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Colchicine Production from *Colchicum* and the Role of *in vitro* Cultures: A Review

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ABSTRACT

In vitro production of naturally-occurring medicinally-important secondary metabolites from plants using callus or cell suspension culture has become an industrially promising project. *In vitro* production of secondary metabolites has many advantages: i) year-round availability of plant material for the production of functional phytomolecules; ii) better avenues for processing and isolation; iii) the possibility of accentuation of chemical reactions leading to other useful secondary metabolites under *in vitro* conditions; and iv) elimination of potential political and geographical boundaries against crop production. (-)-Colchicine is one of the most important and well-studied natural compounds. It occurs mainly in plant species belonging to the genus *Colchicum*. (-)-Colchicine is still in use today as a pharmaceutical agent and as a laboratory tool. Because of the difficulties in seed germination, young corms are used in the propagation of different *Colchicum* species. (-)-Colchicine was produced via tissue culture from calluses and cell suspensions of different *Colchicum* and *Gloriosa* species.

Keywords: Colchicine, *In vitro* cultures, Callus, Cell suspension.

INTRODUCTION

The genus *Colchicum* belongs to the family Colchicaceae; a family of mainly perennial geophytes, although some vines and herbs are also included (Nordenstam, 1998). It is a taxonomically difficult genus. Both leaves and flowers are necessary for species identification (Feinbrun-Dothan, 1986). Plants of the genus *Colchicum* have been known for more than 2000 years for their marked beneficial and poisonous effects (Brickell, 1984). *Colchicum* is native to Europe but had been introduced to Canada and USA, where it is grown in gardens and lives as a wild escapee in meadows and woodlands (Wendelbo and Staurt, 1985; Snyder, 1998).

Species belonging to the genus *Colchicum* are perennial herbs, with a corm or rarely with a creeping stolon. Corm is ovoid, enclosed by brown tunics, convex on one side, flattened and somewhat prolonged downwards on the other, replaced every year by a renewal corm which develops at the side of the previous year's corm at the base of the flowering shoot. Corm tunics, membranous, papery or coriaceous, often extended into a persistent tubular pseudostem, occasionally with horizontal rhizomatous outgrowths. Leaves 2-9 are basal, linear, lorate, lanceolate or elliptic-ovate, smooth, semi-terete to ribbed or plicate, synanthous or hysteranthous, developing with or after flowering (occasionally developing as flowers

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fade), develop leaves and flowers enclosed at the base by a tubular, often white, cataphyll (spathe), which turns into brown tunic in the following year. Scape is very short, subterranean during flowering. Flowers 2-5(-20) are in a short raceme or solitary, hermaphrodite, showy. Perianth is petaloid, pink, purple or white, rarely yellow, infundibular, with a long tube and a 6-partite limb; stamens 6 shorter than lobes of perianth, inserted at the throat of perianth; the 3 inner somewhat higher than the 3 outer; anthers are versatile. Ovary is subterranean, 3-locular; each locule with several ovules; styles 3, free filiform, long, exserted from perianth-tube; stigmas punctiform or oblique. Capsule is septicidal, ovoid to ellipsoid, 3-gonous, tapering or beaked at tip, borne on a lengthened stalk, maturing near ground level. Seeds are globose or angular (Feinbrun-Dothan, 1986). Corm is a solid, vertical swollen stem with somewhat developed internodes. At the top is a terminal bud that produces the flowers and leaves, and on the sides in the axils of the bases of last year's leaves are lateral buds that may form small offsets. The roots are produced from the bottom. A corm uses up all of its stored food to produce a crop of flowers and fruits during the year and then initiates the growth of one or more replacements (Relf and Ball, 2004; Gordon, 1990). During the hot and dry summer, *Colchicum* plants are dormant. Then in the fall, when the soil is cool, new roots grow and their flower bud mature. Vegetative growth and the storage of food for the following year are completed in a short period between flowering and dryness of soil in summer. However, fall–flowering species of *Colchicum* differ only in that they bloom about the time of new roots initiation (Akan and Eker, 2005; Poutaraud and Girardin, 2004; Floridata, 2002).

