SOMATIC EMBRYOGENESIS AND REGENERATION OF

***Polianthes tuberosa* L.**

SAKINAH BINTI ABDULLAH

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Name of Candidate: **Sakinah binti Abdullah**   (I.C/Passport No: **840408-02-5984**)

Registration/Matric No: **SGR 080042**

Name of Degree: **Master of Science**

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ABSTRACT

An efficient protocol was developed for rapid propagation and regeneration of the *Polianthes tuberosa* L. plantlets via somatic embryogenesis pathway using leaf, stem and flower bud as explants. Explants were cultured on MS media supplemented with various combinations and concentrations of BAP and NAA to induce callus formation. Leaf explants cultured on MS media supplemented with 2.0 mg/l NAA was the best to produce optimum callus. Within 5 months the percentage of explant produce callus was $100.00 \pm 0.00\%$. Stem explants started to produce callus earlier (4 weeks) than other explants. 100.0 ± 0.00 % of stem explant produced callus in MS media supplemented with 2.0 mg/l NAA, MS media supplemented with 3.0 mg/l NAA and MS media supplemented with 0.5 mg/l BAP in combination with 2.0 mg/l NAA. Flower bud explants was suitable when cultured on MS media supplemented with 2.0 mg/l NAA and MS media supplemented with 0.5 mg/l BAP in combination with 2.0 mg/l NAA. All the explants (100.0 ± 0.00 %) produced callus. Green and white creamy in colour and soft watery structure of callus from leaf explant were then identified whether it is embryogenic or non embryogenic callus using double staining method. Embryogenic callus was stained in red and non embryogenic callus was stained in blue. Embryogenic callus was then subculture onto solid and liquid somatic embryos induction media. MS media supplemented with 2,4-D at concentration 2.5 mg/l combine with 0.1 mg/l BAP is the best media, where an average of 26.67 ± 0.42 somatic embryos was obtained from 0.5 cm of embryogenic callus from liquid media and 20.53 ± 0.50 somatic embryos was form on solid media. Globular, heart shape, torpedo and cotyledonary stage of somatic embryos were observed in this media. Somatic embryos were then transferred to regeneration media. A combination of 2.0 mg/l Kin with 2.0 mg/l NAA

yield the best shoot regeneration from somatic embryos, producing 26.23±0.74 number of microshoot. MS media supplemented with 0.5 mg/l Kin and 2.0 mg/l NAA is the most suitable media for root formation with 4.23±0.40 number of roots formation. Complete plantlets were then transferred to greenhouse. Plantlets response positively when acclimatized in garden soil (combination of black soil and red soil at ratio 2 to 1) with 63.33±0.09 % of survival rate.

ABSTRAK

Satu protokol yang efisien telah dihasilkan untuk regenerasi dan propagasi pesat pokok *Polianthes tuberosa* melalui proses pembentukan somatik embrio menggunakan eksplan daun, batang dan kudup bunga. Eksplan telah dikultur ke atas MS media yang ditambah dengan beberapa kombinasi dan kepekatan BAP dan NAA untuk menggalakkan pembentukan kalus. Eksplan daun yang dikultur dalam MS media yang dibekalkan 2.0 mg/l NAA adalah yang terbaik untuk menghasilkan kalus yang optimum. Dalam masa 5 bulan, peratus kalus yang terbentuk ialah $100.00 \pm 0.00\%$. Eksplan batang mula menghasilkan kalus lebih awal (4 minggu) berbanding eksplan yang lain. $100.00 \pm 0.00\%$ eksplan batang menghasilkan kalus dalam media MS yang dibekalkan dengan 2.0 mg/l NAA, media MS yang dibekalkan dengan 3.0 mg/l NAA dan media MS yang dibekalkan dengan 0.5 mg/l BAP dikombinasikan dengan 2.0 mg/l NAA, Eksplan kudup bunga adalah sesuai bila dikultur ke atas media MS yang ditambah dengan 2.0 mg/l NAA dan media MS yang ditambah dengan 0.5 mg/l BAP dan 2.0 mg/l NAA. Semua explan (100.0 ± 0.00 %) menghasilkan kalus. 100.0 ± 0.00 % daripada explan batang menghasilkan kalus di atas media MS yang ditambah dengan 2.0 mg/l NAA, media MS yang ditambah dengan 3.0 mg/l NAA dan media MS yang ditambah dengan 0.5 mg/l BAP dikombinasikan dengan 2.0 mg/l NAA. Kalus yang bewarna putih kekuningan dan hijau serta mempunyai struktur yang lembut berair daripada eksplan daun dikenalpasti sama ada kalus tersebut embriogenik atau pun tidak menggunakan teknik pewarnaan berganda. Kalus embriogenik akan diwarnakan dengan warna merah dan kalus bukan embriogenik akan diwarnakan dengan warna biru. Selepas itu, kalus embriogenik di subkultur ke atas media penggalak pembentukan embrio somatik pepejal dan cecair. Media MS yang dibekalkan dengan 2.5 mg/l 2,4-D dan 0.1 mg/l

BAP adalah media yang terbaik, dimana 26.67±0.42 bilangan embrio somatik diperolehi daripada 0.5 cm kalus embriogenik dalam media cecair dan 20.53±0.50 bilangan embrio somatik dalam media pepejal. Peringkat-peringkat pembentukan embrio somatik seperti 'globular', 'heart shape', 'torpedo' dan 'cotyledonary' dapat diperhatikan dalam media ini. Embrio somatik kemudian dipindahkan ke dalam media regenerasi. Kombinasi antara 2.0 mg/l Kin dengan 2.0 mg/l NAA menghasilkan regenerasi pucuk yang terbaik dari embrio somatik dengan menghasilkan 26.23±0.74 bilangan pucuk mikro. Media MS yang dibekalkan 0.5 mg/l Kin dan 2.0 g/l NAA adalah media yang paling sesuai untuk pembentukan akar dengan bilangan 4.23±0.40 akar. Plantlet kemudian dipindahkan ke rumah hijau. Plantlet memberi respon positif bila di aklimitasi pada tanah kebun (gabungan tanah hitam dan merah dengan nisbah 2 kepada 1) dengan 63.33±0.09 % kadar keterushidupan.

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LIST OF ABBREVIATIONS

BAP	Benzylaminopurine
2, 4-D	2, 4-Dichlorophenoxyacetic acid
HCL	Hidrochloric acid
IAA	Indole-3-acetic acid
1BA	Indolebutyric acid
Kinetin	6-Furfurylaminopurine
kPa	Kilo Pasca
mg/l	Milligram per Liter
min	Minute
MS	Murashige and Skoog
NAA	Naphthalene acetic acid
NaOH	Sodium Hydroxide
Tween 20	Polyoxyethylene sorbitan monolaurate
v/v	Volume per volume
w/v	Weight per volume

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction

1.1.1 Plant tissue culture

Plant tissue culture includes all the aspects of the *in vitro* culture of cell, tissue and organ. According to Debergh and Read (1991), tissue culture is alternatively known as cell, tissue and organ culture through *in vitro* condition. Such tissue cultures have been used in five broad areas of research and application (Thorpe, 1990). These are studies on clonal propagation, cell behavior, secondary product formation, plant modification and development and production of pathogen free plants and germplasm storage. According to Dirr (1987), tissue culture has four potential applications, the production of natural products, genetic improvement of crops and germplasm storage, the production of disease free plants and rapid multiplication which offers the greatest significance to the commercial propagator.

The history of plant tissue culture started in 1756 when Henry-Louise Duhamel du Monceau's pioneering experiments on wounding healing plants demonstrated spontaneous callus formation on the decorticated region of elm plants. According to Gautheret (1985),

Henry-Louise Duhamel du Monceau's studies could be considered a 'foreword' for the discovery of plant tissue culture.

During 1838-1839, Schleiden and Schwann established the 'Cell Theory' which become the foundation of plant tissue and cell culture. In this theory, plantcell was described as the smallest biological unit, autonomic and capable of generating to give a complete plant. This theory was called totipotency theory. This cellular totipotency concept was than popularized by Virchow (1858) with his famous aphorism 'every cell from a cell'. In 1878, Vochting was successful in dissecting plants into smaller and smaller fragments and keeping the fragment viable and growing.

In year 1902, German Botanist Gottlieb Haberlandt developed the concept of *in vitro* cell culture. He was the first to culture isolated, fully differentiated cells in a nutrient medium containing glucose, peptone and Knop's salt solution (Razdan, 1993). The first attempt by him was failed. His paper entitled 'Experiment on the Culture of Isolated Plants Cells' was set forth clearly the totipotency concept. Haberlandt was known as the father of tissue culture.

Although the first attempts at initiating cultures of plant cells were made by Haberlandt at the turn of this century, it has only been during the last three decades that rapid developments in plant cell, tissue, and organ culture have occurred. In 1922, Kotte from Germany and Robbins from USA postulated that a true *in vitro* culture could be made

easier by using meristematic cells. White (1934), was the first that successfully to cultured excised roots. White used yeast extract in a medium containing inorganic salts and sucrose but later replaced yeast extract by pyridoxine, thiamine and nicotinic acid. This media later proved to be one of the basic media for the variety of cells and tissue culture (Razdan, 1993).

The discovery of plant hormone, make the true plant tissue culture was achieved. Snow, (1935), showed that IAA can stimulate cambial activity. Gautherat found that the addition of auxin enhanced the proliferation of his cambial cultures. In 1939, Gautherat in Paris, Nobecourt in Grenoble and White in Princeton independently succeeded in obtaining the first real plant tissue culture. Later on in 1948-1957, the discovery of various types of cytokinin helps in the tissue culture progression. Skoog and Miller (1957) published the study that clarified the interrelationship of auxin and cytokinins in the control of shoot and root regeneration. In 1962, Murashige and Skoog, published the formulation of new media known as MS medium. This media later on used by many researchers in tissue culture. Thus from here, rapid progression of tissue culture can be observed. Many plants can be regenerate through tissue culture technique using this media or modified media.

Clonal propagation of plants refers to the production of genetically identical plants through nonsexual method. *In vivo* vegetative propagation has for many years played an important role in agriculture. The most widely used *in vivo* methods of cloning agricultural crops include cuttings of vegetative parts, layering, grafting and budding. This method

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often fall short of the required which mean too slow, too difficult, too expensive or a completely impossible (Pierik, 1987).

Clonal propagation through tissue culture (*in vitro* propagation) also called micropropagation is the alternative method to overcome this problem. It has now become possible to clone species by *in vitro* culture techniques that are impossible to clone *in vivo*. According to Tisserat (1995), the procedures of plant tissue culture have developed to such a level that any plant species can be regenerated *in vitro* through several methodologists. The list of plants that can be propagated *in vitro* is extensive and encompasses ornamental, woody, vegetable and crop species (Jonas and Karp, 1985).

Micropropagation is an alternative method of vegetative propagation, which is well suited for multiplication of elite clones. It is accomplished by several means, i.e., multiplication of shoots from different explants such as shoots tips or axillary buds or direct formation of adventitious shoots or somatic embryos from tissues, organs or zygotic embryos. The goal of micropropagation is to obtain a large number of genetically and physiologically uniform plantlets (true to type) with high photosynthetic potential and able to survive the transfer to *ex vitro* conditions (Jeong et al., 1993; Solarova and Pospisilova, 1997).

There are 5 stages of micropropagation. Murashige, (1974) proposed three (i-iii) stages. Debergh and Maena, (1981) added first stage which is stage 0. This stage involves

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the selection and preparation of the starting materials. According to Debergh and Maena, (1981) this stage is important and crucial in order to ensure the starting materials are free from contaminants. Second stage is initiation of the culture. This stage involves the shoot multiplication. Usually plant growth hormones are used. Roots induction from the shoots involve in stage 3. Stage 4 is hardening process which is involving acclimatization of the *in vitro* regenerated plantlet. This stage is carried out under green house environment or plantlets were transferred to the soil.

In vitro propagation is the same as multiplication of plants from vegetative parts except that plants are produced by culturing explants (small pieces of leaf, stem, flower, embryo, cotyledon and axillary bud) on medium instead of soil under aseptic conditions. All the instruments use also must be sterile. Micropropagation has many advantages over conventional method of vegetative propagation which suffer from several limitations (Nehra and Kartha, 1994).

To propagate plants *in vitro,* it is necessary that they are capable of regeneration. According to Vasil and Vasil (1972), plant cell and tissue culture have two important characteristics which are the ability to regenerate normal adult plant from single cells or group or highly specialized cells and in many cases to recapitulate various stages of cell division, differentiation and morphogenesis undergone by the zygote after normal gametic fusion.

Morphogenesis refers to the development of organs (shoots, roots or flowers) and overall plant shape and structure. Plant morphogenesis *in vitro* can be achieved via two pathways, organogenesis or somatic embryogenesis. In plants, differentiated of somatic cells reinitiated the ontogenic programme: when given the proper stimuli, they develop into adventitious meristem (De Klerk, 2003). Adventitious meristem will generate adventitious roots, adventitious shoots and embryo. The formation of adventitious shoots and adventitious roots is referred as organogenesis and the formation of embryo from somatic cells is referred as somatic embryogenesis.

There are three phases of organogenesis. According to Sugiyama (1999), in the first phase, cells in the explants acquire 'competence' which is defined as the ability (not capacity) to respond to hormonal signals of organ induction. This process of acquisition of organogenic competence is referred to as dedefferentiation. Through the second phase, the competence cells in cultured explants are canalized and determined for specific organ formation under the influence of the phytohormone (hormone like substances). During the third phase, the morphogenesis proceeds independently of the exogenously supplied hormone.

The concept of hormonal control of organ formation was proposed by Skoog and Miller (1957). In their experiment, they found that bud and root initiation were controled by a balance between auxin and cytokinins. High concentration of auxin promoted the formation of roots while high concentration of cytokinins promote bud and shoots formation. Gibberallin and abscisic acid in the medium are also reported to inhibit roots

formation. Auxins, cytokinins and auxin cytokinins interaction are usually considered to be the most important for regulating growth and organized development in plant tissue and organ cultures as these two classes of hormones are generally required (Evans et al., 1981; Vasil and Thorp, 1994).

Organogenesis can occur directly or indirectly. In direct organogenesis, plant organs such as shoots and roots were formed directly from the explants cultured whereas in indirect organogenesis, plant organ were formed from callus.

Callus is a mass of unorganized cell. According to Pierik, (1987) callus is an actively dividing non-organized tissue of undifferentiated and differentiated cells often developing from injury (wounding) or in tissue culture. The process of callus formation is known as callogenesis. Callus can be subdivided by random dissection or by placing in a homogeniser to produce many thousands of propagules in a single operation. Each propagule can be used in mass propagation of multiple shoots. The production of many thousands of plantlets from callus either derived from cell suspension or isolated protoplast constitutes unique cases of cloning such as calliclones and protoclones (Razdan, 1993). Calliclones are variants selected in tissue culture from callus culture (Skirvin, 1978). Such clones commonly exhibit somaclonal variation.

An exogenous supply of plant growth regulators is often recommended to initiate callus formation on explant. Callus can be used for regeneration of organs and somatic

embryos. There are two types of callus which is embryogenic callus and non embryogenic callus. Embryogenic callus have the potential to regenerate the formation of somatic embryo and can be induce to regenerate a whole plant under appropriate conditions. Non embryogenic callus will die after sometimes in culture.

Suspension culture or liquid culture is used to produce more callus. Liquid cultures provide a system for rapid growth and multiplication of plants *in vitro*, reduce labor-intensive manipulation required for media replenishment and propagules transfer and facilitate scaling- up of the culture systems (Ammirato and Styer, 1985; Ziv, 1989, 1992; Ilan *et al*., 1995). This suspension cultures can be started either from compact or from friable callus. Friable callus which is soft and highly embryogenic is regarded as the best source for initiating a fast growing suspension culture (Remotti, 1995). Phillips *et al*., (1995) quoted that the first step towards de novo regeneration is to establish callus or suspension culture. Cell in suspension cultures receive more homogeneous stimuli in a defined medium supplemented with the requisite amount of inducers such as sugar or auxin (Razdan, 1993).

Somatic embryogenesis is defined as the process in which bipolar structure arises through a series of stages characteristic for zygotic embryo development and having no vascular connection with the parental tissue (Ammirato, 1987; Sharp *et al*., 1980; Terzi and Loschiavo, 1990 and Raemakers *et al*., 1995). These bipolar embryos also called embryoids. Somatic embryogenesis was first induced in suspension culture (Stewart *et al.,* 1958) and in callus culture (Reinert, 1959) of carrot.

There are two types of somatic embryogenesis which are direct somatic embryogenesis and indirect somatic embryogenesis. The term 'direct' somatic embryogenesis is applied to explants that undergo a minimum proliferation before forming somatic embryos, whereas 'indirect' somatic embryogenesis refers to explants which undergo an extensive proliferation before the development of somatic embryos (Sharp *et al*, 1980). Yeung (1995) suggested that in direct somatic embryogenesis, embryogenic cells a present and simply require favorable conditions for embryo development, while indirect embryogenesis requires the re-determination of differentiated cells. Evans *et al* (1981) also distinguished direct and indirect somatic embryogenesis. According to him, direct somatic embryogenesis proceeds from already pre-embryonic determined cells and indirect somatic embryogenesis from cell which require redifferentiation before they can express embryogenic competence.

Cells capable of direct somatic embryogenesis are physiologically similar to those in zygotic embryos. They are frequently found in tissue before the onset of embryogenesis (i.e. in the flower organ) or in the developing zygotic embryo (Reamakers, 1995). In these cells the genes necessary for zygotic embryogenesis are active in varying degrees (Carman, 1990; Sharp *et al*., 1980).

In indirect somatic embryogenesis, callus formation precedes the formation of embryos. In most cases, somatic embryos develop up to pre-embryogenic masses (PEM's) or globular embryos, without differentiation into organs before they are subjected to

secondary embryogenesis. According to Raven and Johnson (2001), the globular somatic embryo will later turn into heart and torpedo shaped somatic embryos before converting to cotyledon and finally form shoots and further develops into new plants.

Direct and indirect should be considered as two extremes of a continuum (Carman, 1990; Wann, 1988; William and Maheswaran, 1986). It is not always clear which types occurs or both direct and indirect can be observed. Emonds (1994) argued that in many systems where embryogenesis has been described as indirect, the embryogenic callus is organized in young embryos (pre-embryogenic masses or (pre-) globular embryos) and that the type of embryogenesis is applied. If that period of embryogenesis is short the process will be direct and if it is long than the process will be indirect (Reamakers, *et al*., 1995).

All somatic cells within a plant contain the entire set of genetic information necessary to create a complete and functional plant. The induction of somatic embryogenesis consist of the termination of the existing gene expression pattern in explant tissue, and its replacement with an embryogenic gene expression program in those cells of the explant tissue which will give rise to somatic embryogenesis (Merkle, *et al*.,1995). A treatment with the ability to down regulate gene expression can stimulate somatic embryogenesis.

One possible mechanism to down regulate gene expression is DNA methylation, which has been found in study to correlate with the amount of exogenous auxin. The

effects of cytokinins in the somatic embryogenesis usually negative effects. High endogenous of cytokinins levels in leaves tissue have associated with lack an embryogenic response in both napier grass and orchard grass (Ling *et al*., 1989; Ranga Swamy, 1961). The frequency of embryogenesis is decrease when a cytokinin is used in conjunction with an auxin (Kochba *et al*., 1972; Raj Bhansali and Arya, 1977; Dhillon *et al*., 1989).

Somatic embryogenesis is the most promising technique for plant multiplication, because of its high proliferation potential and the fact that the risk of chimeric plant development in this case can minimize or eliminated (Stefaniak, 1994). Direct somatic embryogenesis has a lower probability of genetic variation than embryos from intervening callus (Kim *et al.,* 2003) because maintenance of a callus for prolonged period *in vitro* results in greater genetic variability (Lloyd *et al*., 1998; Arene *et al.,* 1993). When plant occurs via somatic embryogenesis it has several advantages such as the probable single cell origin of the regenerated plants and the high rate of plant regenerated, even from long term cultures (Vasil, 1983).

There are advantages and disadvantages of somatic embryogenesis in large scale plant multiplication (Jain, 2002). According to Rout *et al*., (2006), the major advantages are large scale somatic embryo production in bioreactors, encapsulation, cryopreservation, genetic transformation and clonal propagation and the major limitation are genotype dependence of somatic embryo production and poor germination rate.

Somatic embryogenesis can be coated with synthetic gel to produce synthetic seed or artificial seed. Synthetic seed technology has advanced quite dramatically in recent years since the first description of systems which be used to clonally propagate plants using somatic embryos (Kitto and Janick, 1980; Redenbaugh *et al*., 1984; Kitto and Janick, 1985; Mc Kersie *et al*., 1989). Artificially encapsulated somatic embryogenesis can be sown under *in vitro* or ex conditions, producing uniform clones (Aitken *et al*., 1995).

One prerequisite for the application of synthetic seed technology in micropropagation is the production of high quality, vigorous somatic embryos that can produce plants with frequencies comparable to natural seed. Standardi and Piccioni, (1998) reported other than synthetic seed from somatic embryo there are advance in using non embryogenic (unipolar) structures for encapsulation as synthetic seed. There seems to be a lower risk of somaclonal variation using unipolar structures such as microbulbs, microtubers, rhizomes, corms, shoots or nodes containing either apical or axillary buds, meristemoid and bud primodia for encapsulation, and the synthetic seed technology can be extended to a wider variety of genotypes (Phillips, 2004).

According to Razdan (1993), the practical applications of plant tissue culture technology are based on advancement made in the areas of morphology, biochemistry, pathology and genetics. In areas of biochemistry, plant tissue culture technology also involved in production of secondary metabolites. It has been clearly demonstrated that secondary metabolites play a major role in the adaptation of plants to their environment. They have been described as being antibiotic, antifungal and antiviral thus therefore able to

protect plants from pathogen (phytoalexin) and also anti germinative or toxic for other plants (Bourgaud *et al.*, 2001).

The last two decades have witnessed a dramatic rise in the incidence of life threatening systemic fungal infection. The majority of clinically used antifungal have various drawbacks in terms of toxicity, efficacy and cost and their frequent use has led to the emergency of resistance strains. One approach might be used to overcome this problem is testing of plants traditionally used for their antifungal activities as potential source for drug development. There are many studies shows plants extract have antifungal activities. Muschietti *et al.* (2005) investigate on methanol extracts from eleven traditionally medicinal plants for antifungal against yeast, hialohypomycetes as well as dermatophyte and the plant that most effected the yeast growth are *Eupatorium bunifolium* and *Terminalia triflora*.

Plant secondary metabolites usually classified according to their biosynthetic pathway (Harborne, 1999). Three large molecule families are generally considered: phenolics, terpenes and steroids and alkaloids (Bourgaud *et al.*, 2001). Phenolics compound is a widespread metabolite family in plant.

Most valuable phytochemicals are product of plant secondary metabolism (Nehra, 1993). Due to their large biological activities, plant secondary metabolites have been used for centuries in traditional medicine. Even today, the World Health Organization estimates

that up to 80% of people still rely mainly on traditional remedies such as herbs for their medicine (Tripathi and Tripathi, 2003). There are surveys that in westerns countries, where chemistry is the backbone of the pharmaceutical industry, 25% of the molecules used are of natural plant origin (Payne *et al*., 1991). It is estimated that approximately one quarter of prescribed drugs contain plants extracts or active ingredients obtained from or modeled on plant substances (Tripathi and Tripathi, 2003). The most popular analgesic, aspirin was originally derived from species of *Salix* and *Spiraea* and some of the most valuable anti-cancer agents such as paclitaxel and viblastine are derived solely from plant source (Katzung, 1995; Taxol, 1996; Roberts, 1988).

The production of secondary metabolite *in vitro* can be possible through plant cell culture (Barzn and Ellis, 1981; Deus and Zenk, 1982). According to Razdan (1993), the industrial production of secondary metabolites was initiated during the period 1950-1960 by the Pzifer Company with the assistance of Nickell, a distinguished expert in tissue culture. Zenk, (1978) established the successful of cell lines capable of producing high yields of secondary compounds in cell suspension cultures. Cephaelin and emetine (alkaloids) were isolated from callus cultures of *Cephaelis ipecacuanha* (Jha, 1988). According to Kanetkar *et al*., (2006) *in vitro* developed callus tends to produce various active compounds, including gymnemic acid and gymnemaganin. Srividya *et al*., (1998) compared the production of azadirachtin and nimbin *in vivo* and *in vitro*. They found that the production of azadirachtin and nimbin has been shown to be higher in cultured shoots and roots of *Azadirachta indica* compared to field grown plant.

Nowadays, bioreactor is used in the large scale production of economical secondary metabolite. Bioreactors have several advantages for mass propagation of plants. It gives better control for scale up of cell suspension cultures under defined parameters for the production of bioactive compounds and large number of plantlets are easily produce and can be scale up (Tripathi and Tripathi, 2003). Bioreactor offer optimal conditions for large scale plant production for commercial manufacture (Hahn *et al*., 2003). The bioreactor also has been applied for embryogenic and organogenic cultures of several plant species (Levin *et al*., 1988; Preil *et al*., 1988).

1.1.2   Medium selection and preparation

Minerals play an important role in the regulation of plant morphogenesis and growth. Successful plant tissue culture depends on the choice of nutrient medium (Gamborg *et al*., 1976). The cells of most plant species can be grown on completely defined medium. MS (Murashige and Skoog, 1962) media is the widely use media for tissue culture. According to Gamborg and Phillips (1995), MS medium is among the best media for plant regeneration for most species. LS (Linsmaier and Skoog, 1965) medium contains the same salt composition as MS. Nowadays, there are numerous media in use such as B5 (Gamborg *et al*.,1968), NN (Nitsch and Nitsch, 1969), N6 (Chu *et al*., 1975), McCown woody plant (Lloyd and McCown, 1980) and DKW/Juglans (Driver and Kuniyuki, 1984).

Most of all the plant tissue culture media contained inorganic nutrient consist of macronutrients and micronutrients, organic nutrient, carbon source, gelling agent and plant growth regulators. Mineral elements that required by plants in concentration which is

greater than 0.5 mmol l^{-1} are referred to as macronutrients and those less than 0.005 mmol l^{-1} as micronutrients. Macronutrients contained include six major elements: nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), Magnesium (Mg) and sulphur (S). Micronutrients contained iron (Fe), manganese (Mn), zinc (Zn), boron (B), copper (Cu), molybdenum (Mo), cobalt (Co), iodine (I) and sodium (Na).

Macronutrients and micronutrients are the essential mineral for tissue culture media. To be classified as essential the mineral must fulfill the following criteria: (1) an obligatory requirement for normal growth and reproduction; (2) it is not possible to replace the mineral with another mineral or substance; (3) have a direct or indirect role in plant metabolism (Arnon and Stout, 1939). Macronutrients are important components of macromolecules such as protein and nucleic acids as well as constituents of many small molecules (Carl and Richard, 2002).

Micronutrients are required in much smaller quantities than macronutrients and function in various roles such as enzymes cofactors or components of electron transport protein (Marschner, 1995). Organic nutrients that usually added into the media are vitamins, amino acids, activated charcoal, antibiotics and other organic supplements like protein (casein) hydrolysates, coconut milk, yeast and malt extracts, ground banana, orange juice and tomato juice.

Vitamins are added in small quantities into the tissue culture media to achieve the best growth of the tissue. Vitamins like myoinositol, Thiamin (B$_1$), Nicotinic acid (B$_3$), and Pyridoxine (B$_6$) usually used in tissue culture. Thiamine is the basic vitamin required by all cells and tissue (Razdan, 1993). Most plants are able to synthesize vitamins *in vitro*. Amino acid is important for stimulating cell growth. Amino acids added singly prove inhibitory to cell growth while their mixtures are frequently beneficial (Razdan, 1993). Supplemented the medium with adenine sulphate can stimulate cell growth. Skoog and Tsui, (1948) was the first scientist used adenine in the tobacco tissue culture. Adenine can stimulate the adventitious shoot formation.

Sometimes activated charcoal was added in tissue culture media to stimulate growth and differentiation. Studies were reported on orchids, tomato, ivy and carrot. Activated charcoal also helps to reduce toxicity by removing toxic compounds like phenolic compounds produced during the culture and permits unhindered cell growth.

Hormones are organic compounds naturally synthesized in higher plants, which influence growth and development. Plant hormones have also been referred to as phytohormones though this term is seldom used. Synthetic compounds have been developed which correspond to the natural one. Auxin, cytokinins, gibberellins and abscisic acid are the most important hormones use in tissue culture.

Auxins usually added into the media to induce root formation. There are various kinds of auxins: 1 naphthaleneacetic acids (NAA), 1H-indole-acetic acid (IAA), 1H-indole-3-butyric acid (IBA) and 2,4-dichlorophenoxyacetic acid (2,4-D). IAA is the main auxin in most plant. Auxin usually cause cell elongation and swelling of tissues, cell division (callus formation) and the formation of adventitious roots and the inhibition of adventitious and axillary shoots formation, often embryogenesis. Commonly used synthetic auxin in tissue culture are IAA, IBA (tend to denatured in media and rapidly metabolized within plant tissue), 2,4-D (often used for callus induction and suspension culture), and NAA (when organogenesis is required). Among others, dicamba (3,6-dichloro-o-asinic acid) and picloram (4-amino-3,5,6-trichloropyridine-2-carboxylic acid) are often effective in inducing the formation of embryogenic tissue or in maintaining suspension cultures (Gray and Conger, 1985; Hagen et al., 1991). BSAA (benzo(b)selenienyl-3-) acetic acid) is another synthetic auxin with powerful auxin like activities (Lamproye *et al*., 1990; Gasper, 1995). In several systems, auxin, particularly at high concentration is inhibitory. With low auxin concentrations, adventitious roots formation is predominates whereas with high auxin concentrations root formation fail to occur and callus formation takes places (Pierik, 1987).

Cytokinins are adenine derivatives which are often used to stimulate cell division, modification of apical dominance and shoots dedifferentiation in the tissue culture. The most common use cytokinins in tissue culture are the substituted purines, BAP (6-benzylaminopurine) or BA (6-benzyladenine) and kinetin (N-1-H-purine-6-amine). Adenine, adenosine and adenylic acid may have the cytokinin activity although less than that of the cytokinins (Gaspar *et al*., 1996). Adenine can be used to bring about or reinforce response normally attributed to cytokinin action. In higher concentration (1-10 mg/l)

adenine can induce adventitious shoot formation but root formation is generally inhibited (Pierik, 1987). The ratio of auxin and cytokinin is important with respect to morphogenesis in tissue culture system. For embryogenesis, callus initiation and root initiation the requisite ratio of auxins to cytokinin is high while the reverse leads to axillary and shoot proliferation (Razdan, 1993).

Gibberellins usually used to promote the growth of cell culture, enhanced callus growth and induced dwarf of stunted plantlets to elongate. Gibberellins also break dormancy of isolated embryos or seeds and inhibit adventitious roots formation. Gibberallins are a family of compounds based on the ent-gibberellane structure. The most widely available compounds is gibberellic acid, GA_3 which is a fungal product. The most important gibberellins in plant is GA_1, which is the gibberellins primarily responsible for stem elongation. GA_3 is the most commonly used gibberellins in tissue culture. Gibberellins have numerous interactions with other hormones. Gibberellins induced α-amylase activity is antagonized by ABA. Ethylene blocks promote the stem response to Gibberellins. Gibberellins antagonized the senescence promoting effect of ABA and in leaves and petals.

Abcisic acid (ABA) is a single compounds used in the tissue culture medium to promote callus growth. ABA is synthesized from mevalonic acid in roots and mature leaves, particularly in response to water stress. ABA is exported from roots in the xylem and from leaves in the phloem. ABA is often regarded as being an inhibitor, as it maintaines bud and seed dormancy, inhibits auxin-promoted cell wall acidification loosening and slows cell elongation. In tissue culture, at low concentration ABA can effect

callus growth and organogenesis. Some ABA is essential for the maturation and normal growth of somatic embryos and only in its presence do they closely resemble zygotic embryos in their morphological and biochemical development (Roberts *et al*., 1990; Rock and Quatrano, 1995). Other plant hormones that usually used in tissue culture are ethylene, polyamines, jasmonates, oligosaccharins and salicylates.

Gelling agent is added into the media to prepare solid or semisolid media. Agar, gelrite, alginate, phytagel and methacel can be used as a gelling agent. Agar, a polysaccharide obtained from seaweed used in most nutrient media. Agar has several advantages over other gelling agent. Agar does not react with media constituent and agar is not digested by plant enzyme and remains stable at all feasible incubation temperature.

Sugar usually sucrose also was added into the media as an energy source for *in vitro* growth and development. Sugar is important in tissue culture because photosynthesis is insufficient, due to growth taking place in conditions unsuitable for photosynthesis or without photosynthesis. Sugar also used to maintain the osmolarity of the culture medium. According to Pierik (1987), it is striking that a high sugar concentration is needed for adventitious root formation, especially in the case of woody plants.

Plant cells and tissue need optimum pH for growth and development. pH can affect the ions uptake of the cell. pH that suitable for tissue culture for most of the plants is

around 5.00-6.00. pH higher than 6.00 is likely to give hard medium while a low pH result in unsatisfactory solidification of the agar.

1.1.3 Incubation and culture conditions

Environmental factors have great influence to the process of differentiation and growth of tissue in cultures. All cultures must be kept in a room which light, temperature, air circulation and humidity that can be controled. According to Peirik (1987), it is extremely difficult to indicate whether a particular culture should be grown in the light or the dark, at high or low temperature but the best to choose is those light and temperature conditions that are the best for the growth and development of the experimental material *in vivo*.

A diurnal illumination of 16 hours day and 8 hours night is generally found satisfactory for multiplication and proliferation of shoots although there are exceptions such as cauliflower which require 9 hours daylight regimes (Razdan, 1993). In most cultures, the temperature is maintaining around 25ºC. The optimal temperature for *in vitro* growth and development is generally 3-4 ºC higher than *in vivo* (Peirik, 1987).

The volume of culture container also can affect the growth. The volume of the culture container can affect the constitution of the gas phase within the culture vessel. Ethylene, oxygen, carbon dioxide, ethanol and acetaledehyde are metabolically active

gases with possible effects on morphogenesis and may promote unorganized growth of cell (Razdan, 1993).

1.1.4 Acclimatization

Micropropagation on large scale can be successful only when plants after transfer from culture to the soil show high survival rates and the cost involved in the process is low (Razdan, 1993). After *in vitro* rooting stages plants are transfer to the soil. This transfer process were done step by step. Environmental condition for *ex vitro* growth is quite different from those used for *in vitro* cultivation (Kozai *et al*., 1997; Hazarika, 2006).

In vitro acclimatization is one of the key factors in producing healthy plantlets before they are transplanted to *ex vitro* conditions (Pospisilova *et al*., 1999; Hazarika, 2003). Kozai *et al*. (1990) suggested increasing in photosynthetic efficiency of *in vitro* plantlets would be helpful during acclimatization to *ex vitro* conditions. Humidity and temperature play important roles in the growth and development of plantlets metabolism during acclimatization (Jeon *et al*., 2006). Kranz (1996) and Remigio *et al*. (2003) stated that high humidity often causes shoot elongation and increase fresh weight and leaf area. High humidity can be built up around transplanted plants by covering them with clean transparent plastic bags having a small hole for air circulation (Razdan, 1993).

1.1.5 Different types of plant tissue culture

Every part of plant that undergoes dedeferentiation can be used for tissue culture. Organ (Leaf, stem, flower), meristem, embryo, anther, ovary, ovul and endosperme can be used as explants. Meristem culture was used to obtain virus free plants and it is the best mean to produce a large numbers of plants in a short span of time. Quak, (1966) suggested that the absence of vascular elements in the meristem greatly hinders the transport of virus particle. Shoot apex culture methods leading to plant regeneration have also been adopted for plant propagation and production of virus free plant.

Zygotic embryo culture is an aseptic isolation and growth of sexually produced immature or mature embryos *in vitro* with the objective of obtaining viable plant. There are two types of embryo cultures which are culture of immature embryos and culture of mature embryos. Immature embryo is originating from unripe seed and mature embryo derives from ripe seed. Tukey (1933a,b), succeeded in getting normal plants from thousands of abortive embryos of early ripening cultivars of different stone fruits.

Anther culture was used to produce haploid plant (Tsay *et al*., 1992) and partially sterile diploid plant (Woo and Su, 1975; Mok and Wu, 1976; Hsu, 1978; Chung, 1987; Zhang, 1989). Kameya and Hinata (1970) is the first scientist to report on the tissue formation from isolated pollen of an angiosperm. Peirik, (1987) summarized that most of the haploid plants which are obtained *in vitro* have arisen from isolated anthers or sometimes from pollen.

Ovary culture often use for embryo rescue, when the embryo culture and ovule culture fail or not feasible due to very small ovules. Ovary culture also use for *in vitro* pollination and fertilization. *In vitro* pollination is the process of seed formation following stigmatic pollination of cultured whole pistils (Bhojwani and Razdan, 1983). The technique of ovary culture was developed by Nitsch (1951) who successfully reared ovaries of *Cucumis* and *Lycopersicon* excised from pollinated flowers *in vitro* to develop into mature fruits. Ovary culture can be used to produce haploid and diploid plant.

Ovule culture usually prefer when embryo aborts very early and embryo culture is not possible due to difficulty of its excision at very early stage. Haploid plant also can be produce using ovule culture. Haploids provide a relatively easier system for the induction of mutation; therefore they can be employed in rapid selection of mutants having traits for disease resistance (Razdan, 1993).

