CHAPTER III

Materials

and

Methods

3.1 Study location

The study was carried out at the tissue culture laboratory (B.2.5), Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur.

3.2 Micropropagation of *Asparagus officinalis*

3.2.1 Plant materials and Explant sources

Seeds of *Asparagus officinalis* cv. Mary Washington were purchased from NEW TRIO PRODUCTS (Selangor, Malaysia) and were cultivated in the green house of Institute of Biological Sciences, Faculty of Science, University of Malaya (Fig 3.1). The nodal explants from four-week old plants were collected and used as explants.

Fig 3.1: *Asparagus officinalis* cv. Mary Washington grown in the green house at Institute of Biological sciences University of Malaya, Kuala Lumpur

3.2.2 Explants sterilization

Explants collected from field-grown plants are usually contaminated by various microorganisms. The nodal explants collected from four-week old plants were placed in jam jar and covered with net and these were rinsed in a stream of running tap water for 30 minutes to remove all the adhering dust particles and microbes from the surface. The explants were then thoroughly washed (4-5 washing) with distilled water. All explants were manipulated aseptically under a laminar flow hood for the remaining procedures. Hands and arms which were to be used inside the laminar flow were scrubbed with 70% alcohol. Explants were surface sterilized with 6% commercial sodium hypochlorite solution containing tween 20 (sigma) (2 drops per 100 ml) for 15 min and then washed thoroughly in sterile distilled water 5 times. The explants were then immersed in 70% ethanol for 1 minute and lastly rinsed again five times with sterile distilled water and blotted on sterilized filter paper.

3.2.3 Media and culture vessels

The type of medium on which cultures are grown has a major influence on the degree of success achieved in initiating and growing cultures. Media used in all experiments were prepared in the plant tissue culture laboratory. According to the literature, media formulations such as MS (1962) are suitable for micropropagation a wide range of different species from Liliaceae family (Peng and Wolyn, 1999; Mamiyaa and sakamoto, 2000). Media (Murashige and Skoog, 1962) supplements include PGRs, sucrose (Sigma) and sigma phytogel agar (gelling agent). The MS media from murashige and skoog (including vitamins) with the weight of 4.4g was used for media preparation. Media was prepaid for one liter (L) of water. For making a liter of MS medium, 800 ml distilled water

was poured in a beaker and placed on the stirrer (using magnetic beads for stirring) and stirred while adding 4.4 g MS powder (Sigma with vitamins) and 30 g sucrose. During this step the PGRs (BAP, NAA, Kn, IBA) was added to the media based on required concentrations. In order to perform the micropropagation test, four different hormones were engaged in the experiment; BAP (6-Benzylaminopurine) and Kn (Kinetin) from Cytokinins and NAA (1-Naphthaleneacetic acid) and IBA (Indole-3-buteric acid) from Auxins. Different trials were done to obtain the best concentration. The stock solution for each hormone was prepared.

The routine concentration for PGRs, 1 mg/ml (1000 mg/L), was used to make it in an easy and fast calculating way of using the formula *M1V1 = M2V2* (M as concentration and V as volume), e.g., 3 mg/L BAP can easily be made up by dispensing 3 ml of a 1 mg/ml BAP stock.

As plant grows, hormones hardly adopt the aqueous solution and they need to be dissolved as solvent. 1M NaOH was prepared as the solvent. To prepare 1M NaOH, 40g NaOH was dissolved in one liter of distilled water. Solution was stirred to dilute all the solutes. It took about an hour for this process.

After adding hormone to the media the media PH was adjusted to 5.8 for all formulations using 1.0 N and 1.0 N concentrations of HCL and NaOH. Subsequently, the volume of the medium was made up to one liter (L) by using a measuring cylinder.

For solidifying the media, 2.8 grams phytogel agar was added while turned on the heating button in order to melt the agar.

The formulation of MS medium is shown in the Appendix 1. The following combination of media and hormones were used as shown in Table 3.1 and 3.2.

