DEVELOPMENT OF STERILISATION PROCEDURES AND IN VITRO STUDIES OF *Nymphaea lotus*

ELIXON SUNIAN@ELIXSON BIN SULAIMAN

Thesis Submitted to the School of Graduate Studies, University Putra Malaysia in Fulfilment of the Requirements for the Degree of Master of Science.

October 2004

BIODATA OF THE AUTHOR

The author was born in Kg. Masolog, Kota Marudu, Sabah on 19th May 1976. He attended his primary school at Sekolah Rendah Kebangsaan Masolog, Kg Masolog Kota Marudu, Sabah (1983-1988). He continued his Junior High School at Sekolah Menengah Kebangsaan Tandek, Kota Marudu (1988-1991), and completed his Senior High School at Sekolah Menengah Kebangsaan Kota Marudu, Sabah (1989-1995). In 1996, he pursued his Bachelor of Bioindustry Science at the Faculty of Agriculture, Universiti Putra Malaysia, Serdang, Selangor and graduated in 1999. After graduation, he was accepted as a research assistant at the Department of Agro-Technology, Faculty of Agriculture, Universiti Putra Malaysia, to work on tissue culture of water lily under the supervision of Assoc. Prof. Dr. Mohd. Salleh bin Kamarudin for 8 months. In May 2000, he enrolled as a full time Master Degree candidate at Universiti Putra Malaysia.

CHAPTER 1

INTRODUCTION

Background

Water lilies (*Nymphaea* sp.) are one of the most valuable aquatic ornamental plants which have a bright potential to be a multimillion-dollar commodity in the floriculture trade.   They could be exploited as cut flowers (Master, 1974), ornamental plants for water garden, urban landscape for aquascaping and restoration projects (Kane and Philman, 1992) and sources for pharmaceutical and cosmetic products (Perry, 1987).

Water lily family consists of approximately 50-60 species found in tropical to cold temperate region (Halijah, 2000).   Flowers of water lilies are available in many colours including red, pink, yellow and white.   Water lilies are not only beautiful but they are also useful in creating a balanced environment in ponds or lakes as well as improving the water quality.   They function as surface vegetation in controlling the amount of direct sunlight that penetrates the water surface, thus, stabilising the water temperature particularly during dry season, and also control the algal growth from over blooming which causes the green water problem (Dawes, 1989).   In Putrajaya Wetland Garden in Malaysia, water lilies are planted in open water as ornamental plants (Radiah, 2000).

Many years ago, Europeans, Asians and Africans consumed the seeds and tubers of water lilies as food in time of emergency. *Nymphaea alba* at one time was used by the French in the preparation of beer, while Irish and Scottish highlanders used it as a source of dye for dyeing wool (Perry, 1971). During the Egyptian civilization (approximately 4000 B.C), water lilies were used in religious ceremonies (Perry, 1987).

Conventionally, water lilies are propagated vegetatively through tuber production or from new plants sprouting from the underground rhizomatous stem. However, such propagation methods are restricted due to the slow and limited number of plants produced, diseases, large propagation space needed and an extended period to produce saleable plants. These factors contribute to a high production cost (Kelly and Fret, 1986) and often prevent an efficient and rapid production of planting materials to meet the market demand (Kane, 1991).

Significance of tissue culture method for water lily propagation.

Apart from the conventional method of propagation, an efficient approach is needed for rapid and mass multiplication of planting materials of water lilies. At present, no biotechnological method is available that could be adopted by propagators to overcome the current barrier in water lilies propagation. A useful approach is through the application of tissue culture technique and the use of plant growth regulators (Defeo, 1987; Jenks, *et al.,* 1990; Kane *et al*., 1991). The use of cytokinins in *in vitro* culture breaks up apical dominance and enhances development of lateral buds into high number of proliferated shoots. Besides mass production of planting materials within a short period, the *in vitro* technique increases the

availability of disease free stock plants and reduces culture space requirement as well as lowers the cost of production. Once the appropriate medium, type and concentration of cytokinins for shoot proliferation are defined, shoots can be rooted *in vitro* on rooting medium supplemented with auxins prior to acclimatisation and transfer of plantlets to the glasshouse. Growth regulators are also beneficial in *in vitro* microrhizomal growth and development of water lilies for efficient production of planting materials. *In vitro* microrhizomes production have many advantages compared to *in vitro* plantlet regeneration. Microrhizomes can be planted directly in the soil without acclimatisation and convenient to be transported and could facilitate the national germplasm exchange better than *in vitro* plantlets (Mrudul *et al.,* 2001). The microrhizomal growth and development of *Nymphaea* sp. using *in vitro* methods have not been reported by researchers. Microrhizomal formation has been documented in *Nelumbo* species (Kane *et al.,* 1990). Kane *et al.* (1990) have proposed that this technique could be efficient for the production of *Nymphaea* planting materials.

