In Vitro Propagation of Medicinal Plant *Orthosiphun Stamineus* (Misai Kucing) Through Axillary Branching and Callus Culture

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Abstract: *Orthosiphun stamineus* is a herbaceous plant that is popularly known as Misai Kucing. It is widely used in traditional medicine as diuretic agent. This study was divided into two parts that was the *in vitro* production of complete plantlet through axillary branching and callus culture derived from leaf explant. In axillary branching method, sterilization was conducted using 0.02mg/100ml of mercuric chloride followed by rinsing with 20% and 50% of Clorox for 20 minutes and 5 minutes respectively. This sterilization method was able to remove the contaminants from the surface of the axillary stem and almost 70% of the explants were survived. Axillary bud was placed on Murashige and Skoog (MS) basic medium and cultured for 1 month. The *in vitro* shoot was inoculated on MS medium which was supplemented with different concentrations of BAP and NAA. The medium that contained 1.0mg/L of BAP gave the best shoot multiplication (13.25) and shoot length (6.23cm) after 8 weeks in culture. Root formation in term of percentage of root (70%) and the number of root produced (10.50) were the best when shoot inserted into medium contained 6mg/L IBA after 3 weeks in culture. However, MS medium that was supplemented with 2 mg/L IBA enhanced in the root length (3.85 cm). Meanwhile, in callus culture, the leaf explant was placed on MS medium containing with various concentrations of 2,4-D for induction of callus. The optimum level of callus induction and proliferation rate (0.42) were obtained with 4mg/L 2,4-D. The callus cells were tested in medium with Evan's Blue staining and the result showed that the cells were embryogenic. However, the shoot induction from the callus was failed in all tested mediums containing different combinations of BAP and 2,4-D.
[Kamaludin Rashid, Arash Nezhadahmadi, Roihan Mohsin, Shamrul Azhar, Shahril Efzueni. ***In Vitro*** **Propagation of Medicinal Plant *Orthosiphun Stamineus* (Misai Kucing) Through Axillary Branching and Callus Culture.** *Life Sci J* 2012;9(4):5283-5294] (ISSN:1097-8135). http://www.lifesciencesite.com. 787

Keywords: In Vitro; Propagation; Axillary branching; Callus

1. Introduction

Orthosiphun stamineus is one of the most valuable medicinal plants which provide rich medicinal compounds. It has been object of interest among researchers since early 20th century (Khamsah et al., 2000). *Orthosiphun stamineus* is distributed from India through Malaysia to tropical Australia, South East Asia, Soviet Union and Cuba and in early 1920 to Europe. It is also called as *Orthosiphun aristalus* Benth with common names of Java tea (Indubala, 2000), kidney tea or bladder tea. It also has local or vernacular names including Misai Kucing or Kumis Kucing.

1.1. Plant Taxonomy and Morphology

Orthosiphun stamineus is a perennial herb which can grow to the height of 150 cm (Ahamed Basheer and Abdul Majid, 2010) with quadrangular, poorly ramified and ascending stem. The leaves are regulated in opposite pairs. They are glabrous, simple, green, and with a lanceolate leaf blade and a serrate margin. The leaf apice is acuminate with an acute leaf base (Ahamed Basheer and Abdul Majid, 2010). The petiole is partially short about 0.5 to 2cm in length with cuneate at base, acute or acuminate at leaf apical. The stem is quadrangle, reddish in color, and erect with profuse branching. Flowers are borne on verticals about 16cm in length and have campanulate shape. They are white to bluish in color with long exerted filaments that make the flowers look cat's whiskers. Bracts are green, minute (1-2mm) and caudiform in shape. In nature, the flowers are hermaphrodite, about 6.2cm in length (including the stamen). There are two calyx lobes which are greenish red in color, having about 6 mm length and partially gamosepalous. One of the calyx margins is toothed and the other one is entire, both covered with white hairs. There are also two corolla lobes that are partially gamopetalous and covered with minute hairs. The corolla is light violet in color with lobes that are much shorter than the corolla tube. The corollas are bilabiate in shape with fringed margin. The labellum is light violet in color, hairy and pinkish on the under surface. There are 4 stamens which are inserted near the base of the corolla tube. There is a single, central, terete style with a clavate stigma. The fruit splits into 4 oblong-ovoid nutlets with 1.5 to 2.0 mm long.

1.1. Growth Condition

Orthosiphun stamineus can grow well in wet soil and be found in both temperate and tropical garden (Hsuan, 1986). In traditional breeding and

propagation, they are generally propagated by stem cutting in about 15-20 cm which has some buds. For plantation, planting in a nursery for a period of 45 days with cutting placed vertically with only one bud visible is preferred. Every 2-3 weeks the upper 4-6 leaves of the shoots are plucked by hand. In modern breeding and propagation, plant tissue culture is widely used as a modern propagation for commercial purposes in many species. Rapid multiplication with the use of tissue culture provides an alternative method for mass propagation of plant to obtain a large number of propagates originating from a small number of elite plants within a short period (Wirjodarmodjo et al., 1988).

1.2. Medicinal uses of *Orthosiphun stamineus*

Higher plants not only are the most important producers of natural products including food, wood, fibers, and oil but also they are the richest sources of medicinal substances (Tabata, 1976). Medicinal plants and herbs contain substances that are known to modern and ancient civilizations for their healing properties. Traditional medicine is well known because of its high nutritional value and its ability to cure illnesses (Muhammad and Mustafa, 1994). Furthermore, it is widespread all over the world (Akerele, 1988). Determination of major phytochemicals from the extract of *Orthosiphun stammineus* leaves confirmed its strong antioxidant potency and total phenolic content (Akowuah et al., 2004). According to Hamann (1988), around 75 to 90% of people in rural areas rely on herbal traditional medicine. It is used as strong diuretic (Englert and Harnischfeger, 1992) against kidney complaints and illnesses, bladder stones problems, rheumatism, abdominal pain, kidney, edema and gout, and urinary tract infection (Ahamed Basheer and Abdul Majid, 2010). It is consumed as tea which cures diabetes. The leaves of this plant are boiled together with *Andrographis paniculata* and consume as tea to bring down diabetes level (Khairina et al., 2000). Moreover, studies have shown that the *Orthosiphun stamineus* leaves exhibit a range of pharmacological properties such as, anti-inflammatory, antioxidant, anti-bacterial, anti-angiogenetic properties (Ahamed Basheer and Abdul Majid, 2010; Abdelwahab et al., 2010).