Table 3.1: MS supplemented with Different Combinations of BAP and NAA during callus induction and shoot culture of *Asparagus officinalis*

NAA **mg/L**	**BAP** **mg/L**						
	0.0	**0.2**	**0.4**	**0.6**	**0.8**	**1.0**	**1.5**
0.0	T1	T2	T3	T4	T5	T6	T7
0.05	T8	T9	T10	T11	T12	T13	T14
0.1	T15	T16	T17	T18	T19	T20	T21
0.2	T22	T23	T24	T25	T26	T27	T28
0.3	T29	T30	T31	T32	T33	T34	T35
0.4	T36	T37	T38	T39	T40	T41	T42
0.5	T43	T44	T45	T46	T47	T48	T49

Table 3.2: MS supplemented with Different Combinations of Kn and IBA during callus induction and shoot culture of *Asparagus officinalis*

IBA mg/L	**Kn mg/L**						
	0.0	**0.2**	**0.4**	**0.6**	**0.8**	**1.0**	**1.5**
0.0	T1	T2	T3	T4	T5	T6	T7
0.05	T8	T9	T10	T11	T12	T13	T14
0.1	T15	T16	T17	T18	T19	T20	T21
0.2	T22	T23	T24	T25	T26	T27	T28
0.3	T29	T30	T31	T32	T33	T34	T35
0.4	T36	T37	T38	T39	T40	T41	T42
0.5	T43	T44	T45	T46	T47	T48	T49

After the preparation of the medium, water was poured out of the autoclaved glassware. Definite aliquots of the medium were then added depending upon the capacity of the culture vessel. Generally 40 ml of the medium was distributed into the 100 ml flasks. After covering the flasks with aluminum foil, media were steam-sterilized at 121ºC for 20 minutes. After autoclaving, flasks were placed on the table. These were then left to cool and solidify.

3.2.4 Aseptic Manipulation and Inoculation

Aseptic manipulation is a method performed to ensure all equipments used and surrounding area are always clean and sterile, thus minimizing the contamination occurrence. All the experimental manipulations were carried out under strictly aseptic conditions in laminar air flow bench fitted with a bactericidal U. V. tube (15 W, peak emission 2637 Aº). At the beginning of every culturing process, the surrounding of laminar air flow cabinet was sprayed with 70% ethanol, wiped with cotton wool and let to dry for 15 to 20 minutes.

The surfaces of all the vessels and other accessories such as gas burner, tube containing absolute alcohol etc were also cleaned with 70% alcohol. The instruments such as forceps, scalpels, blades etc. were autoclaved in 121ºC for 20 minutes. The fresh material to be inoculated was kept in a petri dish covered with a piece of aluminum foil in order to protect it from the harmful effects of U. V. rays. Alcohol was then sprayed in the chamber with the help of an atomizer. The chamber was then sterilized with U.V. rays continuously for 20 minutes.

Hands and arms were scrubbed with 70% alcohol before inoculation. The sterilized nodal explants were cut with sterile blade. The explants were then cultured on variously augmented MS medium. Each culture flask generally contained 2 explants. All the cultures were maintained for 6 weeks in an air conditioned culture room at a temperature of 25 ± 1ºC. The source of illumination consisted of 2.5 feet wide fluorescent tube (40 watt). The intensity of illumination was 1000 lux at the level of cultures and a 16 hour light regime was followed by 8 hour darkness.

3.2.5 Treatments

The study was conducted with 97 treatments consist of control, MS medium with different combination of BAP and NAA and different combination of Kn and IBA, under light condition, and also 97 treatments with same combinations of hormones under dark condition. There are 10 replications for each treatment with a total of 485 experimental units (conical flask) under light condition and also 485 experimental units in dark condition. The treatments were as indicated in Tables 3.1 and 3.2.

3.2.6 Callus induction and shoot multiplication

The nodal explants from four-week old plants were collected. Each nodal cutting was approximately the same size. Two cuttings were placed vertically into each flask containing 40 ml of medium (Figures 3.2). The basal end of the cutting was inserted ~ 1mm into the medium and each was spaced equal distances apart in the flasks. This study was designed to identify a medium with the optimal combination of PGRs to support shoot multiplication. Media containing various concentrations of BAP, NAA, Kn and IBA were tested. Specifically, 97 formulations were examined with different combinations of BAP (0.0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5 mg/l) and NAA (0.0, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 mg/l) (Table 3.1) and also different combinations of Kn (0.0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5mg/l) and IBA (0.0, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5mg/l) (table 3.2). Each of the 97 treatment was replicated 10 times (5 conical flasks for each treatment, each flask containing 2 explants). Each of the 485 flasks was randomized on trays and placed on a shelf in the growth chamber under light condition (1000 Lux) and at a temperature of 25±1ºC (Figure 3.3). This experimental was repeated for the same 97 treatments with the same conditions but flasks were placed in the growth chamber in darkness.

