producing callus although no significance among the treatment.

***Keywords**:* Establishment axenic explants, callus induction, *Annona muricata* L., micropropagation, 2,4-dichlorophenoxy acetic acid.

ABSTRAK

Soursop *(Annona muricata L.), telah dieksploitasi secara komersial bagi nilai-nilai perubatan. Secara besar-besaran penanaman spesies memerlukan jumlah besar menanam saham yang tidak boleh dicapai dengan menggunakan benih kerana benih memiliki tahap dormansi. Oleh itu, tisu kultur memiliki potensi besar untuk menghasilkan penanaman saham berkualiti. Kajian ini telah dibuat untuk menentukan protokol kerja untuk axenic daun explants penubuhan dan induksi callus A. muricata. Untuk percubaan permukaan pensterilan, daun dipotong dan dibasuh dengan pencuci Tepol, diikuti Rendam dalam 200ml etanol 70% selama 30 saat, sebelum ini ia telah direndam di dalam kepekatan berbeza Chlorox® untuk pendedahan yang berbeza. Percubaan kedua bertujuan melihat kepekatan 2,4-D yang terbaik untuk callus induksi. Hasil kajian menunjukkan bahawa 15% Chlorox® dengan pendedahan 30 minit menghasilkan 100% explants axenic. Kedua, daun explants diletakkan di atas MS + 4.0 mg/ml 2,4-D + 1.0 mg/ml BAP dan MS + 6..0 mg/ml + 1.0 mg/ml BAP merupakan explants yang terbanyak menghasilkan callus walaupun tiada perbezaan yang significant di antara rawatan.*

***Kata kunci**: Penubuhan eksplant axenic, callus induksi, Annona muricata L., mikropropagasi, asid asetik 2,4-dichlorophenoxy.*

1.0 INTRODUCTION

Annona muricata L. or soursop belong to the family of Annonaceae. The family Annonaceae comprises approximately 132 genera and more than 2000 species, approximately 50 species have edible fruit and some of them have commercial value. These subtropical and tropical trees are part of the natural flora in Central and South America (Hannia, 2000). All of species are originally from the Americas and most are undomesticated. Based on previous study of Hannia (2000) the commercial acceptance of *A. muricata* as exotic fruits is widespread worldwide, therefore there is a rising interest to expand *A. muricata* plantations in countries where this plant have been introduced such as Australia, California, Chile, Israel and Spain. The trees of *A. murica*ta are many importance which is the fruit of *A. muricata* is found to be edible and used commercially for the production of juice, candy, ice cream and sherbets. Seed and leaves were used in the traditional medicine to treat various disease (Gajalakshmi, *et al*., 2012; Salaeh *et al*., 2013).

According to the Orwa *et al.* (2009), *A. muricata* have their own local names based on their languages which is English (durian blanda, soursop, custard apple), Filipino (atti, llabanos, guayabano), French (corossol, corosselier), Malay (durian belanda, durian Makkah, durian benggala) and Spanish (guanabana, graviola, curassol).

A. muricata has normally been propagated from seeds, since vegetative propagation through conventional methods is very slow and costly because the collections of these plants in the field were exposed generally to plagues, diseases, climate problems and space. The culture *in vitro* was used to establish banks of germplasm and to conserve thus the necessary diversity for plant breeding programs (Sandoval and Muller, 1989). Thus, *in vitro* method could be important for propagation of this species (Bejoy and Molly, 1992).

Additionally, the traditional method of vegetative propagation was inefficient and inadequate, due to the low morphogenetic potential of this species and the low rooting rate of stem cuttings. The clonal propagation in *Annona* sp is carried out by grafting and budding which are time consuming, while seedling rootstocks are highly variable resulting in the decreased productivity (George and Nissan, 1987). The seed propagation result immense variability affecting yield, size and quality of fruits. Due to lack of germplasm of improved varieties its cultivation is limited. The *in vitro* tissue culture methods of micropropagation can be applied successfully to cherimoya and other *Annona* sp to overcome these problem (Encina *et. al*., 2014). Micropropagation has been used in other species of *A. cherimoya* and *A. muricata* (Lemos and Blake, 1996). After the first success with the micropropagation of cherimoya and after the studies Bejoy and Hariharan (1992), Lemos and Blake (1996) recorded similar results micropropagating another two important species which are *A. muricata* and *A. squamosa*. However the major obstacles in tissue culture is getting enough axenic explants since most of the stock plants are collected from the field that expose to the various contaminats.