The function of endosperm tissue is to store reserve food substance for seed germination and growth. Endosperm is regarded as the second embryo due to the lacks of any organogenic or vascular differentiation. During the last few decades the technique of endosperm culture has been applied to raise triploid plantlets which have a significant role in plant improvement (Razdan, 1993). Regeneration of plant from endosperm tissue provides an easy and direct approach to triploid production (Bhojwani and Razdan, 1996). Triploid plants are useful for production of seedless fruits like watermelon without seeds and for the production of trisomics for cytogenetic studies.

1.1.6 Genetic stability in micropropagated plant

Plant regeneration isolated from tissue culture could have substantial application in agriculture. Plants regenerated from populations of single cell may retain all the essential characters of a cultivar or clone but selectively alter undesirable traits. Heredity variations can be observed in cell colonies or plants regenerated *in vitro* which may later express at the time of vegetative multiplication or sexual reproduction (Razdan, 1993).

One of the important aspects of plant breeding is for selection and introduction of better trait qualities in existing crop species. The example heredity variations are haploid, triploid and polyploidy plant production. Plant pathogen or insect attacks have become a great problem in agricultural. To overcome this problem, tissue culture technique for produce virus free plant and plant that resistance to antibiotic and insect attack were introduce.

Somaclonal variation involves all forms of variation among regenerated plants derived from tissue culture (Larkin and Scowcroft, 1981; Jain et al., 1998; Jain and De Klerk, 1998), such as: i) physical and morphological changes in undifferentiated callus; ii) differences in the ability to organize and form organs *in vitro*; iii) changes manifested among differentiated plants and chromosomal changes.

1.1.7 Advantages of Plant tissue culture

Micropropagation can create a large number of clones from a single seed or explant. It can produce many copies of the same plants then which may be used to produce plants with better flowers, odors, fruits or any other properties of the plants that are beneficial to the human beings.

It is easy to select desirable traits directly from the *in vitro* culture. Protoplast fusion is one of the methods, offers the potential of combining genomes that cannot be combined sexually and combination of genomes in different ways (Evans, 1983). Mutant and somaclonal variants will be exploited and changes in flower colour or growth habit can result in novel varieties (Jones, 1976). Mutants may also contribute in genetic engineering by identifying important genes, provide marker genes and recipients for gene transfer or increase knowledge for plant functioning (Bright *et al*., 1985).

Tissue culture technique can help in eliminating plant diseases through careful stock selection and sterile techniques. Viruses can be eliminated through *in vitro* culture by excision and culture of small meristematic parts of plants such as shoot tips which are usually protected and free from infection. Usually in plant tissue culture, the starting materials or source of explant were selected from healthy mother plant and sometimes combined with heat or chemical treatment.

The time required is much shortened, no need to wait for the whole life cycle of seed development. For species that have long generation time, low levels of seed production, or seeds that do not readily germinate, rapid propagation is possible. It overcomes seasonal restrictions for seed germination. To produce plants anytime we want although the climates are not appropriate to produce a plant. It enables the preservation of pollen and cell collections form which plants may be propagated (like a seed bank). It allows for the international exchange of sterilized plant materials (eliminating the need for quarantine.) It enables cold storage of large numbers of viable plants in a small space.

Tissue culture technique very useful solution for the prevention of starvation in third world countries since the process is highly efficient, by using only one plant, it is possible to produce more than one thousand of the same plant with higher productive if its genome changed.

1.1.8 Disadvantages of Tissue Culture

If large scale production is being thinking, the costs of the equipment and labour are very expensive. The procedure is very variable and it depends on the type of the species so sometimes it needs trial-and-error type of experiments if there is not any review about that species. The procedure needs special attention and diligently done observation. There may be error in the identity of the organisms after culture.

1.2 General Description of *Polianthes tuberosa* L.

1.2.1 History of *Polianthes tuberosa* L.

Polianthes tuberosa L. is a well known ornamental plant because of its floral scent that is described as a complex, exotic and hypnotic sweet. Most flowers begin to lose their scent when they are picked but not so with tuberose, the flower scent continues to produce itself like jasmine. The tuberose is a night blooming plant. The flowers have a mild fragrance during the day and a strong fragrance at night.

Polianthes tuberosa L. is a flower with centuries of romantic history. The legend of *Polianthes tuberosa* L. in France warns that young girls should not breathe in its fragrance; for fear that it would put them in a romantic mood. In India, *Polianthes tuberosa* L. is known as "raat ki rani" which is means queen of the night for the similar reason. In parts of South India, it is known as "Sugandaraja", which translates to "king of fragrance/smell". In Persian, it is called "Maryam" and is a popular name of girls. In Cuba, it is called "azucena" which is the name given to amaryllis in Mexico. In Indonesia, it is called "bunga harum sedap malam" which means fragrant night flower. In Singapore it is called "Ye Lai Xiang", which means "fragrance that comes at night" in Mandarin. In Malaysia we have known it as "bunga harum sundal malam". In Hawaii it was used traditionally for wedding ceremony. Bride wears a wreath of *Polianthes tuberosa* L. and pikaki flowers around the head called "a haku". This costume is still popular until today. In the Mekong Delta of Vietnam, *Polianthes tuberosa* L. is today a symbol of purity and spirituality and is grown

commercially for cut flowers used in temples and pagodas in the worship of ancestors during weddings or funerals (Nguyen and Marc, 2007).

Polianthes tuberosa L. is endemic to Mexico and surrounding countries. The Aztects called it as "Omixochiltl" or bone flower from the words "omitl", bone and "xocitl", flower. In Mexico today one also hears the names "nardo", "azucena", "amole" and "amigo de noche" applied to the white *Polianthes tuberosa* L. sold in the Indian markets of Mexico city, neighbouring towns and especially in and near Guadalajara where these flowers are cultivated extensively (Emily, 1973). Da Oarta, (1908) reported that in the early 16th century *Polianthes tuberosa* L. was one of costly items which the Portuguese merchants brought from India following the route of their early from the Cape of Good Hope.

Now this plant grows over much of tropical and temperate world. De Hertogh and Le Nard, (1993) stated that this plant was cultivated in tropical and subtropical regions. The cultivation of tuberose usually in Morocco, the Cameron Island, France, Hawaii, South Africa, India and China. *Polianthes tuberosa* L. is cultivated on large scale in France, Italy, South Africa, North Carolina in USA and many tropical and subtropical India. In India the commercial cultivation of tuberose in confined mainly to west Bengal, Karnataka, Maharashtra, Tamil Nadu and Uttar Pradesh.

1.2.2 Classification and Nomenclature

Kingdom : Plantae

Division : Magnoliophyta

Class : Liliopsida

Subclass : Liliidae

Order : Amaryllidales

Family : Agavaceae

Subfamily : Agavoideae

Genus : Polianthes

Species : tuberosa

Name : *Polianthes tuberosa* L.

Polianthes tuberosa L. is a perennial plant belonging to the family Agavaceae. The genus of *Polianthes* consists of 14 species, 3 varieties and 2 cultivars (Solano and Feria, 2007). Most species in this genus are used for ornamental and ceremonial purposes. The example of species are *P. bicolor, P. densiflora, P. geminiflora, P. howardii, P.logiflora, P. montana, P. multicolor, P. palustris, P. sessiliflora* and *P. venustaliflora.* The best known taxon is *Polianthes tuberosa* L., which has been cultivated and used for medicinal, ornamental and ceremonial practices since prehispanic times (Solano and Feria, 2007).

Species of *P. tuberosa* L. have been recorded in 18 Mexican states, where most occur in pine forest, oak forest or pine oak forest, some in grassland and a few tropical dry or semidecidous forest or pine oak forest (Solano, 2000). Five species of *Polianthes* which are *P. densiflora, P. howardii, P. logiflora, P. palustris* and *P. platyphylla* are listed by IUCN (IUCN, 1997) and are considered to be in the category of special protection according to Mexican Law (SEMARNA, 2002).

1.2.3 Morphological Description of *Polianthes tuberosa* L.

Polianthes tuberosa is an herbaceous species consist of grass like leaves arising from underground tuberose structure produce offsets that result in small clump of leaves. It is a half hardy, bulbous perennial perpetuating itself through the bulblets. Although some different botanical terms such as tuberose rhizome and tuberose rootstock have been suggested for the underground storage of this flower, practically is called bulb (Bryan, 1989). Bulbs are made up of scales and leaf base and the stem is condensed structure which remains concealed within scales. Roots are adventitious and shallow.

This plant can reach about 30 to 46 cm tall. It has dull green leaves often with slight reddish cast, thin, linear and slightly succulent. These leaves are borne in sets of six arising from the tuber. Usually leaves length are between 30 and 50 cm long. The inflorescence is a spike which is 3 to 4 meter tall. This spike produce clusters of waxy pure white flowers on the upper part of the stalk. There are some 20 fragrant white florosets (Morris, 1984; Huxley et al., 1992). They are consisting of a funnel shape tube in 3.6 to 6 cm long. The tube is narrow at its base and separates into six segments approximately.

Single, semi double and double flower varieties are available. Single flower variety has one row of corolla segments. Semi-double flower variety bearing flowers with two or three rows of corolla. Double for which crops has to be grown flowers variety having more than three rows of corolla. Selection of suitable cultivar depends on purpose for which crops has to be grown i.e use in loose flower, cut flower, extraction of essential oil , pot culture beautification of surrounding. Single cultivars are more suitable for loose flower and essential oil.

1.2.4 Propagation and planting

Polianthes tuberosa L. is typically propagated through bulbs. Each bulb has potential to produce one flower. The yield of flowers depends much on the size of bulbs used at planting (Kale and Bhujbal, 1972; Sadhu and Bose, 1973; Pathak *et al*., 1980; Yadav *et al*., 1984) and the environmental conditions (Brundell and Steenstra, 1985; Sadhu and Das, 1978).

Plant height (leaf length) and number of leaves per clump also show gradual increase with the increase in bulb size (Dhua et al., 1987). This was due to better vegetative growth of the plants and sufficient stored food materials in such bulbs.

After planting the bulb, the terminal bud sprouts and develops into a flowering shoot (Kosugi and Kimura, 1961). Axillary buds sprouts a few weeks later, produce foliage

shoot (bud seldom flowers), and eventually thicken at the base of the shoot to form 15-20 new bulbs per plant in a sympodial or clump form (Ruth, 1992).

To cultivation, this plant requires a warm sheltered position and a well-drained soil. When grown in pots it is best to use a fibrous loam enriched with compost and some silver sand for drainage. This plant requires copious amounts of moisture when starting into growth. Bulb should be planted at a distance 30 cm from one another.

In vitro propagation of *Polianthes tuberosa* L. is more efficient than conventional propagation for building up aseptic stocks of varieties especially for the establishment of new cultivars and the production of pathogen-free stock materials. Furthermore, because of large number of propagation cycles in the field, conventionally produced bulbs may become easily infected (Anbari *et al*., 2007; Chen *et al*., 2005; Gang *et al*., 2007; Sochacki and Ozlikowska, 2005). According to Staikidou *et al*., (2005) and Ziv and Chen (2003), the application of tissue culture techniques allow rapid and large scale propagation of uniform plants for field culture.

1.2.5 The Economic Importance of *Polianthes tuberose* L.

Polianthes tuberosa L. can be used in landscapes in all white gardens or with other plants. *Polianthes tuberosa* L. in pots can be put in containers and use for entranceways and interiors decoration. It is also widely grown commercially as cut flowers and as a source of

perfume as they have a powerful fragrance. India, Mexico, Japan and New Zealand commercially grown *P. tuberosa* for its fragrant cut flowers (Naidu and Reid, 1989).

According to Benschop (1993), *tuberose* is one of the most important cut flowers in tropical and subtropical area. Being fragrant has made it a favourable flower and also one of the most important cut flowers in Iran, where it holds 5 % of the total cut flower production (Anonymous, 2002). In Taiwan, it occupies a prime position in the floriculture industry (Huang and Huang, 1982; Shen et al., 1987; Shen et al., 1991). In Kenya, *P. tuberosa* is an important export crop among small scale farmers. Nguyen and Marc (2007), stated that many farmers in Mekong Delta, Vietnam dedicate up to a quarter of their land for *P. tuberosa* culture, often in intercropping or rotation with rice or vegetables. The crop is highly valuable, fetching as much as 10 times the value of a rice crop over a given area.

Essential oils, known as nature's living energy are the natural aromatic volatile liquids found in shrubs, flowers, trees, roots, bushes and seeds. Essential oils are extracted from aromatic plant. Essential oils often have pleasant aroma, their chemical makeup is complex and their benefits vast which make them more than something that simply smells good. Tuberose essential oil is very expensive because it is hard enough to get the essential oil from the flower. The natural flower oil of *P. tuberosa* remain today as one of the most expensive of the perfumer's raw materials.

According to Guenther, (1957) it requires 150 kg of flowers to yield one kg of absolute of effleurage which is brown, semisolid, alcohol soluble liquid pomades. The purified "absolute" is used today in perfumes of the highest grade and is usually blended with other perfume. The pomade is used as a base in nearly all the heavier types of perfumes such as that of gardenia, (Emily, 1973). In the Latin manuscript it has referred to by Pliny (2379 AD) as an ingredient in royal perfume. *Polianthes tuberosa* was cultivated in France and India for perfume industry (Naidu and Reid, 1989). The exotic smell of *Polianthes tuberosa* was included in several worldwide known perfumes such as 'Pison' by Cristian Dior and 'Chole' by Karl Lagerfeld.

Beside the essential oil from the flower also can be used as a food flavour and chocolate flavour. Nguyen and Marc (2007) stated the Aztec in Maxico used the essential oil of the plant to flavor chocolate. The most common constituents of tuberose concrete are geraniol, nerol, benzyl alcohol methyl benzoate and methyl anthranilate. The flower buds contain an alkaloid lycorine which causes vomiting. Two steroidal sapogenins namely becogenin and small amount of licogenin, a poly-fructose are isolated from the bulbs. Dried tuberose bulbs in the powdered form are used to treat gonorrhea.

The luminous white flowers also contain anti-inflammatory and antispasmodic properties. Tuberose is known to improve one's capacity for emotional depth. It also amplifies artistic inspiration as it stimulates the creative right side of the brain and it brings serenity to the mind and hearts. *Polianthes tuberosa* L. are gaining an importance in

pharmaceutical and perfume industries because of their peculiar of various commercial valuable compounds (Sangavai and Chellapandi, 2008).

1.2.6 The limitation of *Polianthes tuberose* L.

Polianthes tuberosa L. is a plant which does not produce seeds. It is propagated commercially through bulb. There are a lot of problems faced when using this propagation technique. The main challenges facing production and marketing of good quality *Polianthes tuberosa* cut flowers in the lack of clean planting materials as the resources poor farmers multiply their own propagules.

Polianthes tuberosa L. facing a lot of diseases problems include botrytis, erwina, fusarium and anthracnose. *Polianthes tuberosa* L. is subjected to infection by several fungi and among them tuber rot is an important disease incited by *Fusarium oxysporum* as it causes a considerable damage to the crop and reduces the flower yield. The main symptoms of the disease are the rotting symptoms observed from the neck region of tubers. The affected plants were stunted in growth, resulting in poor flower setting. The affected tubers showed reddish brown discolouration. *Botrytis* is economically important on soft fruits and bulb crops. *Botrytis cinerea* is a *necrotrophic fungus* that affects many plant species. Anthracnose caused by *Colletotrichum*. Anthracnose is usually a problem during period of high humidity.

In the Mekong Delta, Vietnam, the culture of *Poliantes tuberosa* L. is a risky business; more often than not, the crop is severely damaged or completely destroyed by nematodes of the genus *Aphelenchoides* (Nguyen and Marc, 2007). Nguyen *et al.*, (2010), found that the nematode is *Aphelenchoides besseyi* Christie. This is an ectoparasite that can persist for several months on the harvested bulbs or dried flowers (Cuc and Pilon, 2007). *A. besseyi* has also previously been shown to be a parasite of *Polianthes tuberosa* L. in West Bengal, India (Khan, 2004; Khan and Pal, 2001), as well as in Hawaii (Holtzmann, 1968). Besides that, this plant very susceptible to aphid, mite and thrips infestation.

Virus also becomes one of the big problems faces by conventional propagation of *Polianthes tuberosa* L. More recently, a serologically and biologically distinct potyvirus, *Tuberose mild mosaic virus* (TuMMV), has been reported from Taiwan (Chen and Chang, 1998; Chen *et al.*, 1998) and and the sequence of its 3-terminus, including the coat protein gene, was determined (Chen *et al.*, 2002). All the major tuberose cultivars have been found to be infected by TuMMV and a virus-free bulb propagation programme is becoming established (Chen *et al.*, 2002). Lin *et al.*, (2004) reported that a new genus of potyvirus (TuMMoV) has infecting Hangzhou, China tuberose plant.

Weeds also become a serious problem in the successful cultivation of tuberose, as heavy manurial and irrigation requirement and perennial nature of the crop create conductive condition for growth and development of different species of weed. In India weeding is generally being done by hand. If done frequently, it is effective but this procedure is highly consuming thereby increasing cost of cultivation.

1.3 Micropropagation of *Polianthes tuberosa* L.

Regeneration of whole plants from cells, tissues or segments from different parts of the plant body is a relatively common phenomenon in most plant groups and is an important means of vegetative propagation in nature as well as in horticulture and agriculture (Vasil and Vasil, 1972). Little studies consider tissue culture has been done for *Polianthes tuberosa*. A few reports were done on micropropagation of these species. Sangavai and Chellapandi, (2008) has worked on the effect of IAA and IBA for the proliferation of callus and shoot.

1.4 The objectives of the present work

Due to many advantages that can be obtained from *Polianthes tuberosa* L. and in order to improve the productivity and to fulfill the consumer demands, studies concerning propagation of this species is very important. In the present studies, experiments on callus induction and formation in *Polianthes tuberosa* L. were carried out. Different types of hormones such as NAA, BAP, and 2,4-D were used to induce callus formation. Leaf, stem and flower buds of *Polianthes tuberosa* L. were used as explants. The best explant and the best combinations of hormones for callus formation will be determined. Further study will be carried using the callus.

Other objective of this study was to differentiate and identify embryogenic and non-embryogenic callus using 'double staining' technique. Callus was induced using plant growth hormone like NAA, BAP and 2, 4-D from leaf, stem and flower bud explants. Embryogenic callus developed into somatic embryos. Somatic embryos can be induced by subculture the embryogenic callus cells into suspension media for about 4-5 weeks.

The process of somatic embryos and their stages were also studied. Somatic embryos are structurally similar to zygotic embryos found in seeds. Somatic embryos also have the ability to grow into complete plants as zygotic embryos. The different between these two types of embryos is that the somatic embryos develop from somatic cells but the zygotic embryos develop from the fusion of male and female gametes. Somatic embryos have the specialty to produce more plantlets and can be potentially used for clonal propagation system.

The main objective of this study was to establish *in vitro* propagation of *Polianthes tuberosa* L. Regeneration of *Polianthes tuberosa* L. was investigated in this study. Indirect regeneration from somatic embryo was observed. Shoot and root formation were induced using plant growth regulators. The effect of plant growth regulators was investigated. Different combination of auxin (NAA) and cytokinin (BAP and IBA) at various concentrations were used for this purpose.

Finally acclimatization process were carried out after complete plantlet was obtained from tissue culture. The successful of acclimatization process is an assurance that the whole micropropagation process, from the beginning of selecting the starting materials, initiation of *in vitro* cultures and establishment of an efficient acclimatization system has been achieved. During this period, the plantlets were observed to study the capability of plantlets to adapt to new environments. This study also was carried out to determine whether the plantlets could grow into healthy and vigorous plants.

Figure 1.1: Intact plant of *Polianthes tuberosa* L. grown at University of Malaya green house.

CHAPTER 2

CALLUS INDUCTION FROM VARIOUS EXPLANTS OF *POLIANTHES TUBEROSA* L.

2.1 EXPERIMENTAL AIMS

Callus is basically a more or less non organized tumour tissue which usually arises on wounds of differentiated tissues and organ. Callus from different plant species may be different in structure and growth habit. There are white or coloured callus, free (easily separated) or fixed, soft (watery) or hard, easy or difficult to separate into cells and aggregates in liquid media. In exceptional conditions, and sometimes spontaneously, the regeneration of adventitious organs and or embryos can occur from a callus.

The initiation of callus formation is referred to as callus induction. All types of explants and tissue can be used for starting material for callus induction. A lot of research has been done using various parts of plant as explants. Salehzadeh *et al*., (2008) used scale, leaf primordial and immature floret explant for callus induction of *Hyacinthus orientalis* L. Khawar *et al*., (2005) used bulb scale of Madonna lily (*Lilium candidum* L.) as an explant for callus formation. Slabbert *et al*., (1995) used immature floral stem of *Crinum macowanii* as an explant for callus induction.

Exogenous provided plant growth regulators are often recommended to initiate callus formation on an explant. Some explant required only auxin, some need cytokinin alone and some need booth auxin and cytokinin to start callus formation. According to

Pierik (1987), monocotyledons react differently when considering callus induction generally being less likely to form callus tissue than dicotyledons, it is often only necessary to add auxin as the hormonal stimulus for callus induction.

In this chapter, the main aim of this experiment was to investigate the effect of plant growth regulators on callus formation from leaf, stem and flower bud explants of *P. tuberosa*. BAP and NAA with different concentration were used in this experiment.

2.2 MATERIALS AND METHODS

2.2.1 Source of Explants

Intact plants of *Polianthes tuberosa* L. was collected from Cameron Highlands, Malaysia and purchased at nurseries in Sungai Buluh, Selangor. The same size of the bulbs was selected and grown in University of Malaya. Five-month-old plants with flower spike (stem) and flower bud were used in this tissue culture studies.

2.2.2 Types of Explant

Polianthes tuberosa L. produce no seeds. Intact explants were used as source for explant in this study. Three different explants were used which are leaf, stem and flower bud.

2.2.3 Explants Sterilization

Explants (leaf, stem and flower) from intact plant of *Polianthes tuberosa* L. were sterilized. First explants were surface sterilize under running tap water for 30 minutes to remove contaminants and any residue that found on the explants. Then explants were soaked in different concentrations (70%. 50%, 30%, 20%, and 10%) of sodium hypochlorite (clorox). At the first soaked of 70% sodium hypoclorite, two drops of Tween 20 was added. After that, the explants were rinsed with sterile distilled water to get rid of any trace of sodium hypochlorite that was used earlier. Each rinse lasting approximately for three minutes. The explants were then soaked in ethanol 70% for three minutes. Finally the explants were washed 3 times in sterile distilled water for three minutes.

2.2.4 Media Preparation

2.2.4.1 Preparation of Basic Media

Murashige and Skoog, MS (1962) was used as a basic medium for callus induction. MS media powder from Sigma was used. To prepare the basic media, first 1000ml conical flask were filled up with 800ml of distilled water. 30 g/l sucrose, 4.4 g/l MS powder and 8 g/l technical agar were diluted in distilled water. The media solution was stirred until all the sucrose, MS powder and technical agar were dissolved. After that, distilled water was made up to 1 Litre in the conical flask. pH of the media solution was adjusted to 5.8 using 1.0 M HCl (hydrochloric acid) and 1.0 M NaOH (sodium hydroxide). The media was autoclaved at a pressure of 104 kPa (15 Psi^2) and temperature of 121ºC for 20 minutes. After the media has been autoclaved and cooled (50ºC), the media were dispensed into 60 ml sterile universal container.

2.2.4.2 Preparation of Media with Hormones

Culture media with hormones was prepared using the same method but before the solution was autoclaved, hormones were added into the media. For hormones that are sensitive to heat like GA_3 and Abscisic acid, the hormones were added into the media solution after the autoclaved process and the media was cooled until 50ºC. The hormones need to be filtered sterilize before added into the media. Sterile membrane filtered was used to filter the hormones.

2.2.4.3 Media Sterilization

The media was autoclaved at internal steam temperature of about 120°C and steam pressured was allowed to build up to 1.2 kg per square metre for about 20 minutes. After autoclaving, the media was left to attain a temperature of about 50°C before it was poured

into sterile universal container which has dimensions of about 4.5 mm in diameter and 60 mm in height. Media was dispensed under aseptic condition in laminar flow chamber.

2.2.5 Culture Conditions

All equipments and apparatus used for tissue culture process must be sterilized before used. Laminar flow chamber, which all tissue culture work was done in, was sterilized by spraying the area with 70% ethanol and then wiped with autoclaved tissue. Before that, the shortwave length ultraviolet (UV) lamp was switched on in the chamber for about 15 minutes. All tissue culture apparatus like forceps and scalpels were autoclaved first before used. Sterile blades, universal containers and Petri dishes purchased from the supplier, Megalab Company were used. All apparatus and related tools must be wiped with 70% ethanol prior to culturing. Scalpels and forceps were dipped into hot bead sterilizer and cooled in sterile distilled water before used to excise the explants. The hot bead sterilizer was switch on for about 15 minutes before used (to get the desirable temperature, 250ºC).

2.2.6 Callus Induction

Leaf explants were cut into segments (1 cm x 1 cm) stem and flower bud explants were cut into 1 cm in length. All the explants were cultured on MS media (Murashige and Skoog, 1962) containing 30 gL^{-1} sucrose and 8 gL^{-1} agarose gel. MS (Murashige and Skoog, 1962) culture medium was used together with different types, concentrations and combinations of hormones. All cultures were kept in the culture room with photoperiod of 16 hours light and 8 hours dark.

The list of media used in this study:

1. MS basal

2. MS + 0.5 mg/l BAP

3. MS + 1.0 mg/l BAP

4. MS + 1.5 mg/l BAP

5. MS + 2.0 mg/l BAP

6. MS + 0.5 mg/l NAA

7. MS + 1.0 mg/l NAA

8. MS + 1.5 mg/l NAA

9. MS + 2.0 mg/l NAA

10. MS + 2.5 mg/l NAA

11. MS + 3.0 mg/l NAA

12. MS + 3.5 mg/l NAA

13. MS + 4.0 mg/l NAA

14. MS + 0.5 mg/l BAP + 0.5 mg/l NAA

15. MS + 0.5 mg/l BAP + 1.0 mg/l NAA

16. MS + 0.5 mg/l BAP + 1.5 mg/l NAA

17. MS + 0.5 mg/l BAP + 2.0 mg/l NAA

18. MS + 0.5 mg/l BAP + 2.5 mg/l NAA

19. MS + 0.5 mg/l BAP + 3.0 mg/l NAA

20. MS + 0.5 mg/l BAP + 3.5 mg/l NAA

21. MS + 0.5 mg/l BAP + 4.0 mg/l NAA

22. MS + 1.0 mg/l BAP + 0.5 mg/l NAA

23. MS + 1.0 mg/l BAP + 1.0 mg/l NAA

24. MS + 1.0 mg/l BAP + 1.5 mg/l NAA

25. MS + 1.0 mg/l BAP + 2.0 mg/l NAA

26. MS + 1.0 mg/l BAP + 2.5 mg/l NAA

27. MS + 1.0 mg/l BAP + 3.0 mg/l NAA

28. MS + 1.0 mg/l BAP + 3.5 mg/l NAA

29. MS + 1.0 mg/l BAP + 4.0 mg/l NAA

30. MS + 1.5 mg/l BAP + 0.5 mg/l NAA

31. MS + 1.5 mg/l BAP + 1.0 mg/l NAA

32. MS + 1.5 mg/l BAP + 1.5 mg/l NAA

33. MS + 1.5 mg/l BAP + 2.0 mg/l NAA

34. MS + 1.5 mg/l BAP + 2.5 mg/l NAA

35. MS + 1.5 mg/l BAP + 3.0 mg/l NAA

36. MS + 1.5 mg/l BAP + 3.5 mg/l NAA

37. MS + 1.5 mg/l BAP + 4.0 mg/l NAA

38. MS + 2.0 mg/l BAP + 0.5 mg/l NAA

39. MS + 2.0 mg/l BAP + 1.0 mg/l NAA

40. MS + 2.0 mg/l BAP + 1.5 mg/l NAA

41. MS + 2.0 mg/l BAP + 2.0 mg/l NAA

42. MS + 2.0 mg/l BAP + 2.5 mg/l NAA

43. MS + 2.0 mg/l BAP + 3.0 mg/l NAA

44. MS + 2.0 mg/l BAP + 3.5 mg/l NAA

45. MS + 2.0 mg/l BAP + 4.0 mg/l NAA

2.2.7 Data Analysis

Thirty replicates were used for each treatment and the data were analyzed statistically using Duncan's Multiple Range Test (DMRT). The statistical analysis based on mean values per treatment was made using the technique of analysis of variance. The comparative LSD *multiple range test* (p=0.01) was used to determine the differences between treatments.

2.3 RESULTS

2.3.1 Callus induction

Callus induction from leaf, stem, and flower bud explants of *Polianthes tuberosa* L. has been successfully achieved. Callus initiation was observed from the cut end surfaces of leaf, stem and flower bud explants within 3 months in all media tested with the exception of the media MS without hormone and MS supplemented with BAP alone. Explants in the MS media without hormone become necrotic after some time in the media and explants in the MS media supplemented with BAP alone showed no active growth and died after 4 weeks.

Stem started to produce callus earlier than other explants. Within 4 weeks, a small clump of green undifferentiated cells (Figure 2.3) could be observed but the callus proliferation was slow in stem explants. The percentage of callus formation was lower compared to other explants tested after 5 months in the culture media. Flower bud explants produced more callus compared to stem explants but the initiation of callus was started late compared to other explants. Only after 12 weeks in culture callus could be observed. Leaf explant was the best explant for callus formation. Explants started to produce callus after 5 weeks in the culture and the callus proliferation was fast. After 5 months the whole explant was covered with callus.

Two types of callus could be recognized according to the colour. The first type is the callus which is nodular, friable, soft (watery) and greenish. The second type is the callus which is nodular, friable, soft, wet looking surface and yellow whitish and cream in colour. Most of the callus induction from flower bud explants (Figure 2.4) is the second type callus and the callus formation from leaf explant (Figure 2.2) was the first type.

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MS medium supplemented with 2.0 mg/l NAA, MS media supplemented with 0.5 mg/l BAP in combination with 2.0 mg/l NAA, MS media supplemented with 3.0 mg/l NAA and MS media supplemented with 0.5 mg/l BAP in combination with 2.5 mg/l NAA are suitable for callus formation from leaf explant. 100.0±0.00 % of the explant tested produced callus. MS media supplemented with 1.5 mg/l BAP in combination with 2.5 mg/l NAA, MS media supplemented with 1.5 mg/l BAP in combination with 3.0 mg/l NAA and MS media supplemented with 2.0 mg/l BAP in combination with 4.0 mg/l NAA are inappropriate for the formation of callus from leaf explant. Only 60.0±0.09 % of the explants cultured produced callus (Table 2.1).

100.0±0.00 % of stem explant produced callus in MS media supplemented with 2.0 mg/l NAA, MS media supplemented with 3.0 mg/l NAA and MS media supplemented with 0.5 mg/l BAP in combination with 2.0 mg/l NAA. These 3 media were the right media for callus callus induction from stem explant. MS media supplemented with 1.5 mg/l BAP in combination with 2.5 mg/l NAA and MS media supplemented with 2.0 mg/l BAP in combination with 4.0 mg/l NAA were not suitable for callus formation from stem explant of this species, only 50.0±0.09 % of the explant produced callus (Table 2.1).

Flower bud explant was suitable when cultured on MS media supplemented with 2.0 mg/l NAA and MS media supplemented with 0.5 mg/l BAP in combination with 2.0 mg/l NAA. All the explants (100.0±0.00 %) produced callus. MS media supplemented with 2.0 mg/l BAP in combination with 2.0 mg/l NAA and MS media supplemented with 0.5 mg/l BAP in combination with 4.0 mg/l NAA were not suitable media for callus

formation from flower bud explants. Only 56.67±0.09 % and 50.0±0.09 % of the explants produced callus (Table 2.1).

MS media supplemented with 2.0 mg/l NAA alone and MS media supplemented with 0.5 mg/l BAP in combination with 2.0 mg/l NAA were the best media for callus formation. 100.0±0.00 % of explants from leaf, stem and flower buds produced callus after 5 months in culture. In MS media supplemented with 3.0 mg/l NAA alone, all explant cultured (100.0±0.00 %) from leaf and stem produced callus and 90.0±0.06 % of flower bud explants produced callus (Table 2.1).

Higher percentage of explants produced callus could be observed in MS media supplemented with NAA combination with lower concentrations of BAP. 100.00±0.00 of explant produced callus could be observed in MS media supplemented with 2 mg/l NAA and 0.5 mg/l BAP. On MS media supplemented with NAA and higher concentrations of BAP (1.0 mg/l, 1.5 mg/l and 2.0 mg/l) lower percentage of explants produced callus. 50.00±0.09 % of callus formation was the lowest percentage obtained from this study. This percentage was obtained from stem and flower bud explants cultured on MS media supplemented with 2.0 mg/l BAP and 4.0 mg/l NAA and stem explant cultured on 1.5 mg/l BAP and 2.5 mg/l NAA (Table 2.1).

Table 2.1: Callus induction from leaf explant of *Polianthes tuberosa* L. cultured on MS supplemented with various concentrations of BAP and NAA maintained at 25±1 ℃ under 16 hours light and 8 hours dark. Thirty replicates were used in each treatment. Data were recorded after 5 months of culture.

MS + Hormone (mg/l)		Explants	Percentage of explant produced callus (mean ± SE)	Observations
BAP	**NAA**			
0	0 (control)	Leaf	0.00 ± 0.00^{e}	No response. Explants became necrotic after 2 weeks in culture.
		Stem	0.00 ± 0.00^{f}	No response. Explants became necrotic after 2 weeks in culture.
		Flower bud	0.00 ± 0.00^{g}	No response. Explants became necrotic after 2 weeks in culture.
0	0.5	Leaf	76.67 ± 0.08^{abcd}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
		Stem	70.00 ± 0.06^{abcde}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 1 month.
		Flower bud	86.67 ± 0.06^{abcde}	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
0	1.0	Leaf	90.00 ± 0.06^{abcd}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
		Stem	90.00 ± 0.06^{abc}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft

				(watery) callus was observed after 1 month.
		Flower bud	83.33±0.07^{abcde}	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
0	1.5	Leaf	96.67±0.03^{ab}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
		Stem	93.33±0.05^{ab}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 1 month.
		Flower bud	96.67±0.03^{ab}	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
0	2.0	Leaf	100.00±0.00^{a}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
		Stem	100.00±0.00^{a}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 1 month.
		Flower bud	100.00±0.00^{a}	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
0	2.5	Leaf	96.67±0.03^{ab}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.

		Stem	90.00 ± 0.06^{abc}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 1 month.
		Flower bud	96.67 ± 0.03^{ab}	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
0	3.0	Leaf	100.00 ± 0.00^{a}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
		Stem	100.00 ± 0.00^{a}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 1 month.
		Flower bud	90.00 ± 0.06^{abcd}	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
0	3.5	Leaf	86.67 ± 0.06^{abcd}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
		Stem	93.33 ± 0.05^{ab}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 1 month.
		Flower bud	96.67 ± 0.03^{ab}	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
0	4.0	Leaf	86.67 ± 0.06^{abcd}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft

				(watery) callus was observed after 2 months.
		Stem	83.33±0.07[abcd]	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 1 month.
		Flower bud	83.33±0.07[abcde]	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
0.5	0	Leaf	0.00±0.00[e]	No response. Explants became necrotic after 2 weeks in culture.
		Stem	0.00±0.00[f]	No response. Explants became necrotic after 2 weeks in culture.
		Flower bud	0.00±0.00[g]	No response. Explants became necrotic after 2 weeks in culture.
0.5	0.5	Leaf	83.33±0.07[abcd]	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
		Stem	76.67±0.09[abcde]	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 1 month.
		Flower bud	80.00±0.07[abcdef]	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
0.5	1.0	Leaf	86.67±0.06[abcd]	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.

		Stem	83.33±0.07[abcd]	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 1 month.
		Flower bud	83.33±0.07[abcde]	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
0.5	1.5	Leaf	93.33±0.05[abc]	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
		Stem	90.00±0.06[abc]	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 1 month.
		Flower bud	90.00±0.06[abcd]	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
0.5	2.0	Leaf	100.00±0.00[a]	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
		Stem	100.00±0.00[a]	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 1 month.
		Flower bud	100.00±0.00[a]	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
0.5	2.5	Leaf	100.00±0.00[a]	Explant still fresh but swollen after 3 weeks.

				Green, friable, and soft (watery) callus was observed after 2 months.
		Stem	83.33 ± 0.07^{abcd}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 1 month.
		Flower bud	93.33 ± 0.05^{abc}	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
0.5	3.0	Leaf	86.67 ± 0.06^{abcd}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
		Stem	90.00 ± 0.06^{abc}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 1 month.
		Flower bud	83.33 ± 0.07^{abcde}	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
0.5	3.5	Leaf	83.33 ± 0.07^{abcd}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
		Stem	76.67 ± 0.08^{abcde}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 1 month.
		Flower bud	80.00 ± 0.07^{abcdef}	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.

0.5	4.0	Leaf	76.67 ± 0.08^{abcd}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
		Stem	76.67 ± 0.08^{abcde}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 1 month.
		Flower bud	90.00 ± 0.06^{abcd}	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
1.0	0	Leaf	0.00 ± 0.00^{e}	No response. Explants became necrotic after 2 weeks in culture.
		Stem	0.00 ± 0.00^{f}	No response. Explants became necrotic after 2 weeks in culture.
		Flower bud	0.00 ± 0.00^{g}	No response. Explants became necrotic after 2 weeks in culture.
1.0	0.5	Leaf	73.33 ± 0.08^{abcd}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
		Stem	66.67 ± 0.09^{bcd}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 1 month.
		Flower bud	66.67 ± 0.09^{bcdef}	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
1.0	1.0	Leaf	73.33 ± 0.08^{abcd}	Explant still fresh but swollen after 3 weeks.

