CALLUS AND CELL CULTURE OF *Gynura procumbens* (Lour.) Merr. (compositae) USING STATIONARY CULTURE SYSTEM

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by

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
g/L	Gram per litre
MS	Murashige and Skoog
rpm	Revolutions per minute
WHO	World Health Organisation

LIST OF PUBLICATIONS

1.0 Conference

1.1 Khaw Mei Lin, Chan Lai Keng and Derek Chan Juinn Chieh. (2013). Application of membrane (fabric) as potential supporting agent for the cell culture of *Gynura procumbens*. Oral presentation presented at "2nd Seminar on Sustainable Agriculture and Natural Resources". Universiti Sains Malaysia. 9th April 2013. (APPENDIX 1)

KULTUR KALUS DAN SEL
***Gynura procumbens* (Lour.) Merr. (compositae)**
MENGGUNAKAN SISTEM KULTUR STATIK

ABSTRAK

Potensi kegunaan kain sebagai unit imobilisasi untuk kultur kalus dan sel *Gynura procumbens* telah dinilai. Empat jenis kain yang berlainan (linen, kain kasa dan kain kapas 1 dan kain kapas 2) dipilih untuk kajian ini dan saiz liang kain yang diuji juga diteliti. Linen didapati sebagai agen sokongan yang terbaik untuk kultur kalus *G. procumbens* apabila dikultur atas kepingan kain persegi yang dipotong menjadi 5 cm x 5 cm. Hasil biojisim segar adalah 3.022 ± 0.108 g. Kalus yang dihasilkan daripada pengkulturan atas kain adalah berwarna kekuningan dan berciri rapuh dan berbutiran. Saiz liang linen pada umumnya meliputi lingkungan yang lebih luas dari 0.012 mm^2 - 0.071 mm^2 berbanding dengan tiga jenis kain yang lain apabila diperhatikan di bawah mikroskop stereo. Apabila kalus *G. procumbens* dikulturkan dengan menggunakan kain linen dengan rekabentuk berbeza (beg atau kepingan) dan isipadu medium yang berbeza (10 mL, 15 mL, 20 mL, 25 mL dan 30 mL) dalam mod statik, hasil biojisim kalus tertinggi (2.733 ± 0.153 g) diperolehi daripada kalus yang dikulturkan atas kepingan linen dengan 10 mL medium cecair. Kalus adalah berwarna kekuningan dan teksturnya adalah rapuh dengan butiran. Secara umumnya, kuantiti medium kultur cecair yang lebih kecil dapat menyokong penghasilan biojisim kalus segar *G. procumbens* yang lebih tinggi dengan pertumbuhan kalus yang sihat. Kalus yang dikulturkan di bawah pancaran cahaya berterusan dengan keamatan cahaya 2000 – 2500 lux adalah lebih rapuh dan kurang padat. Penghasilan biomass tertinggi (1.381 ± 0.458 g) kalus berciri rapuh dan sihat diperolehi daripada kalus yang dikulturkan dalam 10 mL kultur cecair dengan mod statik dalam beg kain

kasa (5 cm x 5 cm). Saiz bekas didapati memberikan kesan yang bererti terhadap pertumbuhan sel *G. procumbens*. Sel-sel yang dikulturkan di dalam bekas 500 mL selama 12 hari menghasilkan sel *G. procumbens* yang sihat dan biojisim sel segar (5.161 ± 0.747 g/L) yang tertinngi. Maka, kajian ini menunjukkan bahawa kalus *G. procumbens* boleh dikekalkan atas kepingan kain linen dalam 10 mL medium MS dengan mod statik. Ia juga menunjukkan bahawa 500 mL adalah saiz bekas yang paling sesuai untuk pertumbuhan optimum sel *G. procumbens*.

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USING STATIONARY CULTURE SYSTEM

ABSTRACT

The potential use of fabric as immobilization units for the callus and cell culture of *Gynura procumbens* was evaluated. Four different types of fabrics (linen, muslin and cotton 1 and cotton 2) were chosen for the study and their pore size was also examined. Linen was found to be the best support agent for the callus culture of *G. procumbens* when cultured on square pieces of fabric cut into 5 cm x 5 cm. The fresh biomass yield was 3.022 ± 0.108 g. The callus produced from culturing on fabric was yellowish, friable and granular in nature. The pore size of linen generally covers a wider range from 0.012 mm^2 – 0.071 mm^2 compared to other three types of fabrics when examined under stereomicroscope. When the callus of *G. procumbens* was cultured using linen of different design (bag or piece) and different medium volume (10 mL, 15 mL, 20 mL, 25 mL and 30 mL) under stationary mode, the highest callus biomass yield (2.733 ± 0.153 g) was obtained from callus cultured using linen pieces in 10 mL liquid medium. The callus was yellowish in color and their texture was friable with granule. A lesser amount of liquid culture medium generally supported higher callus fresh biomass gain of *G. procumbens* coupled with a healthy callus growth. The callus cultured under continuous light with light intensity of 2000 – 2500 lux was more friable and less compact. The highest biomass yield (1.381 ± 0.458 g) and healthy friable callus were obtained from callus cultured in 10 mL culture medium under stationary mode using muslin bag (5 cm x 5 cm). The vessel size was found to have significant effect on the cell growth of *G.*

procumbens. The cells cultured in 500 mL vessel for 12 days produced the highest and healthy fresh cell biomass (5.161 ± 0.747 g/L) of *G. procumbens.* Hence, this study indicated that the callus of *G. procumbens* could be maintained on pieces of linen in 10mL of MS medium under stationary mode. It also showed that 500 mL vessel is an ideal size for the optimal cell growth of *G. procumbens.*

CHAPTER 1. INTRODUCTION

Plants have been used for medicinal purposes is not just a custom of the distant past. Many of these plants have been identified and collected from the wild. Knowledge about the uses of plants was compiled by trial and error and passed orally down from one generation to another. Each part of medicinal plants has its own uses for the treatment of certain disease or incorporates in our meal for maintaining good health.