The application of tissue culture method for *Nymphaea* sp. offers not only a rapid and mass multiplication of the existing germplasm stock but is also beneficial for the conservation of important, exotic, rare and endangered species which may be useful for future genetic manipulation/engineering programmes. The technique is also valuable for long-term storage of elite plants and for the clonal production of disease free plants. Such advances have been reported in several species of aquatic ornamental plants such as *Nelumbo* sp. (Kane *et al.*, 1990), *Nymphaea* sp. (Jenks *et al.*, 1990; Lakshmanan, 1994), *Myriophyllum* sp. (Kane *et al.*, 1991) and *Cryptocoryne* sp. (Kane *et al.*, 1999).

However, the establishment of an aseptic culture of *Nymphaea* sp. is usually hampered by frequent occurrence of microbial contamination. Being in an aquatic environment, *Nymphaea* is easily exposed and contaminated by large population of aquatic microbes. Swindells (1990) had attempted to establish an *in vitro* culture of *Nymphaea* sp. using the rhizome bud, leaf blade and petiole segment but faced with an extremely high rate of microbial contamination. Kane *et al*. (1990) had reported the first successful *in vitro* establishment of water lily but obtained a low rate of sterile culture and shoot branching. An efficient sterilisation technique for *in vitro* culture of *Nymphaea* sp is therefore critically needed. Once a contamination-free culture is established, plant growth regulators requirement at the different stages of micropropagation could be investigated.

Objectives

This research was undertaken with two main objectives:

1)   To develop an efficient sterilisation procedure for *in vitro* culture of water lily (*Nymphaea lotus*).

2)   To evaluate the *in vitro* responses of water lily to growth regulators (cytokinin and auxins) and nutrient salt strength with respect to shoot formation and adventitious root formation and microrhizome growth and development.

CHAPTER 2

LITERATURE REVIEW

Taxonomy and morphology of water lily

Water lilies (*Nymphaea* sp.) are grouped under the division Spermatophyta in the sub-division Angiospermae. They are classified as Dicotyledonous plants since their sprouting seeds received nourishment through two large leaf-like cotyledons. *Nymphaea* (including *Nymphaea* sp., *Numphar* sp. and *Nelumbo* sp.) belongs to the Nymphaeaceae family. *Nymphaea sp*. is considered as one of the most primitive flowering plants. Some botanists consider water lily or *Nymphaea* sp. as a link between the flowering plants and cycads. The latter was dominant on earth 75-100 million years ago (Master, 1974).

Naturally, water lilies have a short and stout rhizome that bears a rosette of several leaves. The flowers may be floating or growing on short stalks close to the water surface (Plate 1). The flowers range from white, pink, blue, purple and yellow in colour and most of them are aromatic and scented (Halijah, 2000). The flowers are bisexual and regular and have four sepals and many petals arranged spirally and with many stamens (Plate 2). The flowers have various shapes and sizes. Some of them are beautifully cup-shaped, some are horizontally open like a bowl while others are star shaped with large petals tapering to a point (Master, 1974; Perry, 1987; Thomas, 1988).

Plate 1:   The flowers of water lily (*Nymphaea lotus*) floating or growing on short stalks close to the water surface.

Plate 2:   T..lour and
                  st............

Under native conditions, water lilies are characterised by their partially submerged leaves and their laminae are normally held on long petiole and floating on the water surface. The leaves are mostly orbicular in shape with a cleft almost to the centre where a petiole is attached. In others, the leaves are oval, cordate or ovate in shape depending on the species (Master, 1974; Lavid, *et al.*, 2001).

Types of water lily

Water lilies include tropical and hardy types. Typically, tropical water lilies require more space because they produce larger flower and leaves. Tropical water lilies usually hold their blooms on stiff, strong stems above the water surface. Water lilies are subdivided into day bloomers and night bloomers. Tropical day bloomers usually open their flowers in the morning and close in the late afternoon. Day bloomers practically have a fine fragrance. Tropical night bloomers open their flowers around dusk, and close around noon the following day (Thomas, 1988). Many beautiful day bloomer varieties are available such as the blue water lilies (Blue Beauty, Charles Thomas and Daubeniana), and pink water lilies (Evelyn Randig, General Pershing and Enchantment). Examples of night bloomer varieties are red water lilies (Antares, Maroon Beauty and Red Flare), pink water lilies (Emily Grant Hutchings) and white water lilies (Bali Night, Missouri, and Trudy Slocum) (IWGS, 2000).