Species of the genus *Colchicum* are known commonly as Autumn crocus, Meadow Saffron or Naked Ladies (Clapham, 1962). The name Meadow Saffron (old English name), strictly belongs to the species *C. autumnale*, the only native species in Britain. Previously, it has been Autumn crocus, due to the superficial resemblance of its flower to a crocus, but this confusion is not any more present (Wendelbo and Staurt, 1985). In Jordan, it is locally known as Al-Zafran (Al-Eisawi, 1998).

Out of the 225 species of the genus *Colchicum* (Nordenstam, 1998), nine are found in Jordan, namely: *C. brachyphyllum* Boiss. & Haussk. ex Boiss., *C. crocifolium* Boiss, *C. heirosolymitanum* Feinbr, C. tauri Siehe ex Stefanov, *C. ritchii* R. Br., *C. shemperi* Janka & Stefanov, *C. stevenii* Kunth, *C. triphyllum* G. Kunze, and *C. tunicatum* Feinbr (Feinbrun-Dothan, 1986; Al-Eisawi, 1998; Oran and Al-Eisawi, 1998).

The pharmacological activity of this genus is attributed to the presence of colchicinoids, mainly (-)-colchicine (Samuelsson, 1992), which was first isolated by Pelletier and Caventou in 1820 (Pelletier and Caventou, 1820). (-)-Colchicine; (*S*)-*N*-(5,6,7,9-tetrahydro-1,2,3,10-tetramethoxy-9-oxobenzo-[a] heptalen-7-yl) acetamide, occurs as yellowish-white amorphous scales with a very bad bitter taste and darkens on exposure to light (Dollery *et al*., 1999; Trease and Evans, 1989). It has a molecular weight of 399.4 and a *p*Ka value of 1.7, m.p. 155–157°, $[\alpha]_D$ – 121° ($CHCl_3$) (Dollery *et al*., 1999; Cordell *et al.*, 1989). (-)-Colchicine is freely soluble in water, alcohol and chloroform but only slightly soluble in ether or petroleum spirit (Dollery *et al*., 1999; Trease and Evans, 1989).

(-)-Colchicine is still the drug of choice for treatment of gout (Terkeltaub, 2003) and is used for the treatment of a number of pro-inflammatory disorders, such as familial Mediterranean fever (Drenth and Van Der Meer, 2001), and Behcet's disease (Sakane and Takeno, 2000). In addition, clinical studies have proved (-)-colchicine to possess a potent antitumor activity. But due to the lack of tumor selectivity and high toxicity, (-)-colchicine use as an anti-neoplastic drug is limited (Eigsti and Dustin, 1955). Among (-)-colchicine analogues, (-)-demecolcine has a wider margin of safety than (-)-colchicine and is used for the treatment of myelocytic leukaemia and malignant lymphoma (Samuelsson, 1992). (-)-Colchicine is also used in biological and breeding studies to produce polyploidy, or for multiplication of the chromosomes in the cell nucleus (Trease and Evans, 1989).

(-)-Colchicine has also been used as a selective neurotoxin and in animal models of Alzheimer's disease and epilepsy. Although the mechanisms mediating the neurotoxic action of (-)-colchicine have not yet been established, most studies have attributed these effects to its microtubule actions (Feng and Kaplowitz, 2000) and inhibition of cellular division (Trease and Evans, 1989). In addition, (-)-colchicine suppresses tubulin polymerization, with free tubulin serving as (-)-colchicine receptor (Weakley *et al*., 2001).

(-)-Colchicine can significantly inhibit the function of several ion channels, including voltage–gated sodium, calcium channels and ligand–gated nicotinic (Weiner *et al*., 1998). Chakerbarty *et al*. (1983) have reported the presence of a unique (-)-colchicine binding activity in the polysomes of rat brain.

In addition to the genus *Colchicum*, (-)-colchicine was reported from species belonging to the *Merendera* and *Gloriosa* genera (subfamily: Wurmbaeoideae, Colchiaceae). (-)-Colchicine and its congeners are chemotaxonomic markers for the subfamily Wurmbaeoideae (Nordenstam, 1986).