2. Materials and Methods

2.1. Preparation of medium

Media used in this study was Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). This is prepared by preparing stock solutions for micronutrients, macronutrients, and vitamins. The procedure began by weighing their premixed powder respectively. Each of them was mixed together with additives like sugar, plant growth regulators and distilled water and then poured up to one liter. Then, PH of the medium was adjusted to 5.8 with Sodium Hydroxide (NaOH) and HCl so that 4.0g agar was dissolved into the solution. The solution was later mixed using a magnetic stirrer on a hotplate until it is dissolved and melted. The medium was then poured into each of the test tubes, petri dishes and jars respectively. For petri dishes, after the entire agars were clearly dissolved, the media were poured with 1000 ml bottles and then autoclaved. After that, medium was poured into petri dish before the medium was started to gel. Once medium gelled, petri dishes were closed and the mouth was sealed with parafilm in order to culture. Three types of plant growth regulators were used like BAP, NAA, and IBA. Stock solution of all growth regulators were prepared by weighing 100 mg of each component that was dissolved in a few drops of 1.0M NaOH or 1.0M HCl. This solvent was carefully added drop by drop into a beaker of swirled ultra-pure water, which was then made up to 100 ml volumetric flask. All the stock were stored in cold room and defrosted later for using in the medium.

2.2. Preparation of aseptic condition

Culturing was carried out in a laminar air flow cabinet under aseptic condition. The laminar was exposed to the UV light for 30 minutes to sterilize the surface of working area in the laminar air flow cabinet. The fan blower of the laminar flow was switched on and all the items were swabbed with 70% alcohol. The long-handle forceps and scalpels used were sterilized by dipping them for 10 seconds in 90% alcohol. Then, they were heat sterilized with head bead during the work. The explants were transferred into sterile petri dishes or test tubes using sterile forceps. After that, the plastic cap of test tubes and jars were opened and the mouth was quickly flamed. The explants were then placed on the agar surface. The mouth of vials was then re-flamed before capping and sealed with parafilm before transferring them to the incubation room.

2.3. Source of explants

One year explants were provided by Golden Hope Research Centre Sdn. Bhd, Banting, Selangor and Institute of Biological Sciences Garden, University of Malaya, Kuala Lumpur (Plate 1)

2.4. Sterilization Technique

Two sterilization techniques were used in this study, labeled as A1 and A2.

2.4.1. A1 Technique

The nodal stem segment was washed by detergent for several times and rinsed under running tap water for 30 minutes. It was pretreated by immersion in absolute ethanol for some time and the surface disinfected by 0.02g/100ml mercury chloride ($HgCl_2$) under constant agitation for 5 minutes. After that the stem was rinsed for three times with sterile distilled water and each of them soaked respectively

for at least 5 minutes. The explants then were sterilized with 20% Clorox with addition of three drops of tween 20 for 20 minutes for the first time and sterilized with 5% Clorox for 5 minutes for the second time. Before inoculation, they were rinsed for three times with sterile distilled water to be ready for culturing.

2.4.2. A2 technique

The explants were treated with 0.08g/100 ml mercury chloride ($HgCl_2$) and rinsed for three times with distilled water. Then, they were soaked in 15% Clorox for 15 minutes and rinsed thoroughly three times in sterile distilled water to be ready for culturing.

2.5. Production of *in vitro* explants

The cleaned explants were aseptically cultured into test tube containing basin MS medium. Explants were cut into 15 to 20 mm of nodal stem that were consisted an axillary bud before culturing. After that the mouth of test tube was flamed and closed with plastic cap so that the test tube was sealed with parafilm and kept in 26 to 27^0C under light condition for one month. Then, observation was made weekly to check the contamination.

2.6. Induction of multiple shoots in proliferation culture medium

Segment with three nodes was excised from one month old *in vitro* axillary shoots. The nodes were separated individually to be as explant sources. The explants were cultured in medium supplemented with BAP at 0, 0.5, 1.0, 1.5, 2.0 mg/L and NAA at 0.0, 0.5, 1.0 mg/L levels and were arranged in factorial design. Ten replications were conducted for each treatment. The culture was maintained at temperature 25 $\pm$ 2^0C with 24 hour at 3500 lux photon density. Every week, the responses of the explants and the number of formed shoots were noted.

2.7. Production of *in vitro* complete plantlet

Well-developed shoots from proliferating shoots were separated and rooted on media supplemented with different concentrations of NAA at 0.0, 2.0, 4.0, 6.0 mg/L. Ten replications were conducted and the cultures were maintained at 25 $\pm$ 2^0C for 24 hours at 3500 lux photon density. After that, the number of roots produced was recorded for a period of two weeks.

2.8. Callus culture and sterilization technique

The leaves were washed with detergent and rinsed with sterile distilled water. Then, they were dipped in 90% alcohol for several seconds. Surface sterilized using 0.02/100 ml mercury chloride ($HgCl_2$) for 5 minutes and rinsed with distilled water for three times. After that, they were soaked in 20% Clorox for 10 minutes and rinsed again with sterile distilled water for three times. Following this stage, they were dried on sterile filter paper to be ready for culturing in culture medium.

2.9. Induction of callus

The explants were cut into disk at size 2-3mm and cultured on petri dishes containing 2, 4-D at 0.0, 1.0, 2.0 and 4.0 mg/L. The replication for each concentration was 10. All the cultured explants were placed in dark condition with temperature regulated at 25$\pm$$2^0$C. Diameter of callus was recorded at week two and week eight and the morphology of callus was also recorded. The ability of callus to form somatic embryo in every treatment was confirmed using Evan's blue.