According to the World Health Organization (WHO, 2009), the usage of traditional medicine expands widely in developed countries as complementary or alternative medicine. There are about 10,000 plant species used medicinally and most of these are used in traditional systems of medicine (Kuipers, 1997). A recent conceptual framework by Smith-Hall *et al.* (2012) indicated that the number of people that relied on medicinal plant products to maintain their health or treat illnesses was unlikely to decrease in the foreseeable future. Modern pharmacopoeia contains at least 25% of drugs derived from plants and many others were synthetic analogues built on prototype compounds isolated from plants (De Silva, 1997). An attempt had been made by Farnsworth *et al.* (1985) to correlate 119 substances extracted from well-known traditional plant species with pharmacological actions. Many medicinal plants with medicinal values were investigated for useful active compounds for example artemisinin in *Artemisia annua* L., was used for the treatment of fever, malaria, skin diseases, jaundice and hemorrhoids (Das, 2012; Dalrymple, 2013). Taxol (plaxitaxol), found in the bark of the *Taxus* tree, was one of the most promising anticancer agents (Vinisree *et al*., 2004).

Gynura procumbens, commonly known as sambung nyawa by Malaysian locals, is a fast growing medicinal herb found in various part of Southeast Asia. Its leaves are eaten raw. In folk medicine, the plant is widely used for the treatment of kidney problems, skin rashes, fever and hypertension. The leaf extracts of *G. procumbens* was found to exhibit anti-hyperglycaemic, anti-hyperlipidaemic, anti-inflammatory activities and reduction of high blood pressure capabilities (Zhang and Tan, 2000; Akowuah *et al*., 2001; Akowuah *et al*., 2002; Iskander *et al*., 2002; Kim *et al*., 2006; Hoe *et al*., 2011).

World markets are now turning to plants as the sources of ingredients in healthcare products. The increasing demand for medicinal plants will definitely reduce the sustainable supply of medicinal plants in the future. Plants are endangered by a combination of factors such as over-collecting, unsustainable agriculture practices, urbanization, pollution and climate change and lack of proper regulation on management and conservation. The bulk of the material traded is still harvested from the wild and only a very small number of species is cultivated (Kuiper, 1997). Besides, due to the lack of knowledge and interest among the younger generations, some of the traditional medical information was buried together with the previous generations. The expanding trade in medicinal plants has serious implications on the survival of several plant species, with many under serious threat of becoming extinct.

Therefore, plant cell and tissue culture techniques can be as the alternative approach to maintain sustainability supply of plant materials for producing bioactive compounds under artificially controlled conditions (Thorpe, 2007). The widespread use of plant *in vitro* culture techniques has many advantages when classical methods of vegetative propagation prove inadequate. One of the advantages of *in vitro* culture techniques is that it enables production of a more predictable, consistent, and

healthier crop. *In vitro* culture also allows for mass production of plantlets and making plants more commercially viable. They can also process in large numbers throughout the year irrespective of season and weather.

However, micropropagation technology is expensive as compared to conventional methods of propagation by means of seed, cutting and grafting (Hussain *et al*., 2012). Laboratory shake flask system at small scale also needs at least a shaker for success establishment of a cell culture system. The application of bioreactor from shake flask for scaling-up might not be affordable for many research groups (Paek *et al*., 2001). In addition, liquid medium was found to be cheaper than solid medium for the cost of agar has increased in recent years (Nene and Sheila, 1994; Mbiyu *et. al*., 2012). Therefore it is desired to adopt measures to reduce the production cost of micropropagation and production of useful active compounds. This can be achieved by improving the process efficiency and better utilization of resources.

Hence to ensure sustainable production of plant material for *G. procumbens*, this study was carried out with the following objectives:

1. To test and evaluate an alternative method of using fabric (linen, muslin and cotton) as a potential support agent in the stationary culture system for callus and cell line culture and maintenance of *G. procumbens*.
2. To determine the optimum vessel size for sustainable production of *G. procumbens* cells using stationary culture system.

CHAPTER 2. LITERATURE REVIEW

2.1 Medicinal plants in Malaysia

According to Rao (2010), there are approximately 1000 - 2000 species of medicinal plants found in Malaysia. Documentation of traditional knowledge on medicinal plants has been carried out in various part of Malaysia. A study done in Kampung Bawong, Perak, West Malaysia showed that majority of local Orang Asli there still depend on local plants for treating various diseases and ailments. A total of 62 species of plants used by Orang Asli are described in this study based on field survey (Samuel *et al*., 2010). According to Ong *et al*. (2011), leaves are the commonly used part in preparing herbal medicine in Kampung Mak Kemas, Terengganu, Malaysia. The medicinal plants harvested in the wild were pounded, decocted or infused to treat various ailments, such as dermatological complaints, reproductive, abdominal problems and fever. Many studies of similar kind were conducted as reported by Alsarhan *et al*. (2012) in Kangkar Pulai, Azliza *et al*. (2012) in Ulu Kuang, Selangor, Johor, Ong *et al*. (2012a) in Kampung Pos Penderas, Pahang and Ong *et al*. (2012b) in Tapah, Perak.

