EFFICIENT DIRECT REGENERATION OF TRUE-TO-TYPE *POGOSTEMON CABLIN BENTH* FROM LEAF EXPLANT AND PROFILE OF ESSENTIAL OILS

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ABSTRACT

Pogostemon cablin Benth is one of the best patchouli species, which has huge potential to serve the cosmetic and pharmaceutical industries due to its fragrance and aromatic essential oils. The purpose of the present study is to establish an efficient and reproducible protocol for direct multiple shoot induction from patchouli leaves with clonal fidelity of the desired cultivar. Shoot induction from leaf culture of patchouli was tested on Murashige and Skoog (MS) basal medium supplemented with 2.26 µM 2,4-D (Dichlorophenoxyacetic acid) and Benzylaminopurine (BAP) at 0, 1.11, 2.22, 3.33, 4.44 µM. The highest frequency of shoot induction (88 %) and number of shoots per explant (102) was observed on solid MS medium supplemented with 3.33 µM of BAP. The addition of 2,4-D did not favour shoot induction but induced callus formation. For elongation of shoots, MS basal medium gave a better response compared to half-strength MS medium and the average length of shoot was 3.3 cm. Elongated shoots gave good rooting on MS basal liquid medium with an average of 12 roots per shoot, with an average length of 2.75 cm. The regenerated tissue cultured plantlets were acclimatized and successfully established in soil (90%). Genetic fidelity of the tissue cultured plants was assessed using the technique of Random Amplified Polymorphic DNA (RAPD). Nine arbitrary decamers displayed same banding profile in all the tissue cultured plants and in mother plant. The molecular analysis confirmed the true-to-type nature of the tissue cultured plantlets. The profile of essential oils determined by gas chromatography / mass spectrometry in tissue cultured plants and mother plants showed similar patterns. However, the levels of major essential oils were higher in all tissue cultured plants characterized. This study provides the pre-requisite to improve the traits of the desired cultivar of patchouli by genetic transformation in the future.

ABSTRAK

Pogostemon cablin Benth merupakan salah satu spesies di dalam keluarga pokok nilam yang terbaik di mana ianya mempunyai potensi yang besar bagi menyumbang kepada industri kosmetik dan farmasi kerana keharuman dan kandungan minyak pati aromatiknya. Tujuan penyelidikan ini adalah untuk membina satu protokol yang efisien dan dikembangkan untuk induksi tunas secara terus yang dilakukan ke atas daun nilam dengan klonal yang konsisten dari kultivar yang dikehendaki. Induksi tunas dari kultur daun nilam diuji ke atas medium asas Murashige-Skoog (MS) yang mengandungi 2.26 µM 2,4-D (Dichlorophenoxyacetic asid) dan Benzilamino purin (BAP) pada 0, 1.11, 2.22, 3.33, 4.44 µM. Frekuensi tertinggi induksi tunas (88%) dan jumlah tunas pada eksplan (102) diamati pada medium padat MS yang mengandungi 3.33 µM BAP. Penambahan 2.4-D tidak menyokong induksi tunas tetapi menyokong pembentukan kalus. Untuk pemanjangan tunas, medium asas MS memberikan respon yang lebih baik berbanding dengan medium setengah-MS dan panjang purata tunas adalah 3.3 cm. Tunas yang memanjang memberikan perakaran yang baik pada media MS cair dengan purata 12 akar pada setiap pucuk, dengan panjang purata 2.75 cm. Rangkaian regenerasi *plantlet* diaklimatisasi dan berjaya tumbuh di tanah (90%). Konsistensi genetik dari rangkaian tanaman kultur diuji menggunakan teknik *Random Amplified Polymorphic DNA (RAPD).* Sembilan *decamer* rawak memaparkan profil pita yang sama di semua rangkaian tanaman kultur dan tanaman induk. Analisis molekul menunjukkan sifat semulajadi untuk jenis rangkain tanaman kultur. Profil minyak pati yang ditentukan dengan GC/MS pada rangakaian tanaman kultur dan tanaman induk menunjukkan pola yang serupa. Walau bagaimana, kadar minyak pati utama lebih tinggi pada semua rangkaian tanaman kultur yang diuji. Kajian ini memberikan pra-syarat untuk memperbaiki sifat-sifat kultivar pokok nilam yang dikehendaki melalui transformasi genetik untuk kajian di masa hadapan.