2.10. Induction of shoots

A small portion of friable callus was cultured in medium supplemented with NAA at 0.0, 1.0, 2.0, 3.0, 4.0 5.0 mg/L and 2, 4-D at 0.0, 0.5mg/L and were arranged in factorial design. All the cultured materials were kept at 25$\pm$ 2^0C for 24 hours at 3500 lux photon density. Then, the number and height of shoots produced were recorded.

3. Results

3.1. Production of *in vitro* complete plantlet from axillary bud explants

3.1.1. The effect of using different sterilization techniques on the explants

Figure 1 shows the result of sterilization experiments based on using different concentrations of mercuric chloride ($HgCI_2$), Clorox, and the length of time exposed to the explants. Contamination was visually detected 3 to 4 days after sterilization treatment was given. It was observed that treatment with 0.02mg/100ml of $HgCI_2$ and rinsing with 20% and 5% of Clorox for 20 minutes and 5 minutes were able to remove the surface contaminant on the axillary bud which made almost 70% of the explants to be survived (Plate 2). Meanwhile, in A2 sterilization technique, only 20% of the explants were free from the contaminants and some of the explants were not tend to grow as the tissues of the explants had sign of death after 1 day in the culture media. The contamination could easily note at the edge of the implanted axillary bud which a small white, black or red spot occurred and spread quickly to cover the whole surface of the medium (Plate 3). Fungi and bacteria were believed to be the main causes of the contamination of the explants.

3.1.2. *In vitro* multiplication of shoots

The explants to be used for *in vitro* propagation started to give growth response after 10 days culture in a basic medium. Adventitious buds were in rise at leaf axil and shoot apex. After 2-3 weeks, the vigorous explants were obtained from the survived explants (Plate 4). Besides, it was noticed that some of the explants produced root in the culture medium (Plate 5). The vigorous explants were left for 5 weeks to develop more shoots. Then, the suitable

and healthy segment consisting of three nodes were excised from the 5 week old *in vitro* axillary shoots and the nodes were separated individually to be as explants sources.

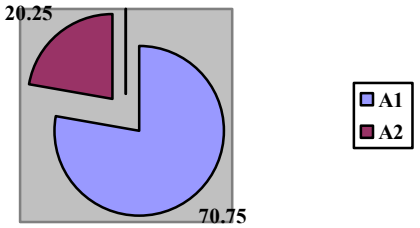

Figure 1. The percentage of explants survived in A1 and A2 sterilization technique

The growth of multiple shoots in various treatments tested in medium containing the combinations of BAP and NAA was recorded as shoots number and shoot length over a period of 4 and 8 weeks (Figures 2 and 3).

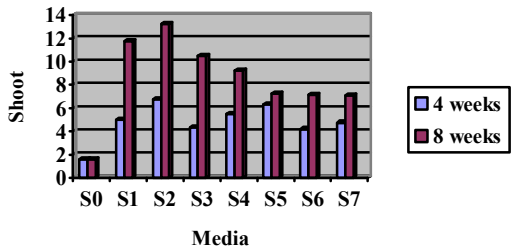

Figure 2. Multiple shoots formation in nodal segments in MS medium supplemented with different concentrations of BAP and NAA

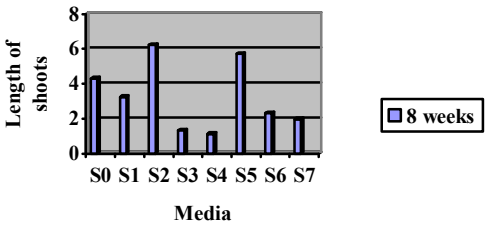

Figure 3. Shoot length in MS medium containing different concentrations of BAP and NAA

The explant cultured in the media produced a cluster of shoots in the all media supplemented with BAP and NAA except in the basic medium. Multiple shoots were initiated from axillary bud explant after 1 week in culture (Plate 6).

The nodal segment of *Orthosiphun stamineus* produced the highest number of multiple shoots per explant when it was cultured in MS + 1.0mg/L BAP alone (Plate 7) where an average 13.75 shoots per explant was obtained after 8 weeks. The number of multiple shoots was increased with the duration of culturing time. The shoots obtained from week 4, resulted an average of 6.75 per shoots showed two-fold greater increase in the next 4 week periods.

On the other hand, the poor growth was seen in MS media containing 1.5mg/L and 2.0mg/L BAP. It was observed that the shoot length was in average 1.34 and 1.13 per shoots (Plate 8). However, the multiple shoots produced in the media are up to almost 10 shoots per explants.

Addition of NAA in the presence of BAP in the MS medium induced callus formation at the base of explants and decreased the formation of multiple shoot number (Plate 9). In the medium without any hormone showed the lowest shoots formation but some of the explants produced complete plantlets with roots obtained after 8 weeks of culturing.

3.1.3. Effect of rooting on explants

The *in vitro* shoots when separated as individual shoot from the multiple shoots produced roots when cultured in basic MS medium or MS medium supplemented with different concentrations of IBA (Figures 4, 5, and 6).

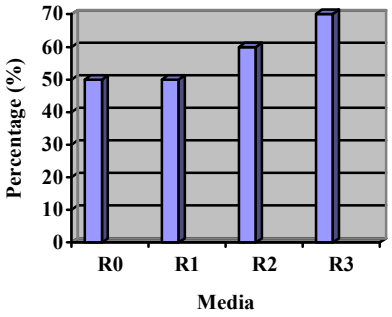

Figure 4. Percentage of shoots producing roots in different concentrations of IBA

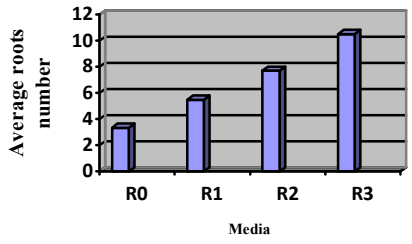

Figure 5. Average number of roots produced per shoot in different concentrations of IBA

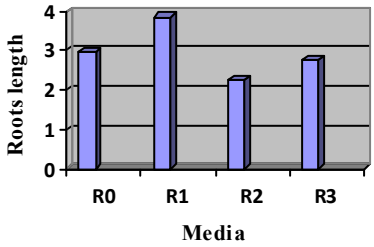

Figure 6. Average length of roots produced in different concentrations of IBA

From the result, the percentage and number of roots formed in each shoot were increased when the concentrations of IBA were enhanced (Plate 10).

