CHAPTER V

Discussion

5.1 Regeneration

The present investigation was done on an important medicinal plant, *Asparagus officinalis* with a view to develop a reliable protocol for its clonal propagation under *in vitro* conditions. Successful plant tissue culture protocol development for Asparagus can be partially measured by the ability to establish cultures free of contamination. Media composition performed, for the most part, exceptionally well in all stages of protocol development. This research strived to customize techniques, hormone concentration and pathogen control to produce healthy Asparagus clones.

The application of molecular approaches to medicinal plants would also benefit from the development of cell, tissue and organ culture systems for *in vitro* growth and regeneration of medicinal plants. In addition, such tissue culture systems could also prove useful for large-scale biotechnological production of medicinal plant phytochemicals (Briksin, 2000). Furthermore, uniform plant growth with consistent plant materials can be achieved, plants can be grown in sterile, standardized conditions and are free from biotic and abiotic contamination.

Micropropagation has been successfully used for production of many medicinal plants belonging to Liliaceae family such as *Chlorophytum arundinaceu* (Samantaray and Maiti, 2011), *Hyacinthus orientalis* L. (Salehzadeh *et al*., 2008), *Asparagus cooperi* (Ghosh and Sen, 1996). To our knowledge, this is the first work for *in vitro* culture of edible wild Asparagus in Malaysia. Indirect organogenesis was applied to obtain preliminary information on preserving and mass propagation of this plant. The data present a relatively efficient method for *in vitro* culture of this limited and valuable vegetable in Malaysia. *Asparagus officinalis* were cultured on MS medium containing different combination of BAP and NAA and also different combination of Kn and IBA for callus induction. The

basal medium without plant growth regulators was used as a control. In comparison of all treatments of two cytokinins and two auxins with control group, it was observed that the medium should be supplemented with both cytokinins and auxins hormones for callus formation of *Asparagus officinalis*. Most of the callus formation occurred on the medium with the amount of cytokinin was more than auxin. Benmoussa *et al*. (1996) reported efficient establishment of callus in *Asparagus densiflorus* cv. Sprengeri on MS medium supplemented with several auxins and cytokinins. Krishna and Sanu (2009) described that NAA at many of the concentrations either singly or in combinations with BAP or Kn is effective in the induction of callus in *Asparagus racemosus.* They reported that NAA singly showed an increase in callus induction from nodes, internodes and shoot tips of *Asparagus racemosus*.

Inagaki *et al.* (1980) reported the optimum callus formation (72%) for *Asparagus officinalis* on MS medium supplemented with 1.0 mg/l BAP and 5.0 mg/l NAA. Harada and Yakuwa (1983) observed that induction of callus from Cladophylls of *Asparagus officinalis* was hardly observed or not recognized at all with both NAA and IBA. They reported a fairly high percentage (51 to 90%) of callus induction in a combined addition of auxins (NAA or IBA) and cytokinin (BAP). They also showed that the growth of callus was better in medium with NAA than with IBA. In their experiment, no callus formation was seen in the MS medium without either auxins or cytokinins. Cytokinins as well as auxins play an important role in the callus induction in the tissue culture of Asparagus as reported by Yakuwa *et al*. (1971). In the present study also it was observed that the amount of cytokinin should be more than auxin for callus induction of *Asparagus officinalis*.

In the present study among the two groups of hormones (BAP+NAA, Kn+IBA concentrations), Kn and IBA was reported to be more efficient than BAP and NAA in promoting callus formation (Figures 4.4 and 4.5). Also, between dark and light condition,

dark condition was found to be more efficient than light condition in promoting callus growth. However the callus was formed under dark condition is yellow in color (Figure 4.2) but the callus formed under light condition is green color (Figure 4.1).

In Asparagus, multiple shoot proliferation from nodal explant taken from *in vivo* raised plants occurred on MS medium supplemented with different concentration of BAP either alone or in combination with lower concentration of NAA or MS medium supplemented with different concentration of Kn either alone or in combination with lower concentration of IBA. However, the best result was obtained on MS medium supplemented with 1.5 mg/l BAP and 0.05 mg/l NAA under dark condition (Figure 4.11). For shoot proliferation, growth regulators especially cytokinins are one of the most important factors affecting the response (Garland *et al.,* 1981). Sarabi *et al.* (2010) reported highest average number of shoot proliferation of size 3 mm or more per explant in *Asparagus officinalis* on MS medium supplemented with 0.5 mg/l BAP and 0.015 mg/l NAA after 3 months of culture under light condition. In contrast to my finding the best results with highest average number of shoot proliferation of size 4 mm or more per explant was obtained on MS medium supplemented with 1.5 mg/l BAP and 0.05 mg/l NAA after 6 weeks under dark condition. The present findings are compatible with those obtained by Sarabi *et al.,* (2010), who reported that in *Asparagus officinalis*, BAP and NAA were found to be most efficient in shoot formation. Harada and Yakuwa (1983) reported that shoots differentiated from the callus of Asparagus in the media with auxin (NAA or IBA) combined with BAP. Benmoussa *et al.* (1996) reported the effects of different concentration and combination of BAP and/or Kinetin on shoot regeneration of *Asparagus densiflorus*. In their report Kinetin was found to be less effective than BAP in the initiation of shoots. They reported that the high numbers of shoots were produced in the presence of 0.4 µM BAP alone. Mehta and

Subramanian (2005) reported a very high rate of multiple shoots from nodes of *Asparagus adscendens* using 0.27 μM NAA and 0.46 μM Kinitin.