2.2 *Gynura procumbens*

2.2.1 Habitat and distribution of *Gynura procumbens*

Gynura procumbens (Lour.) Merr. is a common medicinal plant found in various parts of Southeast Asia (Perry and Metzger, 1980). *Gynura* was first described in 1838 (Chen and Henny, 2013). It can be found abundantly in Peninsula Malaysia since reported by Burkill (1966) and widely distributed in Indonesia,

Malaysia and Thailand (Zahra *et. al*., 2011). It comprises about 44 species native to the humid tropics of Africa to Southeast Asia (Vanijajiva, 2009).

Gynura procumbens belongs to the Compositae (Asteraceae) family (Burkill, 1966). It was also called *G. sarmentosa* DC. and *Calacia procumbens* Lour. (Perry and Metzger, 1980; Wiart, 2002). Locally it is known as sambung nyawa, kelemai merah, kacham akar, akar sebiak or Daun Dewa in Malaysia (Burkill, 1996; Wiart, 2002; Asean Herbal and Medicinal Plant, 2010). It is also known as 百病草 in Mandarin, 'Pyar Mee Swae' in Myanmar (Thet *et al*., 2008) and Pra kum dee kwai in Thai (Gale *et al.*, 2007).

Gynura procumbens is an evergreen herbaceous plant (Figure 2.1). It occurs in a wide range of habitats but it is best grown under partly-shaded area and moist soil. The simple leaves are succulent and stipulate with a short petioles (0.2 - 0.8 cm). The leaf size ranges from 2.5 - 7.5 cm long and 0.8 - 2.5 cm wide. The leaves are arranged in an alternating fashion along the stem. The shape of the leaves is elliptical to lanceolate, cuneate base with shallowly dentate margins (Wiart, 2002). The matured stems are green tinged with purple and somewhat woody when old. It produces fibrous to tuberous roots. The erect, scrambling to climbing stem can trail on the ground or twine around other plants for support (Vanijajiva, 2009). Overall plant height can be several feet if allowed to grow unattended.

Gynura procumbens produces flowers in long hanging inflorescence. The flowers head are panicled, orange to yellow in colour, bisexual with narrow bracts and purple involucres. The style is slender and hairy. The achenes are narrow and puberulous and the pappus (modified calyx) are white, fine and silky (Wiart, 2002; Vanijajiva, 2009; Asean Herbal and Medicinal Plants, 2010; Rahman and Asad, 2013).

Figure 2.1: One year old potted mature plant of *Gynura procumbens*.

2.2.2 Traditional uses of *Gynura* species

Gynura procumbens is used for kidney problems in Indonesia and as febrifuge in Indo-China (Perry and Metzger, 1980; Wiart, 2002). The dried and mashed leaves are mixed with oil and applied as a poultice for rashes (Perry and Metzger, 1980). In Thai folk medicine, semi-succulent leaves of *G. procumbens* are applied externally to treat topical inflammation, rheumatism, viral infection of the skin and general body-pain. In Indonesia, it has been used to treat fevers, skin rashes and as tonic to treat ringworm infection. In the Philippines, particularly in Mindanao, the plant is grown in the rice fields to control insect pests. In Malaysia *G. procumbens* is used as folk medicine to treat diabetes. *G. procumbens* is generally regarded to be a relatively harmless herb to consume. In fact, the young shoots of this plant are eaten raw as 'ulam'.

Other species of *Gynura* have also been reported for their medicinal properties. The other most known species is *Gynura aurantiaca*, so called because of its orange inflorescences. This plant is also commonly known as "Purple Passion" because of the velvety purple leaves. *G.aurantiaca* is sometimes cultivated as an ornamental plant and grown as house plant in the Malay Peninsula (Burkill, 1966) or as interiorscape plants due to their colorful velvety foliage (Chen and Henny, 2013). Crushed leaves of *G. aurantiaca* made into paste to apply to ringworm spots (Perry and Metzger, 1980).

Gynura bicolor (Roxb & Willd.) DC. is widely distributed in Asia and is very popular for vegetarian cuisine in Taiwan. *G. bicolor* vegetable juice contains complex mixture of antioxidants and is therefore responsible for many health benefits. *G. bicolor* can be a commercially viable product in the food industry (Lu *et al*., 2012).

The leaves of *G. cusimbua* can be used as tincture to treat stomach ulcer (Meetei and Singh, 2007). The juice of the plant stem and leaves were used as antiseptic for wound healing (Sanglakpam *et al*., 2012).

Gynura divaricata helps to combat craving in opium addiction (Perry and Metzger, 1980) and lowering blood glucose level (Keeratikajorn *et al*., 2012). A patent application has also filed for *G. divaricata* in preparing liver protecting drug or health care food by Li (2013).

In Taiwan, the juice of the leaves and roots of *G. formosana* is taken as an antihemorrhagic and applied externally to wounds and snake bite (Perry and Metzger, 1980).

The juice of *G. japonica* is applied to poisonous insect bites like scorpions and centipedes. The leaf and the root also applied to bites of poisonous snakes. The dried tuberous root can be grated and cooked with chicken to serve as tonic (Perry and Metzger, 1980).