According to the Hannia (2000), the *A. muricata* is the regarded as great delicacy in areas where *A. cherimola* cannot grow. In contrast is regarded with the *Annona* spp., the *A. muricata* flowers and fruits more or less continuously during the whole year. The market of soursop is potential at the tropics and undetermined in the word season areas. Thus, *in vitro* propagation has been used for seedlings production in many fruiting species, principally due to its advantages in terms of the possibility of obtaining large numbers of healthy seedlings in short periods of time.

1.1 Problem Statement

Nowdays, *Annona muricata* L. becomes high value to the economy especially in medicinal purpose. Normally *A.muricata* propagated through seedlings, usually takes three weeks, but under suboptimal condition can be delayed for up 2 to 3 month due to the seeds dormancy. The problem of getting high germination from seeds which is due to seed dormancy is common phenomenon face by soursop grows including local growers in Malaysia. Germination of *A. muricata* usually takes three weeks, but under suboptimal conditions can be delayed for up 2 to 3 month. *A. muricata* also propagated by grafting. The most pronounced symptom of graft failure is smooth, clean braking off a tree at the graft union (University Of Maryland, 2016). Hence tissue culture is one of the promising technique for *A.muricata* propagation. However, there has been insufficient information about tissue culture of *A.muricata* were published.

1.2 Objectives

a) To establish an effective surface sterilization to obtain axenic explants for *Annona muricata* L. from leaves.

b) To determine the effects of 2,4-D on callus induction of *Annona muricata* L. leaves.

2.0 LITERATURE REVIEWS

2.1 *Annona muricata* L.

Annona muricata L. is the most tropical semideciduous tree with largest fruits of the *Annona* genus, widespread at the tropic areas of Asia, Central and South America including the Amazon basin (Hannia, 2000). *A. muricata* also an evergreen tree, native of tropical America, that is widely cultivated in the tropics for its fruit (Samson, 1986). This species widespread through tropical distribution also as a native species in southern Thailand which is known as Durian-thet (On-Usa *et al*., 2014). *A. muricata* is native to the Antiles. *A. muricata* is a lowland tropical fruit-bearing in the Annonaceae family and compromising approximately 130 genera and 2300 species (Sejal & Jayvadan, 2016). It grows well below 1,150m in the most tropical micro-ecosystem of Central and South America. In Colombia there are reported more than 1,134 ha of soursop with 15 tons/ha yield (Hannia, 2000). There are established *A. muricata* plantations in Argentina, Bahamas, Brazil, Bermudas, British Guiana, Colombia, Cuba, Curaçao, Dominican Republic, Florida (USA), French Guiana, Haiti, Hawaii, India, Jamaica, Malaysia, Mexico, Panama, Pacific Islands, Peru, San Salvador, Santo Domingo, South East China, Surinam, Philippines, Trinidad and Tabago, Venezuela and Vietnam (Morton, 1987). The most important species at commercial level (nourishment, pharmacy industry) belong to the genera Annona, being the most interesting and used species *A. squamosa, A. muricata, A. cherimola* and hybrid *A. squamosa x A. cherimola* (George and Nissen, 1993). The soursop is adapted to areas of high humidity and relatively warm winters. Temperatures below 5°C (41°F) will cause damage to leaves and small branches, and temperatures below 3°C (37°F) can be fatal (Sejal & Jayvadan, 2016).

2.2 Taxanomic Classification and Botanical Description

The taxanomic and nomenclature of *Annona muricata* L. according to United Stated Department of Agriculture (USDA) is as below:

Kingdom : Plantae

Class : Magnoliopsida

Order : Magnoliales

Family : Annonaceae

Genus : Annona L.