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α	Alpha
β	Beta
δ	Delta
γ	Gamma
°C	Centigrade degree
1 kb	1 kilobase
µl	Microliter
µM	Micromolar
%	Percentage
ppm	parts per million
2,4-D	2,4-dichlorophenoxyacetic acid
ANOVA	Analysis of variance
amu	atomic mass unit
BAP	N^6-Benzylaminopurine
BA	N^6-Benzyladenine
cm	centimeter
ca	About [Latin: circa]
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleoside- 5'-triphosphate
dATP	2'-deoxyadenosine- 5'-triphosphate
dCTP	2'-deoxycytidine-5'-Triphosphate
dGTP	2'-deoxyguanosine-5'-Triphosphate
dUTP	2'-deoxyuridine- 5'-triphosphate
EDTA	Ethylenediaminetetraacetic acid
eV	electron Volt
GC-MS	Gas Chromatography - Mass Spectrometry
g	gram
IAA	Indole-3-Acetic Acid
IBA	Indole Butyric Acid
i.d.	The same [Latin: idem]
mg	Milligram
ml	mililiter
M	Molar
MS	Murashige and Skoog
MW	Molecular weight
NAA	α-Napthtalene acetic acid
PCR	Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA

CHAPTER 1

INTRODUCTION

1.1 Commercial significance of essential oils

Natural resources, including the wide variety of plants, have economical value. Some plants in the plant kingdom have been extensively exploited for commercial reasons. For example, patchouli and eaglewood are exploited because of the essential oil content; oil palm as an agricultural crop in the production of palm oil used for cooking; teak is used in the furniture and building industries; the oil from *Jatropha* seeds can be extracted and processed to produce biofuel; the fiber from pinus and acacia is used for the pulp and paper industries (Torres *et al*., 2005; Widjaya, 2001; Sayyar *et al*., 2009; Goh *et al*., 2007; Noormahayu *et al*., 2009; Soehartono and Newton, 2000; Bhatia *et al*., 2008). The production of essential oils from patchouli, nutmeg, cananga, citronella, sandalwood, cajuput, ginger, and clove, is constantly used in the pharmaceutical, perfumery and food industries (Bakkali *et al*., 2008).

1.2 Plant species producing essential oils

An estimated 200 species of aromatic plants are currently reported to produce essential oils and they belong to the Labiatae, Compositae, Lauraceae, Graminae, Myrtaceae, Umbiliferae and other families (Table 1.1).

Table 1.1 Taxonomic distribution of plants producing essential oils

No	Plant	Order	Family	Genera	Species	Source of essential oil
1	Fennel	Apiales	Apiaceae	Foeniculum	*Foeniculum vulgare*	Fruit and seed
2	Kuss-kuss grass	Poales	Gramineae	Vetiveria	*Vetiveria zizanioides*	Roots
3	Anis	Sapindales	Rutaceae	Clausena	*Clausena anisata*	Fruit and seed
4	Bangle	Zingiberales	Zingiberaceae	Zingiber	*Zingiber purpureum*	Roots
5	Cempaka	Magnoliales	Magnoliaceae	Michelia	*Michelia champaca*	Flower
6	Sandalwood	Santalales	Santalaceae	Santalum	*Santalum album*	Wood
7	Cloves	Myrtales	Myrtaceae	Syzygium	*Syzygium aromaticum.*	Flower
8	Eucalyptus	Myrtales	Myrtaceae	Eucalyptus	*Eucalyptus sp.*	Leaf
9	Gaharu	Malvales	Thymelaeaceae	Aquilaria	*Aquilaria sp*	Wood
10	Wintergreen	Ericales	Ericaceae	Gaultheria	*Gaultheria sp.*	Leaf
11	Ginger	Zingiberales	Zingiberaceae	Zingiber	*Zingiber officinale*	Roots
12	Sweet flag	Acorales	Acoraceae	Acorus	*Acorus calamus*	Roots
13	Kieffer lime	Sapindales	Rutaceae	Citrus	*Citrus hystrix*	Fruit
14	Cardamom	Zingiberales	Zingiberaceae	Amomum	*Amomum cardamomum*	Fruit and seed
15	Cinnamon	Laurales	Lauraceae	Cinnamomum	*Cinnamomum cassia*	Stem
16	Cajeput	Myrtales	Myrtaceae	Melaleuca	*Melaleuca leucadendron*	Leaf
17	Tulsi	Lamiales	Lamiaceae	*Ocimum*	*Ocimum sanctum*	Leaf
18	Cubeb	Piperales	Piperaceae	Piper	*Piper cubeba*	Fruit
19	Cananga	Magnoliales	Annonaceae	Canangium	*Canangium odoratum*	Flower
20	Galangal	Zingiberales	Zingiberaceae	Kaempferia	*Kaempferia galangal*	Roots
21	Coriander	Apiales	Apiaceae	Coriandrum	*Coriandrum sativum*	Fruit and seed
22	Klausena	Sapindales	Rutaceae	Clausena	*Clausena anisata*	Seed
23	Turmeric	Zingiberales	Zingiberaceae	Curcuma	*Curcuma domestica*	Roots