Gynura pseudo-china leaves are used as poultice for pimples. It is also pounded in brine and taken for irregular menstruation. They are used in Indo-China as poultice for breast tumours, skin in erysipelas and irregular menstruation (Burkill, 1966). The sap of the leave is used as a gargle to treat throat inflammation. The root is used as a remedy for uterine hemorrhages, dysentery and inflamed wounds (Perry and Metzger, 1980). A recent case study by Nakbanpote *et al*. (2010) reported that *G. pseudo-china* has properties of a zinc hyperaccumulator that can remove contaminating chemicals in polluted soil.

Gynura pinnatifida roots are astringent and are employed in China for wounds healing and stopped hemorrhages (Lewis and Elvin-Lewis, 1977). In

traditional Chinese medicine, the roots of *Gynura* species have been consumed for promoting microcirculation and relieving pain (Dai *et al*., 2007).

2.2.3 Pharmacological properties of *Gynura procumbens*

Due to the wide application of this plant in traditional medicine, a number of studies have been conducted to investigate its pharmacological activities. A study conducted in Singapore used diabetic rat to prove that an alcoholic extract of *G. procumbens* was able to reduce elevated blood cholesterol and triglyceride levels as anti-hyperlipidemia agent (Zhang and Tan, 2000). They discovered that the leaf extracts of *G. procumbens* significantly suppressed the level of elevated serum glucose and reduced serum cholesterol and triglyceride levels in diabetic rats.

Kim *et al.* (2006) reported that *G. procumbens* possesses anti-hypertensive properties in hypertensive rat model. It was also found to decrease blood pressure via inhibition of the angiotensin converting enzyme (Hoe *et al*., 2007).

The phytochemical work on *G. procumbens* showed that the ethanol extract of its aerial plant parts has antireplicative action against herpes simplex virus. Several antiherpetic compounds were isolated from the extract, namely, caffeoylquinic acid derivatives, phytosteryl glucosides, and glycoglycerolipids. The laboratory evidence and the reduction of the infection incidence in patients supported the antiherpetic effect of *G.* procumbens (Jarikasem *et al*., 2013).

Gynura procumbens leaf extract exhibited chemopreventive properties for tumour inhibition on breast cancer (Nurulita *et al*., 2011). Agustina *et al*. (2006) also demonstrated that ethanol extract of *G. procumbens* leaves could inhibit the progression of 4NQO induced rat tongue carcinogenesis in the initiation phase. The

protein fraction, SN-F11/12, of *G. procumbens* was found to inhibit the growth of a breast cancer cell line, MDA-MB-231 (Hew *et al*., 2013).

Recently, pharmacological studies have indicated that phenolic extract of *G. procumbens* is a good source of natural antioxidant with radical-scavenging action (Puangpronpitag *et al.*, 2010). The total phenolic contents and free radical scavenging activity of *G. procumbens* leaf extract was found to be affected by temperature. The expression of antioxidant activity is lesser at higher temperature due to the degradation of bioactive polyphenolic antioxidants in the extracts (Akowuah *et al*., 2009).

Gynura procumbens ethanolic leaf extract promoted ulcer protection indicated by significant reduction of ulcer area in the gastric wall, reduction or inhibition of edema and leucocytes infiltration of submucosal layer in induced gastric lesions of rats (Mahmood *et al.*, 2010). The application of *G.* procumbens extract also significantly accelerated the rate of wound healing in experimental rats, less scar on the wound enclosure and the granulation tissue contain markedly less inflammatory cells and more fibroblast, collagen fiber and blood capillaries compared to gum acacia-treated rats (Zahra *et al*., 2011).

The anti-diabetic properties of *G. procumbens* were studied in many scientific researches. Zhang and Tan (2000) reported that the hyperglycemic activity of *G. procumbens* leaves extract might have biguanide-like activity on experimental rats. According to Lee *et al*. (2012), ethanolic extract of *G. procumbens* leaves showed promising antidiabetic effect and was comparable to metformin, suggesting its high potential to be developed as a plant-derived antidiabetic agent. *G. procumbens* also reported to contain substances that are able to stimulate insulin secretion, thus contributing to the anti-diabetic property of the plants (Thet *et al.*, 2008). Besides,

the hypoglycemic action of *G. procumbens* could be due to direct or indirect effects on the activities of one or more of the upstream components of the insulin biosignaling pathway (Chong *et al*., 2012).

The leaves of this plant are often consumed in diet and study had shown that the leaves did not have any toxic effects (Rosidah *et al.*, 2009). Study on antibacterial activity indicated that *G. procumbens* extract did not show antibacterial activity on *Staphylococcus aureus, Salmonella typhi, Bacillus cereus, Pseudomonas aeruginosa* and *Escherichia coli.* A phytochemical screening showed that *G. procumbens* extract is a good natural source of bioactive compounds and that they may have beneficial health effects for consumption (Kaewseejan *et al*., 2012).

In a proteome analysis study by Hew and Gam (2011), 92 unique proteins were identified from the leaves of *G. procumbens*. Amongst the proteins was miraculin, a flavor enhancer with high commercial value and peroxidase with a potential useful natural resource in cosmetic and skin care industry.

The benefits of the traditional use of *G. procumbens* have also been supported by the isolation and identification of several possible active chemical constituents from this plant, including flavonoids, saponins, tannins, and terpenoids (Akowuah *et al*., 2002). Besides, Tan *et al*. (2013) also reported a few chemical constituents of the genus *Gynura* which include flavonoid, phenolic acid, cerebrosides, polysaccharide, alkaloids, terpenoids and sterols. Phenolic acid and flavonoid are the major components of the *Gynura* genus.