Cultures were observed regularly and the contaminated flasks were replaced with fresh cultures. After a period of 6 weeks, the media and explants were screened and the responses recorded.

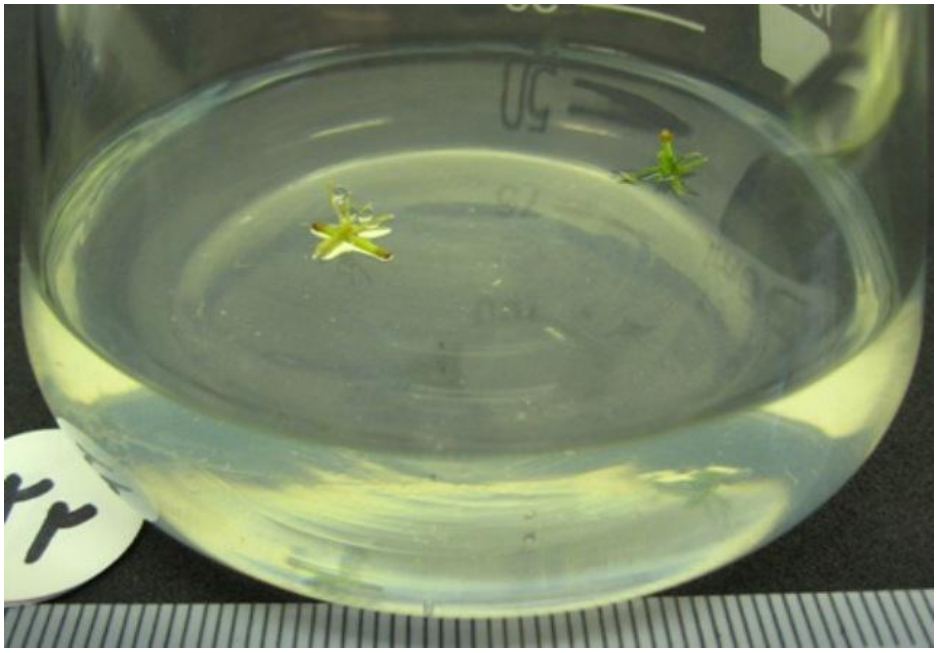

Figure 3.2: The nodal explant from 4 weeks old plant, cultured in MS medium

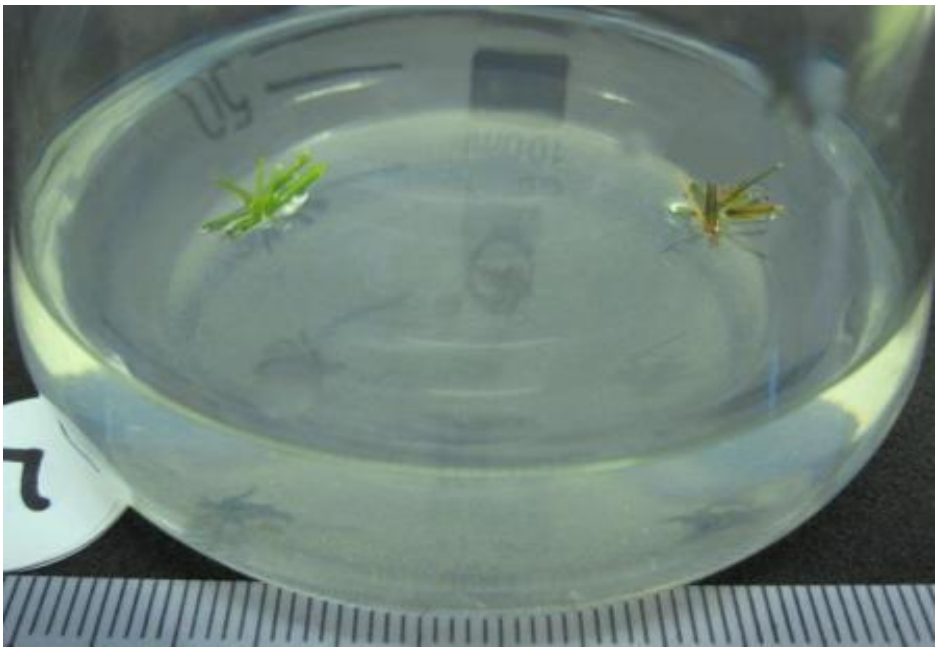

Figure 3.2: The nodal explant from 4 weeks old plant, cultured in MS medium

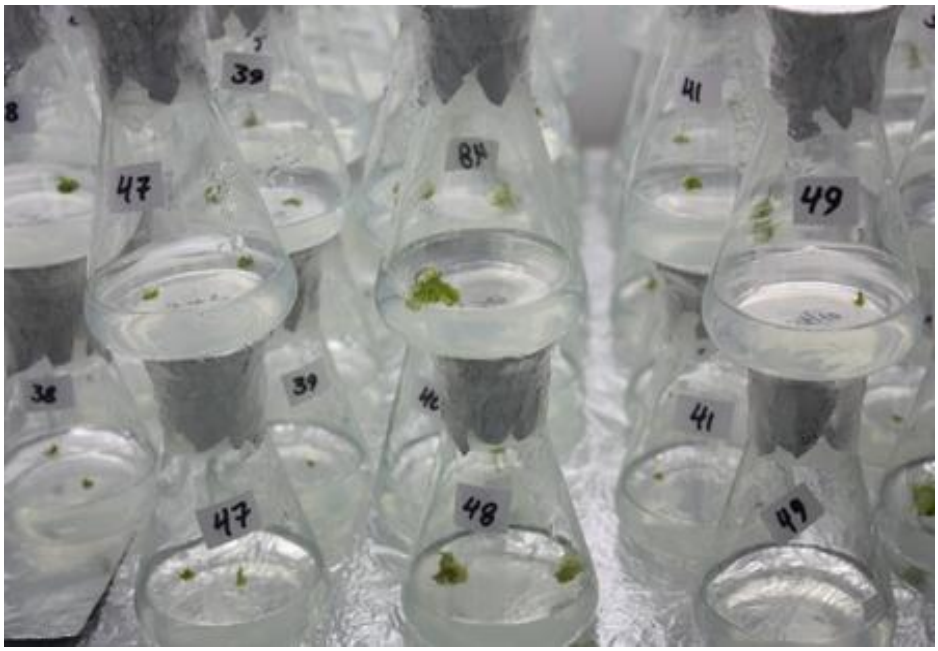

Figure 3.3: Flasks contain cultured explants in MS medium, placed in the growth room

3.2.7 Rooting of shoots

Explant materials for the rooting study were obtained from plantlets derived from the multiplication trial experiments. Uniform cuttings were excised aseptically and placed vertically in flasks containing 40 ml of medium (Fig 3.4 and 3.5). No cytokinin was used, however, eight different concentrations of IBA (0.1 mg/l, 0.2 mg/l, 0.3 mg/l, 0.4 mg/l, 0.5 mg/l, 0.75 mg/l, 1.0 mg/l and 1.5 mg/l) were examined to determine the optimal root development. Each of the eight treatment was replicated seven times for a total of 21 explants per treatment. All flasks were randomized on trays and placed on a shelf in the growth chamber. Data was recorded with respect to the number and length of root after six weeks.