Hardy water lilies are perennials. They open their flowers in the morning until mid or late afternoon. Hardy blossoms are quite small compared to tropical bloomers. They do not bloom as abundantly as the tropical types and do not hold their flowers high over the water surface. Some examples of the hardy varieties are orange water lilies (Carolina Sunset, Florida Sunset, Peaches and Cream), and yellow water lilies (Colonel A.J. Welch and Gold Medal) (IWGS, 2000).

Propagation of water lily

There are three conventional methods of propagating water lilies which are from seed, plants arising from mature floating leaves and tuber or rhizome division.

Seed production is slow, painstaking and rarely practiced. The plants produced are not always true-to-type and non-uniform in maturation (Master, 1974; Squire, 1995).

Certain water lilies produce miniature plants directly from the mature floating leaf. Small dark coloured swelling usually develops in area where the petiole joins the lamina between the leaf lobes (sinus). After a few days, this swelling seems to break open and rudimentary leaves which may be perfectly proportionate begin to form and develops a small tuber and root system on the under side of the mature leaf. Mostly, only tropical day bloomers have this viviparity characteristic (Thomas, 1988).

Most nurseries or water lilies propagators practiced the vegetative division of tubers or rhizome as a method of propagating water lilies. The tubers or underground stem (rhizome) of water lilies are extremely tough and capable of surviving in many adverse conditions. The tubers of hardy types are irregularly cylindrical whereas the tropical ones are large and rounded in shape. The tropical water lilies can grow in dense patches and can spread to cover a huge area displacing, shading and excluding other species. One planted rhizome can cover an area of about 4.6m diameter in about five years. For this reason, planting them in lakes is not recommended (IWGS, 2000).

Tissue culture of water lily (*Nymphaea* sp.) and other aquatic plants.

Tissue culture method is being used to propagate horticultural crops in hundreds of small or large-scale nurseries and laboratories throughout the world. Millions of plants especially ornamental plants have been produced and sold locally or exported to other countries using this method. The technique is also widely

exploited for agricultural/plantation crops, medicinal and aromatic plants, woody

plants and a numbers of aquatic plants (Huang and Tan, 1988; Skene and Barlass,

1989; Kane, 1991; Everitt and Lockwood, 1995; Jenks *et al*., 2000).

Recently, there is a significant increase in the market demand for native
herbaceous and aquatic ornamental plants. In Florida, many of the species are
planted as ornamental plants, for marginal plants in aquascaping and for re-
vegetation of the wetland restoration project (Brown, 1988). This leads to the
requirement of high number of planting materials (Kane and Philman, 1992).
According to Pategas (1992), the increasing market demand for aquatic plants have
resulted from the expansion of wetland plant industry in Florida.

The domestic market has also a bright future for water lily as well as the

other ornamental aquatic plants due to the development of many recreational centres

such as wetland areas, botanical gardens, agro tourisms, resorts and golf courses.

Recently, housing development projects particularly in the Klang Valley, Selangor

have rapidly grew. To remain competitive, many housing developers have become

interested in constructing water garden features as a concept in their housing

landscape to attract customers to their projects. Aquatic plants such as water lily are

likely to be the choice to decorate their gardens and this trend will benefit nurseries

to sell their products.

Reports on tissue culture of *Nymphaea* sp. are very few. Jenks *et al*. (1990)

reported that thidiazuron (TDZ) can be used to stimulate growth and development of

epiphyllous buds of water lily *Nymphaea* 'Daubenia'. The epiphyllous buds

produced shoots after 5 weeks of culture on a polypropylene membrane raft

containing 80 ml liquid MS medium supplemented with 3.0 μM TDZ.

Lakshmanan (1994) reported that a combination of 4.0 μM naphthalene acetic acid (NAA), 11.1 μM benzylaminopurine (BAP) and 32.0 μM N^6-2-isopentelnyl adenine (2iP) in MS medium yielded high shoot proliferation as well as satisfactory shoot elongation of *Nymphaea* hybrid James Brydon. Pre-treatment of the shoot with gibberellic acid (GA_3) did not improve shoot multiplication. A further 45 days culture of the shoot on MS basal medium containing 0.5 mg/L activated charcoal produced an average of 3 roots per shoot and 7.4 cm root length.

Kane (1991) had attempted to regenerate hardy water lily *Nymphaea* 'Attraction' through adventitious shoot formation from non-meristematic tissues such as leaf and floral bud tissues. Explants derived from mature leaves showed no morphogenic response when cultured on MS medium supplemented with various TDZ concentrations. Callus formation was observed by culturing unopened flower bud explants (stigmatic lobe and ovary) from the same cultivar on MS medium supplemented with 1.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 1.0 mg/L 2,4-D + 0.5 mg/L 6-benzyladenine (BA). However, the rate of microbial contamination from such culture was very high.