The highest percentage of shoots that produced roots was properly seen in shoot grown in MS medium containing 6mg/L of IBA. This medium produced 70% of root and in average 10.50 roots was formed per shoot. When the culture contained 4mg/L of IBA, it was observed in the basic medium that the shoots were also capable to produce 50% of roots. Meanwhile, in terms of production of root length, the longest root was produced in medium containing 2mg/L IBA (Plates 11, 12, and 13).

3.2 Callus culture

3.2.1 Induction and multiplication

The results of the callus induction and multiplication in MS media supplemented with different concentrations of 2, 4-D is summarized in Table 1.

The callus was initiated from the leaf explant after one week in the culture. It was observed that the whole part of the explant was formed into callus after one week later. The callus in medium supplemented with MS medium containing 4.0 mg/L of 2, 4-D was found to promote good callus proliferation. The results showed that the callus in this medium produced the lowest multiplication at week two but the callus diameter was increased to 1.17cm to give the highest diameter incensement (0.42) (Plate 14). Meanwhile, the callus in MS medium containing 2.0 mg/L and 3mg/L of 2,4-D encourage a longer diameter at the first two weeks (0.92 and 0.97) but the proliferation rate decreased with the duration of culturing time which affected the callus incensement (0.29 and 0.23) (Plate 14). Most of the callus textures in all treatments were friable (Plate 15) and the callus colors were light brown and brown (Plate 16). All the callus tested using Evan Blue staining had response to acetocarmine stained material which gave a bright red colors. So, the calluses were recognized as embryogenic cells (Plate 17).

3.2.2. Induction of shoot

The combinations of 2, 4-D and BAP in all used media was failed to produce shoot from the callus after 6 weeks culture. It was observed that the callus color was changed from brown to green after 2-3 weeks in the culture but the color was suddenly changed into dark brown and black and considered to become senescence. Therefore, no result has been recorded.

4. Discussions

Orthosiphun stamineus is currently gained recognition as an important medicinal plant. Generally, the raw used in the production of healthy food from medicinal plants was collected from the wild. Continuous extraction of this material from the forests has led to the depletion of this important raw material. Conventional propagation is faced problems of poor seed viability, low germination, and delayed rooting of vegetative cuttings. There is an urgent need to apply non-conventional propagation methods for conservation and future commercial delivery. Therefore, biotechnology provides a new method for the mass production of elite plants as well as for the *in vitro* production of plant raw materials (Schumacher, 1988). Thus, tissue culture techniques have been reported for conservation and propagation of several endangered medicinal plants (Sharma et al., 1993; Sudha and Seeni., 1996). This study was conducted to produce *in vitro* plantlet through axillary branching and callus culture for rapidly increase the number of propagates of *Orthosiphun stamineus* for cultivation as well as aid in the replacement of natural population.

Table 1. Callus induction and multiplication in MS media supplemented with different concentrations of 2,4-D

Media	*Week 2	*Week 5	Proliferation rate	Callus texture	Callus color	Evan Blue staining
C0	-.	-	-	-	-	-
C1	0.69	1.02	0.34	Friable	Light brown	Red
C2	0.92	1.21	0.29	Friable	Light brown	Red
C3	0.97	1.20	0.23	Friable	Brown	Red
C4	0.75	1.17	0.42	Friable	Brown	Red

* Diameter of callus (cm)

Plate 1. Source of explants obtained from Institute of Biological

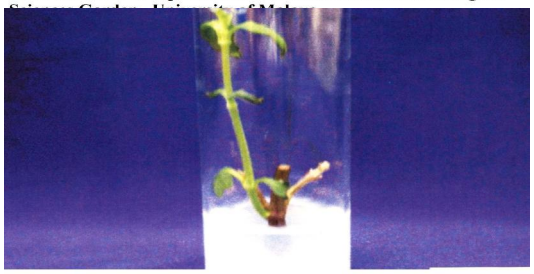

Plate 2. The explant survived after 2 weeks in culture when it was treated with HgCl2 and rinsed with Clorox

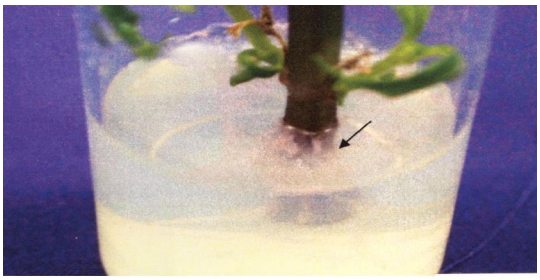

Plate 3. Red spot was spread when contamination occured after 3-4 days sterilization

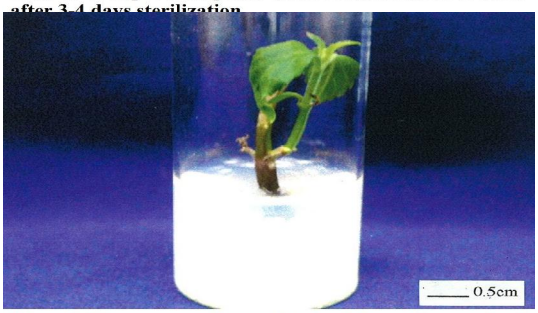

Plate 4. Vigorous explants obtained from the survived explants after 2-3 weeks

Plate 5. The explant produced complete plantlet root in basic medium after 3 weeks

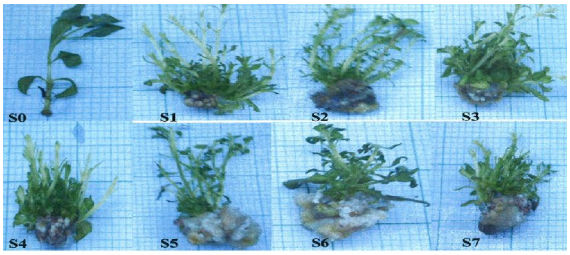

Plate 6. The effect of BAP alone and combination with NAA that produced multiple shoots (S1-S7) compare to the culture in control medium (S0)