Among all species of *Colchicum*, *C. autumnale* is the best source for (-)-colchicine. The richest plant parts in (-)-colchicine are the corms and seeds. *C. autumnale* seeds contain 0.6-1.2%, while corms contain up to about 0.6%. Seeds are mainly used by the pharmaceutical industry for the extraction of colchicinoids (Trease and Evans, 1989).

Several analytical methods were reported in the literature to determine (-)-colchicine content in pharmaceutical preparations, biological fluids and plant extracts (Alexander *et al*., 1994; Al-Fayyad *et al.*, 2002; Ondra *et al.*, 1995; Sütlüpinar *et al*., 1988). (-)-Colchicine and (-)-demecolcine were determined in raw and dried leaves, stems, mother and daughter corms of *C. autumnale* in four stages of its ontogenesis by (Vicar et al., 1993). They found that (-)-colchicine content in raw material varies during plant growth and the content of both alkaloids decreases with drying. In another study by Alali et al. (2004), *C. stevenii* corms, flowers and leaves were reported to contain 0.17, 0.12 and 0.20 (wt/wt) g%, respectively, while *C. hierosolymitanum* corms and flowers were found to contain 0.13 and 0.09 (wt/wt) g%, respectively. In a cultivation study using different NPK fertilizer levels, (Al-Fayyad et al., 2002) have reported the following values for (-)-colchicine in *C. hierosolymitanum*: corms 0.052 wt/wt g%, flowers 0.025 wt/wt g% and leaves 0.013 wt/wt g%, and in *C. tunicatum*: corms 0.07%, flowers 0.07% and leaves 0.036% (Al-Fayyad *et al.*, 2002). Ondra et al. (1995) assayed corms of seven Turkish *Colchicum* species; namely: *C. macrophyllum*, *C. turcicum*, *C. cilicicum*, *C.*

kotschyi, *C. bornmuelleri*, *C. speciosum* and *C. triphyllum* for their colchicinoid alkaloids. (-)-Colchicine content was found to be 222.3 (0.05), 323 (0.04), 300 (0.02), 1058 (0.04), 3063 (0.01), 4245 (0.01) and 958 (0.01) $\mu g g^{-1}$ dried drug (*RSD*, %), respectively. (-)-Colchicine content in *C. brachyphyllum* Boiss. & Haussk. ex Boiss and *C. tunicatum* Feinbr (Colchicaceae), growing wild in Jordan, was determined during different growth stages by Alali et al. (2006). Underground parts in both species and during different growth stages, always showed higher (-)-colchicine content than the above ground parts. In *C. brachyphyllum*, total (-)-colchicine content of underground parts during flowering stage was found to be about 0.15% (wt/wt), while that of aerial parts was only about 0.04% (wt/wt). In *C. tunicatum*, total (-)-colchicine content of underground parts was found to be 0.12% (wt/wt) and 0.13% (wt/wt) during flowering and vegetating stages, respectively, while that of aerial parts was only about 0.04% (wt/wt) and 0.02% (wt/wt), respectively (Alali et al., 2006). Malichova *et al*. (1979) showed that the levels of (-)-colchicine remained the same as in fresh material when leaves and flowers of *Colchicum autumnale* were slowly dried well.

This review is aimed to discuss the role of *in vitro* tissue cultures in (-)-colchicine production with emphasis on *Colchicum* species.

Plant Tissue Culture

Plant tissue culture is a science which refers to the *in vitro* cultivation of all plant parts (single cells, tissues and organs) under aseptic conditions (Pierik, 1997; Torres, 1988). Plant tissue culture systems are often used to study various physiological, biochemical, genetic and structural problems related to plants. Plant tissue culture techniques also have great potential as a means of vegetatively propagating economically important crops and crops of future potential on a commercial basis (Gopi and Vatsala, 2006; Sivakumar *et al.*, 2004; Tokuhara and Mii, 2001; Wang *et al*., 2003). Plant tissue culture includes the various types of cultures, whole plant, embryo, organ, callus, suspension and cell culture, protoplast culture, haploid culture, among others (Zamber *et al*., 2002).