Species : *Annona muricata* Linn

As shown in Figure 1, *Annona muricata* L. is a tropical evergreen semideciduous fruit tree (4-15 m), branched near the base, with all parts evil-smelling when bruised. The features of the plant are open and large roundish canopy. The branchlets are terete, finely wrinkled, scabrous, reddish brown and glabrous, with many round lenticels (Hannia, 2000). The leaves are entires with an acute or cuneate base, biserate, short petioled, dark green and shiny, the upper surface is lustrous and leathery or coriaceous with an obovate, or elliptic-oblong form, shortly acuminate apex and narrow transparent margin (Figure 2). The flowers are cauliflowers, regular and pedicel, strong-smelling and borne on the short hairs, the bract is small (Hannia, 2000). The tree sepals are almost free, dark green, ovate triangular, coriaceous, densely clothed with small hairs. The six petals are placed in two rows, the three outer ones are the largest, thickly coriaceous, cover with short tomentum, they are first green and later on pale yellow, their size is 3-5 cm long and 2-4 cm wide (Hannia, 2000). The numerous stamens are borne in many rows on a raised torus and crowned in whorl around ovaries. Morton (1987) also stated the fruit is largest of *Annonas spp*., weighing up to 7kg. It is syncarpous ovoid or ellipsoid, usually irregular, oblique or curved, heart shaped (Figure

3). It measures between 15-35 cm length and 10-15 cm width (Morton, 1987). The skin is dark green on the immature fruit, becoming slightly yellowish green, glabrous, but bears numerous and fleshy spine like prickles (Morton, 1987). The pulp is creamy white, fleshy, juicy and sub acid with soft, cottony stands that contain many seeds. The seeds are numerous about 100 approximate with dark brown colour (Figure 4). The reticulated leathery looking skin has short spines. Its inner surface is cream-colored and granular and separates easily from the mass of white, fibrous juicy segments which surround the central pithy core (Sejal & Jayvadan, 2016).

Figure 1: tree of *Annona muricata* L.

Figure 2: the leaves of *Annona muricata* L.

Figure 3: the fruits of *Annona muricata* L.

Figure 4: the seeds of *Annona muricata* L.

Source Figure 1: http://www.rarexoticseeds.com/en/annona-muricata-seeds-soursop-seeds-soursap-seeds-graviola-seeds.html
Source Figure 2: http://www.growables.org/information/TropicalFruit/annonamuricatanew.htm
Source Figure 3: http://www.growables.org/information/TropicalFruit/annonamuricatanew.htm
Source Figure 4: http://eastjava.ecrater.com/p/15533094/50-seeds-soursop-guanabana-graviola-annona

2.3 Economic Importance

According to the Orwa *et al*., (2009), *Annona muricata* L. have many economic importance which is divided into products of food, timber, poison and medicine. Thus with this advantages of food product, *A. muricata* can be consumed fresh for dessert when fully ripe or mixed with ice cream or milk to make a delicious drink, as is done in Java and in Cuba and other parts of America. The fruit is of economic value and hence cultivated and used widely as an edible food (Sejal and Jayvadan, 2016). *A. muricata* fruit consist of about 67.5% edible pulp, 20% peel, 8.5% seeds and 4% core by weight. The fruits is a good source of vitamins B and C and a poor to fair source of calcium and phosphorus. The fresh fruit is consumed in the form of ice creams, conserves and drinks. Also with the cooked pulp a candy is prepared to fill up parts of pastry shop to make jellies (Avilan, *et al*., 1992). The product of timber is whitish and heartwood brown. Next, powder of dried leaves and sap from fresh ones are useful in destroying vermin. All trees parts have insecticidal properties and can be used with fruit to kill fish in either hand acts as poison.