The seed production in most *Cryptocoryne* sp. is limited and vegetative propagation by rhizome division is also extremely slow (Rataj and Horeman, 1977). The application of tissue culture technique has significantly improved the vegetative propagation of the species. *In vitro* shoot proliferation of *Cryptocoryne lucens* could be enhanced by the addition of BA (Kane *et al.*, 1990). Maximum shoot proliferation occurred from single and triple-node explants after 35 days of culture on LS medium supplemented with 20 μM BA. The most efficient shoot

proliferation of *C. lucens* (7.7 mean number of shoots per explant) occurred from single node explant on medium with combination of 20 μM BA and 0.5 μM NAA. From 20 single node explants per vessel, almost 150 shoots per vessel can be generated per 35 days culture cycle. Maximum plantlet establishment could be achieved by directly sticking triple-nod microcutting in either soilless polyurethane foam cubes and 4-cell packs containing Vergro.

The different types of culture vessel used can effect *in vitro* shoot proliferation due to differences in gas exchange between the culture vessels and external environment (Jackson *et al.,* 1987). A larger culture vessel gives better shoot multiplication, shoot length and fresh weight (Monette, 1983; Mackay and Kitto, 1988; McClelland and Smith, 1990). In contrast, Kane and Philman (1992) reported that the type of culture vessel used does not significantly affect the quality and number of shoots produced per explant of *Pontederia Cordota* L However, maximum shoot production per vessel could be obtained using a square GA-7 vessel compared to glass culture tubes or a glass baby food jar.

A rapid *in vitro* shoot multiplication using lateral bud explants *of Anubias barteri* Var. *undulata* could be attained on MS medium supplemented with 0.3 mg/L BA, 0.01 mg/L thidiazuron and 0.1 mg/L NAA (Li-Chun, *et al*. 1994). Reculturing the shoot on fresh medium of the same composition produced more shoot clusters and longer shoot length. After six subcultures, the proliferation rate could be increased by five-folds. After a month of culturing them in 100 mL flask containing MS medium supplemented with 0.1 mg/L NAA, rooted shoots were produced.

In vitro culture technique has been used in many ornamental plants to induce random genetic variation through mutation breeding (Boertjes and Van Harten, 1978; Ibrahim et al., 1998). In vitro method could also be adopted as a useful tool for mutation breeding studies in aquatic plant species for the production of new varieties. Induction of direct or indirect shoot organogenesis has been reported in several aquatic plant species (Rao and Mohan Ram, 1981; Kakkar and Mohan Ram, 1986; Kane and Albert, 1989). Jenks et al. (2000) have established a protocol for rapid shoot organogenesis of ornamental aquatic plant Nymphoides indica. Maximum shoot regeneration frequency (80%) occurred on petiole explants placed on MS basal medium supplemented with 10.0 μM BA and 20.0 μM indoleacetic acid (IAA).

Kane et al. (1988) has also demonstrated direct adventitious bud formation of parrot-feather (Myriophylum aquaticum) from stem internode explant placed on MS medium supplemented with 10.0 μM 2iP after 7 days of culture. In another work, Kane et al. (1991) observed maximum adventitious shoot formation (28 shoots per internode) from the same species on MS basal medium supplemented with 40.0 μM 2iP.

Microbial contamination in tissue culture

Total removal of microorganisms from an explant surface is a prerequisite in a tissue culture propagation programme. To survive and grow properly, in vitro plant cultures need to be free of fungal or bacterial infections.

Basically, the laboratory and the explant itself are two sources of microbial contamination (Cassells, 1991). Eplants should be taken from healthy grown mother stock plants and should be well sterilised before culturing them in vitro. The main

microorganisms associated with plant surfaces are fungi (including yeast), bacteria and mollicutes which need to be discarded before aseptic excision of the tissues (Bove, 1988). Microorganisms inhabiting the plant surface deprive nutrients from the plant particularly in areas with abundant of nutrients such as the nectars, wounded parts, and in senescent tissues that are likely to support high population of microorganisms (Preece, 1988). Thus, very careful attention should be given to explant selection to avoid microbial contamination. Beside stage 0 (explant sterilisation) which aims at reducing the donor plant microbial contamination, strategies for collecting explants from plants in the field have been proposed (George and Sherrington, 1984; Duhem *et al*., 1988; Enjalric *et al*., 1988). Once a clean culture is obtained, new contamination may arise which normally comes from the insufficiently clean laboratory system and poor individual skill (Cassells, 1988).

The degree of contamination varies among species and explants segment being used. The use of explant parts from roots and underground portion such as bulbs, rhizomes and tubers, or plants grown in high humidity has high probability of being contaminated by microorganism (Hartmann, *et al*., 1994). Most research and commercial tissue culture laboratories have been using disinfectant solutions for sterilisation of explants. Common disinfectant solution used includes mercuric chloride, sodium hypochlorite, bromine water and silver nitrate (Torres, 1989).