2.3 Plant Tissue Culture

2.3.1 Culture medium and culture condition

Plant tissue culture broadly refers to the *in vitro* culture of all plant parts e.g., cells, tissues, organs, and other components on artificial media under aseptic condition. Plant *in vitro* culture was first initiated by a German scientist, Haberlandt, at the beginning of the 20th Century (Thorpe, 2007). It is a method of quickly multiplying large numbers of plants genetically identical to the mother plant.

Formulation of a suitable medium is very important in plant tissue culture. The basic nutrient requirements in a culture medium are very similar to those require by the whole plants. Generally the mineral nutrient in culture medium is made up of macronutrients and micronutrients. It also contains carbohydrate (usually sucrose), vitamins and plant growth regulators. Sometimes amino acids and other nitrogen supplements and undefined supplements such as fruit juices (Puchooa and Ramburn, 2004), yeast extracts (Hamza, 2013) or coconut water (Mohammad and Ali, 2010) are added. Both solid and liquid medium can be used for culturing.

Optimal growth and morphogenesis of tissues may vary from different plants according to their nutritional requirements. Effective identification of the mineral requirement can better regulate the *in vitro* growth of plant tissue (Niedz and Evens, 2007).

There are several basal medium formulations. Murashige and Skoog medium (MS) is most extensively used for *in vitro* propagation of many plant species. Basal medium plays an important role in optimizing and the production of metabolites. Five different basal media used in a study carried out by Kajani *et al*. (2012) indicated that different concentration and composition of the nutrients in the medium

not only affect the taxanes production, but also had different levels of effectiveness on the production of various taxane compounds from *Taxus baccata* L.

Most plant tissues grow well in culture medium with pH adjusted to 5.4 – 6.0 prior to autoclaving. The effective range of pH for a culture medium is constrained as such that it does not affect chemical reactions catalyzed by enzymes during culture growth. Within the acceptable pH limit, it will ensure the salt in the medium remain in the soluble form for the optimum uptake of nutrient and plant growth regulator. Medium pH also affects the gelling efficiency of agar. The agar gel generally becomes softer in more acidic medium. If the concentration of the agar is increased, the medium becomes hard and does not allow the diffusion of nutrient into the tissues. Hardness of the culture medium greatly influences the growth of cultured tissues. On the other hand, Skirvin *et al*. (1986) reported that there were significant differences between initial pH level and pH levels following autoclaving. The actual pH should be reported before and after autoclaving.

2.3.2 Factors affecting growth of *in vitro* culture

Auxin, cytokinins, gibberellins and abscisic acid are the four broad classes of plant growth regulators (PGRs) commonly used in plant culture. The amount required is determined by the type of intended culture. The auxins commonly used in plant tissue culture media are indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), dichlorophenoxyacetic acid (2,4-D), naphthalene acetic acid (NAA) and picloram. The only naturally occurring auxin found in plant tissue is IAA. Commonly used cytokinins include benzylamino purine (BAP), 6-benzylaminopurine (BA), N6-(2-isopentyl) adenine (2iP), furfurylamino purine (kinetin) and zeatin. BA and kinetin are synthetically derived cytokinin. The high

concentration of auxins generally favors root formation, whereas the high concentration of cytokinins promotes shoot regeneration. Manipulation and variation of auxins and cytokinins levels in the culture media can successfully change the growth behavior of plant cultures (Gopitha *et al*., 2010; Ngomuo *et al.*, 2013).

Physical factor such as light, temperature, aeration rate can also affect metabolite synthesis in cultured plant cells. Singh and Patel (2014) reported that the multiplication and growth of *Punica granatum* L. shoots were significantly influenced by the light intensity. He assessed this factor under continuous artificial light intensity in the range of 1000 - 4000 lux. Maximum number of shoots and shoot length was obtained under 3000 lux light intensity. Light was also found to be an important factor in inducing and maintaining pigmentation in the cells suspension culture of *M. Malabathricum* L. (Koay, 2008). An increment of 10-fold in pigment production was reported in culture incubated under light intensities of 301 – 600 lux compared to 0 - 300 lux.

Speed of agitation directly affects the growth and viability of cells in culture. A study by Srivastava *et al*. (2011) reported the maximum fresh weight and viability was observed for suspended cells of *Lantana camara* L. grown at agitation speed of 120 rpm. The cells died at the remaining two agitation speeds 60 rpm and 240 rpm due to aggregation and rupturing of cells respectively. Besides, agitation speed also plays an important role in influencing enzyme production and the morphology of plant cell *in vitro*. Slow agitation speed lower than 130 rpm resulted in low fungal growth and low tannase production of *Aspergillus niger* RETL FT3. This could be due to the low amount of dissolved oxygen and inadequate mixing of the broth in the culture medium. As the agitation speed was higher than 130 rpm, the tannase activity and the fungal biomass also reduced. This indicated that high agitation

speed could have created shear forces which lead to the destruction of the mycelium and consequently to cell damage (Darah *et al*., 2011).

2.3.3 Callus culture systems

Although there are a wide variety of different media formulations, all plant culture can be broadly divided into two very basic categories — liquid and solid culture system. Solid and liquid media may have exactly the same nutritional composition except that the solid culture system contains gelling agent while the liquid culture system normally require agitation without gelling agents.