				Green, friable, and soft (watery) callus was observed after 2 months.
		Stem	86.67 ± 0.06^{abcd}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 1 month.
		Flower bud	66.67 ± 0.08^{bcdef}	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
1.0	1.5	Leaf	83.33 ± 0.07^{abcd}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
		Stem	73.33 ± 0.08^{abcdef}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 1 month.
		Flower bud	90.00 ± 0.06^{abcd}	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
1.0	2.0	Leaf	80.00 ± 0.07^{abcd}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
		Stem	76.67 ± 0.08^{abcde}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 1 month.
		Flower bud	83.33 ± 0.07^{abcde}	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.

1.0	2.5	Leaf	63.33 ± 0.09^{cd}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
		Stem	73.33 ± 0.08^{abcde}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 1 month.
		Flower bud	73.33 ± 0.08^{abcdef}	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
1.0	3.0	Leaf	70.00 ± 0.09^{abcd}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
		Stem	70.00 ± 0.09^{abcde}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 1 month.
		Flower bud	83.33 ± 0.07^{abcde}	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
1.0	3.5	Leaf	73.33 ± 0.08^{abcd}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
		Stem	63.33 ± 0.09^{bcde}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 1 month.
		Flower bud	70.00 ± 0.09^{abcdef}	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was

				observed after 2 months.
1.0	4.0	Leaf	73.33±0.08[abcd]	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
		Stem	66.67±0.09[bcde]	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 1 month.
		Flower bud	70.00±0.09[abcdef]	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
1.5	0	Leaf	0.00±0.00[e]	No response. Explants became necrotic after 2 weeks in culture.
		Stem	0.00±0.00[f]	No response. Explants became necrotic after 2 weeks in culture.
		Flower bud	0.00±0.00[g]	No response. Explants became necrotic after 2 weeks in culture.
1.5	0.5	Leaf	73.33±0.08[abcd]	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
		Stem	66.67±0.09[bcde]	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 1 month.
		Flower bud	76.67±0.08[abcdef]	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.

1.5	1.0	Leaf	70.00 ± 0.09^{abcd}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
		Stem	63.33 ± 0.09^{bcde}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 1 month.
		Flower bud	66.67 ± 0.09^{bcdef}	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
1.5	1.5	Leaf	76.67 ± 0.08^{abcd}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
		Stem	76.67 ± 0.08^{abcde}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 1 month.
		Flower bud	70.00 ± 0.09^{abcdef}	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
1.5	2.0	Leaf	80.00 ± 0.07^{abcd}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
		Stem	66.67 ± 0.09^{bcde}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 1 months
		Flower bud	73.33 ± 0.08^{abcdef}	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was

1.5	2.5	Leaf	60.00 ± 0.09^{d}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
		Stem	50.00 ± 0.09^{e}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 1 month.
		Flower bud	73.33 ± 0.08^{abcdef}	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
1.5	3.0	Leaf	60.00 ± 0.09^{d}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
		Stem	63.33 ± 0.09^{bcde}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 1 month.
		Flower bud	70.00 ± 0.09^{abcdef}	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
1.5	3.5	Leaf	70.00 ± 0.09^{abcd}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
		Stem	73.33 ± 0.08^{abcde}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 1 month.

		Flower bud	66.67 ± 0.09^{bcdef}	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
1.5	4.0	Leaf	70.00 ± 0.09^{abcd}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
		Stem	76.67 ± 0.08^{abcde}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 1 month.
		Flower bud	70.00 ± 0.09^{abcdef}	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
2.0	0	Leaf	0.00 ± 0.00^{e}	No response. Explants became necrotic after 2 weeks in culture.
		Stem	0.00 ± 0.00^{f}	No response. Explants became necrotic after 2 weeks in culture.
		Flower bud	0.00 ± 0.00^{g}	No response. Explants became necrotic after 2 weeks in culture.
2.0	0.5	Leaf	63.33 ± 0.09^{cd}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
		Stem	56.67 ± 0.09^{de}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 1 month.
		Flower bud	56.67 ± 0.09^{ef}	Explant was swollen after 3 weeks. Green, friable, and

				soft (watery) callus was observed after 2 months.
2.0	1.0	Leaf	63.33±0.09^{cd}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
		Stem	73.33±0.08^{abcde}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 1 month.
		Flower bud	60.00±0.09^{def}	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
2.0	1.5	Leaf	66.67±0.09^{bcd}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
		Stem	66.67±0.09^{bcde}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 1 month.
		Flower bud	56.67±0.09^{bcde}	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
2.0	2.0	Leaf	73.33±0.08^{abcd}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
		Stem	56.67±0.09^{de}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 1 month.

		Flower bud	60.00±0.09^{abcde}	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
2.0	2.5	Leaf	76.67±0.08^{abcd}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
		Stem	66.67±0.09^{bcde}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 1 month.
		Flower bud	60.00±0.09^{def}	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
2.0	3.0	Leaf	66.67±0.09^{bcd}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
		Stem	60.00±0.09^{cde}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 1 month.
		Flower bud	63.33±0.08^{cdef}	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
2.0	3.5	Leaf	66.67±0.08^{bcd}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
		Stem	70.00±0.09^{abcde}	Explant still fresh but swollen after 3 weeks.

|				
				Green, friable, and soft (watery) callus was observed after 1 month.
		Flower bud	63.33 ± 0.09^{cdef}	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
2.0	4.0	Leaf	60.00 ± 0.09^{d}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.
		Stem	50.00 ± 0.09^{e}	Explant still fresh but swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 1 month.
		Flower bud	50.00 ± 0.08^{f}	Explant was swollen after 3 weeks. Green, friable, and soft (watery) callus was observed after 2 months.

Figure 2.1: Effect of NAA and BAP on callus induction from leaf, stem and flower bud explants. The vertical bars represent standard errors.
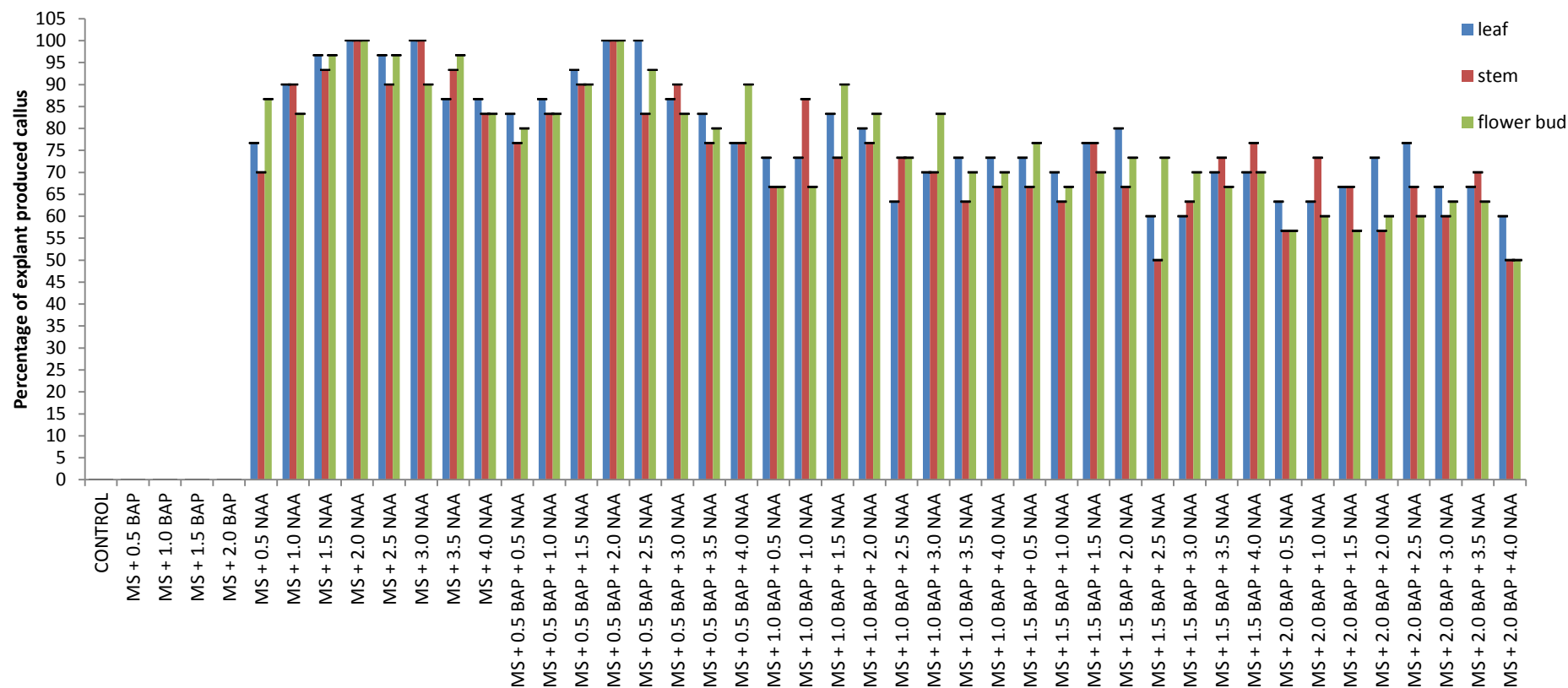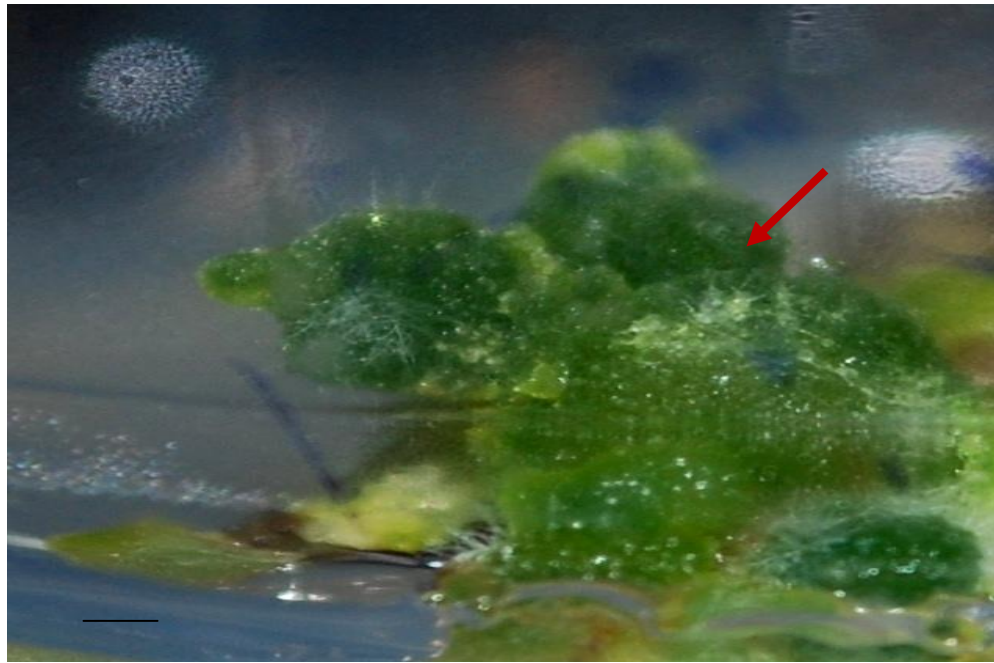
Figure 2.2: Callus derived from leaf explant cultured on MS media supplemented with 2 mg/l NAA and 0.5 mg/l BAP. Bars = 1 mm.
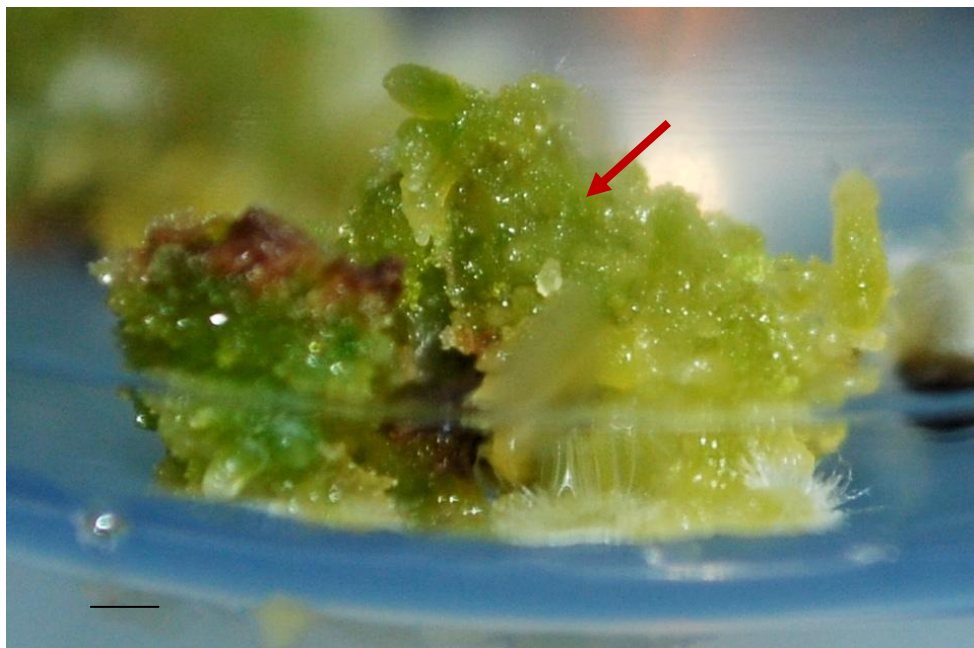
Figure 2.3: Callus derived from stem explant cultured on MS media supplemented with 2 mg/l NAA. Bars = 1 mm.
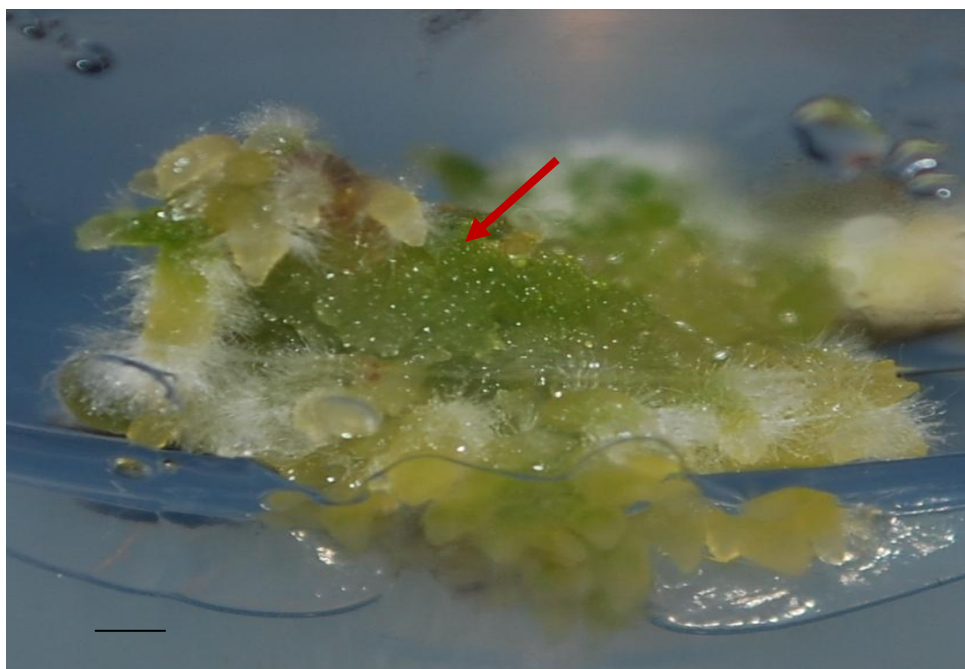
Figure 2.4: Callus derived from flower bud explant cultured on MS media supplemented with 2 mg/l NAA. Bars = 1 mm.

SUMMARY OF THE RESULTS

1. The results indicate that all explants could produce callus.

2. Stem started to produce callus earlier (4 weeks) than other explants. Leaf explants started to produce callus after 5 weeks and flower bud explants started to produced callus after 12 weeks.

3. Flower bud explants produced more callus compared to leaf and stem explant.

4. Two types of callus were obtained from the present study. First type of callus was nodular, friable, soft (watery) and greenish and the other types was compact nodular and creamy yellow.

5. There was no callus formation when explants were cultured on MS medium without hormone.

6. MS medium supplemented with NAA alone was the best media for callus formation in all explants.

7. The frequency of explants produced callus in media supplemented with higher concentrations of BAP was lower compared to explants produced callus in media supplemented with lower concentrations of BAP.

CHAPTER 3

SOMATIC EMBRYOGENESIS INDUCTION IN *POLIANTHES TUBEROSA* L.

3.1 EXPERIMENTAL AIMS

Plant morphogenesis can be achieved via two pathways, organogenesis or somatic embryogenesis. In somatic embryogenesis, a plant with both root and shoots axes arises from actively dividing cells, but does not form any direct vascular connections with the original tissue (Hicks,1980). Somatic embryogenesis is the process of a single cell or a group of cells initiating the developmental pathway that leads to reproducible regeneration of non-zygotic embryos capable of germinating to form a complete plant. This process is the best exemplifies the concept of totipotency, that all normal living cells possess the potential to regenerate the entire organism.

Of the method use for micropropagation, somatic embryogenesis is potentially important as it is capable of providing a large number of plants in shorter period of time than organogenic approaches. Since the 1960s much information has been gathered about the requirements for the manipulation of somatic embryogenesis *in vitro* (Thorpe, 1995). Levine (1947) reported the recovery of *Daucus carota* seedlings from tissues exposed to low level of NAA. Reinert (1958) and Steward (1958) were known as the first scientist to observe somatic embrogenesis in *Daucus carota* cell suspension culture.

The main objective of this experiment was to investigate the effects of plant growth regulators, state of culture media on somatic embryogenesis from leaf explants of *P. tuberose.* In addition, we differentiated embryogenic and non embryogenic callus by double staining method.

3.2 MATERIALS AND METHODS

3.2.1 Preparation of explants

Leaf explants from five-month-old intact plants were used in this experiment. In order to get the sterilized explants, explants sterilization process were done. Firstly explants were surface sterilize under running tap water for 30 minutes to remove contaminants and any residue that found on the explants. Then explants were rinsed in different concentrations (70%. 50%, 30%, 20%, and 10%) of sodium hypochlorite (clorox) and 70% ethanol. At first rinse of 70% sodium hypochlorite two drops of Tween 20 was added. Finally the explants were washed 3 times in sterile distilled water. Each rinse lasting approximately for one minute. Sterilized explants were cultured on culture medium. Embryogenic callus that obtained from the explants were used for somatic embryo induction.

3.2.2 Preparation of culture medium and callus induction

Solid culture media and liquid culture media were used in this study. Solid media was prepared by diluting MS powder with 30 g/l sucrose and 8 g/l agar in distilled water (same as media preparation in chapter 2) and the pH of the medium was adjusted by adding 1.0 M NaOH or 1.0 M HCl to 5.8 prior to autoclaving. Suspension culture media was prepared using the same method but without the gelling agent. Various types and concentrations of plant hormones such as 2,4-D and NAA were added into the culture medium to study the induction and formation of somatic embryo. Solid and liquid media were supplemented with the same concentrations and combinations of hormones. Below is the list of media that was used in this study.

1. MS media (control)

2. MS + 0.1 mg/l BAP

3. MS + 0.5 mg/l BAP

4. MS + 1.0 mg/l BAP

5. MS + 2.0 mg/l BAP

6. MS + 2.5 mg/l BAP

7. MS + 2.0 mg/l NAA

8. MS + 2.5 mg/l NAA

9. MS + 3.0 mg/l NAA

10. MS + 3.5 mg/l NAA

11. MS + 4.0 mg/l NAA

12. MS + 0.1 mg/l 2-4,D

13. MS + 0.5 mg/l 2-4,D

14. MS + 1.0 mg/l 2-4,D

15. MS + 1.5 mg/l 2-4,D

16. MS + 2.0 mg/l 2-4,D

17. MS + 2.5 mg/l 2-4,D

18. MS + 3.0 mg/l 2-4,D

19. MS + 4.0 mg/l 2-4,D

20. MS + 3.5 mg/l NAA + 0.1 mg/l BAP

21. MS + 3.5 mg/l NAA + 0.5 mg/l BAP

22. MS + 3.5 mg/l NAA + 1.0 mg/l BAP

23. MS + 3.5 mg/l NAA + 1.5 mg/l BAP

24. MS + 3.5 mg/l NAA + 2.0 mg/l BAP

25. MS + 3.5 mg/l NAA + 2.5 mg/l BAP

26. MS + 2.5 mg/l 2-4,D + 0.1 mg/l BAP

27. MS + 2.5 mg/l 2-4,D + 0.5 mg/l BAP

28. MS + 2.5 mg/l 2-4,D + 1.0 mg/l BAP

29. MS + 2.5 mg/l 2-4,D + 1.5 mg/l BAP

30. MS + 2.5 mg/l 2-4,D + 2.0 mg/l BAP

31. MS + 2.5 mg/l 2-4,D + 2.5 mg/l BAP

Callus induction studies were discussed in chapter 2. Callus that was obtained were analyzed whether it is embryogenic or non embryogenic.

3.2.3 Identification of embryogenic callus

Callus can be divided into two types, embryogenic callus and non-embryogenic callus. Embryogenic callus has the ability to regenerate new plantlets whereas non embryogenic callus will die after sometimes in the culture. Embryogenic and non embryogenic callus can be identified by using 'double staining' technique (Gupta and Durzan, 1987). First, the callus cells stained with acetocarmine and evan's blue dye and then were observed under microscope with different magnifications.

Double staining technique:

Preparation of 2% acetocarmine

1. Distilled water (55 ml) was measured using a 100 ml cylinder.

2. The distilled water was poured into a beaker containing a stir bar.

3. 45 ml of glacial acetic acid was measured using a pipette.

4. Glacial acetic acid was added to the beaker containing distilled water.

5. This gave a 45% acid solution.

6. The beaker containing the solution was placed on a stir plate in a fume hood.

7. The solution was boiled gently for 5 minutes on highest setting, stirred, cooled and filtered by funnel using Whatmant filter paper.

8. Finally it was stored at room temperature

Preparation of 0.5% Evan's Blue

1. Distilled water (100 ml) was measured out into a 250 ml flask.

2. 0.5 grams of Evan's Blue was added to the 100 ml distilled water in the flask.

3. The solution was swirled to mix properly.

4. Finally, it was stored at room temperature.

Double staining method

1. Small pieces of callus (3-5 mm) were placed on clean glass slides.

2. A few drops of acetocarmine was added until all callus were submerged.

3. The callus was gently divided with forceps into very small pieces in the acetocarmine solution.

4. The specimens were flamed or heated gently for 2 minutes without boiling it.

5. The callus was washed for 2 to 3 times with distilled water to remove all liquid of acetocarmine.

6. 2 drops of 0.5% Evan's Blue was added to Acetocarmine stained cells.

7. After 30 seconds, the slides were washed 2-3 times with water and then all water was removed.

8. One to two drops of glycerol was added to stained cells to prevent drying.

9. The slides were observed under light microscope and the embryogenic and non embrogenic callus were identified.

3.2.4 Embryogenic callus initiation

Leaf explants were cultured on MS medium supplemented with different types and combination of hormones as shown before. The culture was incubated in the culture room. After 2 months, green and white friable callus was observed. These calluses were transferred to other media for somatic embryo induction.

3.2.5 Induction of somatic embryo

Embryogenic callus were subcultured onto solid media or liquid media for somatic embryo formation. Embryogenic callus derived from callus induction medium were cut into small pieces (0.5 cm) and then transferred to solid media or liquid media. Cultures in solid media were maintained on the rack in the culture room at photoperiod of 16 hours light and 8 hours dark. Cultures in liquid media were maintained on a horizontal shaker at 100 rpm in the culture room condition. Different stages of somatic embryogenesis were observed after 2 months.

3.2.6 Data analysis

Three replications with 30 explants in each replication were maintained for each treatment and the data was analyzed statistically using Duncan's Multiple Range Test (DMRT). The statistical analysis based on mean values per treatment was made using the technique of analysis of variance. The comparative LSD *multiple range test* (p=0.01) was used to determine the differences between treatments.

3.3 Results

3.3.1 Induction and identification of embryogenic callus

Callus obtained was soft and watery in structure. The colour was green and white creamy. MS media supplemented with 2.0 mg/l NAA and 3.0 mg/l NAA gave the highest result with the percentage of callus formation (100.00±0.00) (Table 3.1).

Embryogenic callus were identified using double staining method. Embryogenic callus stained red with acetocarmine (Figure 3.1) and non-embryogenic callus cells stained blue with Evan's blue (Figure 3.2).

Table 3.1: Induction of callus formation from leaf explant cultured on MS solid medium supplemented with different concentrations of NAA

MS + NAA(mg/l)	Callus formation (%)	Observation
0.5	76.67 ± 0.08^{abcd}	Embryogenic callus. Green and white creamy in colour and soft watery in structure.
1.0	90.00 ± 0.06^{abcd}	Embryogenic callus. Green and white creamy in colour and soft watery in structure.
1.5	96.67 ± 0.03^{ab}	Embryogenic callus. Green and white creamy in colour and soft watery in structure.
2.0	100.00 ± 0.00^{a}	Embryogenic callus. Green and white creamy in colour and soft watery in structure.
2.5	96.67 ± 0.03^{ab}	Embryogenic callus. Green and white creamy in colour and soft watery in structure.
3.0	100.00 ± 0.00^{a}	Embryogenic callus. Green and white creamy in colour and soft watery in structure.
3.5	86.67 ± 0.06^{abcd}	Embryogenic callus. Green and white creamy in colour and soft watery in structure.
4.0	86.67 ± 0.06^{abcd}	Embryogenic callus. Green and white creamy in colour and soft watery in structure.

Means followed by the same letter are not significantly different at the 0.01 level of confidence
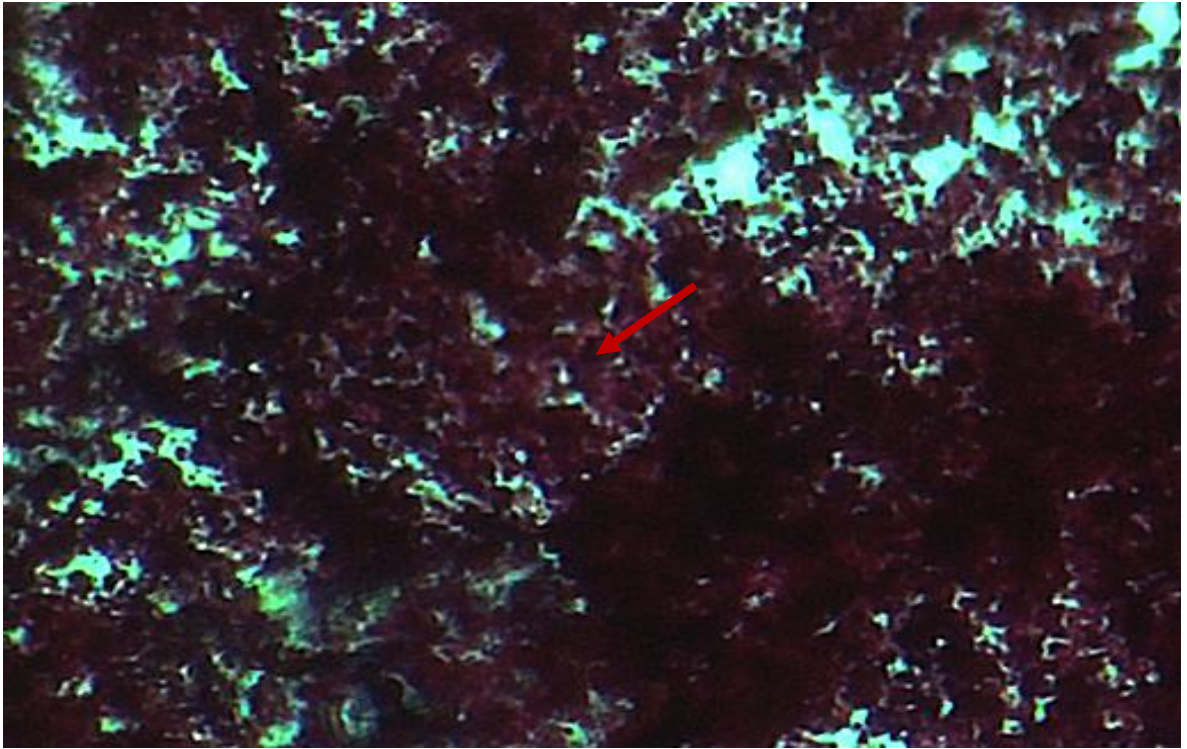
Figure 3.1: Embryogenic callus cells stained red with acetocarmine.

Figure 3.2: Non-embryogenic callus cells stained blue with Evan's blue.

Figure 3.3: Embryogenic callus derived leaf explant cultured on MS media supplemented with 2.0 mg/l NAA.

3.3.2 Somatic embryo development

In order to encourage the production of mature development stages somatic embryos, callus embryogenic (Figure 3.3) must be transferred to other media. 35 combinations of liquid and solid media were used in this study. The best media for somatic embryo formation were determined.

Embryogenic callus after being transferred to MS hormone free medium and MS supplemented with BAP alone did not produce any somatic embryo. Embryogenic callus became necrotic and died after some time in the culture media. However, on the medium supplemented with BAP in combination with NAA or 2,4-D, smooth round structure occurred on the surface of embryogenic callus within 3 months. Embryogenic callus that was subcultured on MS media supplemented with NAA or 2,4-D alone also gave rise to the smooth round structure (Figure 3.5). According to Luo *et al*., (1999) whose done the histological studies of the same structure from callus of *Astaragalus* sp. showed that these structures was somatic embryos at globular stage without vascular connection to the callus.

Somatic embryo at globular stage will become oblong (Figure 3.6) after 13 weeks and developed further into heart shape (Figure 3.7) after 15 weeks. Heart shape somatic embryo elongated (Figure 3.8) after 16 weeks and after 17 weeks torpedo stages (Figure 3.9) were observed. The last stage (Figure 3.10) which is cotyledonary stage was observed after 19 weeks in culture. Similar results was obtained from embryogenic callus cultured on solid media.

Compared to NAA, NAA combined with BAP and 2,4-D alone, 2, 4-D combined with BAP promoted somatic embryogenesis effectively. The high frequencies of somatic embryogenesis occurred on MS medium supplemented with 2,4-D at concentration of 2.5 mg/l combined with 0.1 mg/l BAP where an average of 26.67±0.42 somatic embryos were obtained from 0.5 cm of embryogenic callus from liquid media and 20.53±0.50 somatic embryos were formed on solid media. MS supplemented with 2,4-D alone also gave the best result with 24.47±0.41 of somatic embryo formation on liquid media and 19.67±0.61 on solid media (Table 3.2).

MS media supplemented with 0.1 mg/l 2-4,D was not very suitable for somatic embryo induction. Only 5.77±0.29 was observed on liquid media and 4.63±0.41 on solid media. Liquid media was the best media for formation of somatic embryo compared to solid media. Higher number of somatic embryo was found in liquid media in every test with the same treatment except in MS media supplemented with 2.5 mg/l 2-4,D and 2.5 mg/l BAP. 7.70±0.48 was found in liquid media and 9.03±0.52 in solid media (Table 3.2).

Table 3.2: Number of somatic embryo produced in liquid and solid media supplemented with different concentrations of BAP, NAA and 2,4-D.

MS + Hormone (mg/l)	Number of Somatic Embryo per Explant	
	Liquid media	Solid media
MS media (control)	0.00 ± 0.00^p	0.00 ± 0.00^q
MS + 0.1 mg/l BAP	0.00 ± 0.00^p	0.00 ± 0.00^q
MS + 0.5 mg/l BAP	0.00 ± 0.00^p	0.00 ± 0.00^q
MS + 1.0 mg/l BAP	0.00 ± 0.00^p	0.00 ± 0.00^q
MS + 1.5 mg/l BAP	0.00 ± 0.00^p	0.00 ± 0.00^q
MS + 2.0 mg/l BAP	0.00 ± 0.00^p	0.00 ± 0.00^q
MS + 2.5 mg/l BAP	0.00 ± 0.00^p	0.00 ± 0.00^q
MS + 2.0 mg/l NAA	12.63 ± 0.26^{lm}	7.43 ± 0.29^o
MS + 2.5 mg/l NAA	16.17 ± 0.35^{ij}	9.30 ± 0.29^{lmn}
MS + 3.0 mg/l NAA	19.27 ± 0.48^{def}	11.53 ± 0.32^{ijk}
MS + 3.5 mg/l NAA	24.67 ± 0.32^{b}	19.43 ± 0.23^{a}
MS + 4.0 mg/l NAA	20.43 ± 0.38^{cde}	16.70 ± 0.39^{d}
MS + 0.1 mg/l 2-4,D	5.77 ± 0.29^{o}	4.63 ± 0.41^{p}
MS + 0.5 mg/l 2-4,D	8.93 ± 0.40^{n}	7.57 ± 0.31^{no}
MS + 1.0 mg/l 2-4,D	13.87 ± 0.35^{kl}	10.53 ± 0.65^{klm}
MS + 1.5 mg/l 2-4,D	15.83 ± 0.39^{ij}	12.60 ± 0.54^{hij}
MS + 2.0 mg/l 2-4,D	19.93 ± 0.43^{def}	15.13 ± 0.56^{def}
MS + 2.5 mg/l 2-4,D	24.47 ± 0.41^{b}	19.67 ± 0.61^{a}
MS + 3.0 mg/l 2-4,D	21.00 ± 0.25^{cd}	16.30 ± 0.71^{cde}
MS + 3.5 mg/l 2-4,D	17.70 ± 0.43^{gh}	14.40 ± 0.59^{fgh}
MS + 4.0 mg/l 2-4,D	14.90 ± 0.48^{ij}	11.80 ± 0.64^{ijk}
MS + 3.5 mg/l NAA + 0.1 mg/l BAP	26.93 ± 0.38^{a}	20.10 ± 0.62^{a}
MS + 3.5 mg/l NAA + 0.5 mg/l BAP	23.47 ± 0.38^{b}	17.28 ± 0.60^{bc}
MS + 3.5 mg/l NAA + 1.0 mg/l BAP	20.73 ± 0.38^{cd}	14.20 ± 0.57^{fgh}
MS + 3.5 mg/l NAA + 1.5 mg/l BAP	18.78 ± 0.27^{fg}	13.00 ± 0.51^{ghi}
MS + 3.5 mg/l NAA + 2.0 mg/l BAP	16.43 ± 0.31^{hi}	10.97 ± 0.49^{jkl}
MS + 3.5 mg/l NAA + 2.5 mg/l BAP	11.40 ± 0.31^{m}	8.13 ± 0.49^{no}
MS + 2.5 mg/l 2-4,D + 0.1 mg/l BAP	26.67 ± 0.42^{a}	20.53 ± 0.50^{a}
MS + 2.5 mg/l 2-4,D + 0.5 mg/l BAP	23.53 ± 0.37^{b}	18.90 ± 0.54^{ab}
MS + 2.5 mg/l 2-4,D + 1.0 mg/l BAP	21.63 ± 0.48^{c}	16.00 ± 0.63^{cdef}
MS + 2.5 mg/l 2-4,D + 1.5 mg/l BAP	19.70 ± 0.46^{def}	14.63 ± 0.58^{efg}
MS + 2.5 mg/l 2-4,D + 2.0 mg/l BAP	13.40 ± 0.63^{l}	12.30 ± 0.60^{ijk}
MS + 2.5 mg/l 2-4,D + 2.5 mg/l BAP	7.70 ± 0.48^{n}	9.03 ± 0.52^{mno}
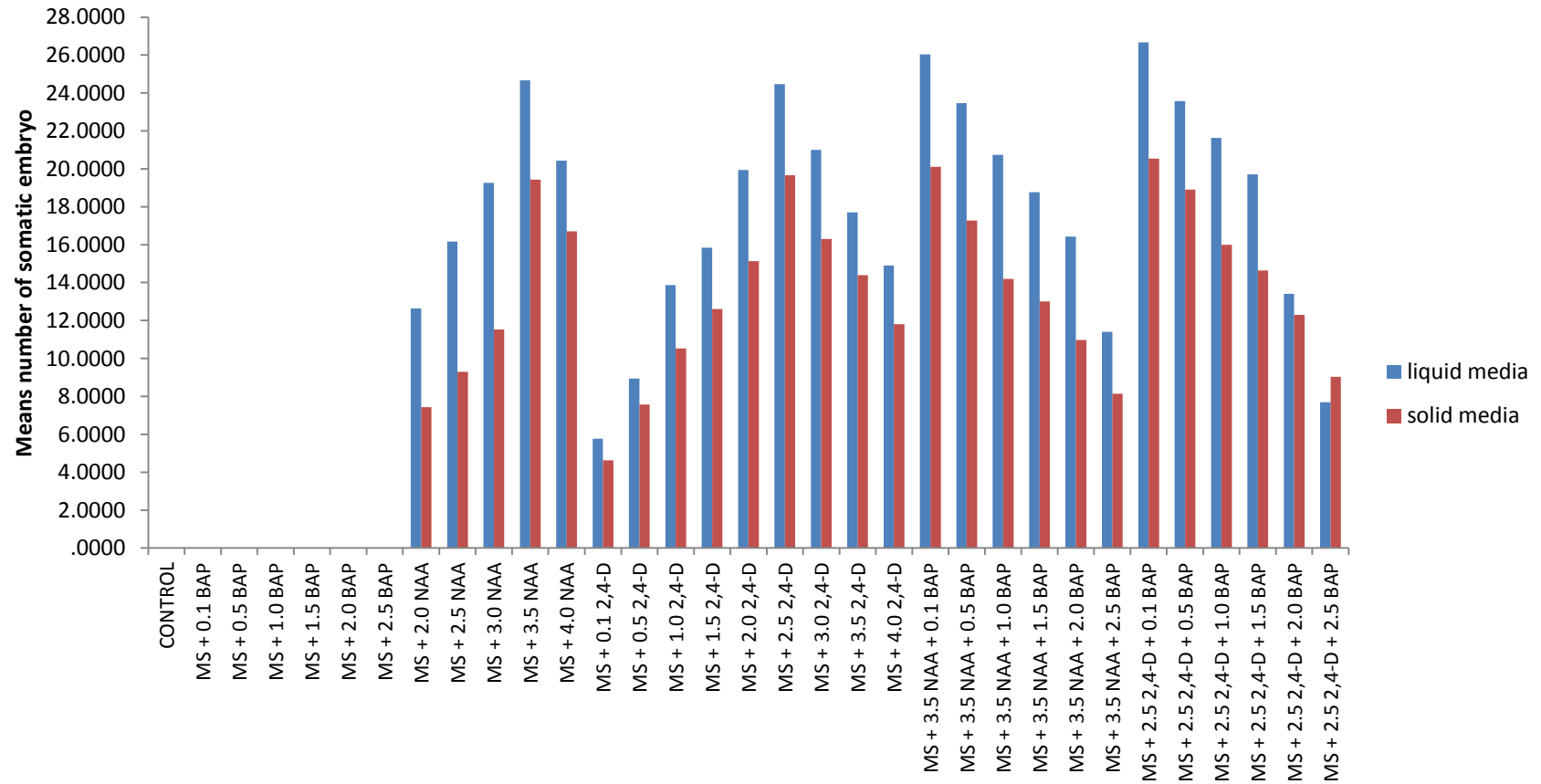
Figure 3.4: Graph showing mean number of somatic embryos produced in liquid media and solid media at temperature 21°C with 16 hours light and 8 hours dark.