Krishna and Sanu (2009) reported that NAA and BAP singly play a good role in shoot induction. They also observed that combinations of NAA and BAP at various levels are effective in shoot induction of *Asparagus racemosus.* This observation is in agreement with the present finding that MS medium supplemented with BAP and NAA is more effective on shoot induction of Asparagus.

Several authors have pointed out that the major obstacle of Asparagus micropropagation protocol is not the establishment of shoot culture and shoot multiplication, but difficult root initiation.

Induction of roots at the base of *in vitro* grown shoots is essential and indispensable step to establish tissue culture derived plantlets to the soil. Chin (1982) reported considerable improvement of shoot and root development in *Asparagus officinalis* with ancymidol. The most efficient auxins for rooting are IBA and NAA (Uddin *et al*., 2005). Kar and Sen (1985) reported that lower concentration of IBA added to the MS medium failed to induce roots in *Asparagus racemosus*. This was true of *Asparagus adscendens*, where IBA did not induce rhizogenesis. Sarabi *et al.,* (2010), reported root initiation in *in vitro* regenerated shoots of *Asparagus officinalis* on MS supplemented with 1.5 mg/l of IBA. Similarly, in the present study, root formation from *in vitro* regenerated shoots of *Asparagus officinalis* occurred on MS supplemented with IBA (Figure 4.15) but in low concentration (0.4 mg/l). These results confirmed that some plant species have enough levels of endogenous hormones and do not require a high level of exogenous growth regulators for plant regeneration (Hussey, 1982).

Afroz et al. (2010) observed induction of roots from regenerated shoots of *Asparagus racemosus* (2-3 roots/shoots) when cultured on half MS with 0.05 mg/l BAP and 1.0 mg/l

IBA. In comparison with the present study induction of roots from regenerated shoots (2 roots/shoot) of *Asparagus officinalis* were observed on MS medium supplemented with 0.4 mg/l IBA alone. Harada and Yakuwa (1983) reported root formation of *Asparagus officinalis* on media supplemented with NAA combined with BAP and also on media supplemented with IBA combined with BAP. They reported that in the case of using NAA as auxin, short and semitransparent roots were formed on the surface of the callus clumps, but when IBA was used, the long and opaque roots, which were akin to a normal root with a physiological function were obtained in higher frequency. This observation is also in agreement with the present study that IBA is more effective on root formation of Asparagus.

The results can be explained on the basis that different species of plants and even different organs of the same plant are characterized not only by their unique intrinsic biochemical make-up but also by the sensitivity of the endogenously supplied chemical stimuli.

5.2 Antioxidant and Antibacterial activities

The present study is in agreement with the previous reports on high antioxidant activity of *A. officinalis* (Makris and Rossiter, 2001; Rodríguez *et al*., 2005; Sun *et al*., 2007). Antioxidant activities of the methanol, acetone and water extracts of *A. officinalis* were examined by Sun *et al*. (2007) and acetone extract showed the highest antioxidant capacity while water extract obtained the lowest activity. The high solubility of antioxidant compounds in ethanol in different plants, such as *DOIspyros kaki* cv. Fuyu (Jang *et al*., 2010) and *Cajanus cajan* (L.) Millsp (Wu *et al*., 2009), was reported. Rodríguez et al. (2005) used ethanol to extract the antioxidant compounds from different cultivars of *A. officinalis*. The results of this study also showed that ethanol can be a suitable solvent to extract the antioxidants of *A. officinalis*. In terms of antimicrobial activity of the plant extracts, Jana and Shekhawat (2010) reported that ethanol extract of different parts of *Anethum graveolens* showed higher antimicrobial activity than aqueous extracts of the plant parts. Among the five solvents (water, methanol, ethanol, diethyl ether, acetone) used to extract *Gevuina avellana* hulls, ethanol and methanol extracts showed the highest antioxidant activity while the water extract had the lowest antioxidant activity determined by DPPH method (Moure *et al*., 2000). Buckwheat was extracted using methanol, acetone, butanol, ethanol, and ethyl acetate (Sun & Ho, 2005). The methanol extract showed the highest antioxidant activity tested by the b-carotene bleaching method and the acetone extract showed the highest antioxidant activity determined by the DPPH method. Thus, the properties of the extracting solvents will significantly affect the antioxidant activity of the extracts.

The major flavonoid antioxidant in Asparagus has been reported to be rutin (Tsushida *et al.,* 1994), with the content of 286.5 ± 6.0 mg/kg fresh weight (Makris & Rossiter, 2001).