The history of *in vitro* culture had been started from the pioneering work of Schwann and Schleiden who, in 1838, put forward the fundamental totipotency theory, stating that cells are autonomous and capable of developing into complete plants (Hutchinson *et al*., 1995). In 1902, Haber Land tried to grow a plant tissue into sterile media, but this attempt failed (Pierik, 1997). Indole-3- acetic acid (IAA) was the first plant growth regulator discovered (Buchanan *et al*., 2000). This hormone (IAA) created great opportunities in culturing plants *in vitro*. The discovery of the regular cytokinin (kinetin) in 1955 caused a tremendous progress at the time in the *in vitro* studies (Buchanan *et al*., 2000; Pierik, 1997). Murashige and Skoog (1962) were able to formulate revised media for culturing tobacco callus. Their media were composed of macro and micronutrients which are needed by the plant to propagate a huge number of plant species *in vitro*.

Plant tissue culture in Jordan had witnessed a significant advance in the past decades (Shibli, 1995). Various research projects in plant biotechnological fields, preservation of plant genetic resources and producing virus-free plants are standing examples (Arafeh *et al*., 2006; Shibli and Ajlouni, 2000).

Recently, many countries in the world tend to utilize the advantages of plant tissue culture to produce many plant products or secondary metabolites (Ostrolucka *et al*., 2004; Sivakumar *et al*., 2004) either by the massive propagation or by callus and cell culture techniques (Gopi and Vatsala, 2006; Mihaljevic *et al*., 2002). By using plant tissue culture, there are still two major technical challenges which must be faced; the first is the ability to develop a proper propagation protocol in order to provide the required number of plants from which high levels of secondary metabolites or any desired compounds could be produced. The second challenge is to design a bioreactor system that can be manipulated to exploit the genetic and biochemical capabilities of the plant cell culture to increase the production levels (Al-Sane, 2005).

Rapid Multiplication of Superior Plants

Meristem, shoot tip or bud culture provides an effective means for rapid propagation of almost any particular genotype in many plant species. The advantages of employing this technique include efficient clonal propagation of a large number of disease-free plants due to the aseptic procedures, being a safe method for obtaining uniformity and its assurance of the consistent production of true-to-type plants within a short period of time (Chang *et al*., 2000; Chirangini and Sharama, 2005; Ostrolucka *et al*., 2004).

The *in vitro* propagation is started from pieces of whole plants. The small organs or pieces of tissues that are used and obtained depend on the kind of culture to be initiated, the purpose of the proposed culture and the plant species to be used (Bradley *et al*., 2001; Gandonou *et al*.,2005; Li *et al*., 2002; Mihaljevic *et al*., 2002; Ostrolucka *et al*., 2004).

Micropropagation and regeneration techniques are available for the conservation of plant genetic resources of rare and endangered species (Chang *et al*., 2000). Micropropagation of bulb plants as an alternative to the conventional methods for vegetative propagation attracts much attention, because of its advantages (Shibli and Ajlouni, 2000). It increases many times the multiplication level and enables to obtain materials free from disease (Chang *et al*., 2000). Some medicinal herbs are propagated vegetatively through rhizomes, but the rate of propagation is very slow, therefore, tissue culture remains an indispensable tool for rapid multiplication and sustainable growth of slow propagation species (Chirangini and Sharma, 2005; Chang *et al*., 2000).

There is a huge number of publications which report the benefits associated with *in vitro* multiplication of medicinal plants (Chirangini and Sharma, 2005; Elfahmi, 2006; Gopi and Vatsala, 2006; Salvador *et al*., 2003). A very good example is the culture of *Angelica dahurica* (Tsay, 1993); the medicinal herb that has been used in China for thousands of years to prevent headache and heal skin diseases. The herbs collected from plants growing naturally in the mountains are insufficient for medicinal use. Therefore, tissue culture has the potential for clonal propagation for commercial use. Chirangini and Sharma (2005) reported that *Zingiber cassumunar* is used by traditional medical practitioners in the treatment of piles, cough, fever and intestinal disorders. This medicinal herb propagates vegetatively through rhizomes, but the rate of propagation is very slow. Micropropagation is a safe method for obtaining uniform plants to be used by commercial growers as disease-free planting material.