In solid culture system, only part of the callus tissues is actually in contact with the medium; therefore inequalities in growth may arise due to gradients in nutrients, exchange of gases and light intensity. The growth of callus in solid culture may also be limited in certain directions by the medium or walls of the glassware. In addition, accumulation of toxic metabolites or the drying out of the medium is other possibilities that could hamper the *in vitro* growth of callus culture in solid culture system. Therefore, callus cultures have to be subcultured frequently to ensure that they are constantly maintained in an actively growing state. Besides, callus grown in a solid medium cannot be transferred to liquid medium without causing any disturbance to the tissue. It was reported that it is more economical to use liquid culture system compared to the solid culture system using solid media (Mbiyu *et al*., 2012). Despite these limitations, solid culture system is still the method of choice for routine maintenance of callus cultures.

In liquid culture system, callus and cell cultures often showed faster growth rates compared to solid culture system. The cultured tissues in the liquid culture system could be fully or partially immersed in the culture medium but their growth

may be retarded by oxygen deprivation. In cell suspension cultures, the growing cells may reach a very high cell density per unit volume culture, so complete exhaustion of essential nutrients normally occurs by the time the culture enters the stationary phase. The oxygen concentration of liquid media is often insufficient to meet the respiratory requirements of submerged cells and tissues (Torres, 1989). Agitation will help to break the callus into smaller clumps and single cells to obtain a uniform distribution of cell and cell clumps in the medium (Choi, *et al*., 2008). Movement of the culture medium also provides good gaseous exchange between the culture medium and the air. In can be achieved using a rotating platform-orbital shaker.

In callus cultures using solid culture system, gelling agent is often used to provide supporting matrices to keep the plant from being submerged into the medium. Agar was first used by White in 1939 as solidifying agent in plant tissue culture media (Thorpe, 2007). To date, agar is still widely employed as gelling agent for the preparation of semi-solid culture media (Puchooa *et al*., 1999). The stable property of agar at any temperatures makes it a convenient gelling agent in plant tissue culture medium. Agar melts in water at approximately 100^{o}C and solidifies at approximately 45^{o}C. Agar produces a high clarity gel when solidified and this allows better detection of culture contamination and exhibited high level of resistance to plant enzymes during culture (Henderson and Kinneresley, 1988).

Agar is a gelatinous complex polysaccharide obtained from marine algae such as *Gelidiella* and *Gracilaria* species (Nene and Sheila, 1994). Mc Hugh (2002) reported red seaweed species of *Gracilaria* and *Gelidium* are considered as the main sources of seaweeds for the world agar industry. Since agar is derived from a biological source, the property of agar varies with brands and batch, particularly in

their mineral composition, impurities and gelling strength. Indeed in a well-defined medium, agar is the major source of unknown variation.

The agar content and composition showed seasonal variations. According to Givernaud *et al*. (1999), the agar content of *Gracilaria multipartite* was maximal in winter and decreased during the growth periods to minima in June and October during maxima fertility. The gel strength also decreased after the alga reached its maxima of fertility. Seasonal changes in agar properties also reported by Orduña-Rojas *et al*. (2007), from two other potentially economic sources of agar, *Gracilariopsis longissima* and *Gracilaria vermiculophylla*.

The effect of gelling agents on medium pH on six commonly cited inorganic basal media was studied by Owen *et al*. (1991). When the pH of MS medium was adjusted to 5.75 before addition of gelling agent, a post autoclaved pH difference in the culture medium of up to 0.23 was reported. This might in turn result in alterations of plant growth and development *in vitro*. Selby *et al*. (1989) reported that adventitious bud formation on Sitka spruce needle explants was strongly dependent upon the rigidity of the culture medium. There was a significant interaction between agar strength and medium pH. The optimum bud production required acidic medium but adventitious shoot often showed severe vitrification due to the soft gel at lower pH. The survival rate of vitrification shoots or plantlets is low during transfer to green house as they easily lose too much water and very susceptible to infection (Arnold and Eriksson, 1984).

Different agar brands also reported to have different effect on the multiplication rate of marubakaido apple rootstock shoots grown in solid culture system. This might be likely related to different polydispersity and/or amount of high molecular weight fractions in the agar (Pereira-Netto *et al*., 2007). Agar quality

could affect, in principle, all developmental processes, whereby the regeneration of adventitious shoots and roots being the most sensitive (Ahmadian *et al*., 2013).

The relatively high material costs of agar has necessitated search for alternative materials with lower cost. Liquid medium was found to be cheaper than solid media by USD 1.65 for in vitro micropropagation of Kenyan potato cultivar using phytagel as gelling agent (Mbiyu, 2012). The response of seed germination, shoot formation and rooting of *Syzygium cuminii* and anther culture of *Datura innoxia* using culture media gelled with 'Isubgol', a mucilaginous husk derived from the seeds of *Plantago ovata,* was found to be similar to culture media solidified with agar. The price of 'Isubgol' is one-eighth that of the agar used in the study and one-hundredth that of Difco Bacto agar (Babbar and Jain, 1998). Guar gum, being product of plant origin, is biodegradable and poses no threat to the environment on being disposed after used. It was reported to be better in term of axillary shoot proliferation, rhizogenic and embryogenic responses as compared to agar (Babbar *et al*., 2005). Xanthan gum has been proven successfully as an alternative solidifying agent for *in vitro* seed germination, caulogenesis and rhizogenesis of *Albizzia lebbeck*, androgenesis in anther cultures of *D. innoxia* and somatic embryogenesis in callus cultures of *Calliandra tweedii*. It is 3 to 13.5 times cheaper than agar. Besides having cost advantage, it is also biocompatible and biodegradable, highly resistant to enzymatic degradation and extremely stable over a wide pH range. Moreover it is a renewable source that poses no fear of exploitation (Jain and Babbar, 2006).