24	Pepper	Piperales	Piperaceae	Piper	*Piper nigrum*	Fruit and seed
25	Ring Malacca	Zingiberales	Zingiberaceae	Alpinia	*Alpinia Malaccensis*	Roots

Table 1.1. Continued

No	Plant	Order	Family	Genera	Species	Source of essential oil
26	Massoia	Laurales	Lauraceae	Cryptocarya	*Cryptocarya massoia*	Stem
27	Rose	Rosales	Rosaceae	Rosa	*Rosa sp.*	Flower
28	Jasmine	Lamiales	Oleaceae	Jasminum	*Jasminum sambac*	Flower
29	Mentha	Lamiales	Lamiaceae	Mentha	*Mentha arvensis*	Leaf
30	Patchouli	Lamiales	Lamiaceae	Pogostemon	*Pogostemon cablin*	Leaf
31	Nutmeg	Magnoliales	Myristicaceae	Myristica	*Myristica fragrans*	Seed
32	Palmarosa	Poales	Gramineae	Cymbopogon	*Cymbopogon martini*	Leaf
33	Pinus	Pinales	Pinaceae	Pinus	*Pinus merkusii*	Gum
34	Rosemary	Lamiales	Lamiaceae	Rosmarinus	*Rosmarinus officinalis*	Flower
35	Tuberose	Asparagales	Agavaceae	Polianthes	*Polianthes tuberose*	Flower
36	Wild basil	Lamiales	Lamiaceae	Ocimum	*Ocimum gratissimum*	Flower
37	Celery	Apiales	Apiaceae	Avium	*Avium graveolens*	Leaf and stem
38	Lemon grass	Poales	Gramineae	Cymbopogon	*Cymbopogon citrates*	Leaf
39	Betel	Piperales	Piperaceae	Piper	*Piper betle*	Leaf
40	Lemon myrtle	Myrtales	Myrtaceae	Backhousia	*Backhousia citriodora*	Leaf
41	Java turmeric	Zingiberales	Zingiberaceae	Curcuma	*Curcuma xanthorrhiza*	Roots

(adapted from Atsiri Indonesia, 2007).

1.3 Description of Patchouli

Patchouli is a tropical aromatic plant that belongs to the mint family, Lamiaceae (Table 1.2). The plant has erect stems, reaching two or three feet (about 0.75 meters) and is mainly cultivated in Southeast Asia, India and Brazil.

Pogostemon cablin, known as patchouli or nilam in Indonesia and Malaysia, has rounded leaves and is heart shaped. The leaves and stems of patchouli plants are soft and very hairy. The leaves of this plant have the highest content of patchouli oil (2.5-5%) and the fragrance is strong and heavy. For this reason, the oil is used for the perfumery industry (Santoso, 2007; Maeda and Miyake, 1997).

1.4 Patchouli oil

Patchouli oil is a secondary metabolite produced by the plant. The composition of patchouli oil is unique and complex because it consists of more than 24 different sesquiterpenes. The sesquiterpene patchoulol is the major constituent and is the primary component responsible for the typical patchouli aroma. Patchoulol and α-patchoulene are important compounds of patchouli essential oil, and their concentrations determine the quality of the oil. Although α-patchoulene is found in small amounts, it is an important constituent of patchouli oil because together with patchoulol, it also determines the aroma of the oil (Donelian *et al*., 2009).

Patchouli alcohol is synthesized and accumulated in the cells of glandular trichomes of the plant tissue, especially in leaf tissue (Croteau *et al*, 1987), and for this reason, the leaves are used in the distillation process for the extraction of patchouli oil.