As recorded by Sivakumar *et al*. (2004), *Gloriosa superba* L. is an emerging industrial medicinal crop in

South India for its high (-)-colchicine content and is still collected from the wild. Due to over-exploitation of this species in the wild as well as problems faced during field cultivation, *G. superba* is now on the verge of extinction (Sivakumar and Krishnamurthy, 2002). For commercial production of (-)-colchicine and its derivatives, natural production from *in vitro* methods of the source plant are thus of great interest. In the past two decades, focus has been made on plant cell biotechnology as a possible alternative production method. One of the potentially viable methods for the sustainable maintenance of these useful plants is *in vitro* cultivation technology of high-yielding cell lines (Gopi and Vatsala, 2006; Mihaljevic *et al*., 2002; Ogita, 2005; Pan and Staden, 2000). Gopi and Vatsala (2006) conducted a study on *Gymmena sylvestre* R. for high yields of gymnemic acids and gymnemagenin from undifferentiated cells. Arafeh *et al*. (2006) used callus and cell suspension processes to produce thymol oil from *Origanum syriacum* L.

Callus Cultures

Callus culture refers to tissues arising from the disorganized proliferation of cells from segments of plant organs (Torres, 1988). In tissue cultures, it can be defined as an amorphous tissue of loosely arranged thin walled parenchyma cells, arising from proliferating cells of the parent tissue (Dodds and Roberts, 1985).

The callus procedure is a convenient method for starting and maintaining cell lines. However, callus from an explant tissue occurs as a result of dramatic changes in the appearance and metabolism of the cells. Induction of callus and physical disorganization of cultured cells are thought as a result of intercellular physical and chemical communication (Turhan, 2004). The growth potential of established callus lines varies in response to genetic potential and culture medium composition (Mihaljevic *et al*., 2002).The regeneration of plants from callus tissue, especially highly totipotent embryogenic callus, has been recognized as one of the essential techniques for micropropagation and biotechnological applications (Tokuohara and Mii, 2001). Callus tissue may accumulate certain amounts of secondary products, in quantities less or more than the intact plant (Gopi and Vatsala, 2006; Ogita 2005; Sivakumar *et al*., 2004). Plant materials and explants play an important role in the induction of callus tissue (Naik and Nayak, 2005; Shah *et al*., 2003).

Various explants from all organs of the plant can be used as starting materials to induce callus (Zambre *et al*., 2002). In addition, callus cultures were induced from isolated mature zygotic embryos or from segments of juvenile branches (Mihaljevic *et al*., 2002). Friable callus appeared as clumps, varying in color from dark brown to yellow (Elfahmi, 2006).

However, callus induction, referred to as the initiation of callus formation, can be performed via transferring surface sterilized explants aseptically onto a complex semisolid medium supplemented with plant growth regulators, auxin and acytokinin (Chang *et al*., 2000; Mihaljevic, *et al*., 2002; Turhan, 2004; Zambre *et al*., 2002), and subsequent cell proliferation occurs leading to callus formation. Various factors can influence the callus induction ability of the selected explant such as physical factors, chemical factors and genetic constitution of the plant (Naik and Nayak, 2005; Hassawi *et al*., 2005; Bradley *et al*., 2001; Kashyap *et al*., 2005).

Following induction, callus can be removed from the explants and maintained *in vitro* by subsequent subculture on solid media (Hassawi *et al*., 2005; Kashyap *et al*., 2005; Sakhanokho *et al*., 2001; Shah *et al*., 2003) or liquid media (Arafeh *et al*., 2006; Zhigang *et al*., 2005) to improve its friability, and the amounts of callus tissues being subcultured should be relatively constant. Friable callus can be introduced into a complex liquid media and agitated using the rotary shaker leading to cell dispersal through the medium and formation of cell suspension culture (Tokuhara and Mii, 2001; Zhigang *et al*., 2005). However, the callus tissue may differ in structure, color and growth habit (Turhan, 2004; Sakhanokho *et al*., 2001; Shah *et al*., 2003). In addition, habituation (culture loss of PGRs auxins and/or cytokinin requirement for the callus after repeated subcultures) can occur in callus culture systems (Pierik, 1997) and this could be due to mutations or epigenetic changes.