Figure 3.4: Uniform shoot cuttings from the multiplication trial experiments cultured in MS medium for rooting

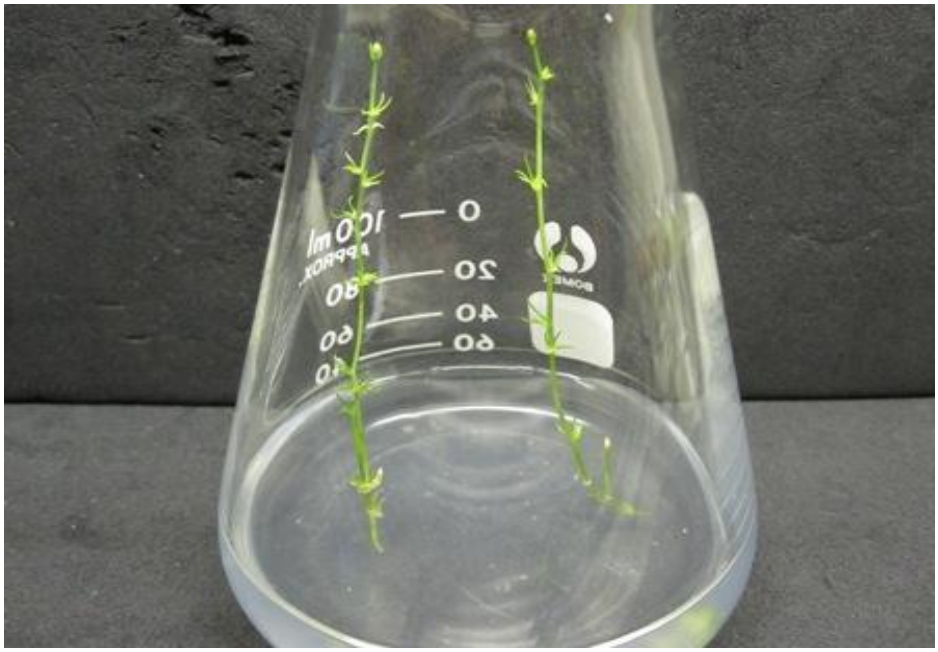

Figure 3.5: Uniform shoot cuttings from the multiplication trial experiments cultured in MS medium for rooting

3.3 Antioxidant and antibacterial activities

3.3.1 Ethanolic extract preparation

The plant samples (*in vivo* and *in vitro* grown plants as well as callus tissues) were dried in an incubator at 40 °C and then were ground to produce fine homogenous powder using an electric blender. The fine powder (3 g) was soaked in 40 ml of 95% ethanol at room temperature in the dark for three days and properly covered and labeled in a conical flask before it was filtered through Whatman® No. 1 filter paper (Whatman International, England). The filtered solution was then evaporated to dryness by placing them in a water bath at 40 °C overnight. The plant extracts (10 and 100 mg/ml) were dissolved in phosphate buffer solution (PBS) and kept at 4 °C until required for the experiments.

3.3.2 Antioxidant activity

3.3.2.1 Superoxide dismutase (SOD) assay

SOD assay kit was used to determine the superoxide dismutase (SOD) activity of prepared extracts. The procedure was carried out based on the protocol in the kit used. Plant extracts (20 µl) of the concentration of 10 mg/ml were added to 200 µl of the kit Working Solution. The mixture, after a gentle shaking was incubated at 37 °C for 20 min after adding 20 µl of the kit Enzyme Working Solution. The absorbance of the mixtures was measured at 450 nm using a microplate reader (BIO-RAD Model 550, USA) and the SOD activity was calculated using the following equation (Xing *et al.*, 2010).

Percentage of Inhibition (SOD Activity) = {[(blank 1 – blank 3) – (Sample A – Sample A's blank 2)] / (blank 1 – blank 3)} × 100

Where blank 1 was a mixture of the Working Solution (200 µl) and Enzyme Working Solution (20 µl) containing 20 µl double distilled water (ddH_2O). Blank 2 contained the plant extract (20 µl) with Working Solution (200 µl) and Dilution Buffer (20 µl) while ddH_2O (20 µl) was added to the plant extract in the blank 3. Ascorbic acid (1 mg/ml) and 1 mg/ml of Tert-butylated hydroxytoluene (BHT) were employed as the positive controls in this study.