A study to evaluate various blends of three alternative gelling agents, viz., guar gum, isubgol or xanthan gum as substitute of agar was carried out by Jain-Raina and Babbar (2011). A total of 55 blends were tested for *in vitro* morphogenic responses, viz., seed germination, caulogenesis and rhizogenesis of *Albizzia lebbeck*.

Xanthagar having xanthan gum and agar (6 : 4) had all desirable characteristic as gelling agent comparable to or better than agar.

Reduction of culture medium cost is a continuously pursued target in large-scale cultures and search for cheap alternatives gelling agent. A significant cost reduction of 42.95 % is possible by a combination of low concentration of agar and corn flour as solidifying agent. It has been shown to be efficient for mulberry micropropagation from single node (Lalitha *et al*., 2014).

Low cost substrate, betel-nut coir, coconut coir and polyurethane foam disk offer a newer possibility as alternatives to agar for non-symbiotic embryo culture of *Cymbidium iridioides*. About 24 % reduction in production cost as alternative to agar (Deb and Pongener, 2013) was reported. The study of using gum kondagogu also reported a cost reduction of 6.5 times as compared to agar for the micropropagation of rough lemon (*Citrus jambhiri* Lush) from nodal explants (Singh *et al.*, 2013).

Moraes-Cerdeira *et al*. (1995) reported that rolled cotton fiber can be used for callus growth of *Artemisia annua* L., *Agrostis stolonifera var. palustris* Farwell and *Taxasxme-dia* Rehd cv. Hichsii at a low cost. The callus that was cultured in 25ml medium compressed with 100% pure, sterile cotton fibers was found grew better than on agar. Others have also reported substitution for agar, including glass fiber-reinforced plastic for the culture of *Chlorococcum* sp. (Shen *et al*., 2014). The water algae will form a thin layer of 'algal film' on the glass fiber-reinforced plastic that acted as a supporting matric in a small volume of culture medium.

The various substrates used as substitutions of gelling agents in a solid culture system enable us to propose a modification of the callus culture of *G.*

procumbens using liquid medium combined with linens as a culture system for the enhancement of callus mass production.

CHAPTER 3. MATERIALS AND METHODS

3.1 Study materials

3.1.1 Callus culture of *G. procumbens*

Callus of *G. procumbens* maintained in the Plant Tissue and Cell Culture Laboratory, School of Biological Sciences, Universiti Sains Malaysia, Penang was used as the plant material for study. The callus was subcultured every 20 days on MS medium supplement with 0.5 mg/L Picloram (Pan, 2011). The cultures were kept in the culture room maintained at 25 ± 2^{o}C and under 24 hours photoperiod of white fluorescent light with intensity of 2000 – 2500 lux.

3.1.2 Preparation and sterilization of cloth materials for callus culture

Four different types of clothes were used, two kind of cotton (cotton 1, cotton 2), muslin cloth and linen (Figure 3.1). Muslin cloth was obtained from the School of Biological Sciences, Universiti Sains Malaysia, Penang while the cotton and linen were bought from Kamdar Departmental Store, Penang. All the fabrics were cut into square pieces of 5cm x 5cm or sewed into bags of the same size. The fabrics were then washed with commercial detergent (Attack, Kao Sdn. Bhd.) and rinsed thoroughly with tap water. They were then disinfected by placing them in boiling water for 15 minutes. They were rinsed again in running tap water to get rid of any residue chemical and finally sun dried in open area. The fabric were then ironed and wrapped with aluminium foil before autoclaved (TOMY SS-325 autoclave) for 30 minute at 121^{o}C under a pressure of 1.05kgcm^{-2}.