1.5 The oil profile of different cultivars of patchouli

Pogostemon cablin, Benth contains around 3-4% patchouli oil, of which 40.23% is patchouli alcohol (patchoulol), which is a major component of patchouli oil. Beside the oil, it also contains more than 20 other compounds (Donelian *et al*., 2009) as listed in Table 1.2.

Table 1.2 Main compounds identified in patchouli essential oil by steam distillation

No	Compounds	Chemical composition (%)
1	1-Octen-3-ol	0.79
2	Limonene	0.08
3	Linalool	-
4	δ-elemene	0.17
5	γ-patchoulene	1.72
6	β-elemene	0.52
7	β-caryophyllene	3.29
8	γ- elemene	-
9	α-guaiene	14.09
10	α-Himachalene	0.11
11	α-patchoulene	4.80
12	Seychellene	1.83
13	9-Epi-caryophyllene	0.48
14	*cis*-β-Guaiene	0.06
15	Ledene	0.03
16	α-selinene	0.49
17	δ-Guaiene	16.79
18	β-Curcumene	0.08
19	7-epi-α-selinene	0.18
20	Longicanfenolene	0.61
21	Caryophyllene oxide	0.15
22	Globulel	0.24
23	Epi-α-cadinel	0.12
24	Patchoulol	31.39
25	δ-Patchoulene	-
26	δ-himachalene	-
27	Ftalete	-
	Total	78.02

1.6 Traditional cultivation of patchouli

Patchouli grows in a moderate environment, between 100-400 meters above sea level. Its productive period can be maintained up to 2 years. First crop can be harvested at 6 months after planting, and the following harvests will be every 4 months. The main part of crop is the leaves, although almost all parts of the plant contain oil. Generally,

patchouli plant is propagated by cutting. This plant does not produce flower, as such, generative propagation is hardly possible (Santoso, 2007). For small scale plantation, traditionally the plant is propagated by cuttings. However, for large scale plantation, a more efficient means of propagation is required for mass production of the plants.

1.7 Problems associated with the use of traditional cultivation

For plantation scale, about 40,000 – 50,000 plants of a particular clone or cultivar is generally required for planting on a hectare (Santoso, 2007). In this context, there must be a continuous supply of desired planting material, such a supply is not achievable using the traditional vegetative method of propagation as it is time consuming. For this reason, an alternative method of propagation using *in vitro* technology should be adopted for mass propagation of the plants with clonal identity (Razdan, 2003).

1.8 *In vitro* propagation of patchouli

The regenerative production of patchouli through *in vitro* culture shortens the generation time and provides for mass production of the desired planting cultivar. This method of propagation could also generate somaclonal variants which could be easily identified and usefully exploited for potential improvements in oil content and profile. The significance of the tissue culture method is that it is able to produce large numbers of plants that are genetically identical to the mother plant.

Generally, there are two types of *in vitro* propagation method: direct regeneration and indirect regeneration. Direct regeneration has been reported by Ling Fei *et al.* (2009) using leaf explants of Lanzhou lily (*Lilium davidii* var. unicolor) and in another case, by Kosmiatin *et al.* (2005) using nodal segment of eaglewood plant. Indirect regeneration goes through a callus phase via somatic embryogenesis with a possibility of generating somoclonal variants.

The success of *in vitro* propagation is affected by several factors, i.e. type of explants (leaf, nodal segment, cotyledon, root, flower, hypocotyls and embryos),

medium employed and type of plant growth regulators used. There are several types of medium that are used for different plant species (Srivastava *et al*., 2004). The media include Murashige and Skoog medium, also known as MS medium is generally and routinely used for a wide variety of plants, Woody Plant Medium (WPM) medium for woody plants, N6 medium for cerealia (especially rice), and White & Knudson medium for orchids. In addition, some other recognized media are Euwens, Gamborg B5, Vacin and Went (VW), Nitsch and Nitsch (NN), Schenk and Hildebrant (SH), and Linsmaeier and Skoog (LS).