3.3.2.2 Erythrocytes haemolysis prevention assay

This assay was based on the method described by Rafat et al. (2010a). Erythrocyte suspension was prepared by centrifugation of rabbit blood at 1000 Xg at 4 °C for 20 minutes. An equal volume of phosphate buffered saline (PBS) was then added with the

collected cells. The erythrocyte suspension (500 µl) was pre-treated using 1000 µl of the plant extract (10 mg/ml) at 37 °C for 40 min before adjusting the mixture volume to 9 ml by adding PBS. Oxidative stress was then induced by mixing 1 ml of 10 mM hydrogen peroxide (H_2O_2). Finally, the released hemoglobins from the hemolysed erythrocytes into the supernatant of the mixtures was determined at 540 nm using a spectrophotometer. Ascorbic acid (1 mg/ml) was applied as the positive control while the non-pre-treated erythrocyte was used as the negative control in this study. Complete erythrocyte hemolysis was acquired by using ultra pure water and labeled as 100% hemolysis and hemolysis of other samples were stated as a percentage of this value.

3.3.2.3 Radical scavenging capacity assay (DPPH)

This method is based on the measurement of the reducing ability of antioxidants to react with DPPH radicals. The method involves determination of a decrease in DPPH radicals. DPPH is a stable and commercially available organic radical and has an absorbance maximum centered at about 515 nm (Huang *et al*., 2005). The radical DPPH is generally used to evaluate the antioxidant properties of synthetic and natural phenols using methanol or ethanol as the most appropriate solvents (Foti *et al*., 2009) because it is more stable than superoxide and hydroxyl radicals. DPPH (2,2-diphenyl-1-picrylhydrazil) free radical scavenging capacity assay was obtained using the protocol described by Rafat *et al*., (2010b). DPPH (950 µl)a concentration of 90 µM was mixed with 50 µl of the plant extracts (10 mg/ml) and the volume was adjusted to 4 ml using 95% ethanol before incubation at room temperature in the dark for 120 min. Scavenging of DPPH reduced the colour of the solution and was measured using a spectrophotometer at 515 nm. Comparison of the reduction of colour in the examined samples with the blank (solution without plant

extract) was used to measure the potential of scavenging capacity of the plant extracts using the following equation (Rafat *et al*., 2010b).

Radical Scavenging Capacity (%) = [(Blank – Sample A) / Blank] x 100

3.3.3 Antibacterial activity assay

The antibacterial potential of *A. officinalis* ethanolic extracts was studied using the paper disc diffusion method of Kil *et al*., (2009). Two Gram-Negative pathogenic bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and two Gram-Positive pathogenic bacteria (*Staphylococcus aureus* and *Bacillus cereus*) were obtained from the Microbiology Division of Institute of Biological Sciences, University of Malaya and then grown in Nutrient Broth medium to yield a final concentration of 10^7 CFU/ml. The test bacteria (0.1 ml) were streaked on Mueller Hinton medium plates using sterile cotton swab by the following steps:

1. A sterile swab was dipped into a inoculam tube.
2. The swab was rotated against the side of the tube (above the fluid level) by using firm pressure in ordet to remove excess fluid. The swab should not be dripping wet.
3. The dried surface of a MH agar plate was inoculated by streaking the swab 3 times over the entire agar surface; the plate was rotated approximately 60 degrees each time to ensure an even distribution of the inoculum.
4. The plate was rimmed with the swab to pick up any excess liquid.
5. The swab was discarded into an appropriate container.
6. The lid slightly ajar was leaved and the plate was allowed to sit at room temperature at least 3 to 5 minutes, but no more than 15 minutes, for the surface of the agar plate to dry before proceeding to the next step.

Next step is placing the appropriate antimicrobial-impregnated disks on the surface of the agar. Sterilized filter paper discs were soaked in ethanolic extracts (100 mg/ml) and were then placed in the center of test bacteria plates. Disks should not be placed closer than 24 mm on the MH agar plate. Each disk must be pressed down with forceps to ensure complete contact with the agar surface. The plates were incubated for 24 h at 37°C and the diameters of the inhibition zones were measured. Tetracycline disc (30 µg) and PBS were used as the positive and negative controls, respectively.

3.4 Statistical analysis

All experiments were carried out in triplicate. One-way analysis of variance (ANOVA) was used to analyze the data using SPSS version 15. The means were compared with Duncan's Multiple Comparison Test (DMCT) and $p<0.05$ was considered to indicate statistical significance.