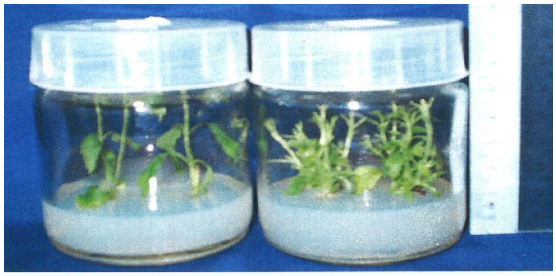

Plate 7. The highest number of multiple shoots and the legth of shoot produced when cultured in MS + 1.0 mg/L BAP alone

Plate 8. The culture with multiple shoots in control medium, MS + 1.0 mg/L BAP and MS + 2.0 mg/L BAP (from left to right

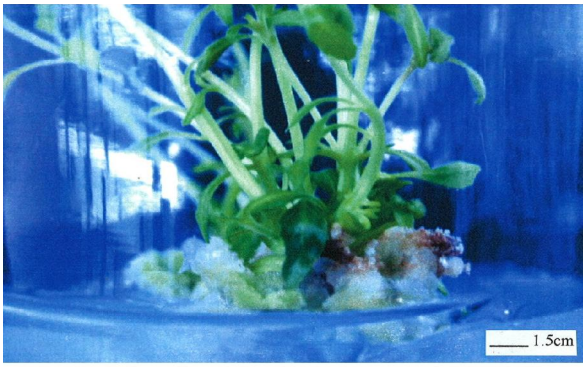

Plate 9. Addition of NAA in the presence of BAP in the MS medium induced callus formation at the base of explants

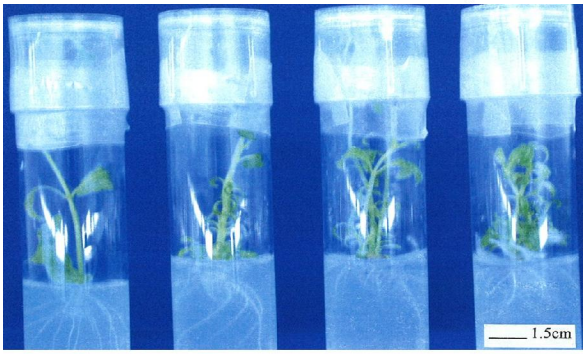

Plate 10. *In vitro* plantlets' root formation in MS medium (control), MS + 2mg/L, MS + 4mg/L IBA and MS + 6mg/L IBA

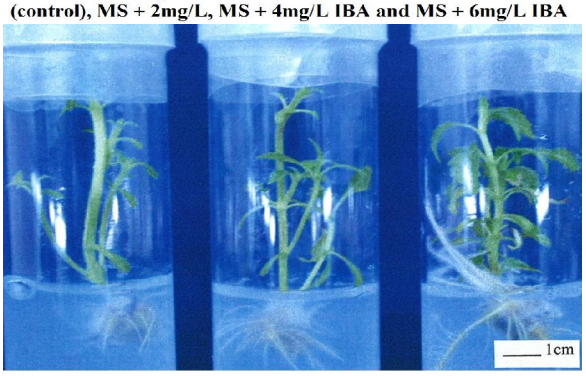

Plate 11. *In vitro* plantlet's root formation in week 1, week 2, and week 3 in MS + 6mg/L IBA (from left to right)

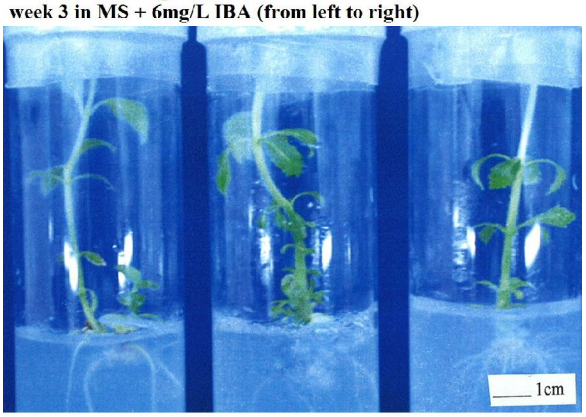

Plate 12. *In vitro* plantlet's root formation in week 1, week 2, and week 3 in MS + 4mg/L IBA (from left to right)

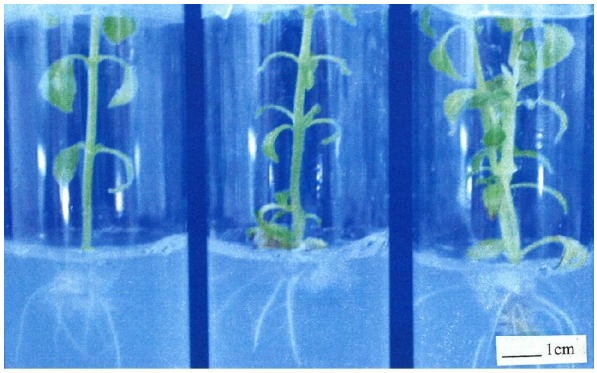

Plate 13. *In vitro* plantlet's root formation in week 1, week 2, and week 3 in MS + 2mg/L IBA (from left to right)

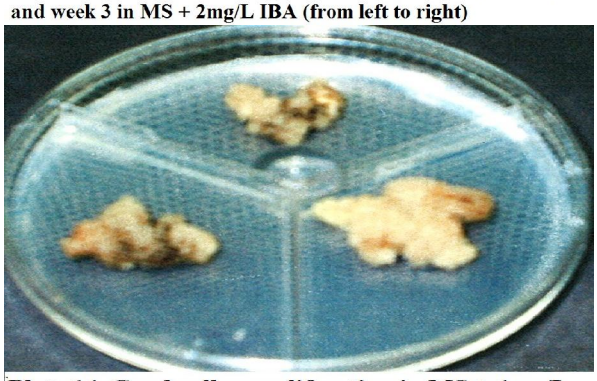

Plate 14. Good callus proliferation in MS + 4mg/L of 2, 4-D after 5 weeks (above) and the lowest proliferation in MS + 3mg/L of 2, 4-D (below)