In vitro micropropagation is an effective means for rapid multiplication of plant species of clonal origin. Many *in vitro* studies have been reported on different patchouli species, using nodal segment explants (Mariska and Lestari, 2003; Misra, 1996; Swamy *et al*., 2010; Zulkarnain, 2004). The genetic variability of patchouli *in vitro* studies were also reported (Mariska and Lestari, 2003; Lestari *et al*., 2001) with genetic improvements (Sugimura *et al*., 2005) and characteristic of patchouli oil (Bunrathep *et al*., 2006; Nuryani 2004; Sugimura *et al*., 1995).

The clonal identification of plant derived from *in vitro* micropropagation can be assessed by analyzing them at the molecular level. Currently, DNA-based polymorphic markers are being increasingly used for monitoring genetic fidelity of plant derived from *in vitro* culture. One such technique is the randomly amplified polymorphic DNA (RAPD) fingerprinting, which is routinely used for detecting somaclonal variation. However, RAPD analysis also has its limitation, particularly the lack of reproducibility of result under certain conditions. In such cases, the use of combination of several techniques is recommended for the evaluation of genetic identity of regenerated plants as this can provide more accurate assessment compared to when any one of them is used exclusively (Kumar, 2009).

1.9 Research objectives

The overall objectives of the project are threefold:

1. To develop a tissue culture protocol for patchouli
2. To verify clonal identity for true-to-type plantlet regeneration
3. To investigate the essential oil content of the plants

The practical objectives of the project are:

1. To study the effects of plant growth regulators on shoot induction from leaf explants
2. To study the effects of concentration of MS medium on shoot elongation
3. To study the effects of plant growth regulators and condition of medium for root induction
4. To verify the clonal fidelity of tissue-cultured plantlets
5. To compare the profile of essential oil between tissue-cultured plant with mother plant.

CHAPTER 2

LITERATURE REVIEW

2.1 Taxonomy of patchouli

2.1.1 Botany of patchouli

Pogostemon cablin Benth known as patchouli or nilam in Indonesia and Malaysia is a tropical plant. These plants included in the mint family Lamiaceae, is a shrub with a height of about 0.3 to 1.3 meters. Patchouli has rounded leaves and is heart shaped (Figure 2.1). The leaves and stems of patchouli plant are soft and very hairy. The leaves of this plant have the highest content of patchouli oil (2.5-5%) and the fragrance is strong. For this reason, the oil is used for the perfumery industry (Santoso, 2007; Maeda and Miyake, 1997).

Figure 2.1 Patchouli plant

2.1.2 Species of patchouli

Generally, there are three species of patchouli, namely:

1. *Pogostemon cablin* Benth (Aceh patchouli)

   This species has leaves that are rather thick and round like a heart and has glandular hairs at the bottom of the leaf. This species does not flower. The oil content is about 2.5 to 5% and the composition of the oil is good.

2. *Pogostemon heyneanus* Benth (Java patchouli)

   This species often grow in the wild and is often referred to as patchouli woods. Leaves are thinner and leaf tip is acute. This is a flowering species. The oil content is low about 0.5 to 1.5% and composition of oil is not favourable.

3. *Pogostemon hortensis* Backer (Soap patchouli)

   This species of patchouli is a substitute for soap. The leaves are thin, with slightly tapered tip and do not flower. The oil content is low about 0.5 to 1.5% and composition of oil is not favourable (Santoso, 2007).

   .

Of the three species of patchouli, *Pogostemon cablin* Benth is the best variety that is usefully exploited for its essential oils for cultivation.

The taxonomy of *Pogostemon cablin* Benth.

Based on morphological characteristics, the classification of *Pogostemon cablin* Benth is classified as follows:

Kingdom	:	Plantae
Division	:	Spermatophyte
Subdivision	:	Angiospermae
Class	:	Dicotyledonae
Order	:	Lamiales
Family	:	Lamiaceae
Genus	:	*Pogostemon*
Species	:	*cablin*

2.2 Importance of patchouli

Patchouli oil is a very important element in fine perfumes, especially oriental perfumes. It blends well with cassia, clove, vetiver and other essential oils used in the soap industry, where patchouli resinoid is being used increasingly. The oil is reported to possess antiseptic activity. The oil has also been used to repel insect, especially moths and leeches.

An infusion of the fresh leaves is used in the Philippines for menstrual disorders. Leaves and tops are added to baths to alleviate rheumatism. The leaf decoction is taken for asthma and cough. Roots of this plant are used for dropsy and rheumatism. Chinese reportedly use the antiseptic plant for cold, diarrhea, enteralgia, halitosis, headache and nausea. Arabs, Chinese, and Japanese believe patchouli is a preventive medicinal plant, used for nervous attacks, insomnia, and loss of appetite (Duke and DuCellier, 1993).