Figure 3.5: Somatic embryo at globular stage (after 12 weeks)
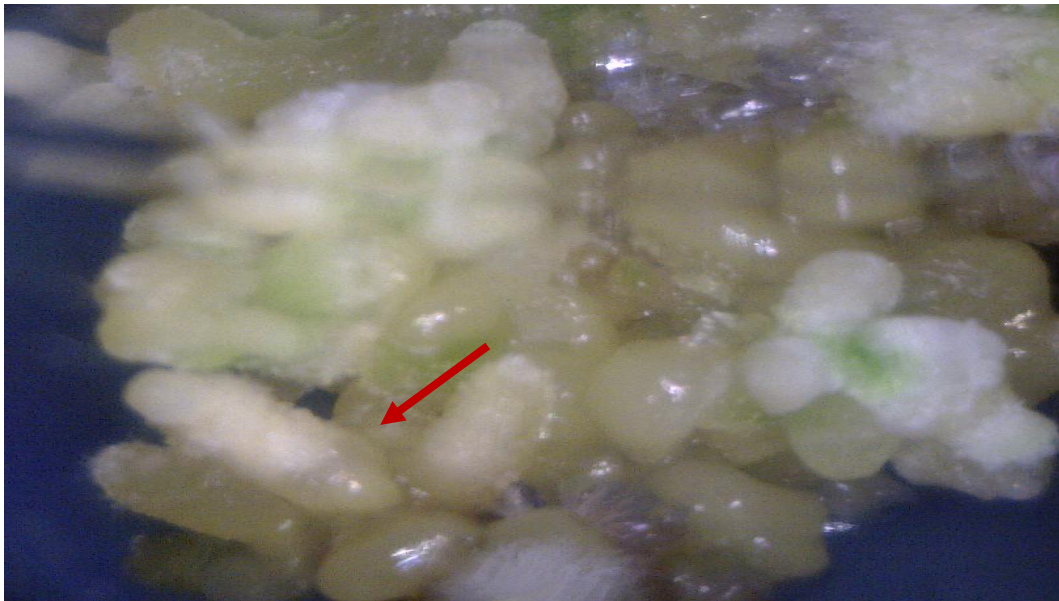
Figure 3.6: Somatic embryo at oblong stage (after 13 weeks)

Figure 3.7: Somatic embryo at heart shape stage (after 15 weeks)
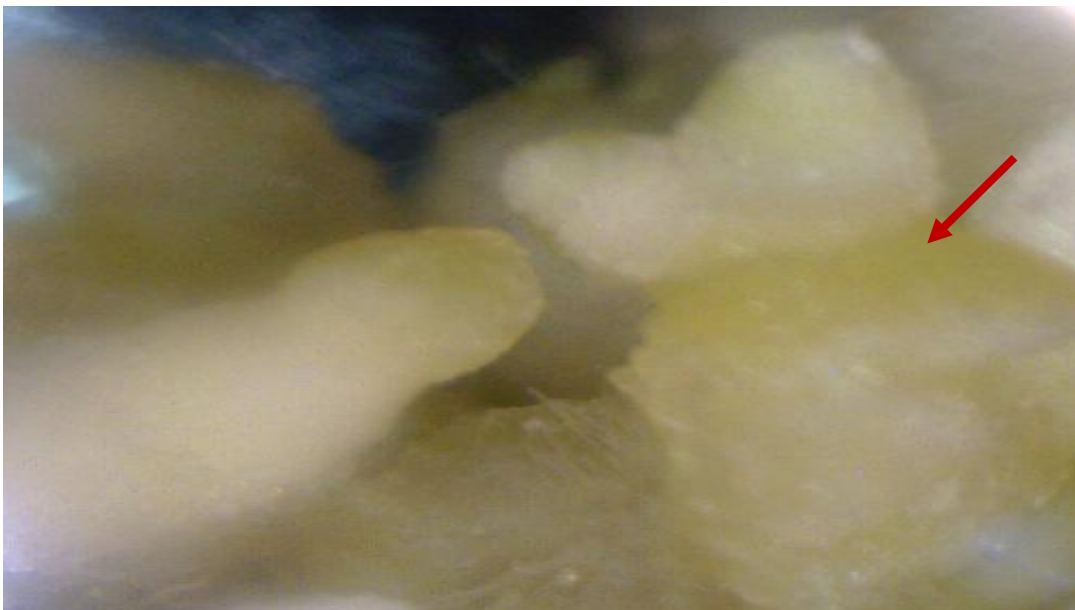
Figure 3.8: Somatic embryo at heart shape elongated stage (after 16 weeks)

Figure 3.9: Somatic embryo at torpedo stage (after 17 weeks)
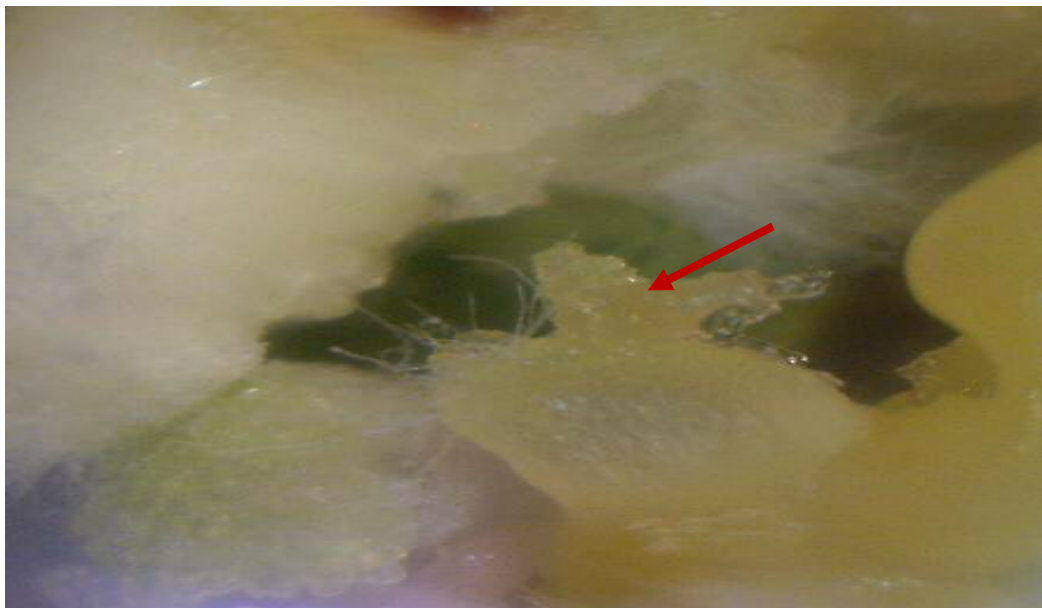
Figure 3.10: Somatic embryo at cotyledonary stage (after 19 weeks)
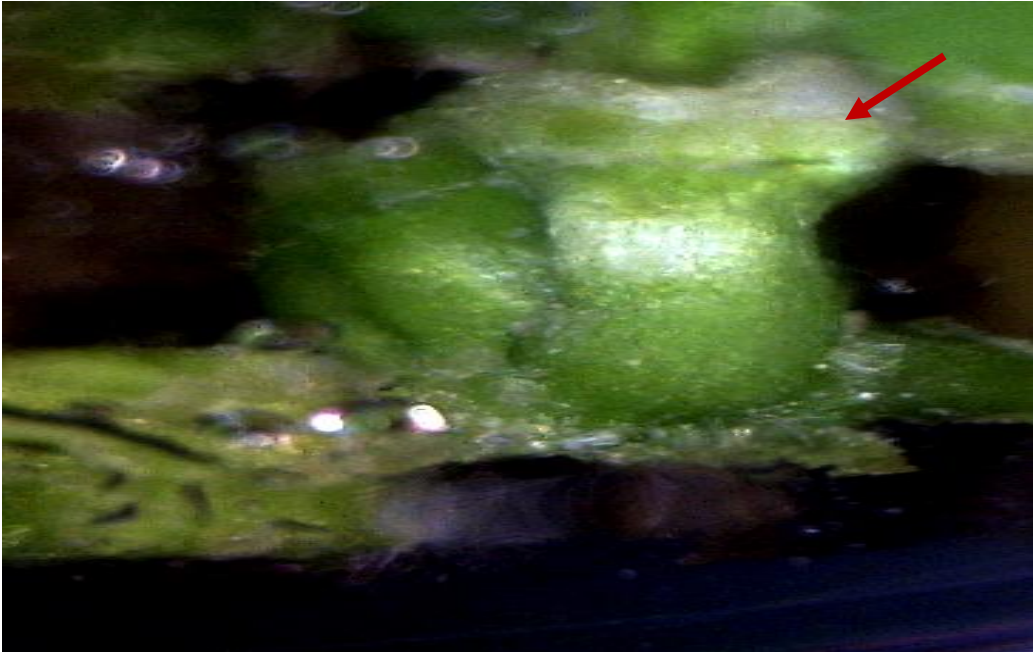
Figure 3.11: Somatic embryo at mature cotyledonary stage

3.4 SUMMARY OF THE RESULTS

1. In this research, four clear development stages of somatic embryo were recognized. The embryos were globular shaped, heart shaped, torpedo stages and the last stage was cotyledonary stage was observed.

2. There were no somatic embryo formation was observed on MS media without hormone and MS media supplemented with BAP alone.

3. MS media supplemented with NAA, 2, 4-D, NAA combined with BAP and 2,4-D combined with BAP gave rise to somatic embryos.

4. MS media supplemented with 2.5 mg/l 2,4-D combined with 0.1 mg/l BAP was the most suitable media for somatic embryo induction in liquid and solid media.

5. The lowest number of somatic embryos was obtained from MS media supplemented with 0.1 mg/l 2-4,D (4.63 ± 0.41).

6. Liquid media is the most suitable media for somatic embryo production compared to solid media.

CHAPTER 4

REGENERATION OF *POLIANTHES TUBEROSA* L. THROUGH SOMATIC EMBRYOGENESIS

4.1 EXPERIMENTAL AIMS

Somatic embryogenesis is a process where group of somatic cells or tissues lead to the formation of somatic embryos which resemble the zygotic embryos of intact seeds and can grow into seedlings on suitable medium (Tripathi, 2003). Few plant species have been shown to regenerate by both organogenic and somatic embryogenic pathways, but many plants species can regenerate by one or the other of these pathways (Phillips, 2004). Both processes, organogenesis and somatic embryogenesis have been reported to occur in the same explant (He *et al*., 1990) but originate from particular tissue layers or cell within explant (Osternack *et al*., 1999). However, somatic embryogenesis is nowadays known as a good pathway to induce regeneration from *in vitro* tissue culture (Victor, 2001).

Finding the right conditions to induce somatic embryogenesis in different species and cultivars is yet, for the greater part, based on trial and and error experiments (Jacobsen, 1991: Henry *et al*., 1994), analyzing the effect of different culture conditions and media modifying especially the types and level of growth regulators. In *Polianthes tuberosa*, there is no organ formation from explants culture. All the explants produce callus (discuss in chapter 2) and somatic embryos after induce the callus the media with high auxin especially 2, 4-D (discuss in chapter 3).

The objective of this study is to develop plant regeneration protocol for *Polianthes tuberosa* via somatic embryogenesis since there is no direct regeneration were observed from three types of explant used. Micro shoots formation will be induce from somatic embyogenesis and formation of complete plant will be discussed. The ability to understand the mechanism involved in the induction of somatic embryogenesis in this species will increase the number of genotypes capable of regeneration by this process.

4.2 MATERIALS AND METHODS

4.2.1 Preparation of explants

Callus was induced using 3 types of explant which are leaf, stem and flower bud (chapter 2). Callus from leaf explant was used to induce embryogenic callus and somatic embryo (chapter 3). Somatic embryo as explants were excised into 1cm x 1cm and cultured onto MS media supplemented with various combinations and concentration of hormones.

4.2.2 Preparation of the regeneration medium

MS media was prepared by diluting MS powder with 30 g/l sucrose and 8 g/l agar in distilled water (same as media preparation in chapter 2). Plant growth regulator (Kin, IBA and NAA) was added to the media before autoclaving except GA_3. GA_3 was added after autoclaving due to heat sensitivity. To sterile GA_3 hormone, sterilization filter 0.05 micropore was used. Autoclaved media was left and wait for the heat to cool down about 70°C. GA_3 was added into the media after that.

4.2.3 Screening for suitable hormones

Several concentration and combination of hormones were tested for regeneration of *Polianthes tuberosa* for somatic embryo. Nine concentrations of Ga_3, eight concentrations of Kinetin combine with 2.0 mg/l IBA and eight concentrations of Kinetin combine with 2.0 mg/l NAA were prepared. The following is the list of media that were used in this experiment.

    1. MS media (as control)

    2. MS + 0.1 mg/l GA_3

3. MS + 0.5 mg/l GA$_3$

4. MS + 1.0 mg/l GA$_3$

5. MS + 1.5 mg/l GA$_3$

6. MS + 2.0 mg/l GA$_3$

7. MS + 2.5 mg/l GA$_3$

8. MS + 3.0 mg/l GA$_3$

9. MS + 3.5 mg/l GA$_3$

10. MS + 4.0 mg/l GA$_3$

11. MS + 0.1 mg/l Kin + 2.0 mg/l IBA

12. MS + 0.5 mg/l Kin + 2.0 mg/l IBA

13. MS + 1.0 mg/l Kin + 2.0 mg/l IBA

14. MS + 1.5 mg/l Kin + 2.0 mg/l IBA

15. MS + 2.0 mg/l Kin + 2.0 mg/l IBA

16. MS + 2.5 mg/l Kin + 2.0 mg/l IBA

17. MS + 3.0 mg/l Kin + 2.0 mg/l IBA

18. MS + 3.5 mg/l Kin + 2.0 mg/l IBA

19. MS + 4.0 mg/l Kin + 2.0 mg/l IBA

20. MS + 0.1 mg/l Kin + 2.0 mg/l NAA

21. MS + 0.5 mg/l Kin + 2.0 mg/l NAA

22. MS + 1.0 mg/l Kin + 2.0 mg/l NAA

23. MS + 1.5 mg/l Kin + 2.0 mg/l NAA

24. MS + 2.0 mg/l Kin + 2.0 mg/l NAA

25. MS + 2.5 mg/l Kin + 2.0 mg/l NAA

26. MS + 3.0 mg/l Kin + 2.0 mg/l NAA

27. MS + 3.5 mg/l Kin + 2.0 mg/l NAA

28. MS + 4.0 mg/l Kin + 2.0 mg/l NAA

4.2.4 Data Analyses

30 replicate were maintained for each treatment and the data was analyzed statistically using Duncan's Multiple Range Test (DMRT). The statistical analysis based on mean values per treatment was made using the technique of analysis of variance. The comparative LSD *multiple range test* (p=0.01) was used to determine the differences between treatments.

4.3 Result

4.3.1 Microshoot formation

Microshoot and roots formation from somatic embryo were observed in 2 months in all media tested with the exception of the medium containing GA_3 alone in which somatic embryo showed no signs of active growth and after some time in the culture media it will become necrotic (Table 4.1).

In MS media without hormone, 4.23 ± 0.22 shoots and 0.57 ± 0.10 roots were observed. MS media supplemented with 2.0 mg/l Kin and 2.0 mg/l NAA was found to be the best media for microshoot formation (Figure 4.1) with the number of microshoot is 26.23 ± 0.74. 2.5 mg/l Kin and 2.0 mg/l IBA give the higher result for microshoot formation with the number of microshoot is 23.63 ± 0.53. For root formation MS media supplemented with 0.5 mg/l Kin and 2.0 mg/l NAA is the most suitable media for root formation (Figure 4.3) with 4.23 ± 0.40 number of roots formation.

Table 4.1: Number of microshoots and roots formation from somatic embryo. Thirty replicates were used in each treatment.

MS + Hormone mg/l	No of shoot formation	No of root formation
MS media	4.23 ± 0.22^j	0.57 ± 0.10^{fg}
MS + 0.1 GA$_3$	0.00 ± 0.00^k	0.00 ± 0.00^g
MS + 0.5 GA$_3$	0.00 ± 0.00^k	0.00 ± 0.00^g
MS + 1.0 GA$_3$	0.00 ± 0.00^k	0.00 ± 0.00^g
MS + 1.5 GA$_3$	0.00 ± 0.00^k	0.00 ± 0.00^g
MS + 2.0 GA$_3$	0.00 ± 0.00^k	0.00 ± 0.00^g
MS + 2.5 GA$_3$	0.00 ± 0.00^k	0.00 ± 0.00^g
MS + 3.0 GA$_3$	0.00 ± 0.00^k	0.00 ± 0.00^g
MS + 3.5 GA$_3$	0.00 ± 0.00^k	0.00 ± 0.00^g
MS + 4.0 GA$_3$	0.00 ± 0.00^k	0.00 ± 0.00^g
MS + 0.1 Kin + 2.0 IBA	6.50 ± 0.21^i	0.67 ± 0.12^{efg}
MS + 0.5 Kin + 2.0 IBA	$6.97\pm0.26h^i$	1.37 ± 0.11^{def}
MS + 1.0 Kin + 2.0 IBA	8.50 ± 0.30^h	2.23 ± 0.20^{bcd}
MS + 1.5 Kin + 2.0 IBA	13.00 ± 0.37^f	3.20 ± 0.23^{ab}
MS + 2.0 Kin + 2.0 IBA	20.23 ± 0.54^c	3.63 ± 0.34^a
MS + 2.5 Kin + 2.0 IBA	23.63 ± 0.53^b	3.63 ± 0.35^a
MS + 3.0 Kin + 2.0 IBA	17.8000 ± 0.55^d	3.30 ± 0.35^{ab}
MS + 3.5 Kin + 2.0 IBA	10.90 ± 0.49^g	3.70 ± 0.41^a
MS + 4.0 Kin + 2.0 IBA	$7.03\pm0.44h^i$	2.23 ± 0.28^{bcd}
MS + 0.1 Kin + 2.0 NAA	4.80 ± 0.41^j	1.03 ± 0.14^{defg}
MS + 0.5 Kin + 2.0 NAA	8.57 ± 0.65^h	4.23 ± 0.40^a
MS + 1.0 Kin + 2.0 NAA	11.13 ± 0.43^g	3.63 ± 0.33^a
MS + 1.5 Kin + 2.0 NAA	17.53 ± 0.79^d	3.00 ± 0.31^{abc}
MS + 2.0 Kin + 2.0 NAA	26.23 ± 0.74^a	3.37 ± 0.29^{ab}
MS + 2.5 Kin + 2.0 NAA	20.80 ± 0.55^c	3.20 ± 1.10^{ab}
MS + 3.0 Kin + 2.0 NAA	18.20 ± 0.59^d	1.90 ± 0.26^{cd}
MS + 3.5 Kin + 2.0 NAA	15.83 ± 0.62^e	1.80 ± 0.26^{cde}
MS + 4.0 Kin + 2.0 NAA	$12.00\pm0.69f^g$	1.97 ± 0.20^{cd}

Figure 4.1: Microshoots formation in MS media supplemented with 2.0 mg/l Kin and 2.0 mg/l NAA.
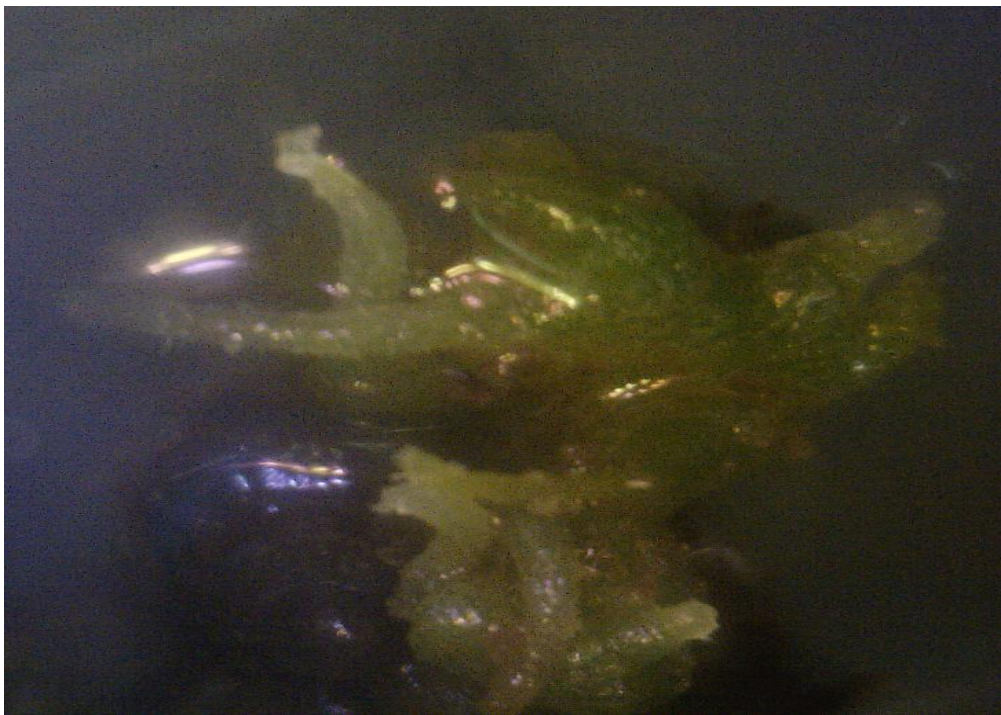
Figure 4.2: Shoots elongation in MS media supplemented with 2.0 mg/l Kin and 2.0 mg/l NAA.
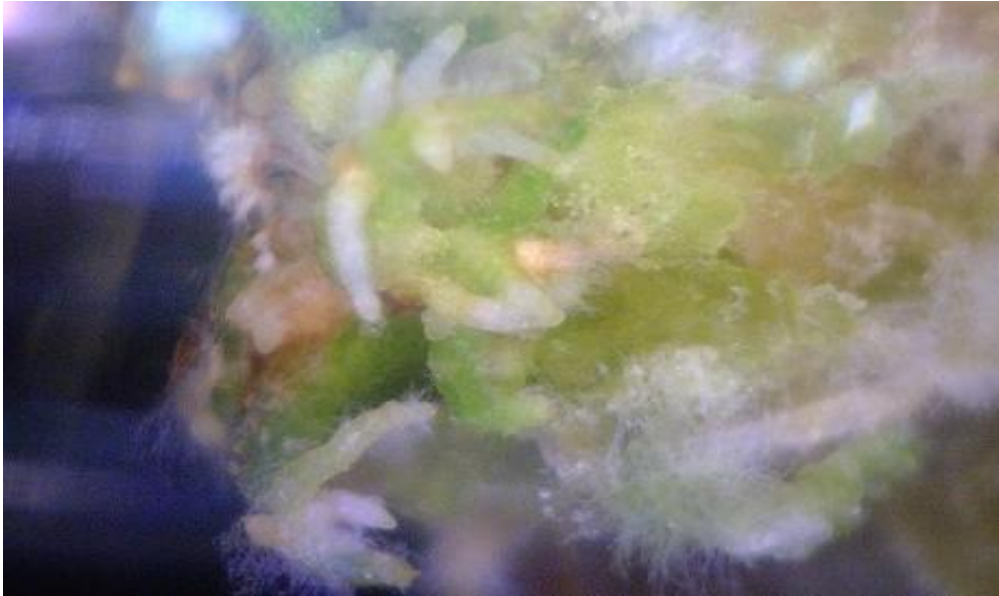
Figure 4.3: Roots formation in MS media supplemented with 0.5 mg/l Kin and 2.0 mg/l NAA
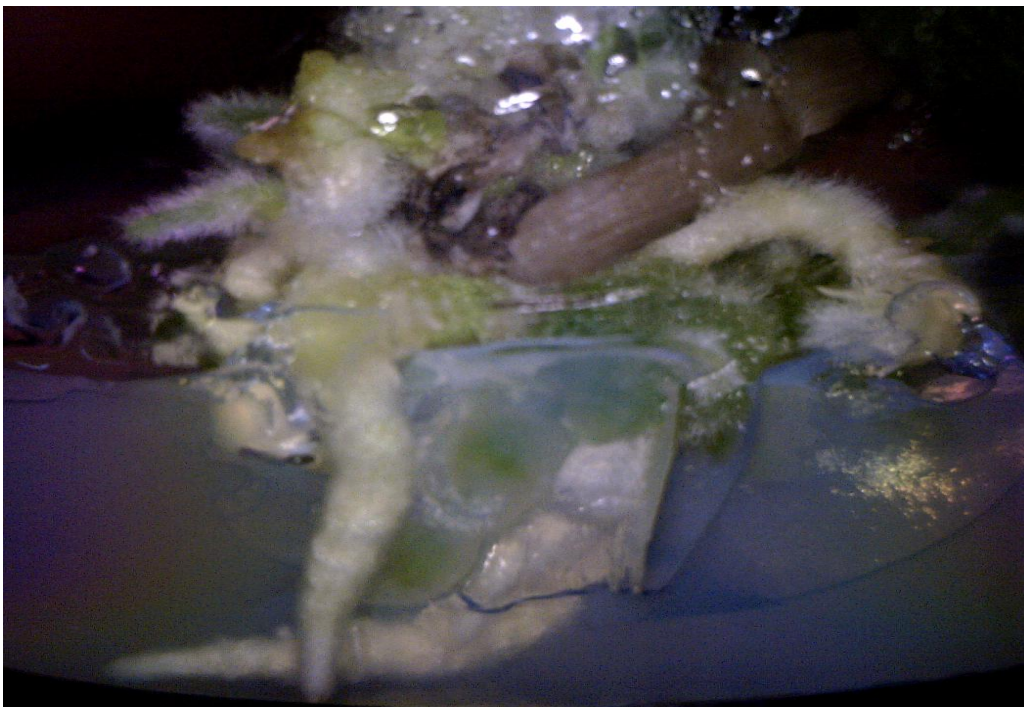
Figure 4.4: Tuber and root formation in MS media supplemented with 0.5 mg/l Kin and 2.0 mg/l NAA

4.4 SUMMARY OF THE RESULT

1. No microshoots and roots formation was observed on MS media supplemented with GA$_3$ alone.

2. MS media supplemented with 2.0 mg/l Kin + 2.0 mg/l NAA was the best media for micro shoot formation.

3. MS media supplemented with 0.5 mg/l Kin + 2.0 mg/l NAA was the best media for roots formation.

CHAPTER 5

ACCLIMATIZATION OF PLANTLETS OF *POLIANTHES TUBEROSA* L.

5.1 EXPERIMENTAL AIMS

Acclimatization is an adaptation process to the natural environment for various plant species which has undergone growth and development process *in vitro* (Preece and Sutter, 1991). Culture induce phenotype cannot survive the environmental condition when directly transfer to the field or green house because plantlets were developed within the culture vessels under low level of light, aseptic conditions, on a medium containing ample sugar and nutrients to allow for heterotrophic growth and in an atmosphere with high level of humidity.

According to Kozai (1991) and Pospisilova *et al*., (1997), the specific *in vitro* environment with artificial medium usually supplied with sugars, the growth of plantlets in small air tight vessels with high air humidity, low gas exchange and thus a CO_2 shortage during almost the whole photoperiod, ethylene production and relatively low photosynthetic photon flux density (PFD), induces disturbance in plant development and photosynthetic performance.

Leaves formed during tissue culture are anatomically and physiologically affected by the culture environment. The physiological and anatomical characteristics of micropropagated plantlets necessitate that they should be gradually acclimatized to the environment of the greenhouse or field. Transfer and acclimatization to the *ex vitro* environment is the final but frequently most hazardous step in successful micropropagation system (Preece and Sutter, 1991).

The plants that developed under lower relative humidity have fewer transpiration and translocation problems *ex vitro*, and persistent leaves that look like normal ones. The low deposition of surface wax, stomatal abnormalities and a non-continuous cuticle are typical anatomical features of herbaceous plants growing under conditions of abundant moisture. This typical *in vitro* anatomy can be prevented by increasing the vapour-pressure gradient between the leaf and the atmosphere.

Varying experiments have been done to lower the relative humidity like opening culture container (Brainerd and Fuchigami, 1981), adjusting culture closure or using culture closure that facilitate water loss (Fari *et al*, 1987) or use of desiccants, by coating the medium with oily materials or both (Sutter, and Langhans, 1982; Ziv *et al*., 1983).

In this study, plantlets from tissue culture container were transferred to the greenhouse to observe the capability of the plantlet to adapt to the natural environment. The aims of this process were to measure the survival rate of acclimatized plantlets from somatic embryos. The successful of the plantlets to survive means the successful of tissue culture of this plant.

5.2 MATERIALS AND METHODS

5.2.1 Plant materials

Micropropagated or regenerated *Polianthes tuberosa* L. plantlets were used in this study. *Polianthes tuberosa* L. was micropropagated through indirect somatic embryogenesis. Somatic embryos were transferred to the medium to induce shoots and roots formation. After 4 months, new plantlets obtained from *in vitro* cultures were ready to be transferred to the greenhouse.

5.2.2 Transfer to *ex vitro* environment

After 3 months *in vitro* plantlets were transferred to pots (80 x 60 mm) filled with a 1 cm layer of sand and a mixture of garden soil and red soil. The agar medium was carefully washed off the roots with distilled water before planted in the pot. The potted plantlets were first kept in the culture room at 25 ± 1 °C under 16 hours light and 8 hours dark for 3 weeks. The plantlets were watered every day.

Plantlets were transferred to 3 different scheme of growth medium:

1) Garden soil – Combination of black soil and red soil at ratio of 2 to 1

2) Autoclaved garden soil – Combination of black soil and red soil at ratio of 2 to 1

3) Red soil

The potted plantlets were covered with a transparent plastic with small holes before transfer to the greenhouse to reduce water lost. Shading was used to reduce irradiance during the first day of acclimatization. After 20 days in the greenhouse, the plastic cover was slowly

lifted up and the shading of the covered reduced. At day 35 the plastic covered was removed completely.

Based on all experiments done, the best acclimatization technique which gave the highest survival rate was identified.

5.2.3 Data analyses

Data obtained were analysed using Duncan Multiple Range Test (DMRT). Mean with different letters in the same column differ significantly at p=0.01.

5.3 RESULTS

5.3.1 Survival rate of *Polianthes tuberosa* L. plantlets

The complete plantlets achieved through shoot formation and root formation from somatic embryos (chapter 4) were successfully transferred to *ex vitro* environment. A great care was taken to minimize exposure to extreme light intensity. High humidity was maintained by covering potted plantlets and the cover was removed in stages, creating partial exposure of the plantlets to the sunlight and full exposure after 35 days in the greenhouse. By this treatment, the plantlets were able to acclimatize progressively.

Plantlets responded positively when acclimatized in garden soil (combination of black soil and red soil at ratio of 2:1) (Figure 5.1 and 5.2). This treatment gave the highest survival rate for acclimatization of *Polianthes tuberosa* plantlets with the percentage of 63.33±0.09 %. Lower survival rate (50.00±0.09 %) was observed in red soil. *Polianthes tuberosa* L. plantlets did not survive when acclimatized in autoclaved garden soil (combination of black soil and red soil at ratio of 2:1) (Table 5.1).

Table 5.1: Responses showed by *in vitro Polianthes tuberosa* plantlets after being acclimatized in various sowing media. Results obtained after 2 months being acclimatized

Methods	Observations	Survival Of *Polianthes tuberosa* (%)
Plantlets were transferred to Garden soil (Combination of black soil and red soil at ratio 2 to 1)	Plantlets survived and showed healthy growth	63.33 ± 0.09^a
Plantlets were transferred to autoclaved garden soil (Combination of black soil and red soil at ratio 2 to 1)	Plantlets did not survive	0.00 ± 0.00^c
Plantlets were transferred to red soil	Plantlets survived with slow growth	50.00 ± 0.09^b

Mean ± SE, n=30. Mean with different letters in the same column differ significantly at p=0.01.

Figure 5.1: Four-month-old *Polianthes tuberosa* L. plantlets after being transferred to the garden soil.

Figure 5.2: Seven-months-old *Polianthes tuberosa* L. plantlets after being transferred to the garden soil.

5.4 SUMMARY OF RESULT

1. *In vitro* plantlets of *Polianthes tuberosa* L. were successfully acclimatized with percentage 63.33±0.09%.

2. Garden soil was identified as the most suitable sowing medium for acclimatization of *Polianthes tuberosa* L. plantlets.

3. Plantlets that were cultured on MS media were removed carefully and the roots were rinsed with distilled water. Plantlets were then acclimazation on garden soil step by step.

CHAPTER 6

DISCUSSIONS

Tissue culture studies have been observed and reported in many monocotyledon species. These include the members of the Gramineae like oats (Carter et al., 1967), rice (Yatawa et al., 1967; Nishi et al., 1968), wheat (Trione, et al., 1968), sorghum (Masteller and Holden, 1970) and a number of temperate grasses (Atkin and Barton, 1973) as well as some bulbous and related species like *Lilium* (Sheridan, 1968), *Asparagus* (Wilmar and Hellerdoorn, 1968), *Allium* (Fridborg, 1971), *Haworthia* (Kaul and Sabharwal, 1972), *Gladious* (Ziv et al., 1970; Simonsen and Hildebrandt, 1971) and *Freesia* (Davies, 1971, 1972). There are many reports of regeneration from cell suspension of cereals and grasses but less attention has been given to monocotyledonous ornamental. Only a few reports exist about cell suspension culture of Agavaceae.

In the present work, tissue culture studies of Agavaceae family, *Polianthes tuberosa* L. are discussed. *Polianthes tuberosa* L. was selected because it is a very unique ornamental plant with attractive long spikes, pleasant fragrant flowers that have strong fragrant at night and mild during the day. This plant has very high commercial values in cut flowers and perfumery industries. Though this plant has been exploited to produce perfume all over the world, however research and application of *Polianthes tuberosa* L. are limited in Malaysia. *In vitro* work was carried out for this species as an alternative method for mass propagation and as a tool to overcome the problems occurring during the conventional propagation. The present tissue culture study was focused on regeneration of complete plantlets of *Polianthes tuberosa* L. This study was separated into several chapters. It encompasses the studies of

callus induction, somatic embryogenesis induction, regeneration from somatic embryos and acclimatization of plantlets.

In chapter 2 studies concerning callus induction of *Polianthes tuberosa* L. was carried out in order to identify and determine the best concentration and combination of hormones for optimum callus formation. Callus could be established from many types of explants. In this study, three types of explants were used which are leaf, stem and flower bud.

From the observation, callus started to initiate from the excision side or cut surfaces of the explants and after some time the whole explant produced callus. According to Haberlandt (1902), induction of callus formation is due to interaction between wound hormones and other hormones present. Wounding can increase uptake of nutrients and growth regulators from the media (Peirik and Steegmans, 1975). It can be considered as a wound response from almost any part of the original plant, both from plant organs and from specific tissue types or cells (Collin and Edwards, 1998). While, according to Yeoman and Aitchison (1977), excision caused destruction of the cells. Slicing eliminates or reduces the anatomical barrier which would cause mechanical resistance, thereby allowing axillary shoots to grown from shoot tips (Pierik, 1987). According to Thorpe (1980) and Wagley et al., (1987), formation of callus from explants tissues involved the development of progressively more random planes of cell division, less frequent specialization of cells and loss of organized structures.

The explants used for callus induction could affect the formation of callus. In the present study, all the explants used, leaf, stem and flower bud produced callus after cultured onto the media.

Rapid callus growth was obtained from flower bud explants cultured on MS media supplemented with 0.5 mg/l BAP combined with 2.0 mg/l NAA. According to Supaibulwatana and Mii (1997), flower head tissue of *Agapanthus africanus* was also the most responsive part to the culture media. These results suggest that flower tissue probably has the undifferentiated meristematic nature as previous suggested by Novak and Havel (1981).

Stem explant started to produce callus early than other explants but the proliferation was slow. In *Digitaria exilis* (L.), stem segments started to produce callus after seven days in culture but no callus formation from leaf explants even after 4 weeks *in vitro* (Ntui et al., 2010). According to Hussey (1975), young, elongating, inflorescence stem proved to be the most consistently reactive tissue. The initiation of callus in the leaf explant was late compared to stem but proliferation was more rapid than flower bud segment.