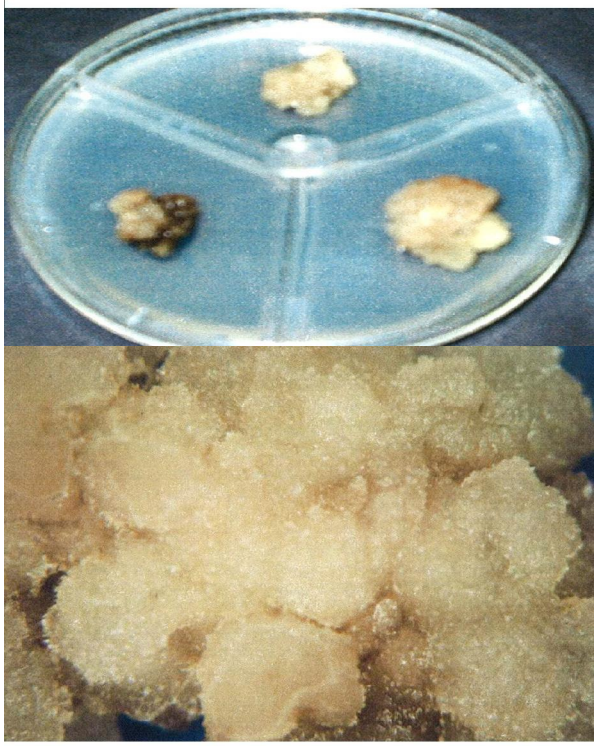

Plate 15. Friable callus

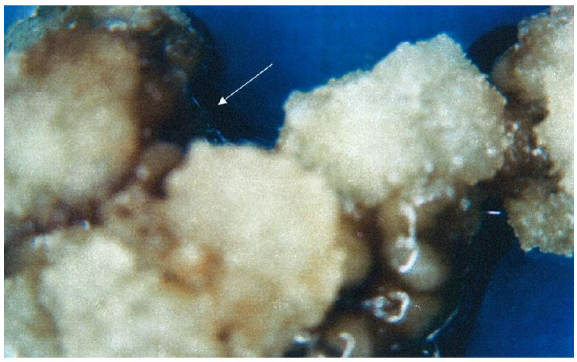

Plate 16. The brown color callus

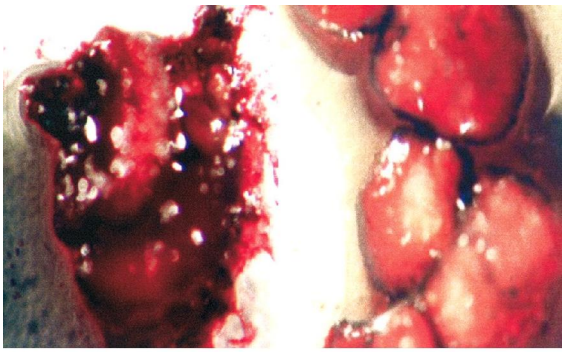

Plate 17. The callus tested using Evan Blue staining which had response to acetocarmine-stained material

4.1. Effect of surface sterilization on explants

In tissue culture, the most important thing to obtain an aseptic culture is the effectiveness of sterilization technique applied to the plant material. The plant material in this study was excised from the axillary stem of *Orthosiphun stamineus* and has been used as a primary explant for the multiplication of shoots and young leaf explants for callus culture. The selected plant materials and the explant should be totally free from any other microbial contaminants. It is because the surfaces of plants carry wide range of microbial contaminants (Bhojwani and Radzan, 1996). Beside the plant material itself which contribute to the contamination of the culture, there are other several possible sources of contaminations such as the medium, the environment of the transfer area, instruments used to handle plant material during inoculation, subculture, the environment of the culture room, and the operator. In this study, it was found that washing with 0.08g/100m1 of mercuric chloride ($HgC1_2$) followed by rinsing with 15% Clorox for at least 10 minutes was failed in eliminating total contaminants in axillary stem of *Orthosiphun stamineus*. On the other hand, 70% of explants became free from the contaminants through using a lower concentration of $HgCI_2$ (0.02mg/1) and rinsing with 20% and 5% of Clorox for 20 and 5 minutes. The contamination on the medium was observed 3-4 days in the culture. The small white, black and red spot of contaminant spread quickly to cover the whole medium. It was believed that bacteria and fungi were the main contaminants. There were various methods to sterilize plants like plant tissue culture, $HgC1_2$ and commercial Clorox which were chosen because they were proven to be effectively decontaminating axillary stem of *Orthosiphun stamieus* (Lee and Chang, 2000). The rinsing processes using 20% and 5% of the commercial Clorox for 20 and 5 minutes have accomplished a sufficient concentration to sterilize the explants with satisfactory result. The commercial Clorox was used containing 99.9% of sodium hypochlorite (NaClO) as the active agent to eliminate the contaminant. The result also showed that application of mercury chloride ($HgC1_2$) at 0.02/100ml was an effective disinfectant; however it contains a danger chemical which poses health risks and disposal problems (Bonga and Aderkas, 1992).

In the case of using higher concentration of $HgC1_2$ (0.08mg/100m1), bleaching of the tissue was caused when the concentration was high enough to kill the microorganism. Dods and Roberts (1982) have stated that the aim in surface sterilization of plant tissue is to remove all microorganisms with a minimum damage to the plant system that need to be cultured. Moreover, they were also indicated that traces of the decontaminant should be removed by several rinsing in sterile distilled water because they can leave toxic residue in the explant and can destroy some components in the medium. Therefore, the concentration of the decontaminant agent and the duration of the treatment should be chosen accurately to minimize the death of the tissue (Bhojwani and Radzan, 1996). Microorganism lodged in crevices in the bark, in leaf axils or at the base of hair may never contact with the decontaminant because air bubbles entrapped in these positions prevent such contact. Furthermore, the tissue surface should be wet firstly by treatment with ethanol. Ethanol partially removes hydrophobic waxes and resins which protect microorganisms from contact with aqueous decontaminants (Bonga and Aderkas, 1992). Pre-cultural treatment can be so essential in that it should be considered as a routine stage of the micro propagation process.