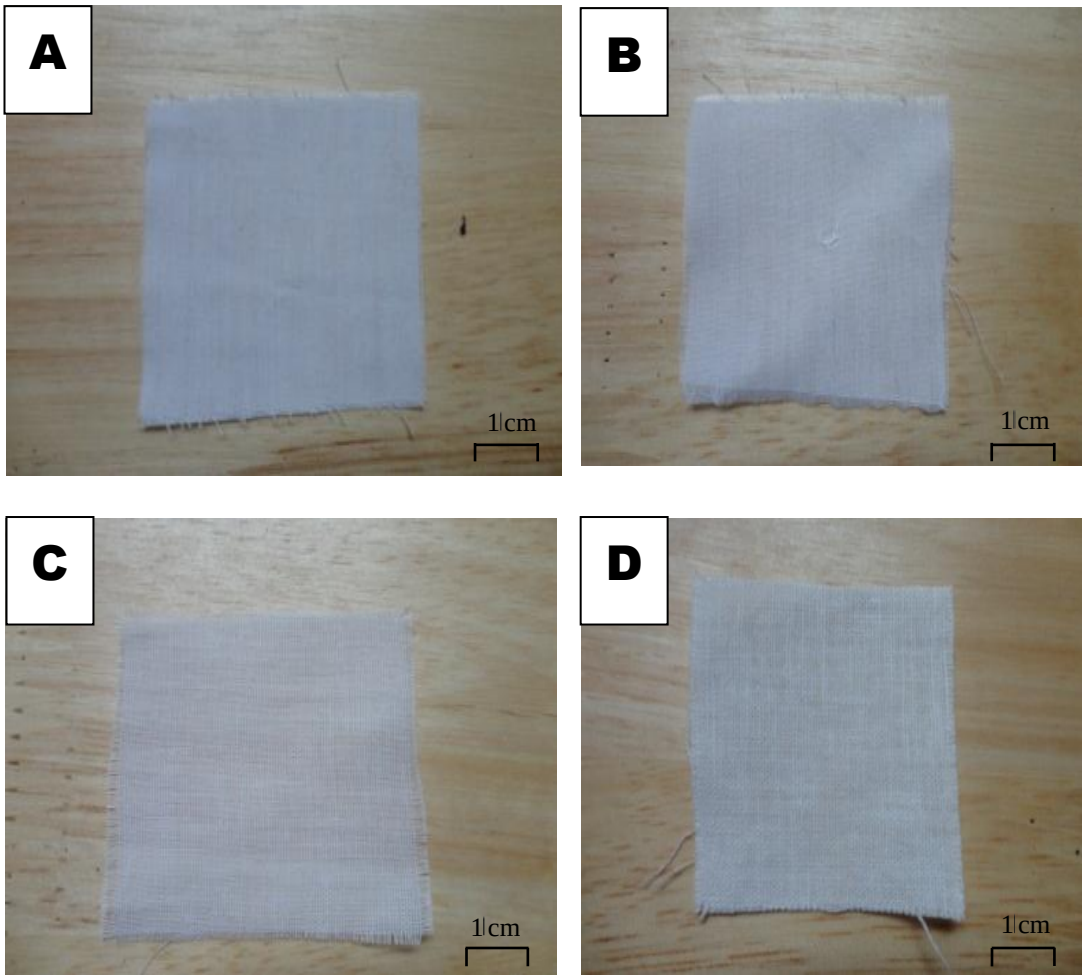

Figure 3.1: Four types of cloth materials used as supporting agent for callus culture of *G. procumbens.*
A: Cotton 1
B: Cotton 2
C: Muslin
D: Linen

3.2 Factors affecting the growth of *G. procumbens* callus culture

3.2.1 Effect of medium volume on callus proliferation of *G. procumbens* in muslin bag under agitation or stationary system

Friable yellowish callus of *G. procumbens* (1.0 g) was placed into the sterilized muslin bags (5cm x 5cm). The bags were then placed into MS medium of different volumes (10 mL, 20 mL, 30 mL, 40 mL, 60 mL, 80 mL and 100 mL) supplemented with 0.5 mg/L Picloram. The callus cultures were placed stationary on a shelf and orbital shaker (Labwit ZHWY-3112) with continuous agitation at 120 rpm respectively. Six replicates were used for each combination treatment. The experiment was carried out using 7 x 2 factorial design. The temperature in the culture room was maintained at 25 ± 2^{o}C with continuous lighting of 2000 - 2500 lux.

After 20 days of culture, the calli were filtered through filter paper (Whatman® No.1, 90 mm diameter) in a filter funnel connected to vacuum pump (Jeio Tech, Korea). The fresh callus biomass was determined by using digital weighing scale (Vibra, Japan). The data were analyzed using Two Way ANOVA and followed by comparison of means using Tukey Test (HSD) at $p \leq 0.05$.

3.2.2 Effect of fabric type on callus proliferation of *G. procumbens* under stationary system

To study the effect of fabric type (muslin, cotton 1, cotton 2 and linen) on callus proliferation of *G. procumbens*, friable callus of *G. procumbens* (1.0 g) was placed onto the square pieces of fabrics cut into 5 cm x 5 cm. The callus was then cultured in 10 mL of MS medium supplemented with 0.5 mg/L Picloram. Complete randomized design was used and 21 experimental units were used for each treatment. The callus cultures were placed on a culture shelf fitted with continuous light

condition supplied by cool white florescent tubes with light intensity of 2000 - 2500 lux. The temperature in the culture room was maintained at 25 ± 2^{o}C.

After 20 days of culture, the calli were harvested by separating the calli from the liquid medium using filter paper (Whatman® No.1, 90mm diameter) that placed in a filter funnel connected to vacuum pump (Jeio Tech, Korea.). The fresh callus biomass was determined by using digital weighing scale (Vibra, Japan). The data were analyzed using One Way ANOVA and followed by comparison of means using Tukey Test (HSD) at $p \leq 0.05$ with the aid of SPSS version 15.0.

3.2.3 Determination of fabric pore size

To study the effect of fabric pore size on callus proliferation of *G. procumbens*, the fabrics (linen, cotton 1, cotton 2 and muslin) were viewed under a stereomicroscope (Olympus SZ61) that equipped with a video camera and digital micrometer. The fabric pore size (area, mm^2) was computed based on width and length measurement from the micrometer calibration bar. Fifteen measurements were taken from each of the fabric samples randomly. The pore size (area, mm^2) calculated was then plotted on a line chart.

3.2.4 Effect of fabric design and medium volume on callus proliferation of *G. procumbens* under stationary system

To study the effect of different medium volume (10 mL, 15 mL, 20 mL, 25 mL and 30 mL) and fabric design (square pieces or bag) on callus proliferation of *G. procumbens*. The bags and square pieces of fabric were made from linen. Linen was chosen because previous experiments conducted indicated that it was the best callus proliferation supporting agent. Friable callus of *G. procumbens* (1.0 g) was placed