Sangavai and Chellapandai (2008), used rhizome of *Polianthes tuberosa* L. as an explant to induced callus formation but low frequency of callus was observed with the maximum percentage of callus formation (37.8±1.2). Hutchinson et al., (2004) used shoots tip explant to investigate *in vitro* propagation of *Polianthes tuberosa* L.

Callus formation in tissue culture of *Polianthes tuberosa* L. is likely resulted from a combination effect of auxin and cytokinin and auxin alone. MS media supplemented with 2.0 mg/l NAA; 0.5 mg/l BAP in combination with 2.0 mg/l NAA; 3.0 mg/l NAA and MS media supplemented with 0.5 mg/l BAP in combination with 2.5 mg/l NAA were suitable for callus formation from leaf explants with 100.0±0.00 % of the explant tested produced callus. 100.0±0.00 % of stem explants produced callus in MS media supplemented with 2.0 mg/l

NAA, MS media supplemented with 3.0 mg/l NAA and MS media supplemented with 0.5 mg/l BAP in combination with 2.0 mg/l NAA. Flower bud explants were suitable when cultured on MS media supplemented with 2.0 mg/l NAA and MS media supplemented with 0.5 mg/l BAP in combination with 2.0 mg/l NAA. All the explants (100.0±0.00 %) produced callus.

In the present study, MS media supplemented with NAA alone was found to be the best media for callus formation (Table 2.1). Khan et al., (2007) also obtained efficient callus induction in *Saintpaulia ionantha* when leaf explants were cultured on MS media supplemented with 1.0 mg/l NAA. Sangavai and Chellapandai (2008) observed that MS media supplemented with 0.5 mg/l BAP with 1AA at concentrations of 0.5, 1.0, 2.0, 2.5, 3.0, and 3.5 could produce callus from rhizome explants of *Polianthes tuberosa* L. MS media supplemented with 0.5 mg/l BAP and 3.0 mg/l IAA was the best media in inducing callus formation of with highest percentage (37.8±1.2).

There is no callus formation in MS media without hormone and MS media supplemented with BAP alone. Similar observation was reported by Paranjothy et al., (1988), whereby callus initiation required an auxin but it may be inhibited by cytokinin. Some researchers found the opposite results compared to this study. In their finding, cytokinin alone could induce callus production. For example, Kathal et al., (1993) found that BAP was the best cytokonin because it induced maximum callus growth in terms of fresh weight in *Cucumis melo*.

Two types of callus could be observed in the culture according to the colour. The first type is the callus which is nodular, friable, soft (watery) and greenish. The second type is the

callus which is nodular, friable, soft, wet looking surface and yellow whitish and cream in colour. According to Lin et al., (2000) plant regeneration in monocots has been achieved from either friable or a compact callus morphotype. In many monocots, the composition of plants growth regulators in the culture medium directed the callus morphotype (Ntui et al., 2010). In *Digitaria sanguinalis*, medium supplemented with 2,4-D alone induced friable callus formation (Le et al., 1997), in *Sorghum bocolour*, medium supplemented with 2,4-D alone produced friable callus whereas 2,4-D together with dicambia induced compact callus formation (Gendy et al., 1996), in *Asparagus densiflorus*, P-chlorophenoxyacetic acid together with BAP induced friable callus and 2,4-D together with kinetin induced compact callus formation (Benmoussa et al., 1996).

Callus can be induced for organogenesis and somatic embryogenesis pathways. According to Hasbullah et al., (2008), the first step towards de novo regeneration is to establish callus or cell suspension cultures.

In chapter 3, embryogenic callus was induced to produce somatic embryos. Embryogenic callus was induced when leaf explants were cultured on MS medium supplemented with 0.5- 4.0 mg/l NAA (Table 3.1). The embryogenic callus was than determined using double staining method. This method was done according to 'double staining' technique prepared by Gupta and Durzan (1987).

In the present study, callus obtained from leaf explant was used as an explant because leaf of *Polianthes tuberosa* is easy to obtain compared with stem and flower buds explant. Suzuki et al., (2002) also used leaf as a source of explant for callus embryogenic induced in *Agaphantus praecox* because of difficulties to get the flower bud explant. Mature vegetative

tissue usually used because they are more convenient and more readily available than immature and floral tissues (Thorpe, 1995). In *Chrysanthemum*, embryogenic callus were produced from mid-rib explants on modified MS medium supplemented with 1.0 mg/l 2,4-D and 0.2 mg/l BAP (May and Trigiano, 1991). Basal and Pandey (1993) obtained efficient callus induction from leaf explant of *Sesbanea aculeate* in MS media supplemented with high concentration of 2,4-D (5.0 to 10.0 mg/l) in combination with different BA concentration.

Some tissue culturists used flower buds to induce callus embryogenic formation. Supaibulwatana and Mii, (1997) used floral organ explants to induce embryogenic callus in *Agaphantus africanus*. Wojciechowicz, (2009) also managed to get embryogenic callus from petal explants of *Sedum* species. Young floral tissues are known to be conducive to *in vitro* culture with good embryogenic potential (Ammirato, 1989). Levels of endogenous growth regulators can be vary among organs and likely effect embryogenesis (Carman, 1990). Myers (2004) founds that the use of entire immature zygotic embryos was more efficient in inducing somatic embryogenesis.

Culture media composition is very important to induce callus embryogenic. In the present study of *Polianthes tuberosa* L., a high concentration level of auxin in the callus induction medium was necessary to enhance embryogenic callus production. MS media supplemented with 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mg/l NAA was successful in inducing embryogenic callus formation, whereas 2.0 and 3.0 mg/l was found to be the best concentration of NAA when 100.0±0.00 of explants cultured managed to produce callus. Nagarajan et al, 1986; Meijer and Brown, 1987; Nolan et al., 1989; Shri and Daris, 1992) also found similar resultsnas they reported for other species. Carman, (1990); Ammirato, (1987);

Luo et al., (1999), also stated that the induction of embryogenic callus was usually promoted by a relatively high concentration of auxins especially 2, 4-D.

Some scientists managed to get callus embryogenic in high level of cytokinins and low level of auxin. Kim et al., (2003) produced callus embryogenic from zygotic embryo of rose at a relatively high concentration of BA (4.44 μM) in combination relatively low concentration of 2, 4-D (1.36 μM). Rout et al., (1991) also obtained the same result as Kim et al., (2003). He also produced embryogenic callus from immature leaf and stem explants of rose at a relatively high concentration of BA (2.22 μM) and a relatively low concentration of NAA (0.05 μM) in addition of 0.3 μM GA$_3$.

In monocotyledonous plants including Liliaceous plants, 2,4-D was predominantly used for the induction of embryogenic callus (Krikorian and Khan, 1984; Van der Valk et al.,1992). In *Medicado truncatula* and *M. sativa*, 2,4-D supplemented with BAP promoted enhanced embryogenic calli and subsequent embryo differentiation (Trinh et al., 1998). In *Digitaria exilis*, somatic embryogenesis was observed higher in medium containing 2,4-D supplemented with BAP compared to medium supplemented with 2,4-D alone (Ntui et al., 2010).

Embryonic callus can be preserved for a long time. Embryogenic callus of *Agaphantus praecox* ssp. orientalis (Leighton) Leighton can be maintained for over 2 years by monthly subculture without apparent loss of their regeneration ability (Suzuki et al., 2002). Bouman et al., (2001) reported that efficiency of embryogenic callus of *Cyclamen* seems to be stable for more than 5 years however suspension cultures can lose embryogenic potential after a number

of subcultures. Pueschal et al., (2003) succeeded in plant regeneration via somatic embryogenesis of *C. persicum* and maintained the regeneration ability for prolong period.

Somatic embryos have the same potential of zygotic embryos to regenerate new plants. Under controlled environmental conditions, somatic embryos germinate readily, similar to their seedlings counterpart. Somatic embryo can provide a useful model to study embryo development in plant (Zimmerman, 1993). Once the induction of an embryogenic state is complete, the mechanisms of pattern formation that lead to the zygotic embryo are common to all other form of embryogenesis (Mordhorst et al., 1997). Thus, somatic and zygotic embryos share similar gross ontogenies, with typically passing through globular, heart shape and torpedo shape stages in dicots or globular scutellar (trasition) and coleoptilar stages in monocots (Gray et al., 1995; Toonen and de Vries, 1996). In contrast to zygotic embryogenesis, somatic embryogenesis can easily be observed, the culture conditions can be controled and large quantity of embryos can be easily obtained (Kawahara and Komamine, 1995).

Somatic embryogenesis is a very valuable tool for achieving a wide range of objectives, from basic biochemicals, physiological and morphological studies, to the development of technologies with a high degree of practical application (Victor, 2001). According to Haccius (1978), somatic embryogenesis has been considered to be a distinct developmental pathway different from shoot or root organogenesis, in which a single cell gives rise to a structure containing bipolar meristem and with no direct vascular connection to the maternal tissue. The mass propagation of plants through multiplication of embryogenic propagules is the most commercially attractive application of somatic embryogenesis (Merkle et al., 1990).

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Somatic embryogenesis potentially offers a promising system for plant regeneration because of the high proliferation capacity and the probable single cell origin, which may avoid the risk of chimeric plants and facilitate the application for mutant selection and recombinant DNA technology (Luo et al., 1999; Ponsamuel et al., 1996; Roberts et al., 1995; Stefaniak, 1994). Thus in this chapter, somatic embryogenesis was induced from embryogenic callus of leaf explant of *Polianthes tuberosa*. Indirect somatic embryogenesis was obtained from this study.

Indirect somatic embryogenesis was observed in this species. Somatic embryos occurred only after callus embryogenic was subcultured onto somatic embryogenesis induce media. According to Sharp et al., (1980) the term indirect is referring to explants which undergo an extensive proliferation before the development of somatic embryogenesis. Indirect somatic embryogenesis generally considered to have a unicellular or multicellular origin (Quiroz- Figuera et al., 2002). Histological studies in different species have described both unicellular (Trigiano et al., 1989; Faure et al., 1996) and multicellular (Taylor and Vasil, 1996; Fernandez et al., 1996) pathways.

A lot of works have been reported in indirect somatic embryogenesis. Lin et al., (2000) observed indirect somatic embryogenesis in *Alstromeria*. Saqlan et al., (2005), Rashid et al., (2003) and Liu et al., (2001) managed to get direct somatic embryogenesis from rice.

Direct somatic embryogenesis can occur when explant cultured produced somatic embryo without callus phase formation. According to Sharp et al., (1980) the term direct is applied to explants that undergo a minimum in proliferation before forming somatic embryos.

Direct somatic embryogenesis was observed in many monocotyledon species. Park et al., (2005) observed somatic embryo in *Eleutherococcus koreanum*. In the absence of growth regulators, roots segment of *Eleutherococcus koreanum* cultured in 1/3, half, full and double strength MS media developed globular embryo directly on surface root without any callus mediation. Castillo and Smith (1997) induced direct somatic embryogenesis from petiole and leaf blade explants of *B. gracili*s on MS medium supplemented with 0.5 mg/l Kin and 2% (v/v) coconut water.

It is not always clear the types of somatic embryogenesis that occurs or both direct and indirect can be observed in the cultures. According to Carman (1990) and William and Mahaswaran (1986), direct and indirect somatic embryogenesis have been considered as two extremes of a continuum. Once induction of embryogenic cells have been achieved, they appear to be no fundamental differences between indirect and direct somatic embryogenesis (Williams and Maheswaran, 1986).

Somatic embryos produced directly from an explant are less subjected to genetic variation (Kim et al., 2003) because maintenance of calluses for a long time *in vitro* results in greater genetic variation (Llyod et al., 1988; Arene et al., 1993). Therefore, it is useful to control the induction of somatic embryogenesis both directly and indirectly from explants in order to produce the maximum number of genetically uniform clonal plant develop from somatic embryogeneis.

To induce somatic embryo formation, embryogenic callus obtained from leaf explant were then transferred to liquid and solid MS media supplemented with thirty combinations of hormone which are NAA alone, 2,4-D alone, BAP alone, NAA combined with BAP and 2,4-

D combined with BAP. When the callus obtained were transferred to somatic embryogenesis induction media, internal cell divisions led the formation of proembryos, distinct from each other in the callus at the beginning of the developmental phase of somatic embryos. This probably indicates the single cell origin of embryos, as in the case of *Agapanthus praecox* spp. orientalis (Suzuki et al., 2002), *Pelargonium x domesticum* (Wilson et al., 1994), *Freesia refracta* (Wang et al., 1990), *Juglans regia* (Polito et al., 1989) and *Tricum aestivum* (Magnusson and Bornman, 1985). Unicellular origin of somatic embryogenesis obtained in the present study may allow the production of non- chimeric plants of *Polianthes tuberosa*.

After 3 months in the culture, round structure of globular stage and other stages like heart shape, torpedo and cotyledonary could be observed. Somatic embryo development was asynchronous in this plant species where different stages of development could be observed within the same treatment and replication unit. Asynchronous was observed also in somatic embryogenesis callus culture of Kampung Royal Poinciana (Myers and Vendrame, 2004), *Astragalus adsurgens* Pall (Luo et al., 1999), and *Azadirachta indica* A. Juss (Medha et al., 1993).

In the current studies, the entire media used produced somatic embryos except for MS media and MS media supplemented with BAP alone (Table 3.2). The highest number of embryos, (26.93±0.38) was obtained from MS media supplemented with 3.5 mg/l NAA and 0.1 mg/l BAP in liquid media. MS media supplemented with 2.5 mg/l 2,4-D and 0.1 mg/l BAP also gave high results with 26.67±0.42 number of somatic embryos.

In MS media without hormone and MS media supplemented with BAP alone, there were no further growth from embryogenic callus. Embryogenic callus were dehydrated after 2 weeks in the media. According to the result (Table 3.2) there is no somatic embryo formation

in MS basal media but MS media supplemented with auxin is the best combination media for somatic embryogenesis. According to Thorpe (1995), over 2000 media formulation has been documented (George et al., 1987; George et al., 1988) but a survey of literature concerning somatic embryogenesis reveals that about half of the embryo induction medium used across all the species is MS based medium.

Many factors including choice of growth regulators, choice of explants and culture medium composition are responsible for successful of somatic embryogenesis. All the result for somatic embryo production shows that liquid media produce more number of somatic embryo compared to solid media with the same treatment (Table 3.2) except only one treatment, MS media supplemented with 2.5 mg/l 2-4,D and 2.5 mg/l BAP, liquid media produce 7.70 ± 0.48 number of somatic embryos while solid media produce 9.03 ± 0.52 number of somatic embryo. This result indicated that maybe some mistake occurred in the handling process of embryogenic callus transferred to the media because other result showed that liquid or suspension media is better in producing more number of somatic embryos.

Liquid medium seems to be more effective which is due to better aeration. Many tissue culturists used liquid suspension culture media for rapid development of somatic embryos. Winkelmann et al., (1998) and Hohe et al., (2001) used cell suspension culture for large scale production of *Cyclamen* somatic embryos. Atanassov and Brown (1984) and Mc Kersie et al., (1989) used suspension culture to induce somatic embryos from *Medicago sativa* L. in MS media supplemented with 1.0 mg/l 2,4-D.

Growth regulators and nutrient component of the media have profoundly influenced the embryogenesis process in plant species and suitable medium composition should be

worked out for embryo induction, development, maturation and conversion. In the present study, combination of auxin and cyctokinin play an important role in somatic embryos production.

MS combined with high auxin and low cytokinin gave the best results compared to MS combined with auxin alone or MS combined with cytokoinin alone (Table 3.2). MS media supplemented with 3.5 mg/l NAA gave rise to 24.67±0.32 number of somatic embryos compared to MS media supplemented with 3.5 mg/l NAA and 0.1 mg/l BAP which gave rise to 26.93±0.38 number of somatic embryo. MS supplemented with 2.5 mg/l 2-4,D gave rise to 24.47±0.41 number of somatic embryos compared to MS media supplemented with 2.5 mg/l NAA and 0.1 mg/l BAP gave rise to 26.67±0.42 number of somatic embryos.

Exogenous applied hormone, mainly auxin such as NAA and 2,4-D play a critical role in the reactivation of the cell cycle and the initiation of the reactivation of the cell cycle and the initiation of the embryo formation. Application of high concentration of 2,4-D in the culture medium itself is a stress signal, since embryogenic induction requires the use of a physiological auxin concentration that inhibit callus growth. Sage et al., (2000) observed that somatic embryos could developed in 0.5, 5.0, and 10.0 mg/l NAA and 2,4-D. More somatic embryos were produced on media supplemented with 5.0 and 10.0 mg/l 2,4-D compared to 0.5 mg/l 2,4-D. In the present studies of *Polianthes tuberosa* L., 2,4-D at concentration 2.5 mg/l produced more somatic embryo compared to 3.0 mg/l 2,4-D.

However, response to auxin and cyatokinins is variable and depends on the species and types of auxin and cyatokinin used (Lakshmanan and Taji, 2000) and relative amounts applied (Myers and Vendrame, 2004). Some species required high auxin and cytokinin

concentration supplemented medium. Luo et al., (1999) reported that the high frequencies (63-74%) of somatic embryogenesis of *Astragalus adsurgens* occurred on MS medium containing 0.1 mg/l NAA in combination with BAP at the concentration of 1.0- 2.0 mg/l, where an average of 280 somatic embryos was obtained from 1 g of embryogenic callus. Similar needs for somatic embryo formation also reported for other species such as *Coffea canephora* (Hatanaka et al., 1991), *Coronilla varia* (Moyer and Gustine, 1984), *Eleusina caracana* (Eapen and George, 1989), *Oryza sativa* (Ram and Nabors, 1984) and *Thevetia peruviana* (Kumar, 1992).

Somatic embryogenesis also occurs in this species when kinetin was applied with the auxin (2,4-D and NAA) in the media. Similar needed was observed in bulb plant *Agave* (Amaryllidaceae). According to Bansude et al., (2003) 0.5 mg/l NAA in combination with 1-2 mg/l BAP can induce somatic embryo in *Agave*. In *Narcissus bulbocodium*, the formation of somatic embryo was stimulated using the combination of IBA and BAP (Salema and Salamak, 2000). Anbari et al., (2007) found that MS media supplemented with 2,4-D (1.6 mg/l), BAP (1.6 mg/l) and GA_3 (0.5 mg/l) managed to induce globular stages in *Narcissus papyraceus* cv Shirazi. The findings of Zive et al., (1995) confirmed the positive effects of 2,4-D and BAP on induction of somatic embryogenesis in bulb plant of *Nerine* (Amaryllidaceae).

In some species, results which were contrast with *Polianthes tuberosa* L.were obtained. The embryogenic callus subsequently gave rise to somatic embryo when growth regulators were removed. Examples include many herbaceous species such as cucumber (Raharjo and Punja, 1994), melon (Gray et al., 1993), squash (Chee, 1992), strawberry (Wang et al., 1984). Reports by Carman (1990) and Ammirato (1987) also indicated that embryo

development was usually associated with a reduction or the omission of auxin from the medium. However, according to Laksmanan and Taji (2000), the removal of plant growth regulators could be critical for somatic embryo differentiation and maturation.

The choice of explants has a great influence on the success of somatic embryogenesis production. Research has been done to observe embryogenic response among young tissue (Punja et al., 1990; Chee, 1990), mature vegetative tissue (Willian and Maheswaran, 1986) and floral tissue (Carman, 1990). According to Thorpe (1995), the relative responsiveness of various immature tissues seems to be species-specific. For examples, cotyledons were more embryogenic than hypocotyls in cucumber (Chee, 1990) while in cotton hypocotyls produced more embryos than seeds or cotyledons (Trolinder and Chen, 1989). According to Carman (1990), floral tissue (ovaries, pedicels, peduncles, buds and inflorescences) may be embryogenic because of their developmental proximity to embryogenesis *in vivo*. According to Razdan (1993), premeiotic inflorescences with the primordia of the individual florosets just beginning to protrude has been observed to be the most suitable material in some systems. In some plant species, induction of embryogenesis from microspores at the uninucleate stage is the most efficient way to induce androgenesis, either from cultured anthers or from isolated microspores (Touraev et al., 2001; Segui Simarro and Nuez, 2005; Litcher, 1989; Testillano et al., 2004). Augustine et al., (2008) use anthers of *Curculogo orchioides* as explants cultured on media with 2,4-D and managed to get small meristematic clumps.

Secondary somatic embryogenesis can occur while inducing somatic embryogenesis. Remotti (1995) observed primary and secondary somatic embryos from cell suspension cultures of *Gladiolus*. No secondary somatic embryogenesis was observed in the present studies of somatic embryogenesis of *Polianthes tuberosa* L. Secondary somatic

embryogenesis is the phenomenon whereby new somatic embryos are initiated from somatic embryos (embryos are form from embryos). It is associated with loss of integrated group control of cells organized in the somatic embryos. Some cells break away from group control and initiate new somatic embryos (William and Maheswaran, 1986).

A major limitation of the embryogenic systems used in *Polianthes tuberosa* L. is the maintenance of embryogenic competence and the low conversion rate of somatic embryos in plants. In a number of species low conversion rates have been shown to be due to poor somatic embryo quality and a lack of maturation and desiccation tolerance (Etienne et al., 1993).

In some other bulbous plant like Liliaceous plant species such as *Agapanthus africanus* (Supaibulwatana and Mii, 1997) *Asparagus officinalis* (Kunitake and Mii, 1990; May and Sink, 1995), *Allium ampeloprasum* (Buiteveld and Creemers-Molenaar, 1994) and *Lilium X formolongi* (Mii et al., 1994; Godo et al., 1996), somatic embryo with high shoot regeneration ability has been utilized as the source of explants which would regenerate into complete plants.

In chapter 4, regeneration of *Polianthes tuberosa* L. through somatic embryogenesis was carried out. No direct regeneration was observed for this species. Indirect regeneration via somatic embryogenesis pathway was observed. Explants cultured produce embryogenic callus (chapter 2), developed into somatic embryo stages (chapter 3) and subsequently were induced to produce shoots and roots.

Several concentrations and combinations of hormones were tested for regeneration of *Polianthes tuberosa* L. from somatic embryos. The development of whole plants with shoots and roots was observed on MS medium without hormone after 2 months. MS medium without growth regulators is a typical medium for somatic embryo maturation and germination in many plants including wetlands monocots like *Phragmites australis* (Straub et al., 1988) and *Sporobolus virginicus* (Straub et al., 1992).

However, in MS medium without hormone, somatic embryo showed weak growth with small number of shoots (4.23±0.22) and roots formation (0.57±0.10) (Table 4.1). Wang et al., (2004) obtained the same result when germinated somatic embryo of monocot *Scirpus robustus* in MS medium, weak shoot growth with both small shoots numbers and shoots no more than 1 cm in height was obtained.

Other than full strength MS medium, half strength MS also can be used for regenerated plantlets from somatic embryogenesis. Jain et al., (2002) used half strength MS medium to regenerated *Phlox paniculata Linn*. plantlets from somatic embryo. Of the somatic embryos, 70 to 75% produced shoot and root meristem after 12 to 14 days in culture. Luo et al., (1999) also used half strength MS to develop somatic embryo of *Astragalus adsurgens* Pall. into complete plantlets within 2 weeks.

The addition of plant growth regulators, auxin and cytokonin to the medium significantly increased the number of generated shoots and roots. The highest number (26.23±0.74) of microshoots were observed in MS media supplemented with 2.0 mg/l Kin and 2.0 mg/l NAA (Table 4.1). According to Preil (2003) and Rout and Jain (2004), many ornamental plants are being propagated by *in vitro* culture on the culture medium containing

auxins and cytokinins. Medha et al., (1993), found similar result using different kind of cytokinin and auxin in regeneration of neem from somatic embryos, 60 to 70% of somatic embryos enlarged and germinated on MS media containing 2.0 mg/l BAP and 0.5 mg/l IAA after 20 to 30 days. According to Wang et al., (2004) addition of cytokinin (BA) into the MS medium can increase shoot regenerated (*Scirpus robustus*) from 8 to 35 and up to 53 depends on BA concentration.

Root initiation typically requires a moderate to high auxin signal but rarely with the use of a more natural source auxin (Gamborg and Phillips, 1995). Highest roots formation was observed on MS media supplemented with 0.5 mg/l Kin and 2.0 mg/l NAA with an average of 4.23 ± 0.40 roots per explant. Root formation occurred when explants were cultured on medium with higher auxin concentration and lower cytokinins concentration (Hasbullah et al., 2008).

Two types of auxin were used in this experiment which is NAA and IBA. NAA is more suitable auxin for plantlets regeneration of this species compared to IBA according to the result obtained. Others also observed the same results for different plant species. Poddar et al., observed 12. 5 plantlets regeneration on MS medium supplemented with 1.0 mg/l NAA alone from somatic embryo of finger millet *Eleusine coracana* (L.) Gaertn. Addition of strong auxin (NAA) with BAP promoted better shoot formation compared to weak auxin like IAA (Pierik et al., 1973).

MS supplemented with cytokinin and auxin was found to be the best media for microshoot formation in all explants. In the present study, no microshoot and root formation was observed on MS media supplemented with GA_3 alone. This result contrary from the

previous research by Philip et al., (2011), when they transferred mature somatic embryos of *Arachis hypogaea* L. to somatic embryo regeneration medium containing MS salts, B5 Vitamins, 0.2mg/l GA_3 and 1.0 mg/l BA and observed 53% plant regeneration from mature somatic embryos after 8 weeks of culture with subculture at 8 day intervals. While, Iyyakkannu *et al*., (2011), observed embryo maturation and germination of *Crocus vernus* on the MS medium with 2.0 mg/l BA and 1.0 mg/l GA_3. When the globular embryos were transferred to the MS medium containing 6% (w/v) sucrose, 2.0 mg/l BA, and 1.0 mg/l GA_3 resulted in the highest frequency of plant regeneration and microcorm formation. The microcorms developed new shoots when they were cultured on the half-strength MS medium with 1.0 mg/l GA_3. These results obtained were contrast with regeneration of *Polianthes tuberosa* L. from somatic embryo.

Gibberellin is generally thought to be inhibitory to rooting. Gibberellin inhibits adventitious root formation in many species especially when applied during the early stages of root formation (Brian et al., 1960; Jasen, 1967; Reinet and Besemer, 1967; Smith and Thorpe, 1975). The present studies, shows that low and high concentrations of GA_3 inhibit root formation in *Polianthes tuberosa* L. as well.

According to Brian et al., (1960) and Reinert and Basemer, (1967), the inhibitory effect of adventitious root formation has been attributed to an inhibition of cell division at an early stage of primordium development. Nanda et al., (1968) suggested that the inhibition of root formation by gibberellins was a result of a change in the partitioning of respirable substrates between shoot and root. Hansen (1976), suggested that GA_3 may inhibit rooting by increasing the soluble carbohydrates. In some species and under special condition, GA_3

promotes root formation (Eriksen, 1971; Varga and Humphries, 1974, Hansen, 1975; Smith and Thorpe, 1975; Bhattacharya et al., 1978).

Tissue culture was considered successful when plantlets obtained successfully acclimatized to the natural environment (*ex vitro*). Thus, in chapter 5, acclimatization of *Polianthes tuberosa* plantlets to the greenhouse was carried out. Three types of soil were used as the substrate for transplanting the plantlets which are garden soil (combination of black soil and red soil at ratio 2 to 1), autoclaved garden soil (combination of black soil and red soil at ratio 2 to 1) and red soil.

Garden soil (combination of black soil and red soil at ratio 2 to 1) was found to be the most suitable substrate for acclimatization of *Polianthes tuberosa* L. plantlets with the percentage of survival rate of 63.33 ± 0.09 %. The percentage of plantlets acclimatized survival rate in red soil was lower compared to the percentage of the acclimatized plants in garden soil (combination of black soil and red soil at ratio 2 to 1) with the percentage of survival rate 50.00 ± 0.09. This is could be due to garden soil (combination of black soil and red soil at ratio 2 to 1) provide good drainage system for the plant and good aeration system. Sink (1984) obtained similar results and he stated that retain water will deplete oxygen level in the soil and thus, this causes failure of the plant rooting system. Debergh et al., (1990) also stated that the soil should not be too wet and the exposure to higher light intensity should be gradual. Da Silva et al., (2005) demonstrated that the greater the aeration the more efficient the survival and the higher the growth and vigour of the plant.

Various substrates were used for plantlets acclimatization. The selection of a suitable substrate can be decisive for acclimatization. Rodrigues et al., (2005) used washed sand and

vermiculate to acclimatize *Heliconia bihai* plantlets. The substrates used for acclimatization revealed that vermiculite has a low yield as compared to washed sand. The low effectiveness of vermiculite as a substrate in the acclimatization of apple tree rootstocks and gloxinia plantlets was reported by Hoffmann et al., (2001) and Silva et al., (2003). Van Huylenbroeck et al., (2000) used a peat substrate for acclimatization of *Calathea louisae* Gagnep. 'Maui Queen' plantlets. Petru and Matous (1984) successfully transferred the plantlets the sterilize peat and perlite (in ratio 1 to 1) substrate and then to a standard horticulture substrate.

Directly after transfer to *ex vitro* conditions, micropropagated plants are very susceptible to various stresses because they have not yet developed adequate patterns of resource allocation and morphological and physiological features required by the new environment (Chaves, 1994). Low photosynthesis rates (Grout and Aston, 1978; Cournac, et al., 1991) and the malfunctioning of the water housekeeping system (Capellades et al., 1990) are the two of the major constrains in tissue cultured plants. When plantlets are in culture, it only used a fraction of CO_2 because it is constantly supplied with a carbon energy source, however when exposed to an *in vivo* environment it becomes autotrophic (Hoe, 1992). Plantlets getting stress because of stomatal development are not complete.

Besides suffer light stress (photoinhibition), plantlets also suffer water stress due to the differences between *in vitro* and *ex vitro* relative humidity. In *in vitro* environment the culture was supplied with high level of humidity but when plantlets were transferred to the greenhouse the level of humidity was decreased because of direct light intensity.

Therefore in this study, gradual decrease of relative humidity was done by keeping the plantlets in culture rooms at 25 ± 1 °C under 16 hours light and 8 hours dark for 3 weeks,

watered every day, covering the plantlets with transparent plastics with small holes and the plastic covered was stepwise opened. By this acclimatization process the *in vitro* plantlets features will slowly develop to the level of intact plant. Lee and Roa, (1987) reported that change in humidity during the process of transfer was the greatest contributing factor to high mortality rate of plantlets. Ziv (1986) stated that by exposing plants to reduced relative humidity the survival rate of plants *in vitro* may be increased during acclimatization. According to Ziv et al., (1983), the low relative humidity condition of the culture vessels is important in order to develop more vigorous plantlets structure and therefore, plantlets could adapt easily when transferred to the field.

A range of methods have been used to lowering the relative humidity *in vitro*. Experiment has been done with varying results. The use of antitranspirants to reduce water loss during acclimatization has had mixed results (Hazarika, 2003). Leaf surfaces covering agents such as glycerol, paraffin and grease promoted *ex vitro* survival of several herbaceous species, but not has evaluated over a long term or examined on woody species (Selvapandian, 1988). ABA sprayer on the leaf surface was done by Wardle *et al*., (1979) to decrease stomatal transpiration of micropropagated cauliflower plantlets. Wardle et la., (1983) used silica gel and lanolin oil to reduce humidity in chrysanthemum. Their experiment recorded high mortality. Ritchie et al., (1991) reported that leaves of chrysanthemum and sugar beet, which were initiated and developed at relative humidity below 100.00 % displayed increased epicuticular wax, improved stomatal functioning and reduced leaf dehydration.

CHAPTER 7

CONCLUSION

Polianthes tuberosa L. gain an importance in pharmaceutical and perfumed industries because of their unique secondary metabolic reactions for the synthesis of various commercial valuable compounds. In Malaysia, cut flower of *Polianthes tuberosa* L*.,* received a lot of consumer demands from cut flower lover because of their beautiful pure white flowers and scented that describe as exclusive. *Polianthes tuberosa* L. have limitation to grow in warm countries, so that a tremendous application of plant tissue culture has been used for *in vitro* cultivation of this plant.

Plant tissue culture is a practice used to propagate plants under sterile conditions, often to produce clones of a plant, which relies on the fact that many plant cells have the ability to regenerate a whole plant (totipotency). Monocotyledonous plants have been regarded as difficult *in vitro* materials. However, an increasing number of monocotyledons have been successfully cultured. Less research have been done on *in vitro* propagation of *Polianthes tuberosa* L. which is monocotyledonous bulbous plant belongs to the family Agavaceae. The present work reported in greater detail on tissue culture studies of *Polianthes tuberosa* L.

Indirect regeneration via somatic embryogenesis pathway occurred in this species. Researchers using cell cultures have brought somatic embryogenesis to the forefront of plant tissue culture activity, in part because of the importance of micropropagation to agriculture, horticulture and forestry. Of the method used for clonal propagation, somatic embryogenesis is potentially the most important as it is capable of providing a larger number of plants in a shorter period of time than organogenic approaches.

Callus was induced on MS media supplemented with various concentrations of BAP and NAA. MS media supplemented with 2.0 mg/l NAA and MS media supplemented with 0.5 mg/l BAP in combination with 2.0 mg/l NAA were the best media for callus induction. All types of explants used which were leaf, stem and flower bud produced 100.0±0.00% callus in these media. Stem started to produce callus after 4 weeks in culture. Leaf explants started to produce callus after 5 weeks and flower bud explants started to produced callus after 12 weeks. Leaf explants was the best explant compared with other explants because callus from this explant proliferated well on all medium tested.

Embryogenic callus was formed from leaf explant cultured on MS media supplemented with 0.5 -4.0 mg/l NAA. Double staining technique has been used to distinguish embryogenic and non embryogenic callus. Embryogenic callus were then transferred to MS media supplemented with BAP, MS media supplemented with NAA, MS media supplemented with 2,4-D, MS media supplemented with NAA combined with BAP and MS media supplemented with 2,4-D combined with BAP to induce somatic embryo formation.

After 3 months in the culture media, various stages of somatic embryos could be observed. Globular, heart shaped, torpedo and cotyledonary stage were obtained in all media excluded MS basal media and MS supplemented with BAP alone. MS media supplemented with 2.5 mg/l 2, 4-D combined with 0.1 mg/l BAP promoted somatic embryogenesis effectively with high frequencies of somatic embryogenesis occurred, where an average of 26.67±0.42 somatic embryos was obtained from 0.5 cm of embryogenic callus from liquid media and 20.53±0.50 somatic embryo was formed on solid media. Somatic embryo

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formations are better on liquid media compared to solid media because of surface interaction between explant and media in liquid media are higher than in solid media.

Somatic embryos were then transferred to regeneration media. Complete *Polianthes tuberosa* L. plantlet with shoots and roots were observed on MS basal media, MS media supplemented with Kin combined with IBA and MS media supplemented with Kin combined with NAA. MS media supplemented with 2.0 mg/l Kin combined with 2.0 mg/l NAA was the most suitable media for shoot regeneration with 26.23 ± 0.74 number of shoot formation. 4.23 ± 0.40 number of roots were obtained on MS media supplemented with 0.5 mg/l Kin combined with 2.0 mg/l NAA.

Regeneration of *Polianthes tuberosa* was successful only when the plantlets obtained from tissue culture can survive in the natural environment. Acclimatization process was then carried out and 89% of the plantlets grown in garden soil (combination of black soil and red soil at ratio 2 to 1) were successfully adapted with the new environment which is very different from tissue culture environment.

Because of the high ability in regeneration and multiplication, the plant regeneration system of *Polianthes tuberosa* established in the present study will be efficiently used for rapid clonal propagation if the micropropagated plants have no aberrant somaclonal variations. Moreover, callus cultures with high regeneration ability may also be used for breeding of this crop through, for example, selection of useful somaclonal variations, polyploid production, somatic hybridization and genetic transformation.

Micropropagation and plant tissue culture scientists have always three important factors to consider when assessing the efficiency of a micropropagation system which are; capacity to induce organogenesis with subsequent successful regeneration, efficient acclimatization and cost. This study of tissue culture of *Polianthes tuberosa* L. was considered a success and the findings of these studies hopefully can be shared with others in the similar field.

For the future studies of this species, a lot of experiments can be done. Analyzing pigment and chemical compound extraction from callus of *Polianthes tuberosa* L. can be done using GCMS (Gas Chromatography Mass Spectroscopy) and HPLC (High Performance Liquid Chromatography). Gas chromatography-mass spectroscopy (GC-MS) is one of the so-called hyphenated analytical techniques. As the name implies, it is actually two techniques that are combined to form a single method of analyzing mixtures of chemicals. Gas chromatography separates the components of a mixture and mass spectroscopy characterizes each of the components individually. By combining the two techniques, an analytical chemist can both qualitatively and quantitatively evaluate a solution containing a number of chemicals.

High performance liquid chromatography is basically a highly improved form of column chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres. That makes it much faster. It also allows us to use a very much smaller particle size for the column packing material which gives a much greater surface area for interactions between the stationary phase and the molecules flowing past it. This allows a much better separation of the components of the mixture. The other major improvement over column chromatography concerns the

detection methods which can be used. These methods are highly automated and extremely sensitive.

Cytology studies also should be done for this species. Cytology, more specifically karyology, is a branch of cytological research that includes studies with particular reference to variation in chromosome morphology and number, evolutionary interpretation of chromosomal changes and genomic relationship. Chromosome counting, mitotic index, meiotic studies and other studies using roots of *Polianthes tuberosa* obtained from tissue culture and from intact plant will be compared. For karyological studies, root tip meristems are commonly favoured and the most convenient. Other actively growing parts of a plant like shoot apex with leaf primodia, developing anthers and ovules in very young floral also can be used for cytological studies.