The important values of this tree also can acts as medicine. The crushed leaves are applied to mature boils and abscesses or are used as remedy for distention and dyspepsia, scabies, and skin diseases, rheumatism, cough and colds. The green bark is rubbed on wounds to stop bleeding (Orwa *et al*., 2009). According to Kedari and Ayesha (2014), the bark, leaves, fruit, roots and fruit seeds of the soursop tree known since long various medicinal uses. The fruit and juice is used against worms and parasites, to cool down fevers, to increase lactation after childbirth (Kedari and Ayesha, 2014). The seeds can be crushed and then used against internal or external parasites, head lice and worms. The tea prepared from the leaves are used as sedative and soporific (inducer of sleep) in the West Indies and Peruvian Andes (Kedari and Ayesha, 2014). This infusion is also used to relief pain for antispasmodic purposes for liver problems a leaf tea is used in the Brazilian Amazon.

Traditionally it issued in medicinal herbal drugs to cure various disease such as for diarrhea (fruit), cough, hypertension, rheumatism, tumors, cancer, asthma, childbirth, lactagogue (fruit), malaria, tranquilizer, skin rashes, parasites (seeds), worms (seeds), liver problems, arthritis (Kedari and Ayesha, 2014). It contains a variety component which attribute to the various biological activities. The roots and bark can be aid for diabetes, but can also be used as a sedative (Kedari and Ayesha, 2014). In Malaysia, the leaves of the soursop are used for high blood pressure and diarrhea. It also used as an astringent and styptic (Kedari and Ayesha, 2014). *A. muricata* has wide potent anti-cancerous agents coined as Acetogenins which play a key role towards many varieties of cancer, Acetogenins are potent inhibitors of NADH oxidase (nicotinamide adenine dinucleotide phosphate-oxidase) of the plasma membranes of cancer cells (Sejal & Jayvadan, 2016).

2.4 Micropropagation

2.4.1 *In vitro* propagation

According to Murashige and Skoog (1974) the *in vitro* plant cell and tissue culture is defined as the capability to regenerate and propagate plants from single cells, tissues and organ under sterile conditions and controlled environment conditions. Plant tissue culture is the one of the most powerful tools for induction of fast crop improvements in modern plant breeding age (Kumlay and Ercisli, 2015). Plant cell culture is promising potential alternative sources to produce secondary metabolites at commercial scale (Rao and Ravishankar, 2002). The basis of plant tissue culture was originally proposed by Gottlieb Harbelandt in the experiment on culture of single cells. He was the Father of plant tissue culture (Trevor, 2007). Micropropagation is a vegetative multiplication system based on the promotion of growth of microcuttings from axillary buds of apical dominant plants, growth of shoots from nodal sections, dissection of axillary shoots on rooting medium and production of small rooted shoots (Drew, 1997). From previous study, C. Haberlant was discover first attempt to culture isolated plant cells in vitro on artificial medium during 1902 (My Agriculture Information Bank, 2015).

As shown in the Table 1 the promotion of *in vitro* plants is divided in stages (Murashige, 1974).

Table 1: Micropropagation steps

Stages	**Description**	**Activity**
0	Stock plant	Identification of the stock or mother plant and source preparation of explants
I	Establishment	Explant selection, elimination of exogenous contaminants and new *in vitro* adaptations.
II	Multiplication	Promotion of in vitro cell or tissue organogenesis and rapid differentiation-multiplication of new shoots.
III	Rooting	The rhizogenesis of the new *in vitro* derived shoots is stimulated under *ex vitro* or *in vitro* conditions.
IV	Acclimatization	Hardening of vegetative structures.
V	Field	Open growth of regenerants.

This type of *in vitro* vegetative propagation has important benefits to produce stable lines in new plants varieties such as *Annona spp.* (Bridg, 1993). The micropropagation of elite or selected plants showed good results which benefit the agriculture. Horticulture and forestry (Conger, 1981). Hypocotyl and nodal cuttings of *Annona muricata* L. have proved to be suitable for *in vitro* culture (Rasai *et al.*, 1994). Shoots from nodal segments and hypocotyl of some *Annona spp.* have been successfully rooted (Rasai *et al*., 1995).