Chemically, patchouli oil contains 35-40% patchoulol (patchouli alcohol), sesquiterpene hydrocarbons and two minor sesquiterpene alkaloids (pyridine derivatives). The components contained in the oil are patchoulol, alpha-patchoulene, beta-patchoulene, Seychellen, alpha-bulnesene, alpha-guaiene, caryophyllene, alpha-elemene, alpha guaiene oxide, D-bulnesene oxide, caryophyllene oxide, potosol, and highly odoriferous norpatchoulenol (Duke and DuCellier, 1993).

2.3 Cultivation of patchouli

This plant can be grown in fertile land with a pH of 6-7. Climatic factors, such as sunlight, temperature, humidity and rainfall are very influential in the growth and development of patchouli. As a tropical plant, patchouli can grow well in tropical regions on altitude 100-400 meters above sea level with optimum temperature of 18°C - 27°C, with rainfall between 2300-3000 mm per year and humidity levels of about 60-70% (Santoso, 2007).

Since *Pogostemon cablin* Benth do not flower, the plants are reproduced by cutting. Cutting with 15-20 cm length are inserted into well prepared soil and shaded until established. The plants are easily rooted without using rooting hormones. However, the horticultural practice of cutting is a slow process and not suitable for large-scale propagation. Therefore, a more rapid method of propagation is required for large scale propagation. One such method of propagation that can be usefully employed to produce relatively uniform plantlets in a short time is via *in vitro* culture. This method ensures a steady supply of planting material for the perfumery and pharmaceutical industries

2.4 Plant tissue culture

Plant tissue culture is the process whereby small pieces of living tissue (explants) are isolated from a desired cultivar and grown aseptically on a nutrient medium. Perhaps the earliest step towards plant tissue culture was made by Henry-Louis Duhumel du Monceau in 1756, who, during his pioneering studies on wound-healing in plants, observed callus formation. Extensive microscopic studies led to the independent and almost simultaneous development of the cell theory by Schleiden and Schwann in 1838-1839. This theory holds that the cell is the unit of structure and function in an organism and therefore capable of autonomy (Smith, 2000).

For successful plant tissue culture, it is best to start with an explant rich in undetermined cells because such cells are capable of rapid proliferation. The usual explants are flower buds, root tips, shoots, nodal segments or leaf, and these are placed on a suitable culture medium where they grow into an undifferentiated mass known as callus. Roots and shoots contain meristematic cells, which is the source of all dividing cells. Root and shoot tips can be excised and cultured directly on medium, and this can initiate new organs (via somatic organogenesis) that can be clonally propagated.

Since the nutrient media used for plants can also support the growth of microorganism, the explant is first washed in a disinfectant such as sodium hypochlorite or hydrogen peroxide. For plant cells, to develop into new organ or a callus it is essential that the nutrient medium contains the correct balance of plant hormones

(phytohormone). There are five main classes of plant hormones: auxins, cytokinins, gibberellins, absissic acid and ethylene (Smith, 2000).

The correct balance of auxins and cytokinins is critical for tissue culture growth and the exact relative amounts need to be determined empirically for each species and explant type. Generally, a low auxin-cytokinin ratio leads to shoot formation whereas a high ratio favours the formation of roots. Requirements for the other hormones vary according to species and explant. Plant tissue culture media contain inorganic salts and trace metals (usually referred to as macroelements and microelements respectively), essential vitamin (thiamine and myoinositol), an organic nitrogen source (usually one or more amino acids), and sucrose as a carbon source. Many plant tissue culture media also include a gelling agent so that plants can grow on the surface of the medium and project roots into the gel (Smith, 2000).

2.4.1 Types of plant tissue culture

In general, a plant consists of different organs, each being composed of different tissues, which in turn are made up of individual cells. If the cell walls of these cells are enzymatically digested, protoplasts are produced. The following cultures are routinely initiated as plant cell cultures.