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APPENDIX I

SEMINARS AND EXHIBITION ATTENDED

1. 1st International Symposium on Medicinal, Aromatic and Nutraceutical Plants from Mountainous Areas at Saas-Fee, Switzerland on 6 – 9 July 2011. Poster entitle 'Induction of somatic embryogenesis for plant regeneration in *Polianthes tuberosa'*.

2. 1st ISHS Symposium on Sustainable Vegetable Production in South East Asia at Central Java, Indonesia on 13 – 17 March 2011. Poster entitle 'Mass Propagation of *Brassica oleracea* var. *botrytis (*cauliflower) Through Tissue Culture System'.

3. 3rd International Conference on Functional Materials and Devices at Terengganu, Malaysia on 14th - 17th June 2010. Poster entitle 'Studies of Anthocyanin Extraction from Roselle Calyces as a Natural Colourant for Coating'.

4. 23rd Eucarpia Symposium on Ornamental Breeding at Leiden, Netherlands on 31st August- 4th September 2009. Poster entitle 'Micropropagation of Selected Ornamental Plants'.

5. 4th International Symposium on Tropical & Subtropical Fruits 2008 at Bogor, Indonesia on 3 – 7 November 2008. Poster entitle 'Tissue Culture, Anatomical and Morphological Studies of *Triphasia trifolia* (Burm.F.) P.Wilson'.

6. 4th Life Sciences Postgraduate Conference at University of Science Malaysia, Penang on 18 – 20 Jun 2008. Poster entitle 'The Effect of Different Hormones on Callus Formation and *In Vitro* Flowering of *Oxalis sp'*.

7. Participated in Innovation And Creativity Expo 2010, University of Malaya on 1st -3rd April 2010. Research title 'Recycling of Spent Mushroom Compost via Cultivation of Snowpea Sprouts (Duo Miao) Artificial Seeds and Vermicomposting'. (Gold Medal)

8. Participated in Malaysia Technology Expo (MTE) 2010, Kuala Lumpur Convention Center, Malaysia on 4th – 6th February 2010. Research title 'Miniature Garden of *In Vitro* Plantlets Derived from Somatic Embryos of Ornamental Plants'. (Bronze Medal).

9. Participated in Invention, Innovation And Technology Exhibition (ITEX) 2009. Kuala Lumpur Convention Center, Malaysia on 15th – 17th May 2009. Research title 'Miniature gardens from in vitro plantlets derived from somatic embryos of ornamental plants'.

10. Participated in Research, Creation And Innovation Expo 2009, University of Malaya on 13th -15th January 2009. Research title 'From Somatic Embryos to In Vitro Plantlets for Miniature Gardens'. (Silver Medal).

11. Participated in Research, Creation And Innovation Expo 2009, University of Malaya on 13th -15th January 2009. Research title 'Artificial Propagules of Economically Important Crops'. (Bronze Medal).

12. Participated in Biomalaysia 2008. Kuala Lumpur Convention Center, Malaysia on 7th – 9th October 2008. (Bronze Medal).

APPENDIX II

LIST OF PAPERS SUBMITTED/PUBLISHED

1. Rosna Mat Taha**, Sakinah Abdullah**, Noraini Mahmad, Azani Saleh, Hashimah Elias. (2012). *In Vitro* Plant Regeneration through Somatic Embryogenesis derived from leaf Explants of *Polianthes tuberosa* L. Industrial Crops and Products-Elsevier. (Submitted) (*ISI-Cited Publication*).

2. Rosna Mat Taha, Rashidi Othman, Noraini Mahmad, **Sakinah Abdullah**, Azani Saleh and Nor Azlina Hasbullah. (2012). Effects of NAA and BAP, Double-Layered Media and Light Distance on *In Vitro* Regeneration of *Nelumbo nucifera* Gaertn. (Lotus), Aquatic Medicinal Plant. Plos one. (Submitted) (*ISI-Cited Publication*).

3. Rosna Mat Taha, Rashidi Othman, Noraini Mahmad, Hashimah Elias, **Sakinah Abdullah** and Sharifah Nurashikin Wafa Syed Mohd Thalal Wafa. (2012). The Effect of Zeatin and Gibberelic Acid on *In Vitro* Flowering of *Phlox paniculata* L. Australian Journal of Crop Science. (Submitted) (*ISI-Cited Publication*).

4. J.S. Yaacob, A.I.M. Yussof, **S. Abdullah**, K. Ramesh and R.M. Taha (2011). Investigation of pH-varied anthocyanin pigment profiles of Agapanthus praecox and its potential as natural colourant. Materials Research Innovations, Vol. 15, Supplement 2: 106-109. (Published) (*ISI-Cited Publication*).

5. **Sakinah Abdullah**, Rosna Mat Taha, Nor Azlina Hasbullah, Normadiha Mohamed, Hashimah Elias, Nor Rafizah Ramli & Noorma Wati Haron (2009). Tissue Culture,

anatomical and morphological studies of *Triphasia trifolia* (BURM. F.) P. Wilson. Acta Horticulturae. (Accepted). (*SCOPUS-Cited Publication*).

6. Normadiha Mohamed, Rosna Mat Taha, Hashimah Elias, **Sakinah Abdullah** & Nor Azlina Hasbullah (2009). Tissue culture studies on *Fortunella polyandra* var. Nagami and Meiwa. Acta Horticulturae. (Accepted) (*SCOPUS-Cited Publication*).

7. Hashimah Elias, Rosna Mat Taha, Nor Azlina Hasbullah, Normadiha Mohamed & **Sakinah Abdullah** (2009). In Vitro Propagation & Cellular Behaviour Studies of *Severinia buxifolia* (Poir) Tenore. Acta Horticulturae. (Accepted) (*SCOPUS-Cited Publication*).

In Vitro Plant Regeneration through Somatic Embryogenesis derived from leaf Explants of *Polianthes tuberosa* L.

Taha, R. M., Abdullah, S.*, Noraini, M., Saleh, A., Elias, H.

Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia.

*Corresponding Author

Email: raihani_84@yahoo.com

Phone number: +60379677121

Fax number: +60379674178

Abstract

Polianthes tuberosa L. is one of the ornamental plants with many uses, such as for medicinal purposes, religious ceremony and cut flower industry. It has white flowers with strong odor. In Malaysia, this species is still considered as underexploited. However, in other countries the essential oil of this plant has been processed into valuable expensive perfumes. Based on the potential uses of *Polianthes tuberosa*, mass propagation of this species through tissue culture is urgently needed. Therefore, in the present study, plant regeneration was established using various plant growth regulators and callus formation especially to obtain embryogenic callus was initiated from various plant organs. The effect of different hormones such as 2,4-D (2,4 Dichlorophenoxy acetic acid), NAA (Naphthalene acetic acid), BAP (Benzyl aminopurine) etc. were investigated on the embryogenic callus formation and regeneration capacity of this interesting species which can be found in highlands of Malaysia. Our results showed that complete plant regeneration could be achieved on MS medium supplemented with 2.0 mg L^{-1} Kin and 2.0 mg L^{-1} NAA from somatic embryos derived from intact leaf explants. Embryogenic callus and subsequent somatic embryogenesis were obtained on MS fortified with 2.5 mg L^{-1} 2,4-D. Various stages of somatic embryos development such as globular, heart-shaped, torpedo and cotyledonary phases were observed. Plant regeneration was also successfully obtained from the subsequent development of these somatic embryos subcultured on MS supplemented with 2.5 mg L^{-1} Kin and 2.0 mg L^{-1} IBA, whereby micro shoots initially formed and root formation was achieved on MS medium supplemented with 0.5 mg L^{-1} Kin and 2.0 mg L^{-1} NAA. Acclimatization of the regenerants was successfully obtained when plantlets were transferred to black soil combined with red soil at a ratio of 2:1 with 63.33 ± 0.03 % survival rate.

Keywords: Tissue culture, Plant growth regulator, *Polianthes tuberosa,* embryogenic callus, somatic embryogenesis, plant regeneration

1.0 Introduction

Polianthes tuberosa L. is a well known ornamental plant due to its floral scent that is described as a complex, exotic and hypnotic sweet. Most flowers begin to lose their scent when they are picked but not so with tuberose, the flower scent continues to produce itself like jasmine.This plant grows over much of tropical and temperate world. This plant was cultivated in tropical and subtropical regions. For instance, Polianthes tuberosa is cultivated on large scale in France, Italy, South Africa, North Carolina in USA and many tropical and subtropical areas in India. Polianthes tuberosa is an herbaceous species consists of grass like leaves arising from underground tuberose structure produce offsets that result in small clump of leaves. Polianthes tuberosa is a half hardy, bulbous perennial perpetuating itself through the bulblets. Bulbs are made up of scales and leaf base and the stem is condensed structure which remains concealed within scales. Polianthes tuberosa is a perennial plant of family Agavaceae. This genus consists of 14 species, three varieties and 2 cultivars (Solano and Feria, 2006). The best known taxon is Polianthes tuberosa, which has been cultivated and used for medicinal, ornamental and ceremonial practices since pre-hispanic times (Solano and Feria, 2006). Although in Malaysia, this plant receives a lot of consumer demands from cut flower lover, however, this species is still considered as underexploited and can be found in the wild. In other countries, the essential oil of this plant has been processed into valuable expensive perfumes. Essential oils, known as nature's living energy are the natural aromatic volatile liquids found in shrubs, flowers, trees, roots, bushes and seeds. Tuberose essential oil is very expensive because it is very difficult to collect the essential oil from the flowers. The natural flower oil of Polianthes tuberosa remain today as one of the most expensive of the perfumer's raw materials. It requires 150 kg of flowers to yield one kg of absolute of effleurage which is brown, semisolid, alcohol soluble liquid pomades. The exotic smell of Polianthes tuberosa was included in several worldwide known perfumes such as 'Pison' by Christian Dior and 'Chole' by Karl Lagerfeld. Based on the potential uses of Polianthes tuberosa, mass propagation of this species through tissue culture is urgently needed. Amongst the methods used for micropropagation, somatic embryogenesis is potentially important and powerful as it is capable of providing a large number of plants in shorter period of time than organogenic approaches. Thus far, somatic embryogenesis from leaf explants of this species has hardly been reported. However, plant regeneration of this species have been achieved using rhizome (Sangavai and Chellapandi, 2008). In the present study, we report and discuss the induction of embryogenic callus from intact leaf explants, subsequent formation and development of somatic embryos and ultimately complete plant regeneration of Polianthes tuberosa L. through somatic embryogenesis process and development of a protocol for successful field transfer.

1.1 Materials and Methods

1.1.1 Preparation of explants

Leaf explants from 3 month-old intact plants were used in the present study. For sterilization process, the explants were surface sterilized under running tap water for 30 minutes to remove contaminants and any residue that were found on the explants. The explants were then rinsed in different concentrations (70%. 50%, 30%, 20%, and 10%) of

sodium hypochlorite (chlorox) and 70% ethanol. At first rinse, 70% sodium hypochlorite and two drops of Tween 20 was added. Finally the explants were washed 3 times in sterile distilled water. Each rinse lasting approximately for one minute. Sterilized leaf explants were cultured on MS media supplemented with various hormones for callus induction, somatic embryogenesis and plant regeneration.

1.1.2 Preparation of culture medium and embryogenic callus induction

Solid culture media and liquid or suspension culture media were used in this study for induction of embryogenic callus. Solid media was prepared by diluting MS media powder with 30 g l^{-1} sucrose and 8 g l^{-1} agar in distilled water. The medium pH was adjusted by adding NaOH or HCl to 5.8 prior to autoclaving. Suspension culture media was prepared using the same method but without the gelling agent. Various types and concentration of plant hormones such as 2,4-D and NAA were added into the culture medium to study the induction and formation of embrogenic callus and somatic embryos. Solid and liquid media were supplemented with equivalent concentrations and combinations of hormones.

1.1.3 Induction of somatic embryos

Embryogenic callus that were formed on MS supplemented with 0.5 – 4.0 mg L^{-1} NAA were subcultured onto solid media or suspension culture media for somatic embryo formation. Embryogenic callus derived from callus induction medium were cut into small pieces (0.5 cm) and then transferred to solid media or suspension culture media. Cultures in solid media were maintained in the culture room under 16 h light and 8 h dark at 25±1ºC. Suspension cultures were maintained on a shaker at 100 rpm in the culture room condition. Different stages of somatic embryogenesis were observed.

1.1.4 Development of somatic embryos

Somatic embryos formed on MS fortified with 3.5 mg L^{-1} NAA and 0.1 mg L^{-1} BAP were selected and cultured onto germination media. Somatic embryos treated as explants were excised into 1cm x 1cm and cultured onto MS media supplemented with various combinations and concentration of hormones. Nine concentrations of GA$_3$, eight concentrations of Kinetin combined with 2.0 mg L^{-1} IBA and eight concentrations of Kinetin combined with 2.0 mg L^{-1} NAA were utilized for culturing of the somatic embryos.

1.1.5 Protocol for field transfer of regenerants/ Acclimatization

After 3 months *in vitro* plantlets were transferred to pots (80 x 60 mm) filled with a 1 cm layer of sand and a mixture of garden soil and red soil. The remaining agar was carefully washed off the roots with distilled water before planted in the pots. The potted plantlets were first kept in the culture room at 25 ± 1 °C under 16 hours light and 8 hours dark for 3 weeks. The plantlets were watered every day.
Plantlets were transferred to 3 different scheme of growth media:
4) Garden soil – Combination of black soil and red soil at ratio of 2 to 1
5) Autoclaved garden soil – Combination of black soil and red soil at a ratio of 2 to 1

6) Red soil

1.1.6 Data analysis

Three replicates consisting of 30 explants in each replication were maintained for each treatment and the data was analyzed statistically using Duncan's Multiple Range Test (DMRT). The statistical analysis based on mean values per treatment was done using the technique of analysis of variance. The comparative LSD multiple range test (p=0.01) was used to determine the significant differences between treatments.

1.2 Results and Discussion

1.2.1 Embryogenic callus induction

All media tested for callus induction produced green and white creamy and soft watery structure of callus. Embryogenic callus were identified using double staining method (Gupta et. al. 1987). All the callus was stained red and was considered as embryogenic because non embryogenic callus would stain blue. All explants produced callus in MS medium supplemented with 2.0 and 3.0 mg L^{-1} NAA (Table 1). Culture media composition is very important to induce embryogenic callus. In the present study of *Polianthes tuberosa*, a high concentration level of auxin (3.0 mg L^{-1}) which was NAA in the callus induction medium was necessary to enhance embryogenic callus production. This is in agreement with the results obtained by previous researchers such as Nagarajan *et al*, (1986), Meijer and Brown, (1987), Nolan *et al*., (1989), Shri and Daris, (1992), Carman, (1990), Ammirato, (1987) and Luo *et al*., (1999). They also found that the induction of embryogenic callus was usually promoted by a relatively high concentrations of auxin, especially 2, 4-D. Ahmed et al. (2011) also induced viable embryogenic callus on MS medium supplemented with 2,4-D and NAA but, with addition of ascorbic acid in *Phyla nodiflora*.

1.2.2 Induction of somatic embryos

In order to encourage the production of mature development stages of somatic embryos, embryogenic callus was transferred to 35 combinations of liquid and solid media as shown in Table 2. Embryogenic callus cultured on MS hormone free medium and MS supplemented with BAP alone did not produce any somatic embryos. Embryogenic callus became necrotic and died after some time in the culture media. However, on the medium supplemented with BAP, in combination with NAA or 2,4-D, callus formed showed smooth round structures which occurred on the surfaces of embryogenic callus within 3 months. Embryogenic callus that was subcultured onto MS media supplemented with NAA or 2,4-D alone also gave rise to smooth round structures (Fig. 1a). According to Luo, (1999), who did the histological studies of the same structure from callus of *Astaragalus* sp. stated that these structures were somatic embryos at globular stage without vascular connection to the callus. After 3 months in culture, round structures of globular stage and other stages like heart shape, torpedo and cotyledonary could be observed. Somatic embryo development was asynchronous in this plant species where different stages of development could be observed within the same treatment. Asynchronous development was observed also in somatic embryogenesis callus

cultures of Kampung Royal *Poinciana* (Myer and Vendrame, 2004), *Astragalus adsurgens* Pall (Luo *et al.*, 1999), and *Azadiracht aindica* A. Juss (Medha *et al.*, 1993).

In the present study, the highest number of embryos, 27±0.4 was obtained on MS media supplemented with 3.5 mg L^{-1} NAA and 0.1 mg L^{-1} BAP in liquid media. MS media supplemented with 2.5 mg L^{-1} 2,4-D and 0.1 mg L^{-1} BAP also gave high results with 27±0.4 number of somatic embryos. Similar nutrients requirement was observed in bulb plant *Agave* (Amaryllidaceae). According to Bansude *et al.*, (2003), 0.5 mg L^{-1} NAA in combination with 1-2 mg L^{-1} BAP could induce somatic embryos in this species. In *Narcissus bulbocodium*, the formation of somatic embryo was stimulated by using the combinations of IBA and BAP (Salema and Salamak, 2000).

Compared to NAA applied singly, NAA combined with BAP and 2,4-D alone, 2 4-D combined with BAP promoted somatic embryogenesis effectively. The high frequencies of somatic embryogenesis occurred on MS medium supplemented with 2,4-D at concentration of 2.5 mg L^{-1} combined with 0.1 mg L^{-1} BAP, whereby an average of 27±0.4 somatic embryos were obtained from 0.5 cm of embryogenic callus from liquid media and 21±0.5 somatic embryos were formed on solid media. MS supplemented with 2,4-D alone also gave quite encouraging results with 24±0.4 of somatic embryo formation in liquid media and 20±0.6 on solid media. In previous study by Siong *et. al.,* (2011) also showed that in cauliflower , 2,4-D applied singly and as well as in combination with kinetin was able to induce somatic embryogenesis. Higher number of somatic embryos were found in liquid media in all experiments with the same treatment except in MS media supplemented with 2.5 mgl^{-1} 2-4,D and 2.5 mgl^{-1} BAP whereby, 8±0.5 somatic embryos were formed in liquid media and 9±0.5 in solid media.

Many factors including choice of growth regulators, choice of explants and culture medium composition are responsible for successful formation of somatic embryogenesis. In the present investigation for somatic embryo production showed that liquid media produced higher number of somatic embryos compared to solid media with the same hormone treatment (Table 2). Somatic embryo at globular stage became oblong in shape after 13 weeks and developed further into heart shape (Fig. 1c) after 15 weeks. Heart shaped somatic embryo then elongated after 16 weeks and after 17 weeks torpedo stages were observed. The last stage (Fig. 1d) which was cotyledonary stage was observed after 19 weeks in culture. Same developmental stages were obtained from embryogenic callus cultured on solid media.

1.2.3 Germination of somatic embryos

Several concentrations and combinations of hormones were tested for regeneration of *Polianthes tuberosa* from somatic embryos. The development of whole plants with shoots and roots was observed on MS medium without hormone after 2 months. MS medium without growth regulators is a typical medium for somatic embryo maturation and germination in many plants including wetlands monocots like *Phragmites australis* (Straub *et al.*, 1988) and *Sporobolus virginicus* (Straub et al., 1992). However, in MS medium without hormone, somatic embryos showed weak growth with small mean numbers of shoots and root formation, 4±0.2   shoots and 1±0.1 root were observed (Table 3). Wang *et al.*, (2004)

obtained similar result when they germinated somatic embryos of monocot *Scirpus robustus* in MS medium. They reported slow shoot growth with both small mean shoot numbers and shoot length no more than 1 cm in height was obtained.

The addition of plant growth regulators, auxin and cytokonin to the medium significantly increased the number of generated shoots and roots. The highest number (26±0.7) of micro shoots were observed in MS media supplemented with 2.0 mg L^{-1} Kin and 2.0 mg L^{-1} NAA (Table 3). According to Preil, (2003) and Rout and Jain (2004), many ornamental plants are propagated by *in vitro* culture on the culture medium containing auxins and cytokinins. Medha *et al.*, (1993), found similar result using different kind of cytokinin and auxin in regeneration of neem from somatic embryos, 60 to 70% of somatic embryos enlarged and germinated on MS media containing 2.0 mg L^{-1} BAP and 0.5 mg L^{-1} IAA after 20 to 30 days. According to Wang *et.al* (2004), addition of cytokinin (BA) into the MS medium could increase shoot regenerated (*Scirpus robustus*) from 8 to 35 and up to 53 depending on BA concentration.

Root initiation typically requires a moderate to high auxin signal but rarely with the use of a more natural source auxin (Gamborg and Phillips, 1995). The highest root formation was observed on MS media supplemented with 0.5 mg L^{-1} Kin and 2.0 mg L^{-1} NAA with an average of 4.23±0.40 roots per explant. In contrast, root formation in sugar beet, occurred when embryogenic callus were transferred onto half strength MS medium supplemented with high auxin (3.0 mg L^{-1} IBA) and in the absence of cytokinin.

1.2.4 Acclimatization

Tissue culture was considered successful when plantlets obtained could be acclimatized to the natural environment (*ex vitro*). Three types of soils were used as the substrates for transplanting the plantlets which are garden soil (combination of black soil and red soil at ratio of 2 to 1), autoclaved garden soil (combination of black soil and red soil at ratio of 2 to 1) and red soil. Garden soil (combination of black soil and red soil at ratio of 2 to 1) was found to be the most suitable substrate for acclimatization of *Polianthes tuberosa* plantlets with the percentage of survival rate of 63.33±0.09 %. The percentage of plantlets acclimatized survival rate in red soil was lower compared to the percentage of plantlets acclimatized in garden soil (combination of black soil and red soil at ratio of 2 to 1) with the survival percentage of 50.00±0.09. This could be because garden soil (combination of black soil and red soil at ratio of 2 to 1) provided good drainage system for the plant and good aeration system. Sink (1984) obtained similar results and stated that by retaining water would deplete oxygen level in the soil and thus, this caused failure of the plant rooting system.

1.3 Conclusion

Callus derived from leaf explants was induced on MS supplemented with 2-3 mg L^{-1} NAA. Embryogenic callus formed when MS was fortified with 3.5 mg/l NAA and 0.1 mg L^{-1} BAP. Various stages of somatic embryo development were observed. Successful *in vitro* regeneration of whole plantlets of *Polianthes tuberosa* through somatic embryogenesis pathway was obtained on MS supplemented with 2 mg L^{-1} kin and 2 mg L^{-1} NAA. The regenerants were transferred to soil with 63.33% success rate. This procedure could be utilised in future for somatic embryogenesis which has higher efficiency to mass propagate

Polianthes tuberosa, especially when very limited *in vitro* work is being reported for this species.

Table 1: Induction of callus formation from leaf explants cultured on MS medium supplemented with different concentrations of hormones after 12 weeks of culture.

MS + NAA(mg L^{-1}) Percentage of explant produced callus (%)		Observations
0.5	77±0.1^{abcd}	Embryogenic callus. Green and white creamy in colour and soft watery structure.
1.0	90±0.1^{abcd}	Embryogenic callus. Green and white creamy in colour and soft watery structure.
1.5	97±0.0^{ab}	Embryogenic callus. Green and white creamy in colour and soft watery structure.
2.0	100±0^{a}	Embryogenic callus. Green and white creamy in colour and soft watery structure.
2.5	97±0.0^{ab}	Embryogenic callus. Green and white creamy in colour and soft watery structure.
3.0	100±0^{a}	Embryogenic callus. Green and white creamy in colour and soft watery structure.
3.5	87±0.1^{abcd}	Embryogenic callus. Green and white creamy in colour and soft watery structure.
4.0	87±0.1^{abcd}	Embryogenic callus. Green and white creamy in colour and soft watery structure.

Mean followed by the same letter are not significantly different at the 0.01 level of confidence

Table 2: Number of somatic embryos produced in liquid and solid media after 8 weeks of culture.

MS + Hormone (mg/l)	No. of Somatic Embryos per Explant	
	Liquid media	Solid media
MS media (control)	0 ± 0^p	0 ± 0^q
MS + 0.1 mg L^{-1} BAP	0 ± 0^p	0 ± 0^q
MS + 0.5 mg L^{-1} BAP	0 ± 0^p	0 ± 0^q
MS + 1.0 mg L^{-1} BAP	0 ± 0^p	0 ± 0^q
MS + 1.5 mg L^{-1} BAP	0 ± 0^p	0 ± 0^q
MS + 2.0 mg L^{-1} BAP	0 ± 0^p	0 ± 0^q
MS + 2.5 mg L^{-1} BAP	0 ± 0^p	0 ± 0^q
MS + 2.0 mg L^{-1} NAA	13 ± 0.3^{lm}	7 ± 0.3^o
MS + 2.5 mg L^{-1} NAA	16 ± 0.4^{ij}	9 ± 0.3^{lmn}
MS + 3.0 mg L^{-1} NAA	19 ± 0.5^{def}	12 ± 0.3^{ijk}
MS + 3.5 mg L^{-1} NAA	25 ± 0.3^b	19 ± 0.2^a
MS + 4.0 mg L^{-1} NAA	20 ± 0.4^{cde}	17 ± 0.4^d
MS + 0.1 mg L^{-1} 2-4,D	6 ± 0.3^o	5 ± 0.4^p
MS + 0.5 mg L^{-1} 2-4,D	9 ± 0.4^n	8 ± 0.3^{no}
MS + 1.0 mg L^{-1} 2-4,D	14 ± 0.4^{kl}	11 ± 0.7^{klm}
MS + 1.5 mg L^{-1} 2-4,D	16 ± 0.4^{ij}	13 ± 0.5^{hij}
MS + 2.0 mg L^{-1} 2-4,D	20 ± 0.4^{def}	15 ± 0.6^{def}
MS + 2.5 mg L^{-1} 2-4,D	24 ± 0.4^b	20 ± 0.6^a
MS + 3.0 mg L^{-1} 2-4,D	21 ± 0.3^{cd}	16 ± 0.7^{cde}
MS + 3.5 mg L^{-1} 2-4,D	18 ± 0.4^{gh}	14 ± 0.6^{fgh}
MS + 4.0 mg L^{-1} 2-4,D	15 ± 0.5^{ij}	12 ± 0.6^{ijk}
MS + 3.5 mg L^{-1} NAA + 0.1 mg L^{-1} BAP	27 ± 0.4^a	20 ± 0.6^a
MS + 3.5 mg L^{-1} NAA + 0.5 mg L^{-1} BAP	23 ± 0.4^b	17 ± 0.6^{bc}
MS + 3.5 mg L^{-1} NAA + 1.0 mg L^{-1} BAP	20 ± 0.4^{cd}	14 ± 0.6^{fgh}
MS + 3.5 mg L^{-1} NAA + 1.5 mg L^{-1} BAP	19 ± 0.3^{fg}	13 ± 0.5^{ghi}
MS + 3.5 mg L^{-1} NAA + 2.0 mg L^{-1} BAP	16 ± 0.3^{hi}	11 ± 0.5^{jkl}
MS + 3.5 mg L^{-1} NAA + 2.5 mg L^{-1} BAP	11 ± 0.3^m	8 ± 0.5^{no}
MS + 2.5 mg L^{-1} 2-4,D + 0.1 mg L^{-1} BAP	27 ± 0.4^a	21 ± 0.5^a
MS + 2.5 mg L^{-1} 2-4,D + 0.5 mg L^{-1} BAP	24 ± 0.4^b	19 ± 0.5^{ab}
MS + 2.5 mg L^{-1} 2-4,D + 1.0 mg L^{-1} BAP	22 ± 0.5^c	16 ± 0.6^{cdef}
MS + 2.5 mg L^{-1} 2-4,D + 1.5 mg L^{-1} BAP	20 ± 0.5^{def}	15 ± 0.6^{efg}
MS + 2.5 mg L^{-1} 2-4,D + 2.0 mg L^{-1} BAP	13 ± 0.6^l	12 ± 0.6^{ijk}
MS + 2.5 mg L^{-1} 2-4,D + 2.5 mg L^{-1} BAP	8 ± 0.5^n	9 ± 0.5^{mno}

Mean followed by the same letter are not significantly different at the 0.01 level of confidence

Table 3: Number of micro shoots and roots formation from somatic embryos. Thirty replicates were used in each treatment.

MS + Hormone mg L^{-1}	No of shoot formation	No of root formation
MS media	4 ± 0.2^j	1 ± 0.1^{fg}
MS + 0.1 GA$_3$	0 ± 0^k	0 ± 0^g
MS + 0.5 GA$_3$	$0\pm0k$	0 ± 0^g
MS + 1.0 GA$_3$	$0\pm0k$	0 ± 0^g
MS + 1.5 GA$_3$	0 ± 0^k	0 ± 0^g
MS + 2.0 GA$_3$	0 ± 0^k	0 ± 0^g
MS + 2.5 GA$_3$	0 ± 0^k	0 ± 0^g
MS + 3.0 GA$_3$	0 ± 0^k	0 ± 0^g
MS + 3.5 GA$_3$	0 ± 0^k	0 ± 0^g
MS + 4.0 GA$_3$	0 ± 0^k	0 ± 0^g
MS + 0.1 Kin + 2.0 IBA	7 ± 0.2^i	1 ± 0.1^{efg}
MS + 0.5 Kin + 2.0 IBA	$7\pm0.3h^i$	1 ± 0.1^{def}
MS + 1.0 Kin + 2.0 IBA	9 ± 0.3^h	2 ± 0.2^{bcd}
MS + 1.5 Kin + 2.0 IBA	13 ± 0.4^f	3 ± 0.2^{ab}
MS + 2.0 Kin + 2.0 IBA	20 ± 0.5^c	4 ± 0.3^a
MS + 2.5 Kin + 2.0 IBA	24 ± 0.5^b	4 ± 0.4^a
MS + 3.0 Kin + 2.0 IBA	18 ± 0.6^d	3 ± 0.4^{ab}
MS + 3.5 Kin + 2.0 IBA	11 ± 0.5^g	4 ± 0.4^a
MS + 4.0 Kin + 2.0 IBA	$7\pm0.4h^i$	2 ± 0.3^{bcd}
MS + 0.1 Kin + 2.0 NAA	5 ± 0.4^j	1 ± 0.1^{defg}
MS + 0.5 Kin + 2.0 NAA	9 ± 0.7^h	4 ± 0.4^a
MS + 1.0 Kin + 2.0 NAA	11 ± 0.4^g	4 ± 0.3^a
MS + 1.5 Kin + 2.0 NAA	18 ± 0.8^d	3 ± 0.3^{abc}
MS + 2.0 Kin + 2.0 NAA	26 ± 0.7^a	3 ± 0.3^{ab}
MS + 2.5 Kin + 2.0 NAA	21 ± 0.6^c	3 ± 1.1^{ab}
MS + 3.0 Kin + 2.0 NAA	18 ± 0.6^d	2 ± 0.3^{cd}
MS + 3.5 Kin + 2.0 NAA	16 ± 0.6^e	2 ± 03
MS + 4.0 Kin + 2.0 NAA	$1\pm0.7f^g$	2 ± 0.2^{cd}

Mean followed by the same letter are not significantly different at the 0.01 level of confidence

Table 4: Responses showed by *in vitro Polianthes tuberosa* plantlets after being acclimatized in various sowing media. Result obtained after 2 months being acclimatized

Method	Observation	Survival Of *Polianthes tuberosa* (%)
Plantlets were transferred to Garden soil (Combination of black soil and red soil at ratio 2 to 1)	Plantlets survived and showed healthy growth	63.33 ± 0.09^a
Plantlets were transferred to autoclaved garden soil (Combination of black soil and red soil at ratio 2 to 1)	Plantlets not survived	0.00 ± 0.00^c
Plantlets were transferred to red soil	Plantlets survived with slow growth	50.00 ± 0.09^b

Mean ± SE, n=30. Mean with different letters in the same column different letters in the same column differ significantly at p=0.01.
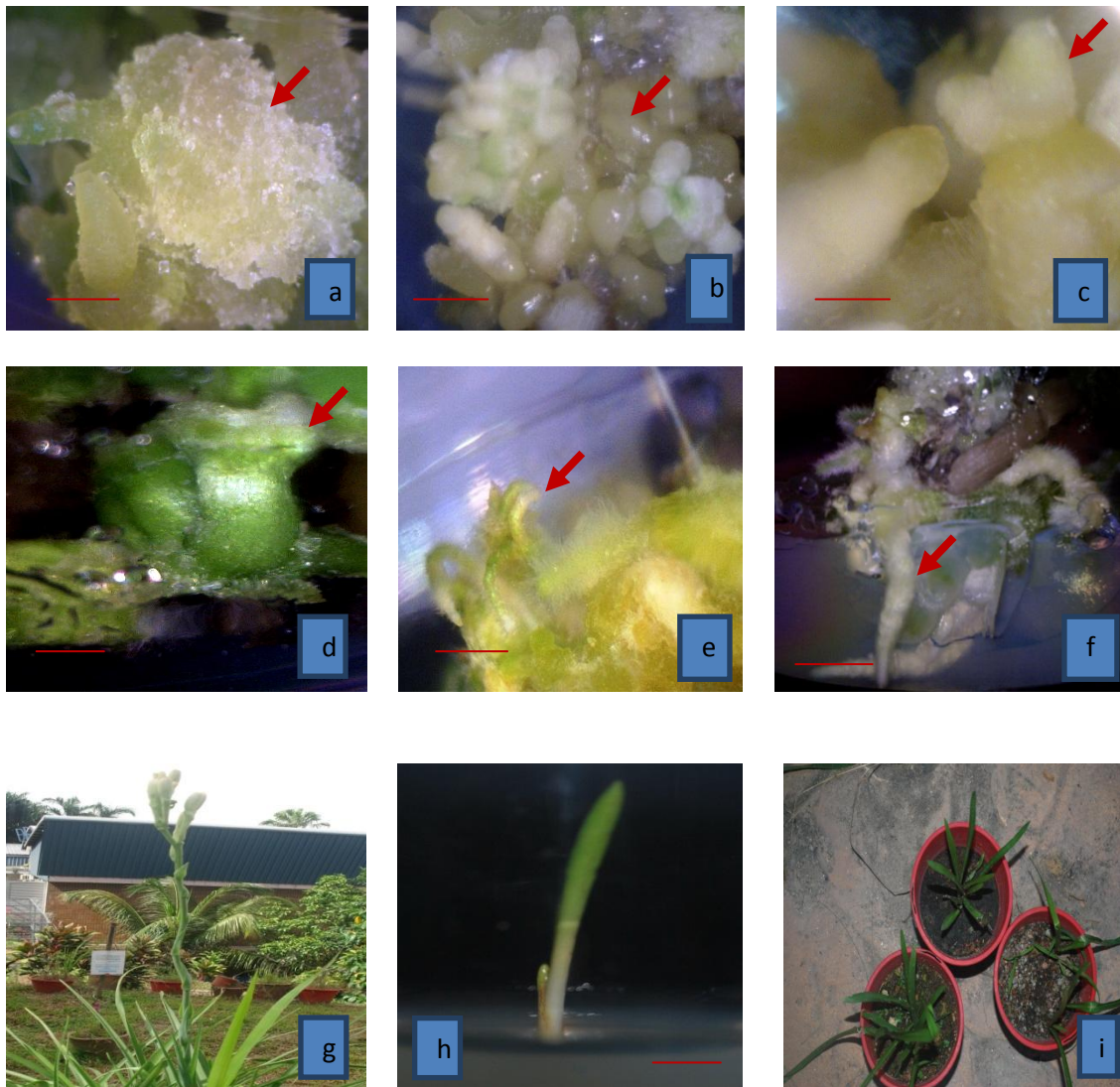
Figure 1: *In vitro* somatic embryogenesis of *Polianthes tuberosa*: a) embryogenic callus; (b) somatic embryos at globular stage; (c) somatic embryos at heart shape stage; (d) somatic embryos at cotyledonary stage; (e) micro shoots formation on MS media supplemented with 2.0 mg/l Kin and 2.0 mg/l NAA; (f) root formation on MS medium supplemented with 0.5 Kin and 2.0 mg/l NAA; (g) Three-month-old intact plant of *Polianthes tuberosa* which were used for explant sources ;(h) Plant regeneration of *Polianthes tuberosa* after 12 weeks of culture. Bars = 1 mm;(i) Four-month-old months old *Polianthes tuberosa* plantlets after being transferred to the garden soil.

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Effects of NAA and BAP, Double-Layered Media and Light Distance on *In Vitro* Regeneration of *Nelumbo nucifera* Gaertn. (Lotus), an Aquatic Edible Plant

Rosna Mat Taha[1], Rashidi Othman[2], Noraini Mahmad[1*], Nor Azlina Hasbullah[3], Azani Saleh[1,4] and Sakinah Abdullah[1]

[1] Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia

[2] Department of Landscape Architecture Kuliyyah of Architecture & Environmental Design, International Islamic University of Malaysia (IIUM), Jalan Gombak, 53100 Kuala Lumpur. Malaysia

[3] Department of Agriculture Sciences, Faculty of Technical and Vocational Education, Sultan Idris Education University, 35900 Tanjung Malim, Perak, Malaysia

[4] International Education Centre (INTEC), MARA University of Technology, Section 17 Campus, 40200 Shah Alam, Selangor, Malaysia.

[*] Corresponding author, email : fara_aid@siswa.um.edu.my

Phone:+603-79677121, Fax:+603-79674178

Abstract (150 words)

The direct regeneration of *Nelumbo nucifera* Gaertn. was successfully achieved from mature explants (green plumule) cultured on a solid MS media supplemented with 1.5 mg/l BAP and 0.5 mg/l NAA with 10.33 ± 0.23 shoots per explant. A new characteristic of layered multiple shoots from immature explants (yellow plumule) cultured on a solid MS media supplemented with combinations of 0.5 mg/l BAP and 1.5 mg/l NAA with higher number of shoots per explant (16.00 ± 0.30). The double-layered media gave the highest number of shoots per explant with a ratio of 2:1 (liquid to solid) with a mean number of 16.67 ± 0.23 shoots per explant from immature explants and 9.00 ± 0.15 shoots per explant from mature explants. In the study involving light distance, the highest shoot obtained (16.67 ± 0.23 mm) from the immature explants was at a light distance of 20 mm compared to the mature explants (9.41 ± 1.11 mm) at a light distance of 250 mm.