4.2. The influence of media and plant growth regulators on shoot multiplication

Growth and morphogenesis of *in vitro* plant tissue are largely governed by the composition of the media culture (Razdan, 1993). A standard

medium consists of a balanced mixture of macronutrient and micronutrient elements, vitamins, carbon sources, a source of reduced nitrogen supply, organic growth factors, and plant hormones (Narayanaswamy, 1977). The choice of particular medium depends mainly on the species of plant, the tissue or organ to be cultured, and the purpose of the experiments (Dodds and Roberts, 1982). The Murashige and Skoog (1962) medium (MS) was chosen in this study as it is proven to be effective for the growth of a variety of dicotyledonous and monocotyledonous plants (Dixon, 1985). The requirement for the growth regulators varies from the system and the mode of shoot multiplication. Higher concentration of cytokinin to auxin ratio is required for the direct of induction of shoots on explants and higher auxin to cytokinin ratio is necessary for callus initiation and growth (George and Sherrington, 1984). The ability of shoots to multiply rapidly in *in vitro* condition is essential for the establishment of economically feasible micro propagation systems. Usually, once proliferating shoot cultures of particular tree species have been stabilized, they can provide a continuous source of micro shoots. In this study, multiplication of shoots from axillary bud of *Orthosiphun stamineus* have been achieved after 8 weeks in culture in MS medium containing different concentrations of BAP and NAA. It was observed that the shoots buds were differentiated from the sides and developed prolific buds, indicating the easy release of the axillary buds. A BAP was selected for micro propagation of wide ranges of medicinal plants for rapid propagation because of its ability to stimulate shoot proliferation (Lee and Chang, 2000; Sudha and Seeni, 1996; Franca et al., 1995; Sharma et al., 1993). The most effective medium in terms of the number of shoots was formed when it was supplemented with 1.0 mg/l BAP alone and the average shoots produced per explant was 13.25. According to Bhojwani and Razdan (1996), to enhance axillary branching, the shoots can be grown in the medium containing suitable concentration of cytokinin with or without auxin. This is also reported by Lee and Chang (2000) indicating that with the addition of BAP alone in MS medium the highest number of multiple shoots of *Orthosiphun stamineus* can be achieved. George and Sherrington (1984) have state that a high concentration of cytokinin in media can induce shoot formation and at the same time inhibits roots formation. This high cytokinin concentration appeared to stop the apical dominance and allows axillary buds to develop. However, as higher levels of BAP added into the media (l.5 mg/L and 2.0 mg/L), the number of shoots was not increased. Although, the satisfactory number of multiple shoots obtained from these media, the length of the shoots did not support good growth of the shoots. This could be due to high levels of cytokinin that were placed in the media which affected the small shoots and failed to elongate (George and Sherrington, 1984). Moreover, by adding a high level of cytokinin, the number of shoots formed was increased but the growth of individual shoots remained arrested (Bhojwani and Razdan, 1996). The result was actually differed from the study done by Lee and Chang (2000) when the greatest responses of multiple shoots formation were obtained from the medium containing 1.5 mg/L of BAP.

Besides, it is important to consider the physiological stage of explants and the effect of stem bud location on the number of shoots per explant bud (Chand et al., 1999). They also found that the rate of shoot multiplication of medicinal plant *Maytenus ilifolia* was affected by the age of axillary bud excised from the plant source. Meanwhile, the addition of NAA in the presence of BAP in MS medium induced callus formation at the base of the explants and decreased the formation of the multiple shoot number. But NAA was proved to be more efficient than IBA in combination with BAP as reported by Lee and Chang (2000) and in this study the combinations produced the formation of 7 shoots per explant in average. For some species, the combination of auxin and cytokinin seem to be positive for the number and length of shoots. Franca et al. (1995) found that *in vitro* derived shoots of *Stryphnodendron polyphythum* was influenced by the action of cytokinin, whereas height was increased by the presence of both auxin and cytokinin. The result from this study showed that the addition of BAP and NAA into the media did not affect the number and height of shoots. Moreover, the height of shoot may not be significant because in time of sub culturing, a shoot placed on the media were not uniformly in size. According to Bhojwani and Razdan (1996) the exogenous requirements of the hormones depend on the levels of the plant system and it is variable with the tissue, plant type, and the phase of plant growth. Consequently, for the shoot multiplication in this study, the presence of NAA in the medium was not obligatory since a BAP alone was enough for effective shoot multiplication. It is needed to emphasize that the superiority of medium containing 1.0 mg/L of BAP was used as the optimal media for shoot multiplication.

4.3. Production of *in vitro* complete plantlet

Plant roots produced by means of tissue culture is an important step in the clonal multiplication of desirable plants. Propagates have to

have an adequate root system before they can be established satisfactorily (Seabrook, 1980). Rooting can be carried by *in vitro* method as well as *in vivo.* In this study, *in vitro* rooting experiment was carried out using only one type of auxin which was IBA. All *in vitro* shoots when separated as individual shoot, rooted in 4 media experiments including free of auxin medium. Razdan, (1993) expressed that roots are mostly induced in the presence of suitable auxin. Although, a mixture of more than one auxin can be particularly effective for root induction but sometimes root formation is better with only one auxin placed into the media (George and Sherrington, 1984).

In this study, IBA was found to be very effective for root initiation of *Orthosiphun stamineus* especially in MS medium supplemented with 6mg/L of IBA when 70% of roots were formed compare to the medium free of auxin. This result was similar to those obtained by Lee and Chang (2000) but they found that percentage of root formed was almost 80%. On the other hand, the root formation in the medium free of auxin was equaled (50%) to rooting percentage in medium containing 2mg/L of IBA. In this case, the production of root in medium of free auxin was mainly depending on the endogenous auxin. It has been reported in another medicinal plant like *Rauwolfia micrantha* (Sudha and Seeni, 1996) and *Exocoecaria agallocha* L. that a similar concentration of IBA (2.46µM) was required to achieve highest frequency of rooting. In *Hydrastis canadensis,* treatment with IBA at range of 1.0-2.0µM produced the lowest formation of roots as another auxin type NAA was needed for successful result (Bedir et al., 2003).