Control of contamination

Reduction of contamination must begin with the donor plant that is used as a source of explants. Screening donor plant from microbial contamination during stage 0 should be a prerequisite. Internal structures such as growing points of bud, seed and fruits are relatively free from contamination. However if a plant grows in a humid environment, mycelia may invade the plant internally and becomes a persistent problem. In general, stock plants grown in containers in a protected environment (glasshouse) are cleaner than those grown out-door (Hartmann *et al*., 1994).

Contamination is not always seen at the establishment stage of *in vitro* culture. Some of the explants may be contaminated internally but appear later after several subcultures in multiplication stage (Thorpe and Harry, 1990). Application of disinfectant solution might be insufficient. In this case, an application of antibiotic may be needed to eliminate persistent microbial infection (Kneifel and Leonhardt, 1992; Barbara *et al*., 1998; Kritzinger *et al.,* 1998). The effectiveness of an antibiotic depends on the accuracy of identification of a target organism. This will allow the selection of suitable antibiotic and determination of the minimum inhibitory concentration of antibiotics to avoid toxicity or mutagenesis (Moutschen, 1985). An application of one type of antibiotic is effective if only one type of contaminant is involved in plant tissue. If more than one species of contaminants are detected, it may be necessary to use a mixture of different antibiotics. According to Barbara *et al*. (1998), an internal bacterial contamination in hazelnut (*Corylus avellana* L and *C. contorta*) could only be eliminated with a combination of timetin, gentamycin and streptomycin.

Factors affecting *in vitro* shoot proliferation.

The effectiveness of *in vitro* method in improving vegetative propagation coupled with a built-in disease protection makes it better alternative to conventional techniques for many plant species. Several *in vitro* procedures have been developed for clonal multiplication of plants which include isolation of axillary buds and terminal buds, induction of adventitious shoots and somatic embryogenesis. Many ornamental plants and woody species are being commercially propagated by axillary bud proliferation (Mantell *et al.,* 1985; Pierik, 1987; Chu, 1992). Shoots can be multiplied rapidly and considerable savings in time and culture space are made compared to conventional methods. Axillary bud proliferation typically gives an average of ten-fold increase in shoot number per monthly culture passage. In a period of 6 months, it is feasible to obtain as many as 1,000, 000 propagules from a single explant (Chu, 1992).

Axillary shoots refer to shoots that emerged from their normal position on the plant in the leaf axils. Multiplication through isolation of axillary buds is the slowest method compared to induction of somatic embryo cells. However, this method is the most commonly used method in *in vitro* multiplication of propagules (Torres, 1989) and plants arising through this method are usually true-to-type and genetically stable compared to those arising adventitiously from disorganised callus (George and Sherrington, 1984; Chu, 1992).

a. Cytokinin

An axillary shoot, depending on the physiological state of the plant, contains a quiescent or active meristem. Leaf axils in vascular plants contain a subsidiary meristem which is capable of growing into a shoot that is genetically identical to the

main axis. However, only a limited number of axillary meristems develop and the

majority of them are inhibited by apical dominance. The capability to produce high

number of axillary bud depends on the cytokinin supply to the meristem. Normally,

an axillary bud cultured on a basal medium with the absence of cytokinin will

develop into a single shoot with a strong apical dominance. When the shoots is

supplied with an exogenous cytokinin, the axillary shoot shows an ability to break

the apical dominance and enhanced development of lateral bud into a high number

of proliferated shoots (Rup and Sukanya, 1990).

The technique of axillary proliferation is applicable to any plant that produces a regular axillary shoot and responds to plant growth regulators. Both auxins and cytokinins are absolutely required in the culture medium in order to induce shoot proliferation. The requirement for hormone varies within species and the ratio of cytokinins and auxins must be determined for each one. Cytokinins often incorporated in medium include 6-benzyladenine (BA), N^6-2-isopentelnyladenine (2iP), 6-4-hydroxy-3-methyl-but-2-enylaminopurine (Zeatin) and 6-furfurylaminopurine (kinetin) and the auxins are indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and α-naphthaleneacetic acid (NAA) (Hughes, 1981). Exogenous auxin does not promote axillary shoot proliferation. However, their presence in the culture medium may improve the culture growth. In many cases, too high concentrations of auxin not only inhibit the axillary branching (Hasegawa, 1980), but also induce callus formation especially when 2,4-dichlorophenoxyacetic acid (2,4-D) is incorporated in the medium.