Abstract

An efficient protocol has been developed for the rapid mass propagation of an edible and medicinal aquatic plant, *Nelumbo nucifera* Gaertn. The direct regeneration of *Nelumbo nucifera* Gaertn. was successfully achieved from mature explants (green plumule) cultured on a solid MS media supplemented with 1.5 mg/l BAP and 0.5 mg/l NAA with 10.33±0.23 shoots per explant (true-to-type). The regeneration was completed with 3.67±0.32 roots per explant after 10-12 weeks in culture. At the same time, direct regeneration from immature explants (yellow plumule) cultured on a solid MS media supplemented with combinations of 0.5 mg/l BAP and 1.5 mg/l NAA resulted in a higher number of shoots per explant (16.00±0.30) and exhibited a new characteristic of layered multiple shoots, while roots formed on the solid MS basal media. The formation of abnormal shoots (pinkish, red and oval leaf) occurred only in mature explants on the solid MS media supplemented with either 1mg/l BAP and 2.5 mg/l NAA, 2.5 mg/l BAP and 2.5 mg/l NAA, 1.5 mg/l BAP plus 2 mg/l NAA or 1.5 mg/l BAP and 2.5 mg/l NAA. The solid MS basal medium was optimum for root formation and occurred within 2-4 weeks for both mature and immature explants. The double-layered media gave the highest number of shoots per explant with a ratio of 2:1 (liquid to solid) with a mean number of 16.67±0.23 shoots per explant with the formation of primary and secondary roots from immature explants. In contrast, a lower mean number of 9.00±0.15 shoots per explant was obtained from mature explants. In the study involving light distance, the highest shoot obtained (16.67±0.23 mm) from the immature explants was at a light distance of 20 mm compared to the mature explants (9.41±1.11 mm) at a light distance of 250 mm.

Keywords : *Nelumbo*; plumule; mass propagation; hormones combination; tissue culture

Introduction

Lotus is in the genus of *Nelumbo* and belongs to the family of Nelumbonaceae. The Nelumbonaceae family consists of a perennial aquatic and emergent angiosperm plant which consists of two species: *Nelumbo nucifera* Gaertn. (the Asian or sacred lotus) and *Nelumbo lutea* (Willd.) Pers. (the American lotus or water chinquapin). The former is distributed in Asia and North Australia and the latter is found in North and South America

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(Borsch and Barthlott 1996, Han et al. 2007). Lotus is an important economic aquatic plant, not only as a dainty and ornamental flower but also as a source of herbal medicine, with strong bioactive ingredients including alkaloids and flavonoids, and antioxidant, anti-steroidal, antipyretic, anti-cancerous, antiviral and anti-obesity properties (Mukherjee et al. 1997, Sinha et al. 2000, Qian 2002, Sridhar and Bhat 2007).

The lotus is usually propagated vegetatively through rhizome division or tuber production, but the normal propagation rate is very low (Shou et al 2008). It can also be multiplied through seeds but, for quick and more efficient germination, the seeds need to be scarified by rubbing the outer hard seed coat gently on sand paper at both ends and finally immersing in water to initiate germination. Scarified seeds germinated after 3-4 days while normal seeds took 10-15 days to germinate. If the hard coating remains intact, the seeds will remain viable for centuries and it may take a few years for the seed to sprout if placed in water (Hartman et al 1990).

Tissue culture methods for the selection of variant types in ornamentals have been documented for many years, especially for flower colour, plant morphology and also physiological characteristics. *In vitro* methods have been known to shorten breeding cycles and therefore reduce the costs of the development of a new cultivar. A few factors that need to be considered in the selection of explants in tissue culture are the source of explants (intact or aseptic), the size of the explants, ontogeny, the age of the explants or the maturation of the stock plant. In addition to factors that are related to explant tissues, there are also other factors that play important roles in the success of micropropagation, including the addition of the growth regulators (plant hormones).The plants produced by *in vitro* propagation were genetically uniform and free from associations with other microorganisms (Alistock and Shafer 2006). Tissue culture has previously been successfully employed for the micro-propagation of a wide range of aquatic plants, but its application in lotus is rarely reported, possibly because of this plant's recalcitrance to regeneration *in vitro* (Zhou 1999). So far, a protocol for lotus flower regeneration (**Arunyanart 1998, Arunyanart and Chaitrayagun 2005)** and the *in vitro* multiplication of lotus plants through shoot proliferation from underground rhizomes (Shou et al 2008) have been reported.

The present study aimed to compare *in vitro* regeneration between mature (green plumule) and immature (yellow plumule) explants on a solid MS media supplemented with different combinations and concentrations of BAP and NAA. To date, this is the first report of succesful *in vitro* regeneration from immature explants (yellow plumule) with new characteristics (layered multi shoots) in double-layered MS media. At the same time, the study aimed to investigate the effect of light distance on the *in vitro* regeneration of this species.

Materials and methods

Nelumbo nucifera Gaertn. were obtained from a natural lake, the Chini Lake, in Pahang, Malaysia. No specific permits were required for the described field studies. The location is not privately-owned or protected in any way and the field studies did not involve endangered or protected species.Two types of seeds were collected from intact plants including mature (green) and immature (yellow) types. These seeds were initially washed with tap water and teepol. The seeds were then sterilised with 99% (v/v) sodium hypochlorite solution for 1 min and rinsed with distilled water three times. In a laminar flow cabinet, the seeds were dipped in 70% (v/v) ethanol for 1 minute and blotted with sterile tissue. The cotyledons were excised into two and plumules were cultured on solid basal Murashige and Skoog (Murashige and Skoog 1962) Murashige and S medium supplemented with 30 g/l sucrose and 8 g/l agar. Growth regulator, α-naphtaleneacetic acid (NAA) and 6-benzyl aminopurine (BAP) were dissolved in NaOH and added to MS media. After two weeks, the plumules were cut into small pieces (3 mm^2) and cultured on MS media with 30 different combinations and concentrations of NAA and BAP. Thirty replicates for each treatment were used. The pH was adjusted to 5.5 by adding 0.1 M of either sodium hyroxide (NaOH) or hydrochloric acid (HCl). Finally, the media was set to pH 5.5 and autoclaved at 104 kPa (15 Psi^2) at 121°C for 21 minutes. The sterilised media was poured into 1/3 of 60ml sterile containers. All cultures were incubated in a culture room at 25±1°C, with a 16 hour photoperiod at 80-85 µmol m^{-2} s^{-1} under white fluorescent light (1000 lux). Subcultures were

performed every 21-28 days to provide new and fresh nutrients under the same conditions.

The completely regenerated plantlets (6-8 weeks) with shoots and roots were transferred onto solid MS media supplemented with a hormone media (bottom layer) and liquid MS basal media (upper layer). Solid media were fixed to 1cm height in the sterile tubes. At the same time the liquid media level was altered to a ratio of either 1:1, 1:2 or 1:3 to a solid level. Cultures were also exposed to the light source at different distances (55 cm, 10 cm, 15 cm, 20 cm, 25 cm and 30 cm). Cultures were incubated at 25±1°C with 16 hours light and 8 hours dark. Thirty replicates for each treatment were prepared.

Results and discussion

Table 1 showed the responses from mature (green) and immature (yellow) plumule explants on 30 different combinations and concentrations of BAP and NAA on a solid MS media. The solid MS basal media (as control) only showed a response for the formation of primary roots in mature explants within 4 weeks without any elongation of the shoots. In contrast, solid MS basal medium was identified for the best rooting formation 2-4 weeks after samples were transferred from the optimum MS media supplemented with BAP and NAA to the MS basal media. Table 1 only shows four treatments which were successful for root formation. The highest number of roots per explant for green explants were on MS basal media with 4.33±0.53 shoots per explant. This was followed by 1.5 mg/l BAP and 0.5 mg/l NAA, 1.5 mg/l BAP and 1.5 mg/l NAA, and 1.0 mg/l BAP and 2.5 mg/l NAA with 3.67±0.32 roots per explant, 0.67±0.09 roots per explant and 0.57±0.35 roots per explant, respectively. Previous studies showed that both direct and indirect shoot organogenesis was demonstrated in several aquatic plant species (Kane et al 1991). Although information which indicates that aquatic angiosperms are adaptable to *in vitro* culture is limited, the results showed vigorous shoots with fewer roots, which was contrary to the report by Christensen (Christensen 1996) where many tissue cultures of water plant species showed more adventitious shoots. The present study successfully developed a direct regeneration from lotus plumule explants through a tissue culture system within 10-12 weeks.

Table 1 also shows that the highest number of shoots per explant from green plumules were found on the MS media that was supplemented with combinations of 1.5 mg/l BAP and 0.5 mg/l NAA, with a mean of 10.33±0.23 shoots per explant. The shoots elongated vertically on the surface of the solid MS within 2 weeks. Rolled leaves formed in week 12, while open or unrolled leaves were visible after another 4 weeks. The lowest number of shoots per explant for green plumules were on the MS media supplemented with combinations of 3 mg/l BAP and 2.5 mg/l NAA, with a mean of 1.33±0.23 shoots per explant, which was slightly lower than in the solid MS without hormone (1.43±0.10 shoots per explant). Only mature explants showed the formation of abnormal shoots (pinkish, red and oval leaf) which occurred on the solid MS media supplemented with combinations of 1.0 mg/l BAP and 2.5 mg/l NAA, 2.5mg/l BAP and 2.5 mg/l NAA, 1.5 mg/l BAP and 2.0 mg/l NAA, and 1.5 mg/l BAP and 2.5 mg/l NAA (Figure 3). Table 1 also shows that the highest number of shoots per explant was found when yellow explants were cultured on MS media supplemented with combinations of 0.5 mg/l BAP and 1.5 mg/l NAA, resulting in 16.00±0.30 shoots per explant. In contrast, no root formation was seen in all treatments from yellow explants. The root formation occurred 2-4 weeks after transferrance to solid MS media. No abnormal shoot formation was seen in the tall treatments of yellow explants. Shou et al. (2008) also reported that shoots derived from lotus buds were cultured on MS medium containing 0.5-2.0 µM NAA, 0.2% activated charcoal, with or without 0.1 µM BA for 1 week, but had to be transferred onto MS basal medium for 4 weeks for root induction (9.2±0.7 number of root/explant).

Comparing the regeneration ability of green plumules with yellow plumules explants, it was found that green plumules explants could regenerate faster (7-8 weeks) than yellow plumules explants (10-12 weeks). The *in vitro* growth of green plumules explants was true-to-type, with dark green horizontal shoots (4 shoots) and root formation (primary and secondary) before the start of the next shoot extension. At the same time, the regeneration of yellow plumules resulted in a new characteristic, with smaller and lighter green vertically-layered shoot and root formation (only primary). Overall, the combination range of 0.5-1.0 mg/l BAP with 0.5-2.5 mg/l NAA was optimum for immature explants and gave the highest (2-16) number of shoots per explant. In contrast, the combination of 1.5-2.0 mg/l BAP with 0.5-2.5 mg/l NAA was optimum for mature explants, and gave the highest (1-10) number of shoots per explant. Among the treatments, the same concentration ratios of BAP and NAA

(0.5 mg/l BAP and 0.5 mg/l NAA, 1.0 mg/l BAP and 1.0 mg/l NAA, 1.5 mg/l BAP and 1.5 mg/l NAA, 2.0 mg/l BAP and 2.0 mg/l NAA, 2.5 mg/l BAP and 2.5 mg/l NAA) were recognised to response twice as well for yellow plumules explants (immature) compared to green plumules explants (mature). Subcultures were needed every 21-28 days to maintain the freshness of the media and the accumulation of shoots and roots. Shou et al. (Shou et al 2008) reported that the maximum number of shoots was induced from lotus bud explants on MS medium containing 8 g/l agar, 30 g/l sucrose, and 4.44 µM benzyladenine (BA) added with 0.54 µM α-naphthalene acetic acid (NAA) for 4 weeks, with low rates of lotus multiplication (3.50±0.05 number of shoots/bud). The different results within a shorter period of response (2-4 weeks), which were influenced by the types of explants and ratios between NAA and BAP and cytokinin and auxin, were considered critical factors for *in vitro* shoot multiplication (Dantu and Bhojwani 1987, Rao and Purohit 2006).

In the present study, the successful direct regeneration of mature explants (green plumule) cultured on solid MS media supplemented with combinations of 1.5 mg/l BAP and 0.5 mg/l NAA gave 10.33±0.23 shoots per explant (true-to-type), whilst immature explants (yellow plumule) cultured on solid MS media supplemented with combinations of 0.5 mg/l BAP and 1.5 mg/l NAA gave 16.00±0.30 shoots per explant. Arunyanart and Chaitrayagun [12] reported that lotus bud explants cultured on MS medium containing 4 µM 2,4-dichlorophenoxy acetic acid (2,4-D) and 1 µM benzyladenine (BA) gave the best callus growth. Shou et al. [7] reported a low rate of shoot multiplication from lotus bud explants cultured on MS medium containing 8 g/l agar, 30 g/l sucrose, 4.44 µM benzyladenine (BA) and 0.54 µM α-naphthalene acetic acid (NAA). In addition, Rahman et al (Rahman et al 2004) reported that cotyledonary explants with and without petiolar gave the best shoot initiation cultured in half strength MS medium supplemented with 1.0 mg/l BA + 0.5 mg/l kinetin + 0.1 mg/l NAA.

The present study also showed that immature explants (yellow plumules) responded better compared to mature explants (green plumules). These results are in agreement with the most frequently reported finding that juvenile explants are most responsive (Merkle et al. 1987,Jain et al. 1995, George 1996). However, the results contrast with Ke et al. (Ke et al. 1987a) and Liu et al. (Liu et al. 2002), who reported that callus formation was only induced from immature embryos, green plumules and young cotyledons. The shoot formation was

influenced by many factors, such as explant types and growth regulators (Jain et al. 1995, Nhut et al. 2001, Guo et al. 2005). Shoot regeneration from cotyledon explants has been achieved in a wide range of plant species using cytokinin in combination with auxin (Bornman 1983).

A few factors that need to be considered in the selection of explants in tissue culture are the source of the explants (intact or aseptic plant), the explant size, ontogeny, the age of explants and the maturation of the plant stock (Murashige 1974a). Explants consisting of young cells and tissues normally show better results compared to older ones. This is because young meristematic cells divide actively. Each desired cultural effect has its own unique requirements, such as cytokinin (high-cytokinin-low-auxin ratio) for the initiation and development of adventitious shoots and auxin for the induction of adventitious roots. In order to enhance the regeneration rate, the MS media was supplemented with NAA and BAP, which induced both shoot and root formation. Historically, auxins (IAA, IBA, NAA, 2,4-D) are well characterized, and have functions in the establishment and maintenance of polarity in organised tissues, as well as in whole plants. Their most marked effect is the maintenance of apical dominance and the mediation of tropisms. In plant tissue culture, cytokinins (BAP, Kinetin, zeatin, 2iP) are commonly used in adventitious shoot bud formation, multiple shoot proliferation, somatic embryogenesis and the inhibition of root formation. Cytokinins are generally considered a critical factor for *in vitro* shoot production, and there are many reports that BAP exhibits a beneficial effect over other cytokinins with regards to shoot multiplication (Dantu and Bhojwani 1987, Rao and Purohit 2006).

Based on the results obtained, some formation of abnormal (red and oval) shoots occurred in MS media supplemented with combinations of 1.0 mg/l BAP and 2.5 mg/l NAA, 2.5 mg/l BAP and 2.5 mg/l NAA, 1.5 mg/l BAP and 2.0 mg/l NAA, and 1.5 mg/l BAP and 2.5 mg/l NAA (Figure 3). Media that was rich in nutrients such as Murashige and Skoog (Murashige and Skoog 1962) were shown to promote vitrification in some plant species (Pâques and Boxus 1987, Pierik 1987), and root formation occurred with the addition of auxin at lower concentrations, while a higher concentration of cytokinin was found to induce the formation of shoots. The ratio between auxin and cytokinin can determine the organ formation (Miller and Skoog 1953, Paulet 1965, Ghautheret 1959). The most common uses of auxins in plant

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tissue culture include the induction of callus growth, organ cultures, somatic embryogenesis and the induction of rhizogenesis. In intact plants, these substances particularly stimulate protein synthesis and participate in cell cycle control. When added to shoot culture media, these compounds overcome apical dominance and release lateral buds from dormancy.

Auxins, together with cytokinins, are involved in the control of morphogenesis in plant tissue culture. Different concentrations and combinations of auxins and cytokinins have different effects on the growth of explants. A balance between the auxin and cytokinin growth regulators is most often required for the formation of adventitious shoot and root meristems. The required concentration of each type of hormone differs greatly according to the kind of plant being cultured, the cultural conditions and the types of hormones used, as interactions between the two classes of regulator are often complex, and more than one combination of substances is likely to produce optimal results. A low concentration of auxin is often beneficial in conjunction with a high level of cytokinin when shoot multiplication is required, while a low concentration of cytokinin (typically 0.5-2.5 µM) is often added to media containing a relatively high concentration of auxin for the induction of an embryogenic callus, especially in broad-leafed plants (Geoge 1993).

The present study showed that the best explants (yellow plumule) cultured on the optimum solid MS media supplemented with 0.5 mg/l BAP and 1.5 mg/l NAA for 20 weeks developed a new characteristic (layered multiple shoots). Within the same period (1-24 weeks) of culture on solid MS media, regeneration from green seed explants was achieved, exactly as for the mother plant, with shoots and roots (complete plantlet). In contrast, regeneration from yellow seeds was different, with smaller elongated shoots. No abnormal shoot formation was seen in the treatment of yellow seed explants. A small percentage of *in vitro* plants showed morphological and cytological changes that can be termed somaclonal variation (Evans and Sharp 1986) . This proved that *in vitro* plantlets could not guarantee the new plant produced, even though the *in vitro* multiplication was true-to-type. The nature of abnormal morphogenesis *in vitro* emphasises the need for the optimisation of plant culture conditions (Gasper et al. 1987). For instance, a tetraploid lotus (4n=32) was produced via *in vitro* culture with colchicine treatment (Yamamoto and Matsumoto 1990).

Table 2 and Figure 4 show the effects of solid and liquid levels in the regeneration of explants from green and yellow seeds. As a control, explants were cultured on solid MS basal media. Green shoots were elongated with a mean number of 4.31±0.80 shoots per explant and the formation of primary roots (Figure 3(a-b)). In contrast, yellow shoots turned green, with a mean number of 9.10±0.51 shoots per explant and the formation of primer roots. The highest number of shoots per explants was found in the liquid to solid ratio of 2:1 with a mean of 16.67±0.23 shoots per explant and the formation of primary and secondary roots for explants from yellow seeds (Figure 3(c)), while a mean of 9.00±0.15 shoots per explant was seen for green seeds with the formation of layered multiple shoots. In media containing a liquid to solid ratio of 1:1, both shoots elongated normally with a mean of 8.33±0.23 and 15.67±0.09 shoots per explant for green and yellow shoots, respectively (Figure 3(d)). Even though the lotus is an aquatic plant, in a liquid to solid ratio of 3:1 (flooded), both shoots turned brown with a mean of 5.33±0.23 shoots per explant for green shoots and 10.33±0.23 shoots per explant for yellow shoots. The better contact between explants and the liquid medium increased the availability of cytokinin and the ability for nutrient uptake (Deberg 1983), increased the dilution of any exudates from explants in the liquid medium (Ziv and Halevy 1983) and made the aeration in the liquid medium more adequate, which enhanced both growth and multiplication (Ibrahim 1994).

In natural habitats, the growth of the lotus is affected by water levels and their fluctuation. The deepest water level recorded was about 2-3 m for the wild lotus (Unni 1971a, Unni 1971b, Unni 1976, Kunii and Maeda 1982, Wang and Zhang 2004) . Rhizomes of some species were killed by anaerobic incubation at 22°C for 7 days, while others survived and showed normal shoot extension upon return to aerobic conditions (Barclay and Crawford 1982). According to Nohara and Kimura (Nohara and Kimura 1997), the maximum depth for lotus growth is 2.4 m (water depth) in an artificial environment of concrete ponds, while no petiole elongation is seen from water depths of 3-5 m.

Table 3 shows the effects of the distance of the light source on the multiplication of lotus shoots. The results showed the highest height of shoots from green seed explants (9.41±1.11 mm) when the light source was at a distance of 250 mm. The lowest height of shoots was at a distance of 50mm with shoots of 1.21±1.01 mm seen from green seed explants. The highest height of shoots (16.67±0.23 mm) obtained from yellow seed explants was at a

distance of 20mm, and the lowest height of shoots (9.12±0.51 mm) was at 50 mm. All of the explants in the nearest light distance (0.0 mm) were dried out and dead. From this experiment, a light distance of 200-250 mm was found to be optimum for the growth of shoots from green seed explants and 100-150 mm from yellow seed explants.

The production of ornamental lotus is on a very small scale in the world, with the plants usually sold in the form of dormant rhizomes. In this research, juvenile shoot explants of *Nelumbo nucifera* Gaertn. were successfully established *in vitro.* Stock plant cultures were increased by repeatedly subdividing cultures at 21-day intervals. Plants cultured in solid MS media produced leaves with elongated petioles. In general, the *in vitro* regeneration of lotus was difficult to maintain on the same solid media for more than 28 days, as the plantlets became yellowish and died. A significant difference in the capability of shoot initiation was found among mature and immature explants, and may be due to the different degree of their sensitivity towards growth regulators, double-layered media and light distances. The results of the present study showed that plantlets survived without subculture for 6-12 months on a double-layer media (liquid basal MS media combined with solid MS added NAA and BAP) that was exposed to light distances of 20-25 mm.

SUPPORTING INFORMATION

Table 1. The effects of different combinations and concentrations of BAP and NAA on mature (green plumules) and immature (yellow plumules) explants, cultured on solid MS media at 25±1°C. This was performed with 16 hours light and 8 hours dark, with 1000 lux intensity of light for 12 weeks.

MS+Hormone		Explant	Observations	No. of shoots per explant (mean±SE)	No. of roots per explant (mean±SE)
BAP (mg/l)	NAA (mg/l)				
0.0	0.0	Yellow	No response	0	0
		Green	Shoots elongation, roots formation after 4 weeks.Adventitious roots after 6 weeks	1.43 ±0.10abc	4.33±0.53d
0.5	0.5	Yellow	Yellow shoots turned green after 1 week	6.00±0.31ef	0
		Green	Shoots elongated	3.00 ± 0.26e	0
0.5	1.0	Yellow	Yellow shoots turned green after 1 week	8.00±0.00h	0
		Green	Shoots elongated	3.67±0.32fg	0
0.5	1.5	Yellow	Layered green shoots after 7 weeks	16.00±0.30m	0
		Green	Shoots elongated	4.33±0.23gh	0
0.5	2.0	Yellow	Yellow shoots elongated	2.67±0.18bc	0
		Green	Shoots elongated	2.67±0.23de	0
0.5	2.5	Yellow	Yellow shoots elongated	2.67±0.67bc	0
		Green	Shoots elongated	2.10 ±0.32bcde	0

1.0	0.5	Yellow	Shoots yellow to green after 1 week	14.20±0.73l	0
		Green	Shoots elongated	6.00 ±0.15jk	0
1.0	1.0	Yellow	Yellow shoots turned green after 1 week	11.33±0.17k	0
		Green	Shoots elongated	4.00 ±0.40g	0
1.0	1.5	Yellow	Yellow shoots elongated	5.77±0.34e	0
		Green	Multiple shoots formation after 8 weeks	6.33± 0.23k	0
1.0	2.0	Yellow	Shoots yellow to green after 1 week	10.67±0.18jk	0
		Green	Multiple shoots formation after 8 weeks	6.33 ±0.17k	0
1.0	2.5	Yellow	Shoots yellow to green after 1 week	6.00±0.30m	0
		Green	Shoots elongated	2.67 ±0.23de*	0.57 ±0.35a
1.5	0.5	Yellow	Yellow shoots elongated	3.67±0.32d	0
		Green	Multiple shoots formation after 4 weeks	10.33 ±0.23m	3.67 ±0.32c
1.5	1.0	Yellow	Shoots yellow to green after 1 week	9.33±0.18i	0
		Green	Multiple shoots formation after 6 weeks	8.67 ±0.18l	0
1.5	1.5	Yellow	Shoots to green Multiple shoots	10.33±0.88j	0
		Green	Shoots elongated	5.33 ±0.38ij	0.67 ±0.09b
1.5	2.0	Yellow	Yellow shoots elongated	2.00±0.00b	
		Green	Shoots elongated	2.33 ±0.32cde*	0
1.5	2.5	Yellow	No response	0	0
		Green	Shoots elongated	2.33 ±0.32cde*	0

2.0	0.5	Yellow	Yellow shoots turned green after 1 week	7.33±0.18gh	0
		Green	Multiple shoots formation after 4 weeks	8.00 ±0.40l	0
2.0	1.0	Yellow	Shoots yellow to green after 1 week	8.00±0.00h	0
		Green	Multiple shoots formation after 4 weeks	6.33 ±0.31k	0
2.0	1.5	Yellow	Yellow shoots elongated	6.00±0.00ef	0
		Green	Multiple shoots after 8 weeks	5.33 ±0.09ij	0
2.0	2.0	Yellow	Yellow shoots elongated	5.33±0.35e	0
		Green	Shoots elongated	1.07 ±0.15a	0
2.0	2.5	Yellow	Yellow shoots elongated	3.33±0.35cd	0
		Green	Multiple shoots formation after 8 weeks	5.67 ±0.17ijk	0
2.5	0.5	Yellow	Yellow shoots elongated	6.07±0.41ef	0
		Green	Shoots elongated	3.67 ±0.18fg	0
2.5	1.0	Yellow	Yellow shoots elongated	3.33±0.18cd	0
		Green	Shoots elongated	4.00 ±0.40g	0
2.5	1.5	Yellow	Yellow shoots elongated	2.00±0.00b	0
		Green	Shoots elongated	5.00 ±0.46hi	0
2.5	2.0	Yellow	Shoots elongated	2.00±0.00b	0
		Green	Multiple shoots	6.33 ±0.35k	0
2.5	2.5	Yellow	Shoots elongated	2.00±0.00b	0
		Green	Shoots elongated	2.33 ±0.35cde*	0

3.0	0.5	Yellow	Shoots elongated	6.67±0.18fg	0
		Green	Multiple shoots	4.33 ±0.31gh	0
3.0	1.0	Yellow	Shoots elongated	2.67±0.18bc	0
		Green	Multiple shoots	4.33 ±0.35gh	0
3.0	1.5	Yellow	Shoots elongated	2.00±0.00b	0
		Green	Shoots elongated	2.00 ±0.26bcd	0
3.0	2.0	Yellow	Shoots elongated	2.00±0.00b	0
		Green	Multiple shoots	5.00 ±0.26hi	0
3.0	2.5	Yellow	No response	0	0
		Green	Shoots elongated	1.33 ±0.23ab	0

*Formation of abnormal shoots. Mean with different letters in the same column differ significantly at $p < 0.05$, as determined by Duncan's Multiple Range Test (DMRT). Mean ± Standard error (SE), replicates (n=30).

Table 2. The effect of double-layered media on the regeneration of shoots. Cultures were maintained at 25±1°C with 16 hours light and 8 hours dark, with 1000 lux intensity of light for 4 weeks.

Ratio liquid:solid (mm)	Explant	No. of shoots per explants (Mean ±SE)	Observations
0:1 (control)	Green shoot*	4.31±0.80	Dark green shoots elongated. Primary root formation.
	Yellow shoot#	9.10±0.51	Yellow shoots turned green and elongated. Primary root formation.
1:1	Green shoot*	8.33±0.23	Shoots elongated
	Yellow shoot#	15.67±0.09	Shoots elongated
2:1	Green shoot*	9.00±0.15	Shoots crept and formed rolled leaves. Primary and secondary root formation
	Yellow shoot#	16.67±0.23	Layered shoots. Primary root formation
3:1	Green shoot*	5.33±0.23	Shoots turned brown
	Yellow shoot#	10.33±0.23	Shoots turned brown

*Double-layered medium (green shoot): combination of Solid MS + 1.5 mg/l BAP + 0.5 mg/l NAA and liquid MS basal.

#Double-layered medium (yellow shoot): combination of Solid MS + 0.5 mg/l BAP + 1.5 mg/l NAA and liquid MS basal.

Each value represents the mean of thirty replicates. Mean ± Standard error (SE), replicates (n=30).

Table 3. The effect of light distance on the regeneration of shoots. Cultures were maintained at 25±1°C with 16 hours light and 8 hours dark, with 1000 lux intensity of light for 4 weeks.

Light Distance (mm)	Height of shoot,mm (Mean ±SE)		Observations
	Green Seeds	Yellow Seeds	
0.0 (control)	0	0	Green and yellow shoots became dried and turned brown
50	1.21±1.01	9.12±0.51	Wrinkle green shoots. Yellow shoots turned green
100	7.67±0.09	10.67±0.09	Green shoots elongation. Yellow shoots turned green
150	7.13±0.72	13.10±1.01	Green shoots elongation. Yellow shoots turned to green
200	9.00±0.15	16.67±0.23	Green and yellow shoots elongated
250	9.41±1.11	9.13±0.50	Green and yellow shoots elongated
300	4.67±0.09	6.33±0.18	Green and yellow shoots turned brown

Each value represents the mean of thirty replicates. Mean ± Standard error (SE), replicates (n=30).
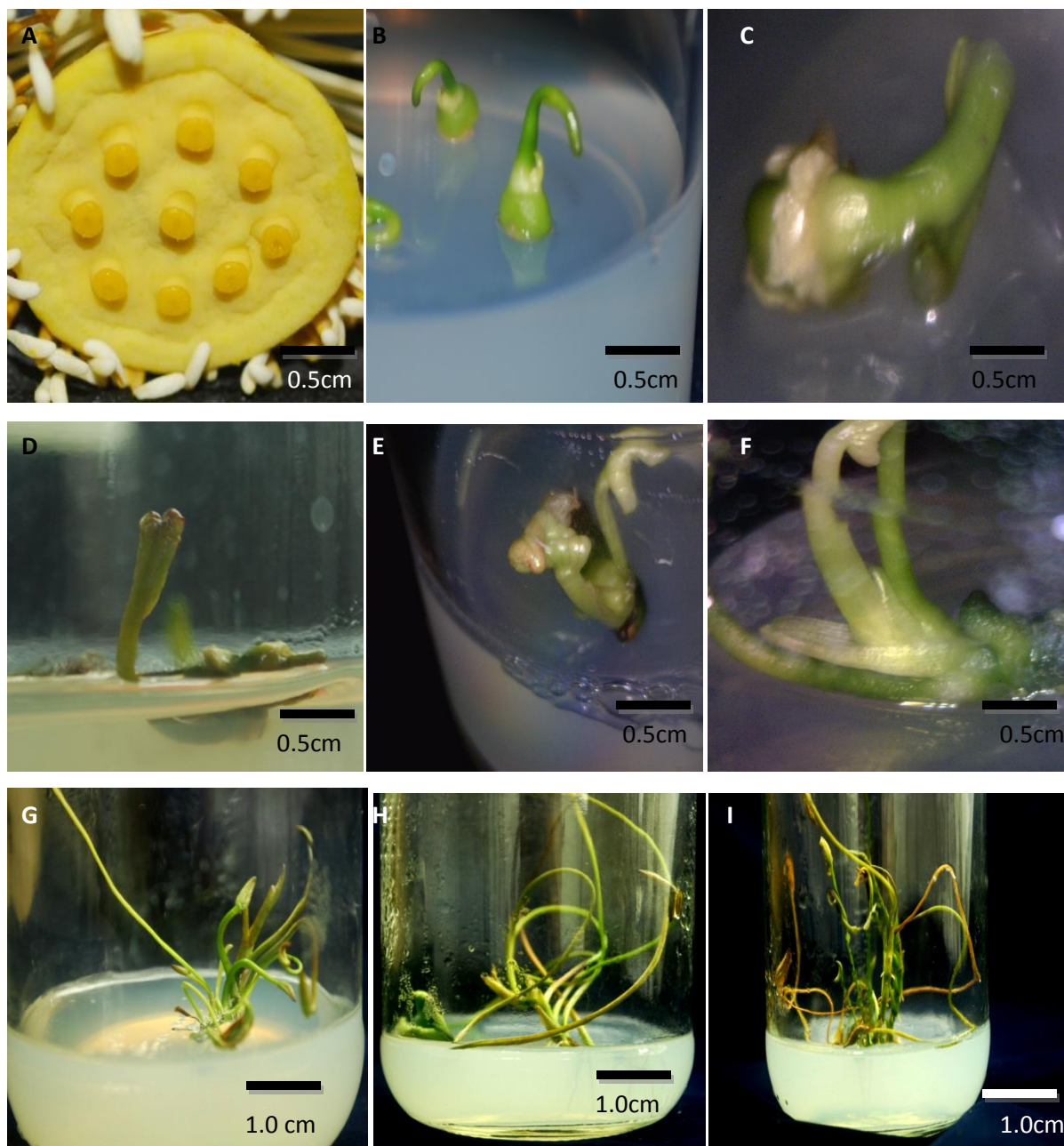
Fig. 1. Effects of BAP and NAA on shoot formation from the immature explants (yellow plumule) of *N. Nucifera* after 24 weeks in culture on MS medium. Yellow plumule cultured on MS solid media supplemented with 0.5 mg/l BAP and 1.5 mg/l NAA. The cultures were maintained at 25±1˚C with 16 hours light and 8 hours dark, with 1000 lux intensity of light for 24 weeks.

A Yellow pod with nine immature seeds.

B Two hinged shoots (explants) on.

C First subculter after 2 weeks cultured on solid MS basal (control).

D Elongation of first shoot.

E Two rolled shoots after 3 weeks.

F Formation of layered shoots after 4 weeks.

G Elongation of shoots after 6 weeks.

H Formation of first unrolled leaf after 8 weeks.

I Layered multiple shoots (vertical position) with formation of primary roots after 12 weeks.
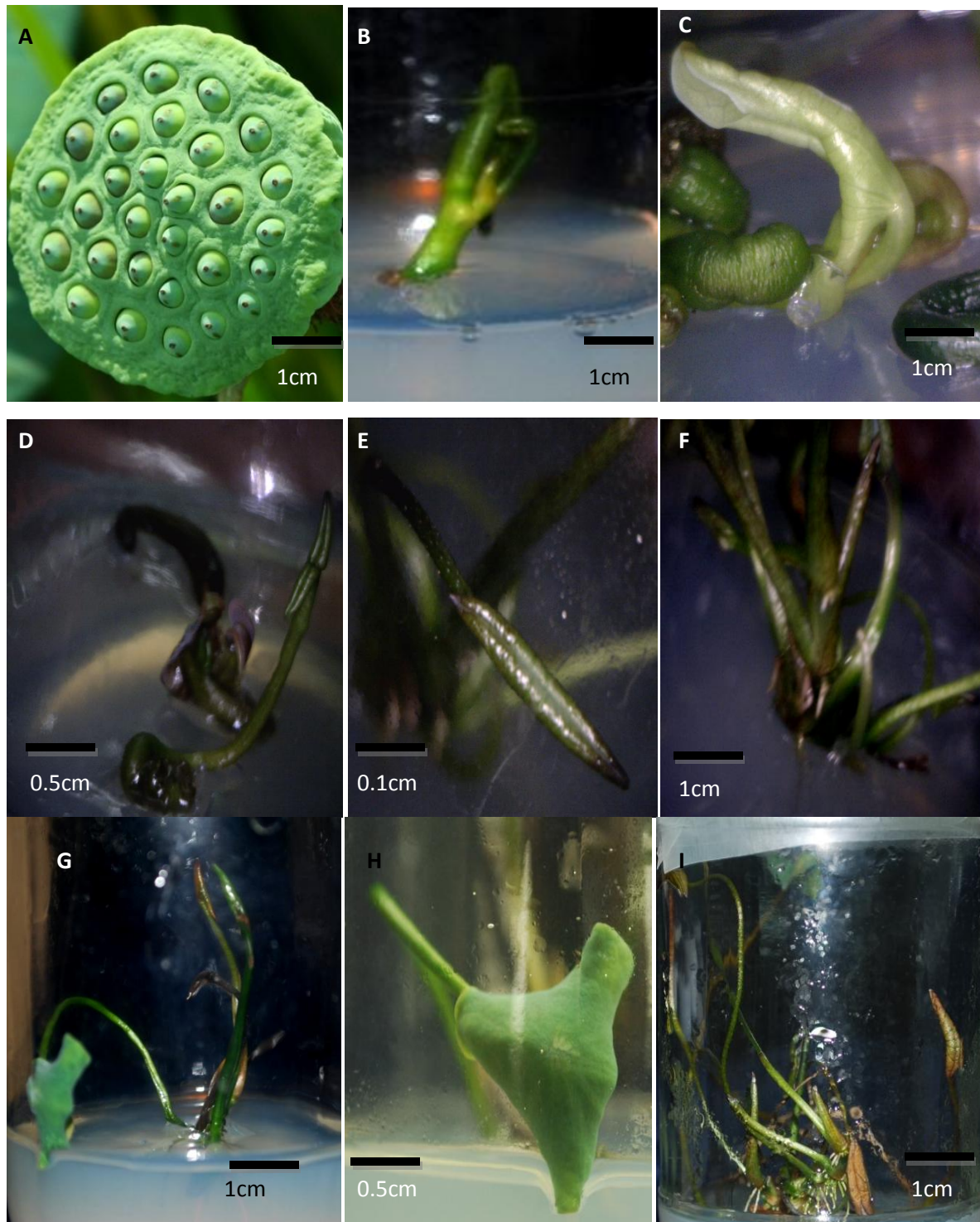
Fig. 2. Effects of BAP and NAA on shoot formation from the mature explants (green plumule) of
N. Nucifera after 24 weeks in culture on MS medium. Green plumule cultured on MS solid

media supplemented with 1.5 mg/l BAP and 0.5 mg/l NAA. The cultures were maintained at 25±1°C with 16 hours light and 8 hours dark, with 1000 lux intensity of light for 24 weeks.