In this study, it was found that the higher concentration of IBA supplemented on media increased the rooting ability of shoots. For example, the number of shoots was increased from 3.33 in medium containing 2mg/L to 10.50 shoots produced in 6mg/L of IBA. High concentration of IBA added to the media stimulated higher degree of cell division and cell elongation of the shoot primordial but if the excessive auxin was applied, it would cause toxicity of the cell. An increase in the concentration of IBA on medium did not influence the root length. Root production in terms of the number of roots was high i.e 10.5 roots per culture in higher IBA level (6mg/L) used but it had shorter root length than those in the lowest medium containing IBA (2mg/L). In other medicinal plant like *Hydrastis Canadensis,* a longer root length in the lower concentration (1.0µm) of IBA has also been reported (Bedir et al., 2003). According to Pierik (1987), if the high auxin concentration failed to produce roots, callus formation takes place. The callus was observed at the base of the shoot in the media except in the medium of free auxin. There are many factors that influence the rooting ability. Franca et al. (1995) found that the most effective way for rooting in S*tryphnoden polyphythum* was using half strength of macronutrients. Nutrient salt in media affected the rooting due to reduced nitrogen level (Bhojwani and Razdan, 1996). The rooting can also be enhanced by improving the number of shoot multiplication cycles. The other factor is to grow the explants in liquid media since it can provide a good aeration beside development of root hairs. Activated charcoal also has been reported to improve root growth where roots have already been initiated (George and Sherrington, 1984).

4.4. Callus culture

The other method of propagation in this study was through callus formation. Leaf explants were used for initiation of the callus. The type of explants used may vary in morphogenesis which failed to regenerate. The effect of the type of explants on other medicinal plant like *Eurycoma longifolia* (Tongkat Ali) for induction of callus was reported by Chand et al. (1999) when the highest callus production was obtained when petiole was used as explants. The presence of 2, 4-D in all media tested was found to be effective in inducing callus proliferation. 2, 4-D is a phenoxy auxin and has been recognized as strong promoters of callus induction and growth (Bonga and Aderkas, 1992). In this study, the tendency of callus to grow and proliferate was measured in the increments in diameter. The results indicated that callus proliferation was affected by the concentration of 2, 4-D in the media. Maximum callus growth was achieved in MS medium containing 4.0 mg/L 2, 4-D. At the initial stage, the proliferation rate was slow but later as the time passed, the callus started to multiply and proliferate to the greatest result. Since 2, 4-D is a strong auxin, the high concentration provided to the media had created a sufficient condition for callus to continue to develop and therefore keep multiplying as the time passes. The produced callus was friable in all treatments and varied in color. This type of callus was considered as non-embryogenic in which it was unable to produce somatic embryos thorough induction of direct somatic embryogenesis. The appearance of embryogenic callus was white, off white or pale yellow, compact or often nodular and translucent. The compact cell later exhibited synchronized cell division whereas the latter cells were bigger and more vacuolated (Aziz et al., 2000). However, the assessment to distinguish embryogenic culture using Evan's Blue staining offered the other results. The callus cells were treated with acetocarmine and Evan's Blue and it was found that the reactions with embryogenic cell remained in bright

red color with acetocarmin stain. According to Dodd and Roberts (1982), the embryogenic cells are characterized by dense cytoplasmic contents, large starch grains and relatively large nucleus with darkly stained nucleolus. In contrast, with embryogenic callus, nucleus is very small so that the acetocarmine-stained red material is difficult to locate and the whole embryogenic callus were blue when stained with Evan's Blue (Gupta and Holmstorm, 2005). Each embryogenic cell is capable of passing through sequel stages of embryo formation. It starts with repeated cell divisions, cell aggregates progressively and passes through globular, heart and torpedo stages respectively before forming plantlet. In this study, there was no evident of any embryo developed in each stage but the callus was considered in the early stage of somatic embryogenesis. In fact, some cells within callus masses are capable of forming embryogenic cell because they are totipotent which means the cell retains all genetic information required for normal development (Butcher and Ingram, 1976). The callus, however, failed to produce shoot in the media containing combination of BAP and 2,4-D. It was observed that the callus showed green chlorophyll pigmentation that was developed but, then, it turned to black, indicating a fail for shoot initiation. There are many possible reasons for these failures such as combination of BAP and 2, 4-D that did not offer a balance of exogenous auxin and cytokinin or either one of these hormones must be omitted from the medium. Besides, the endogenous hormones may be accumulated and their inhibitory effect on organogenesis was not reversed by exogenous hormones. Dodds and Roberts (1982) found that the level of the hormone provided in the medium must be balanced with the residual amounts of the same in the primary explants, in addition to endogenous hormones that may be synthesized by the newly formed callus. Another possible reason is the cultural condition that involves nutritional and physical factors which may block the onset of the process.

5. Conclusions

The sterilization processed with 0.02mg/l00ml of $HgCl_2$ and rinsed with 20% and 5% of Clorox at 20 minutes and 5 minutes were able to remove the surface contaminant on the axillary stem of *Orthosiphun stamineus,* in which almost 70% of the explants survived. The highest shoots multiplication of *Orthosiphun stamineus* was obtained in MS medium containing l.0mg/L BAP. The optimum medium for rooting of *Orthosiphun stamineus* was in MS medium supplemented with 6mg/L IBA. The effective medium for callus induction and proliferation of *Orthosiphun stamineus* from leaf explants was in 4mg/L 2, 4-D. The combination of 2, 4-D and BAP in all media tested were failed to induce shoot from callus of *Orthosiphun stamineus.* However, further studies should be carried out to identify suitable plant growth regulators which can promote shoot induction from the callus. Different types of the medium or modifications of the medium should be considered and supplemented with other types of cytokinin and auxin. Furthermore, acclimatization of the *in vitro* plantlet should be done to ensure the ability of plantlet in the open field. This will provide very useful information in terms of the establishment of *Orthosiphun stamieus* with the use of this technique.

Acknowledgements:

The authors wish to thank to Institute of Biological Sciences, University of Malaya for the grant provided.

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10/21/2012