A medium containing two different cytokinins may alter the number and

quality of shoots formed as compared to a medium with only a cytokinin. The

addition of BA and TDZ in a basal medium formed more axillary shoots among

Vitis rotundifolia Michx than a medium with only a cytokinin (Sudarsono and

Goldy, 1991). A medium supplemented with a combination of two types of

cytokinins also produced longer axillary shoots among *Tilia cordota* Mill, *Sorbus*

aucuparia L. and *Robinia pseudoacacia* L. (Chalupa, 1987).

b. Ethylene

Ethylene has been found to either promote or inhibit shoot multiplication in various plant species. In *Prunus persica* x *P. amygdalus,* incorporation of ethylene concentration of 0.1 and 1.0 ppm for 2 weeks produced more shoots compared to those exposed for 3 or 4 weeks. A treatment without ethylene did not produce any shoot. The highest number of shoots could be obtained in cultures maintained for 2 weeks at 0.1 ppm of ethylene. At a high ethylene concentration (10.0 ppm), leaf clorosis occurred and half of the cultures showed rosette-like appearance while the others dried and died (Kortessa and Athanasios, 1995).

c. Culture vessel

Monette (1983) reported that the size of culture vessel also influence shoot production in a liquid medium. More and longer shoots were produced when *Vitis vinifera* L. cv. Liberger was cultured in a larger 473 mL Mason jar when compared to 125 and 250 mL Erlenmeyer flasks. Similar increases in shoot multiplication, shoot length and fresh weight have also been reported in other species cultured in larger vessels (Mackay and Kitto, 1988; McCleland and Smith, 1990).

d. Genotype

The genotype of explants chosen for propagation may influence the response *in vitro*. Within a species, some genotypes appear to propagate easily while others fail to respond. In *Populus deltiodes*, high production of axillary shoots was obtained with genotype 175 while lowest number of axillary shoot production was observed in genotype 10 (Coleman and Ernest, 1990). This shows that each

genotype has different physiological requirement for maximum shoot proliferation and growth. Earlier studies with *Populus deltoides* demonstrated a significant genotype effect on adventitious shoot regeneration (Coleman and Earnest, 1989) and shoot proliferation (Rutledge and Douglas, 1988).

e. Mineral elements and organic compound

Under *in vitro* condition, plant growth depends upon mineral elements and organic components incorporated in the medium due to low level of photosynthesis and small leaf area of the *in vitro* shoot. Hence, the choice of mineral and organic supplement to be incorporated in a medium is very important (Lumsden *et al.*, 1990). Any deficiency or excess of any minerals and macronutrients in the medium may induce abnormalities in plant growth, vitrification and other undesirable effects. Therefore, the determination of essential elements in the nutrition for *in vitro* plant growth and their optimal concentrations for specific stages or culturing system is prime importance. According to Singha *et al.* (1987), a standard Murashige and Skoog (1962) medium shows deficiency of macro elements for pear and crab apple. Phosphorus content in the MS medium is found to be insufficient for long term culturing of *Actinidia deliciosa* (Mezzetti *et al.*, 1991). Anderson medium (Anderson, 1980) produced more shoot multiplication of red raspberry compared to Murashige and Skoog (1962) medium. The latter medium contains approximately ¼ strength of inorganic NH_4NO_3 of Anderson medium. Chee and Pool (1987) noted an improvement in shoot multiplication of *Vitis* sp. when the concentration of chlorine, iodide and manganese in the original MS medium were reduced.

Adventitious root formation.

Roots that arise from sites other than the normal site in the embryo or the primary root are defined as adventitious roots (Lovell and White, 1986). The word 'adventitious' is derived from the Latin word 'adventicious' which means foreign. Thus roots that arise on stems and leaves are termed as adventitious roots (Debra, 1994).

Generally, microshoots respond to root formation by the presence of auxin in the culture medium. However, the reaction depends on the plant material, its ontogenetic age, the nature of auxin and the time of auxin application (Nemeth, 1986; Gaspar and Coumans, 1987). Auxins such as IAA, IBA and NAA are often incorporated in the medium to induce rooting (George and Sherrington, 1984; Torres, 1989). 2,4-D is rarely used as it is not easily metabolised by the plant and is less active except for monocot species (Robert *et al.*, 1987). In *Draceana fragran*, the presence of 0.5 mg/L IBA is found to increase the number of roots per shoot and root length (Vinterhalter, 1989). However, when too low concentrations of auxins are added to the culture medium, rooting is not stimulated and growth of shoots inhibited (Moncousin, 1982; Jarvis *et al.,* 1983). Reducing the cytokinin concentration from 264.0 to 22.0 µM before a shoot is transferred to a rooting medium can be also beneficial to root development in *Sassafras* (Wang and Hu, 1984).