A Green pod with twenty nine mature seeds.

B Three hinged shoots (explants) cultured on solid MS basal (control).

C After 2 weeks cultured on solid MS.

D Elongation of shoot.

E Rolled shoots after 3 weeks.

F Shoots after 4 weeks.

G First subculter and formation of rolled shoots after 6 weeks.

H Formation of first unrolled leaf after 8 weeks.

I Multiple shoots (cluster) with formation of primary and secondary roots after 12 weeks.
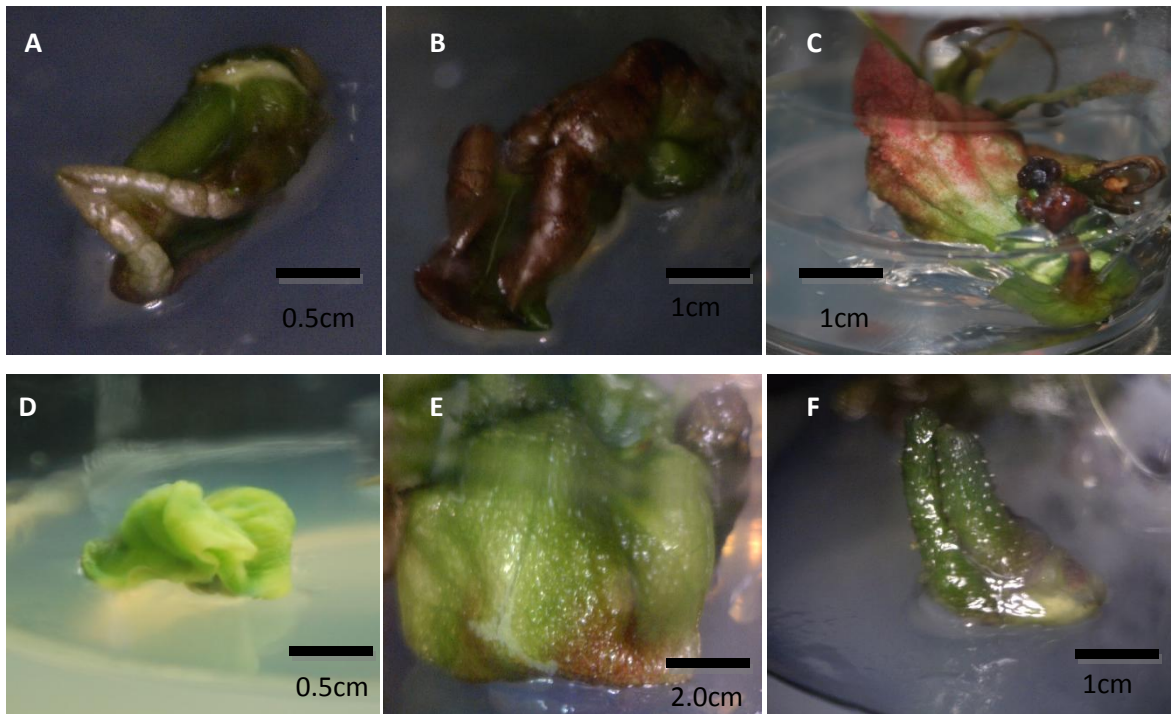

Fig. 3. Abnormal structures occurred when green plumule of *N. Nucifera* cultured on solid MS media supplemented with combination of 1.0-2.5 mg/l BAP and 2.5 mg/l NAA within 4 weeks.

A Pink Shoots

B Red rolled leaf

C Pink and green oval leaf

D Green spongy tissue

E Green and round leaf

F Stem with tiny thorns (prickles)

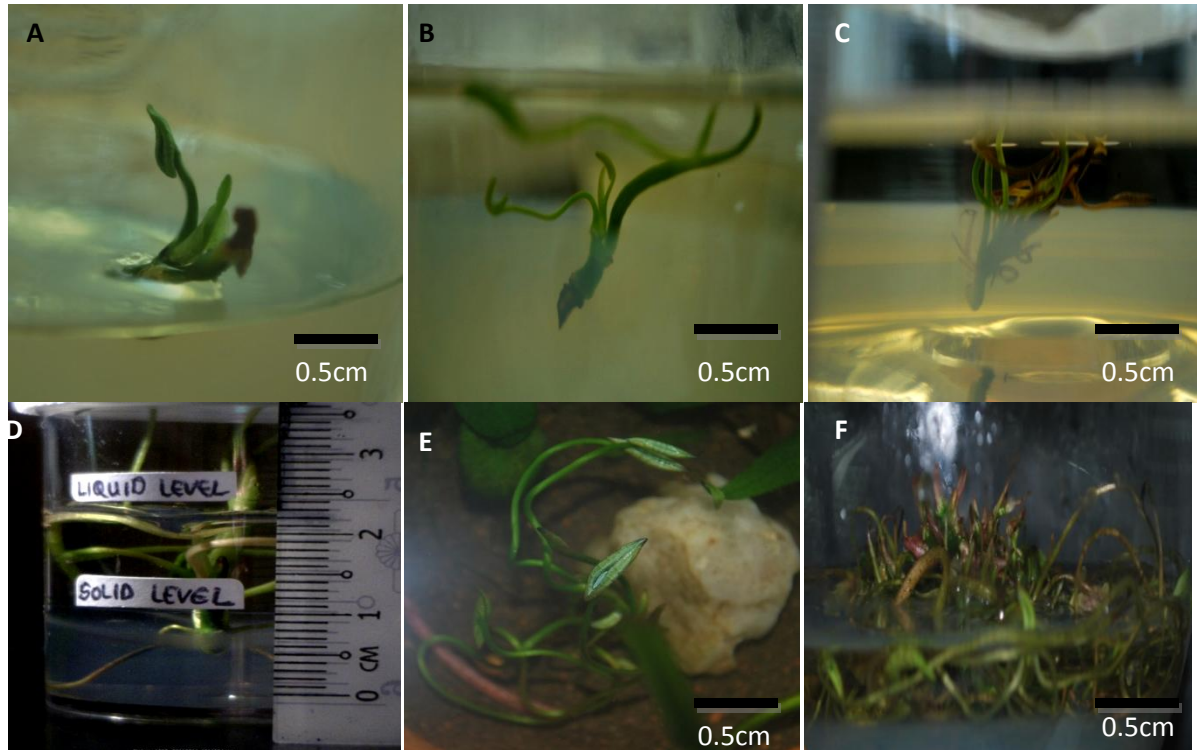Fig. 4. Effects of yellow plumule of *N. Nucifera* on double layered media (on solid MS media and liquid MS media supplemented with 0.5 mg/l BAP and 1.5 mg/l NAA).

A Formation of the first and second shoots.

B Elongation of the first three shoots.

C Layered multiple shoots after 6 weeks.

D Formation of roots after 4 weeks subculture.

E Elongation of leaves after 7 weeks

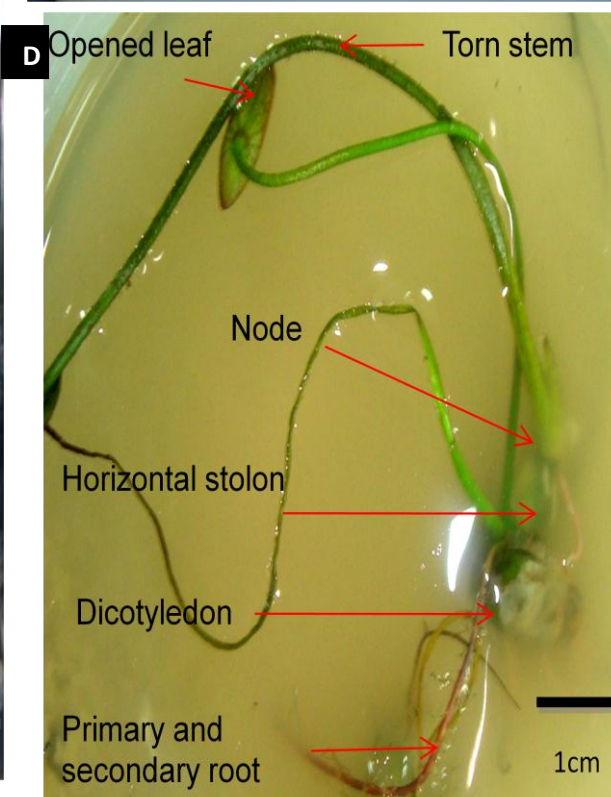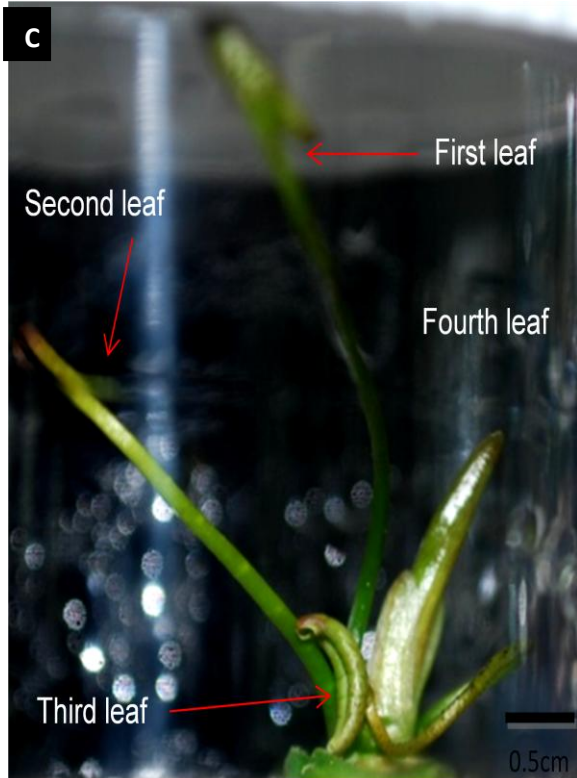F Layered multiple shoots after 10 weeks
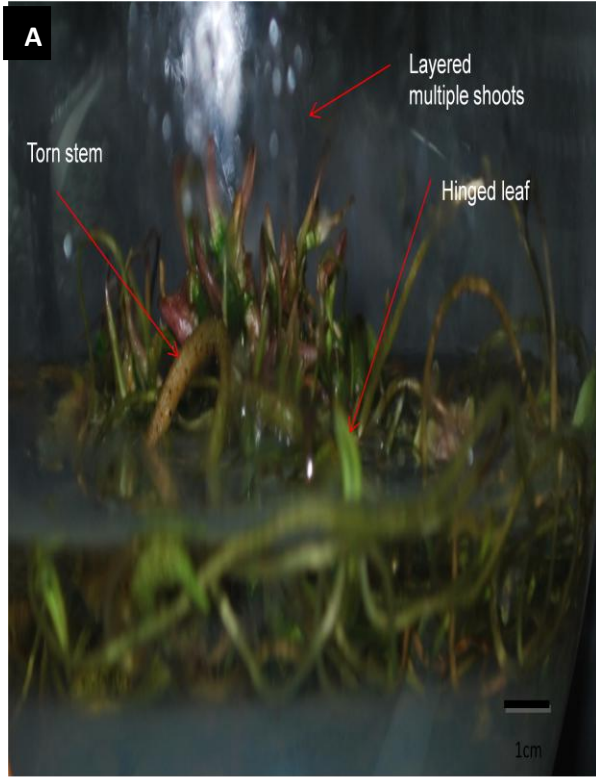
Fig. 5. Comparison of plantlets morphology of *N. nucifera* from various conditions of growth development.

A. *In vitro* multiple shoots from immature explant on double layered MS media

B. Layered multiple shoots transfer to solid MS media for root formation.

C. Multiple shoots from mature on MS media for root formation.

D. *In vivo* of lotus after 4 months.

Acknowledgement

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COVER LETTER

The Effect of Zeatin and Gibberelic Acid on *In Vitro* Flowering of *Phlox paniculata* L.

Rosna Mat Taha, Noraini Mahmad[*], Hashimah Elias, Sharifah Nurashikin Wafa Syed Mohd Thalal Wafa, Fatimah Abdul Rahiman and Sakinah Abdullah

Institute of Biological Sciences, Faculty Science,University of Malaya, 50603 Kuala Lumpur, Malaysia.

[*]Corresponding author, email fara_aid@siswa.um.edu.my

Tel.: (+6)-03-7967-4372

Fax: (+6)-03-7967-4173/78

Suggested Referees:

1. Assoc. Prof. Dr. Shaida Fariza Sulaiman,
   School of Biology, University of Science (USM),
   11800 Penang, Malaysia.
   Email: shaida@usm.edu.my

2. Dr. Norizah Jaafar Sidek,
   Biology Programme, Faculty of Applied Science,
   University Technology MARA, Shah Alam,
   Selangor, Malaysia.
   Email: norri536@salam.uitm.edu.my

3. Dr. .Nor Azlina Hasbullah,
   Department of Agriculture Science,
   Faculty of Technical and Vocational Education,
   Tanjung Malim, Perak.
   Malaysia
   Email: azahhas@yahoo.com

The Effect of Zeatin and Gibberelic Acid on *In Vitro* Flowering of *Phlox paniculata* L.

Rosna Mat Taha, Noraini Mahmad[1*], Hashimah Elias, Sharifah Nurashikin Wafa Syed Mohd Thalal Wafa, Fatimah Abdul Rahiman and Sakinah Abdullah

[1]Institute of Biological Sciences, Faculty Science,University of Malaya, 50603 Kuala Lumpur, Malaysia.

[*]Corresponding author, email fara_aid@siswa.um.edu.my

Tel.: (+6)-03-7967-4372

Fax: (+6)-03-7967-4173/78

Abstract

The present work reports on *in vitro* flowering of *Phlox paniculata* L. Various explants such as leaf, petiole and stem, excised from intact matured plants, aged 4 months old were cultured on MS solid medium supplemented with different concentrations and combinations of 1-napthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP). The range of concentration was 2.6 -10.6 µM for NAA and 2.2 – 8.8 µM for BAP, respectively. Adventitious shoot formation from different explants was obtained depending on the culture conditions. Among the explants tested, leaf segments were found to be the most responsive, and had the highest regeneration frequency (100%) when cultured in the presence of 8.0 µM NAA and 6.6 µM BAP. However, the regenerated plantlets did not form any flower bud in their successive subcultures. *In vitro* flowering was only induced after 4 weeks of culture from shoot tip segments which were maintained on MS solid medium supplemented with zeatin (2.2-11.2 µM) or gibberellic acid (1.4-7.2 µM)). In the present study, treatment with 11.2 µM zeatin showed the highest frequency

of *in vitro* flowering (67.8 ±3.2%) whereas, 5.6 µM GA₃ gave a frequency of 48.8±9.3%. Although the flowers generated *in vitro* were sterile, our study would provide an important step towards future investigation on the essential factors for *in vitro* flowering in *P. paniculata* and to elucidate other developmental and environmental stimuli, which are required for promoting or inhibiting the transition of vegetative state to flowering state in this species.

Key words: *Phlox paniculata L.*, *in vitro* flowering, zeatin, GA₃, micropropagation

Resumo

trabalho atual relata in vitro em flowering do paniculata L. do Phlox. Os vários explants tais como a folha, o petiole e a haste, excised das plantas amadurecidas intatas, envelhecidas 4 meses velho foram cultivados no meio contínuo do ms suplementadas com as concentrações e as combinações diferentes de 1 ácido napthaleneacetic (NAA) e de benzylaminopurine 6 (BAP). A escala da concentração era o µM 2.6 -10.6 para NAAand 2.2 - o µM 8.8 para BAP, respectivamente. A formação Adventitious do tiro dos explants diferentes foi obtida dependendo das condições da cultura. Entre os explants testados, os segmentos da folha foram encontrados para ser os mais responsivos, e tiveram a freqüência a mais elevada da regeneração (100%) quando cultivados na presença 8.0 do µM NAA e 6.6 o µM BAP. Entretanto, os plantlets regenerados não deram forma a nenhum bud da flor em seus subcultures sucessivos. Flowering foi induzido in vitro somente após 4 semanas da cultura dos segmentos da ponta do tiro que foram mantidos no meio contínuo do ms suplementado com o zeatin (µM 2.2-11.2) ou o ácido gibberellic (µM 1.4-7.2)). No estudo atual, o tratamento com o zeatin 11.2 do µM mostrou a freqüência a mais elevada in vitro de flowering (67.8 ±3.2%) visto que, 5.6 o µM GA3 deu uma freqüência de 48.8±9.3%. Embora as flores geradas in vitro fossem sterile, nosso estudo forneceria uma etapa importante para a investigação futura nos fatores essenciais in vitro flowering no P. paniculata e para elucidate outros stimuli developmental e ambientais, que são requeridos promovendo ou inibindo a transição do estado vegetative ao estado flowering nesta espécie.

Palavras-chave : Paniculata L. do Phlox, in vitro flowering, zeatin, GA₃, micropropagation

INTRODUCTION

Phlox paniculata L. is an important flowering plant that belongs to Polemoniaceae, a family with 67 species, of which about 15 species are extensively grown in the gardens (Wherry, 1955). *P. paniculata* L. is a perennial ornamental plant, bears dense terminal clusters of flowers in pink, crimson and mauve and are commonly grown for borders, and also suited for window boxes and tubs (Bailey 1950; Hay and Synge 1969; Massingham, 1971). The species is seed sterile (Bailey 1950; Wherry 1955) therefore, the traditional method of propagation is by root cuttings although roots are often damaged by red ants and sometimes by soil born fungi. Therefore, *in vitro* culture is an effective technique to obtain a large scale clonal propagation of *P. paniculata*. Schnabelrauch and Sink (1979) studied on the clonal propagation of *P. paniculata* through axiliary bud culture. Later, shoot regeneration was induced from adult leaf segments cultured by Declerck and Korban (1995). Those studies revealed potential for inducing multiple shoots in *in vitro* culture of this species. Moreover, multiple shoot regeneration technique is more advantageous for obtaining rapid clonal plants as well as for conservation.

Flowering is a unique developmental event in plants which involves the transition of vegetative shoot apex to form either an inflorescence or a floral meristem, followed by initiation and subsequent maturation of the floral organ (Sim et. al., 2007). The flowering process is one of the critically important stages in plant and is vital for the completion of the life cycle and seed production (Ziv and Naor, 2006). Under natural growth, flower formation usually begins when a plant reaches maturity (Virupakshi et. al., 2002). In plant, the transition of vegetative state to floral stage is considered to be a complex process regulated by a combination of various environmental and genetic factors; some of the important factors are plant growth regulators, carbohydrates, light and pH of the culture medium (Heylen and Vendrig, 1988). However, such transition mechanism from vegetative state to reproductive development is not well understood in most of the plants using the traditional methods. *In vitro* flowering is an important tool to minimize the influence of environmental factors and therefore, this technique clarifies the key

influences on flowering by the precise control of plant growth regulators. Furthermore, *in vitro* flowering also provides an ideal experimental system over *in vivo* grown plants in order to study the biological mechanism of flowering. In the present work, influences of plant growth regulators on clonal propagation from different explants and *in vitro* flowering from shoot apices of *P. paniculata* were examined. So far, the *in vitro* flowering in 'Garden Phlox' has not been reported. In the current work, we used various concentrations of 1-napthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) to induce direct regeneration and at the same time to induce *in vitro* flowering from shoot tips of this species using zeatin and gibberellic acid (GA$_3$).

MATERIALS AND METHODS

Plant materials and culture conditions for *in vitro* shoot regeneration

Three types of explants; leaf, petiole and stem, were collected from intact, garden raised mature plants at the time of their vegetative stages. All explants were initially washed with tap water for 30 min, surface sterilized with 50% commercial bleach (containing 5% sodium hypochlorite) for 1min, then rinsed with sterile distilled water (SDW) for at least three times and finally dipped in 70% (v/v) ethanol for 1 min, followed by three times rinsing with SDW. These sterile explants were cut into segments (approx. 5-10 mm in length for both petioles and stems and around 5x5 mm^2 pieces for leaves) and cultured on MS (Murashige & Skoog, 1962) solid medium (0.8% Agar Technical, No.3, Oxoid Ltd., England) supplemented without (control) or with combinations of 2.6, 5.3, 8.0 and 10.6 μM NAA (Sigma-Aldrich, USA) and 2.2, 4.4, 6.6 and 8.8 μM BAP (Sigma-Aldrich, USA). Prior to sterilization, the pH of the media was adjusted to 5.8 and then autoclaved at 121℃ and 103 kpa for 20 min. All the cultures were maintained at 25±1℃ with a 16 h photoperiod.

Explant preparation and culture conditions for *in vitro* flowering

Shoot apices were collected aseptically from the 4-month-old axenic cultures (vegetative stage) of *P. paniculata* plant. The explants were further aseptically excised into small pieces containing meristems with 3-4 true leaf primordia and cultured on to solid MS medium (prepared as described above) supplemented without (control) or with various concentrations (2.2, 4.5, 6.7, 9.0 and 11.2 μM) of zeatin (Sigma-Aldrich, USA) or gibberellic acid-3 (GA$_3$; Sigma-Aldrich, USA; 1.4, 2.8, 4.3, 5.6 and 7.2 μM). Prior to sterilization, the pH of the media was adjusted to 5.8 and then autoclaved (121ºC, 103 kpa) for 20 min. All the cultures were incubated at 25±1ºC with a 16 h photoperiod.

Morphological analysis

All cultures were continuously observed from one week of treatment, to evaluate their development by counting the total number of shoot buds/leaves initiated by the explant and recording the state of the apical meristem, either vegetative or floral. A meristem was classified as floral when the first sepal primordium of the flower that characterizes the reproductive structure was visible. It was referred to as 'vegetative' when there was no apparent reproductive morphogenesis. The presence or absence of a basal callus and of roots was also registered. Each experiment was repeated at least twice with 10 explants per treatment. Pooled results of the different experiments are presented.

Statistical analysis

All data and variables were statistically analyzed using SPSS statistical package version 11. Values are presented as mean ±SE. One-way ANOVA and Multiple Range Analysis were done on all data using 95% LSD intervals method.

RESULTS AND DISCUSSION

Shoot regeneration from different explants

Initially, three types of explants, leaf, petiole and stem, were collected from mature plant, and they were sterilized and cultured on to MS solid medium supplemented without (control) or with different levels of phytohormones as described in the materials and methods. In the beginning of the culture, direct shoot bud formation was observed in all types of explants when treated with phytohormones (NAA and BAP) but not in the control. However, none of the explants showed callus formation. Within 5 weeks of culture, vegetative buds further developed and regenerated into shoots (Fig. 1A). We observed that the highest frequency (100%) of shoot regeneration was obtained from leaf explants when cultured on medium supplemented with 8.0 µM NAA and 6.6 µM BAP (Fig. 1B). However, at this level of phytohormones, maximum shoot regeneration was also observed in petiole and stem explants showing the rate of 67.8 ±3.2 and 48.8±9.3%, respectively (Fig. 1B). Subsequently, root formation was observed in all clonal shoots within 11 weeks and they were all developed into individual plantlets in the following culture period. When acclimatized and transferred to the field condition, about 90% of clonal plantlets survived under natural environment without showing any morphological variation during their development.

Cytokinin and auxin addition for the *in vitro* shoot regeneration has been studied in many plant species. Various reports have shown that *in vitro* shoot regeneration could be successfully induced by using the combination of BA/BAP and NAA. In the medicinal plant, *Withania somnifera* Dunal, cultured on MS medium supplemented with 8.8 µM BA and 0.5 µM NAA showed multiple shoots regeneration (Saritha and Naidu 2007). Also, in an *in vitro* multiple shoot regeneration protocol of *Boerhaavia diffusa* L. by Roy (2008), showed that maximum frequency (90%) was possible to obtain only when cultured on MS medium containing BA (6.6 µM) and NAA (2.6 µM). Consistent with those previous findings, in the present work, we also showed that both NAA (8.0 µM) and BAP (6.6 µM) treatment generated the maximum frequency of multiple shoots regeneration *in vitro* in *P. paniculata*. Thus, the success in raising plants through direct regeneration and by passing the callogenesis phase has opened up the

possibility for large-scale clonal propagation of *P. paniculata*. When the other combinations of phytohormones, like kinetin and NAA, kinetin and 3-indole acetic acid (IAA), BA and 2,4-dichlorophenoxyacetic acid (2,4-D) or kinetin and 2,4-D were used at different concentrations, profuse shoot regeneration was not evident in all three types of cultured segments. However, in the current work, the successive subculturing of the regenerated plants did not show further morphogenetic differentiation such as floral transition.

In vitro **flowering**

To induce *in vitro* flowering, shoot tip explants were further collected from 4-month-old aseptic plants and cultured on MS solid medium supplemented with various concentrations of singly applied zeatin and GA_3 (Table 1). After 4 weeks of treatment period, development of multiple shoots and initiation of flower buds were observed from cultured explants only when treated with NAA and BAP but not in control (Table 1). Their successive subcultures generated bloomed flowers of white with purple or pink stripes (Fig. 2). In this experiment, 3-4 flowers per plant were developed within 7-8 weeks of culture period. In this study, treatment with zeatin at the concentration of 11.2 μM resulted in the highest frequency (67.8±3.2%), whereas, 5.6 μM GA_3 gave 48.8±9.3% *in vitro* flower induction (Table 1). However, treatment with either zeatin or GA_3 exhibited formation of white or pink flowers occasionally. All the *in vitro* developed flowers were approximately 1.7-2.0 cm in width and extended laterally from the stem (Fig. 2). Each bloom had 5 sepals, slightly extended and pointed shape at their middles with an extended, fused throat that opens into 5 distinct and overlapping lobes. However, the *in vitro* flowers failed to develop other reproductive parts, like stamens, stigmas or pistil even when they were subcultured for a long period of about 10 months.

In earlier studies, the cytokinin requirement for the growth and development of flower buds has been reported in both monocots (Zhong et al. 1992) and dicots (Rastogi and Sawhney 1987, Zhou et al. 2004). Promotion of *in vitro* flowering by cytokinins has been repeatedly reported (Scorza 1982, Dickens and van Staden 1988, van der Krieken et al. 1991, Roberts et al. 1993, Das et al. 1996, Joshi and Nadguada 1997, Kumar and Reddy 1997). The influence of cytokinin on the *in vitro* flowering of *Perilla frutescens* was found remarkable (Zhang 2007). Also, the

beneficial effect of cytokinins on the induction of flowering for other plants was reported in orchids (Kostenyuk et al. 1999), *Fortunella hindsii* (Jumin and Nito 1996) and *Lemna* (Fujioka et al. 1999). The results presented here are similar with those previous findings and revealed an optimum concentration of zeatin (11.2 µM) which could induce the maximum frequency (67.8%) of floral induction in this species (Table 1). However, high concentration of zeatin caused inhibition of *in vitro* flowering (data not shown).

In certain plants, auxin has been reported as either ineffective (Rastogi and Sawhney 1987) or inhibitory (Deaton et al. 1984) for *in vitro* flowering induction process. In this study, absence of *in vitro* flowering induction was observed when different concentrations of 2,4-D and IAA were used in the culture medium. In contrast, gibberellins have been reported as an inducer of flowering process in several long day (LD) and cold-requiring rosette plants (Bernier 1988). Earlier work has been postulated that a GA promotive pathway exists in *A. thaliana* (Koornneef et al. 1998) where GA_3 activates *LEAFY* (*LFY*) transcription (Blåzquez et al. 1998). Considering these findings, our results supported the idea that independently zeatin or GA_3 may form one of the key factors without which floral bud initiation and their subsequent development was not possible in *P. paniculata*. However, detail study on the actual mode of action of zeatin and GA_3 in the *in vitro* flowering process in *Phlox*, remains to be examined. In both cases, two to four flowers were produced for each *in vitro* cultured explant (Fig. 2) of which none of them showed fruiting in the subsequent culture, might be due to the absence of other reproductive organs. In this study, complete flowering was not possible even when they were cultured in the presence of kinetin and IAA . In *Murraya paniculata*, complete plant regeneration was achieved from portions of cotyledons and shoot explants when they were cultured in MS supplemented with 4.44 µM BA. *In vitro* flowering of the same species was observed on subsequent transfer to MS basal (80%) and also on MS fortified with 2.69-10.74 µM NAA (62-72%)(Taha, 1997). In *Begonia x hiemalis*, the best explant for *in vitro* flowering was inflorescence cultured on MS supplemented with 4.44 µM BA, 5.37 µM NAA, 4% sucrose and 40 mg/l adenine (Taha, 2010). It seems that different species require different hormonal regime for *in vitro* flowering.

Flowering is an important transition of the developmental processes for floricultural crops. In this study, an attempt was made to find out the most favorable sets of environmental and nutritional conditions for adventitious shoot regeneration and flower induction *in vitro*. An

interesting feature of the present study was that the potential of shoot apices embarked upon flowering *in vitro* in response to only zeatin or GA_3. The phenomenon assumes significance considering the fact that explants were obtained from axenic cultures and was possible to avoid the maturation period spanning several months before a plant bore flowers. Thus, the observations reported here for *in vitro* flowering of *P. paniculata* are novel and further experiments should lead to a better understanding of the physiological and molecular events underlying the shift from the vegetative state to the flower state, factors related to overcome flower sterility and seed formation *in vitro*. This protocol also can be extended to plant breeding studies for the purpose of quick flowering, fruit and seed formation under *in vitro* conditions and also to overcome problems associated with premature fruit drop or poor seed set.

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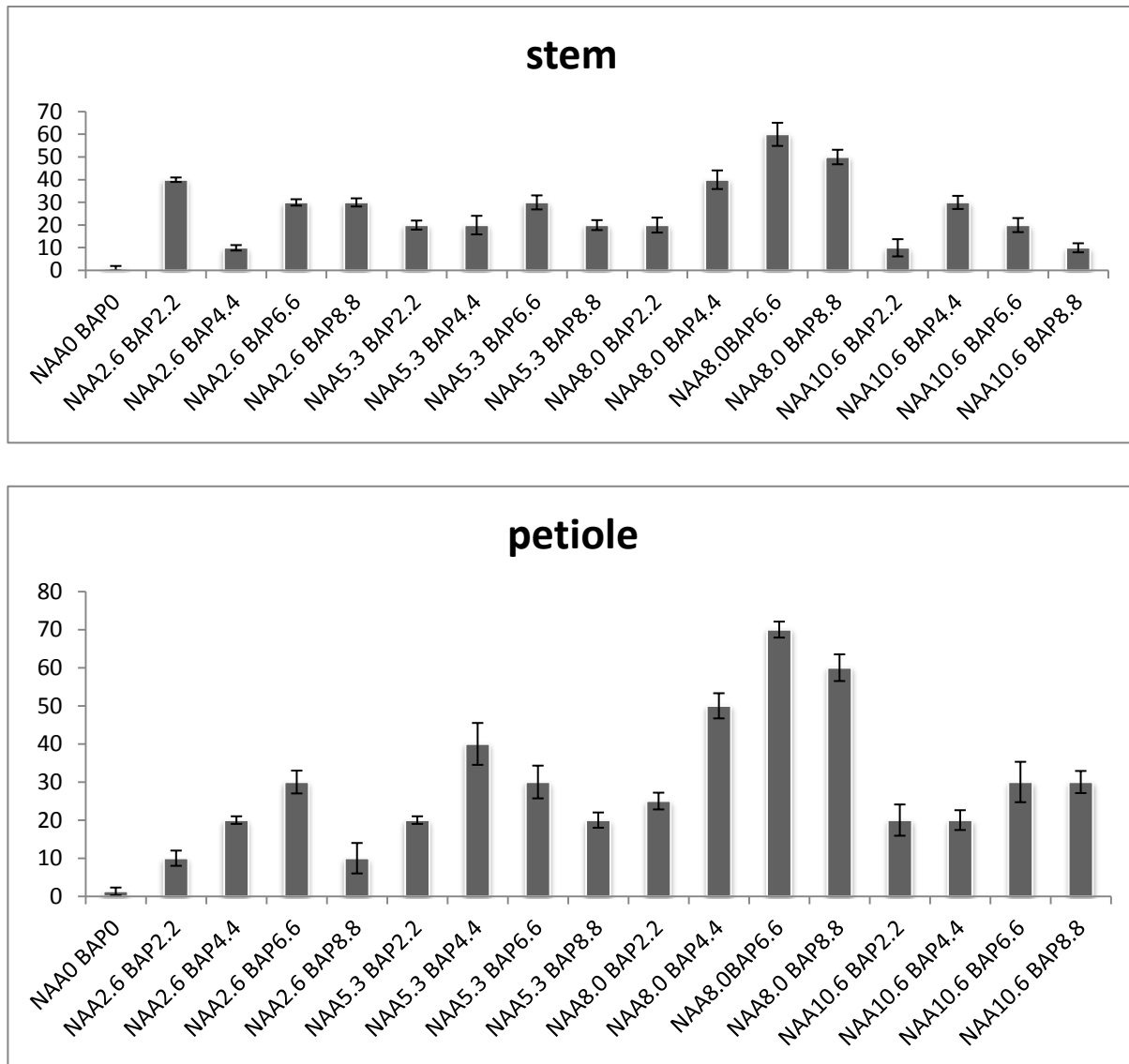Kumar T.A, & Reddy G.M. 1997. Identification and expression of AGAMOUS gene

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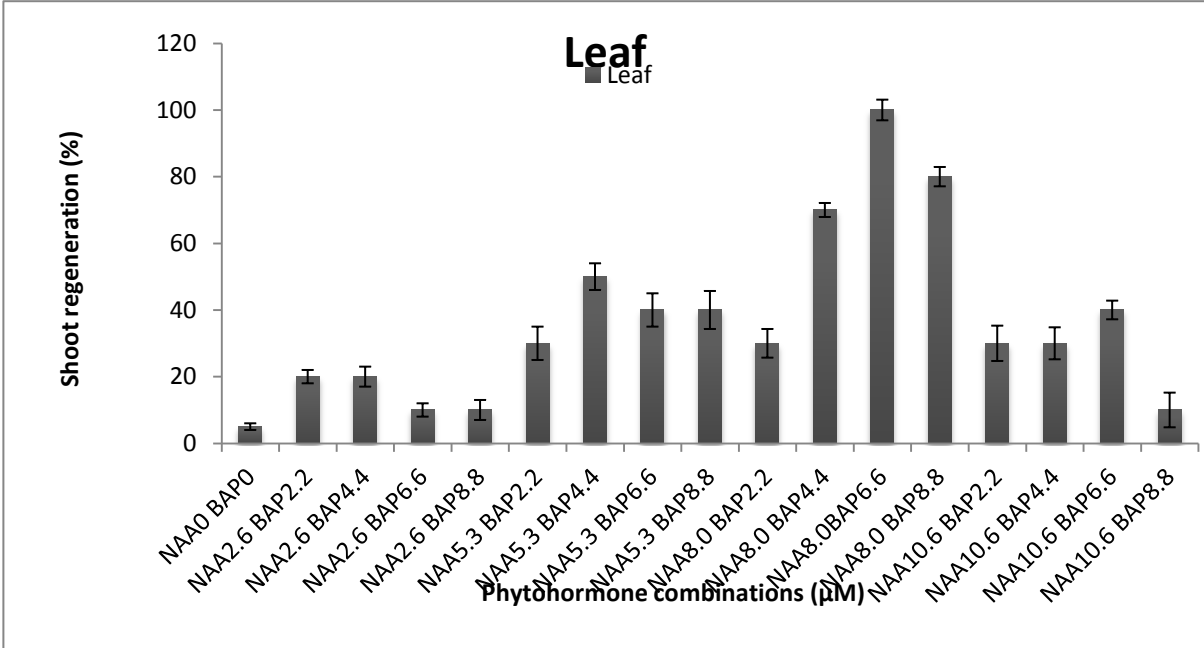
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Figure legends

Fig. 1 *In vitro* shoot regeneration of *Phlox paniculata* L. **A** Profuse vegetative shoot formation from adult leaf explants cultured onto MS solid medium supplemented with NAA and BAP (8.0 and 6.6 μM, respectively) in plastic sterile tube on 5th week. Bar indicates 1 cm. **B** Graph showing *in vitro* shoot regeneration pattern from different explants in response to various concentrations of NAA and BAP, after 8 weeks of culture.

Figure: Bar chart titled "Leaf" showing Shoot regeneration (%) on the y-axis (0–120) versus Phytohormone combinations (μM) on the x-axis. Categories: NAA0 BAP0, NAA2.6 BAP2.2, NAA2.6 BAP4.4, NAA2.6 BAP6.6, NAA2.6 BAP8.8, NAA5.3 BAP2.2, NAA5.3 BAP4.4, NAA5.3 BAP6.6, NAA5.3 BAP8.8, NAA8.0 BAP2.2, NAA8.0 BAP4.4, NAA8.0BAP6.6, NAA8.0 BAP8.8, NAA10.6 BAP2.2, NAA10.6 BAP4.4, NAA10.6 BAP6.6, NAA10.6 BAP8.8.

Fig. 2 Flower development after 6 weeks on shoot tip explant cultures of *Phlox paniculata in vitro*. The MS medium when supplemented with zeatin (11.2 µM) or GA$_3$ (5.6 µM), shoot multiplication with flowers (arrow indicated) of purple (A) or pink stripes (B) were observed. Bars indicate 1 cm.

Fig. 2