Recently, it has been appreciated that there is no simple universal method by which antioxidant activity can be measured accurately and quantitatively (Prior *et al*., 2005). Since application of only one antioxidant activity evaluation assay may not give a reliable result, using a few methods is recommended (Rafat *et al*., 2010). Hence, three different techniques for investigation of antioxidant activity in the present study were employed. The results showed that all applied assays are in agreement that the antioxidant capacity of *in vivo* grown *A. officinalis* was higher than both *in vitro* grown plant and callus tissues (Figures 4.16, 4.17 and 4.18). Perhaps these results show that the amount of antioxidants produced in *in vivo* grown *A. officinalis* is higher than the *in vivo* grown ones. This result was confirmed by the work of Tanwer *et al*. (2010) In their study they showed that antioxidant activity of methanolic extract of the *in vivo* grown *Spilanthes acemella* was higher than the *in vitro* grown callus. In the present study the lowest antioxidant potential obtained from all the three tests belonged to the callus extract but it was not significantly different from *in vitro* plant extract in SOD and erythrocyte haemolysis assays (Figure 4.16).

Poyrazoğlu *et al.* (2009) studied the antimicrobial activity of different extracts of *A. officinalis* against some microorganisms and showed that ethanolic extract of the plant could only inhibit the growth of *Pseudomonas fluorescens* among all the examined yeasts and bacteria. However, the results of this study showed that the callus extract used can inhibit the growth of only one Gram-positive test bacteria (*B. cereus*) (Table 4.1). Based on the statistical analysis, this inhibition is significantly smaller than positive control (30μM of tetracycline). The other *in vitro* and *in vivo* grown *A. officinalis* extracts showed no antimicrobial activity against both groups of Gram-negative and Gram-positive bacteria. The antibacterial activity was more pronounced on the Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus cereus*) than the Gram-negative bacteria (*Escherichia*

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coli and *Pseudomonas aeruginosa*). The reason for the difference in sensitivity between Gram-positive and Gram-negative bacteria might be ascribed to the differences in morphological constitutions between these microorganisms, Gram-negative bacteria having an outer phospholipidic membrane carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to antimicrobial chemical substances. The Gram-positive bacteria on the other hand are more susceptible having only an outer peptidoglycan layer which is not an effective permeability barrier. Therefore, the cell walls of Gram-negative organisms which are more complex than the Gram-positive ones act as a diffusional barrier and making them less susceptible to the antimicrobial agents than are Gram-positive bacteria (Nostro et al., 2000; Hodges, 2002).

The results of well diffusion antimicrobial activity carried out by Jana and Shekhawat (2010) also showed that the ethanolic extract of *A. graveolens* callus inhibited the growth of *Bacillus subtilis* more than the ethanolic extract of the plant roots. The antibacterial activity of the callus extract might be either related to the production of a compound produced in only undifferentiated callus cells or may be produced in higher amounts in these cells compared to differentiated cells. Several quantitative estimations and qualitative studies showed the production of biocompounds can be varied between differentiated and undifferentiated plant cells. For example, Tanwer *et al*. (2010) reported that calli of *Spilanthes acemella* produced a higher amount of sugars compared with stem, leaves and roots of the plant. Jana and Shekhawat (2010) also showed that the callus cells of *A. graveolens* produce saponins while the *in vitro* leaf cells were not able to produce the same compound.

CHAPTER VI

Conclusion Remarks and

recommendations

6. Conclusion remarks and recommendation

In vitro regeneration from nodal explants could be used as an alternative source of raw materials to meet the ever increasing demands of the pharmaceutical industries as clonally propagated plants would also have identical photochemical profiles. The method developed for rapid shoot multiplication of Asparagus is reliable and definitely a promising one for this valuable medicinal plant. For micropropagation studies, various hormones such as BAP, NAA, Kn and IBA were used to obtain plant regeneration and callus formation. The protocol described here may be useful for commercial purposes as it reduced time on callus formation, shoot regeneration and root development at a faster rate in a shorter period. Furthermore, for the potential use of any *in vitro* regenerated plantlets for micropropagation, it is essential to confirm their genetic stability. Random amplified polymorphic DNA (RAPD) analysis and chromosome counting are two important tools that are currently being used on regenerated plantlets to test if they are true to type. The results are valuable for medicinal purposes in the industrial field. Through tissue culture or *in vitro* system (micropropagation) can increase the amount of essential compounds which have antibacterial and antioxidant properties in shorter time compared to the *in vivo* system and consequently save time and are economical in the industry.

This study indicated the antioxidant and antimicrobial activities of *A. officinalis* and also showed that these bioactivities differ between *in vitro* and *in vivo* grown plants. The ethanolic extract from callus, *in vitro* and *in vivo* plants were used to test antioxidant and antibacterial activities of *Asparagus officinalis*. Total antioxidant capacity of *in vivo* grown plant was higher than *in vitro* grown plant while the only antimicrobial activity was obtained from *in vitro* callus tissue against *B. cereus*. Some phytochemical studies are

required to investigate the production of antioxidant and antimicrobial compounds in differentiated and undifferentiated callus cells of *A. officinalis*.