1. Culture of intact plants

   A seed may be cultured *in vitro* from which a seedling is generated.
2. Embryo culture

   An isolated embryo is cultured after removal of the seed coat.
3. Organ culture

   An isolated organ is grown *in vitro*. Different types can be distinguished *e.g.* meristem culture, shoot-tip culture, leaf culture, root culture, anther culture, etc. Often a part of tissue mass or organ, which has been isolated from a plant, is referred to as an explant and the culture of this is an explant culture.
4. Callus culture

   A differentiated tissue is grown *in vitro* that will generate callus.
5. Single cell culture

   The growing of individual cell obtained from a tissue, callus or suspension culture.

6. Protoplast culture

   The culture of protoplasts obtained from cells by enzymatic digestion of the cell wall (Pierik, 1997).

2.4.2 Practical applications and recent advances of plant tissue culture technology

Razdan (2003) pointed out that the principal applications of plant tissue culture technology are based on advancements made in the areas of morphology, biochemistry, pathology and genetics.

1. Propagation aspects

   The important morphological application of plant tissue culture is micropropagation. A small amount of tissue can be used to raise hundreds or thousands of plants in a continuous process. This new method of vegetative propagation is exploited intensively in horticulture and the nursery industry for rapid clonal propagation of many dicotyledons, monocotyledons and gymnosperms.

2. Production of secondary metabolites

   The industrial production of secondary metabolites using cell culture was disappointing and little progress was made on this aspect of applied plant tissue culture. The technology is now available to the industry; the commercial production of shikosin, ginseng saponins and berberidine has been particularly encouraging.

3. Production of pathogen-free plants

   Eradication of virus has been an outstanding contribution of tissue culture technology. The meristem of shoots is deprived of virus and, therefore, it is possible to eliminate the pathogen in cultures provided the transferred part of the explant did not include a sufficient amount of the old tissue. The technique is economical and used very frequently.

4. Germplasm conservation

   Plant tissue culture is being developed as an effective means of germplasm conservation since a low maintenance *in vitro* germplasm storage collection

provides a cost effective alternative to growing plants under field collections, nurseries, or greenhouses.

5. Genetic manipulations

The role of cell and tissue culture in plant genetic manipulations has been increasingly recognized. The principal aspects of genetic manipulation are outlined below:

a. genetic variability due to somoclonal variants
b. *in vitro* pollination for the production of hybrid seeds
c. induction of haploids for diplodisation for breeding programs
d. somatic hybridization via protoplast fusion
e. genetic transformation via gene insertion into explants cultures.

2.4.3 Factors associated with plant cell culture

Success of plant tissue culture is influenced by several factors such as:

a. explant
b. media
c. environment

a. Explant

Explant is a piece of plant tissue placed into tissue culture. An explant can develop a callus as a wound response that consists of unorganized, dividing cells.

Factors in explant selection include consideration of the following:

1. Physiological or ontogenic age of the organ that is to serve as the explant source.

   The age of the explant can be very important, as physiologically younger tissue is generally much more responsive *in vitro*. In addition,

juvenile tissue is generally easier to surface disinfect and establish clean culture (George *et al.*, 2007; Smith, 2000).

2. Season in which the explant is obtained

   The season of the year can have effect on contamination and response in culture. Tissue that is physiology dormant is generally unresponsive in culture until the dormancy requirement is met (George *et al.*, 2007; Smith, 2000).

3. Size and location of the explant

   The explant size has an effect on the response of the tissue. Generally, the smaller the explant, the harder it is to culture. The larger explants probably contain more nutrient reserves and plant growth regulators to sustain the culture. Plants have different hormonal balances throughout the plant and depending on the location of the explant can have different endogenous level of plant growth regulators. Internal differences in hormone balance in the tissue can result in varying *in vitro* responses (George *et al.*, 2007; Smith, 2000).

4. Quality of the source plant

   It is advisable to obtain explants from plants which are healthy as compared to plants under nutritional or water stress or plants which are exhibiting disease symptoms (George *et al.*, 2007; Smith, 2000).

5. Ultimate goal of cell culture

   Depending on what type of a response is desired from the cell culture, the choice of explant tissue will vary. Any piece of plant tissue can be used as an explant. If clonal propagation is the goal, then the explant will usually be a lateral or terminal bud or shoot. For callus induction, pieces of the cotyledon, hypocotyl, stem, leaf, or embryo are usually used. Leaf tissue from the aseptically germinated seed is a good source of tissue for protoplast isolation. To produce haploid plants or callus, the anther or pollen is cultured (George *et al.*, 2007).