Reducing the concentration of macro and micronutrients to half of their normal concentrations during the rooting phase is beneficial for root induction. This practice has been applied for herbaceous plants (Welander, 1979; Maene and Debergh, 1985), woody ornamentals (Hyndman *et al*., 1982; Maene and Debergh, 1985), fruit trees (Simmonds, 1983; Broome and Zimmerman, 1984; Zimmerman, 1984) and forestry trees (Chalupa, 1977; Coleman and Thorpe, 1977; Hammerschlag, 1982; Patel *et al*., 1986). Morte and Honrubia (1997) showed that the rooting percentage of *Helianthemum almeriense* was improved up to 92.0% in the absence of auxins and the MS macronutrients diluted to one quarter or half strength. Similarly, the dilution of macronutrient concentrations improves rooting of explants of *Cistus x purpureus* (M'Kada *et al.,* 1991).

The addition of sucrose in a culture medium undeniably improves rooting in certain plants through biochemical intervention, energetic support or osmotic effect (Greenwood and Berlyn, 1973; Leach 1979; Amerson and Mott, 1982; Hyndman *et al.,* 1982; Aldrufeu and Mele, 1982; Thorpe, 1985). It also favours the development of plant vascular system (Sommer and Caldas, 1981) and accelerates lignification (Thorpe, 1978; Thorpe and Biondi, 1981; Driver and Suttle, 1987). Hyndman *et al.* (1982) reported that the number of rose roots per shoot progressively increased as the sucrose concentration was raised from 1% to 7%. The elongation of sour cherry root on hormone free medium also depends on the presence of sucrose (20.0 mg/L) and its omission completely suppresses root formation (Snir and Erez, 1980; Snir, 1983).

Root formation in grape (Harris and Stevenson, 1979), Norway spruce (Von Arnold and Erickson, 1984) and apple (Werner and Boe, 1980) can be promoted either without or with very low agar concentration. However, virtification has been shown to increase with lower agar concentration (Von Arnold and Erickson, 1984). High water content and succulent leaves of liquid-grown plantlets may be more difficult to acclimatised. Lee *et al.* (1986) noted that the percentage of rooted shoots of sweet gum was higher in liquid medium than in agar solidified medium. Besides, shoots rooted in liquid medium have significantly better fresh weight, dry weight and root elongation.

Light is often considered to inhibit rooting especially at the initial stage of root primordia formation (Moncousin, 1991) or may be required for the root initiation in certain species (Torres, 1989). Druart *et al.* (1981) proved the rooting efficiency of two clones of wild cherry subjected to darkness treatment for 10 days. In another study, pre-conditioning of the shoots in the dark for 9 days, followed by the addition of proline and riboflavin to the medium improved rooting of cherry rootstock (Druart, 1985). The optimal length of the dark pre-conditioning period requirement varies between 3 to 10 days depending on the species and cultivars (Depommier, 1981; Druart *et al*, 1982; Moncousin and Gaspar, 1983; Welander, 1983 and Zimmerman, 1984). In the case of *Prunus tenella*, although dark treatment allowed root formation, it caused defoliation and chlorosis to the microcuttings (Bouza, 1997) which was similar to the finding of Alderson *et al.* (1987).

The effect of temperature during the initiation phase has a marked influence on rooting in juvenile and adult plants during their growth phase. High temperature increases cellular metabolism (Ooishi, *et al.,* 1978; Veirskov and Andersen, 1982) and favours the initiation of primordium (Favre, 1973) or callus (Cheng and Voqui, 1977). It is useful to place cultures either at high temperatures (30-35^{0}C) or lower (20-25^{0}C) depending on whether the cutting is pre-conditioned in light or dark

(Moncousin, 1991). This was particularly true when cutting of apple rootstock M_{26} were maintained *in vitro* and exposed to 3 different day/night temperatures ($22^0/20^0$, $25^0/23^0$ and $28^0/26^0$C) in half-strength Lepoivre medium supplemented with 0.3 mg/L IBA (Le, 1985). When cuttings of apple rootstock M_{26} were maintained at 22^0C, root development was not satisfactory with most cutting having few roots or no root formation. Increasing the temperature from 22 to 25^0C increased rooting percentage and number of roots per shoot. However rooting of M_{26} was reduced at high temperature (28^0C).

Plantlet acclimatisation.

Acclimatisation refers to the climatic adaptation of an organism which has been moved to a new environment (Brainerd and Fuchigami, 1981; Conover and Poole, 1984). Acclimatisation is necessary because *in vitro* plantlets are not adapted or suited to the *ex vitro* environment. *In vitro* plantlets are generally transferred to rooting mixtures to support their growth which include peat, bark, perlite, vermiculate, pumice, sand and soil. The mixture may also be supplemented with lime or fertilizer. Obtaining a high success rate in the transfer of propagules from *in vitro* culture to *ex vitro* condition is one of the most difficult problems in a tissue culture cloning program (Bonga, 1985). The roots developed during *in vitro* culture are sometimes very few in number and often limited in function. The fragile plantlets could be easily injured during washing of the remaining agar. The plantlets not only need to readapt to stress condition due to the change from a saturated to a drier atmosphere but are also susceptible to bacteria or fungus in a non-sterile medium (Brochard, 1991).

The leaves of *in vitro* plantlets have much less cuticle wax on their surface. This causes them to lose water more rapidly than normal plants which may lead to poor survival of plantlets (Sutter and Langhans, 1978, 1979). Placing plantlets in clear plastic bag or boxes under partial shade and high relative humidity for several days and gradually reducing the humidity can generally create a suitable acclimatisation environment (Torres, 1989). Shading is necessary to prevent from direct solar exposure which usually leads to the fluctuation of temperature and relative humidity that causes an excess water loss from the plantlets.

In large-scale commercial productions, misting or fogging systems are often employed to provide better plantlet growth. Plantlets are monitored and watered to prevent desiccation (Matsumoto and Kuehnle, 1997) and should be protected from rain until plantlets are large enough for transplanting into beds or pots (Higaki *et al.,* 1994). Heavy shading and misting are necessary during the daytime to keep the humidity high under natural solar light. However, such situation may cause suppression of photosynthesis thus suppresses the development of autotrophy, root formation and leaf emergence of the plantlet (Kozai, 1991).

Successful acclimatisation can be achieved through careful control of environmental condition and taking physiological changes of micropropagated plants into consideration. The use of computer-controlled acclimatisation units (Kozai *et al.,* 1987) could precisely give a better environment control, higher percentage of survival and more rapid growth of plantlets. Acclimatisation unit, air temperature, relative humidity, light intensity, CO_2 concentration, airflow rate and temperature can be monitored and controlled at the same time.

Factors affecting *in vitro* microrhizome formation

A rhizome is defined as a specialised stem structure of the main axis of a plant that grows horizontally at or just below the ground surface. Conventionally, division method is the usual procedure for propagating a plant that has a rhizome structure. This involves cutting the rhizome to several pieces with each pieces having at least one lateral bud or eye (Hartmann *et al*., 1994). A number of economically important plants such as bamboo, sugarcane, banana, turmeric, and many grasses and aquatic species have rhizome structures.

Sucrose concentration and the strength of MS medium have significant effect on the microrhizomal development of *in vitro* plantlets. The optimal requirements vary among species. Mrudul *et al*. (2001) reported that a half-strength MS basal medium containing 8% sucrose was optimum for the microrhizomal production of turmeric (*Cucurma* sp.). Mrudul *et al.* (2001) also reported that BAP had an inhibitory effect on *in vitro* microrhizomal production of turmeric. Sharma and Singh (1995) found that a full-strength MS basal medium supplemented with 7.5% sucrose and 35.2 µm BAP was optimal for the production of microrhizomes in ginger. Vreugdenhil *et al*. (1998) reported that the development of axillary buds of potato depend on the composition of the medium. They found that a medium with 1% sucrose promoted shoot production, 8% sucrose promoted tuber production and 8% sucrose + 0.5 µm gibberellic acid promoted stolon production.

The formation of minirhizomes has also been reported in *Trillium* sp. (Valerie and Victor, 1993). Minirhizomes occur much readily with *Trillium erectum* compared to *Trillium grandiflorum*. For both species, minirhizome formation was also more responsive from stem segment compared to leaf tissue. Minirhizome formation was more responsive in half-strength compared to full-strength MS medium. Minirhizome formation occurred only in media supplemented with either 2,4-D or NAA and in combination with BA, zeatin, or kinetin. No minirhizome formation occurred from explants placed in media containing 2-isopentenyladenine (IPA), indoleacetylphenylalanine (IAPhe), and indoleacetylalanine (IAAIa).

Callus induction

Callus tissue provides an important means of producing genetic variability and is beneficial for genetic improvement and as selection strategies for evaluating somaclonal variation in plants (Stefania *et al*., 1996). It also acts as a trigger in *in vitro* organogenesis and somatic embryogenesis (Stafford and Warren, 1991). Sachs (1991) described callus as a mass of relatively large cells without obvious shape. This unorganised development usually occurs near the damaged or wounded surface of plants. Under *in vitro* condition, removing a small portion of a plant as explant and placing it on a medium containing nutrients and hormones would normally stimulate the cells to divide and reproduce and eventually form a large collection of cells or callus. Normally, callus formed *in vitro* have similarities with tissues arising *in vivo* after an injury to plant. However they are often different in morphology, cellular structure, growth, and metabolic reaction (Torres, 1989).

Callus formed from explant tissue progressively develop more random planes of cell division and loss of organised structures (Thorpe 1980; Wagley *et al.*, 1987). When subcultured on an agar medium, callus cells will exhibit an S-shape or sigmoidal pattern of growth during each passage. There are five phases of callus