The duration of growth of callus can be monitored by several methods, including fresh weight, dry weight, callus diameter and number of cells per volume of liquid media (Mihaljevic *et al*., 2002). Callus can be maintained and differentiation can be induced when callus is subcultured on appropriate media (Shah *et al*., 2003). However, vitamins, plant growth regulators (PGRs), macro and micro nutrients are key factors in changing the composition of any media used in initiating and growth of calli cultures (Li *et al*., 2002). Some plant species have been targeted and their callus fragments have been grown using different types of media (Zhigang *et al*., 2005).

Cell Suspension Cultures

Cell suspension cultures are widely used in cell culture engineering due to the favorable flow ability and ease of operation as well as higher mass transduction and absorption rates (Zhigang *et al*., 2005). Liquid suspension cultures consist of mixtures of cell aggregates, cell clusters and single cells (Elfahmi, 2006; Shibli and Ajlouni, 2000; Ogita, 2005). The growth rates of such cultures are generally much higher than on agar. The technique offers many advantages for examining the metabolic role of nutrients and their utilization in plant cells. In order to use the suspension culture system for examinations, synchronous growth of cells and a stable morphology are needed to elucidate physiological and molecular biological features of plant cells (Ogita, 2005). Both callus and suspension cultures can be obtained from tissues of most species, but successful starting cultures result from combined interaction of explant source, medium composition, plant genotype and plant physiological condition (Laxmi and Giri, 2003; Manickvelu *et al*., 2006; Salvador *et al*., 2003; Wang *et al*., 2003) . All plant cells and tissue culture publications described the behavior of cells growth in cell suspension culture during growth period, where cells pass through lag phase, followed by a brief exponential growth phase during active cell division (Karam *et al*., 2003; Mitchell and Cowan, 2003). The lag phase could largely depend on the growth phase of the stock culture at the time of subculture and the size of inoculum. After the exponential growth, the growth declines and cultures enter the stationary phase (Arafeh *et al*., 2006; Karam *et al*., 2003; Mitchell and Cowan, 2003).

The degree of cell separation in a suspension culture is greatly influenced by the origin and history of the callus used to initiate the culture and by the composition of the culture medium. Lee *et al*. (2004) and Ogita

(2005) noted that aggregation increases during the period of maximal cell division and the incidence of mitotic index is greater in the cell aggregates than in single cells. Thus the frequency of subculture may affect the degree of aggregation.

Plant Secondary Metabolites

Plants in general biosynthesize natural secondary metabolites including: alkaloids, essential oils, amino acids, terpenes, lignans, polyphenols…etc. (Dewick, 2002; Herbert, 1988). *In vitro* production of these naturally occurring secondary metabolites from a vast range of plants has become an industrially promising project (Salvador *et al*., 2003). Secondary metabolites can be derived from callus, cell and cell suspension cultures (Arafeh *et al*., 2006; Elfahmi, 2006; Shibli *et al*., 1997 and 1999; Sivakumar *et al*., 2004). However, *in vitro* production of secondary metabolites has many advantages: i) year-round availability of plant material for the production of functional phytomolecules; ii) better avenues for processing and isolation; and iii) the possibility of accentuation of chemical reactions leading to other useful secondary metabolites under *in vitro* conditions (Sivakumar *et al*., 2004).

Naturally occurring phytochemicals have been extracted from *in vivo* grown plants several decades ago (Dewick, 2002), and medicinal plants have been, traditionally, grown for their active derived components (Chirangini and Sharma, 2005). The production of secondary metabolites from callus and cell suspension cultures has been reported by so many researchers (Abdullaev *et al*., 2003; Al-Sane, 2005; Arafeh *et al*., 2006; Elfahmi, 2006; Karam *et al*., 2003; Sivakumar *et al*., 2004; Salvador *et al*., 2003; Ogita, 2005; Gopi and Vatsala, 2006). Several factors influence the accumulation of secondary metabolites in callus and suspended cells. The most important factors are the chemical constitution of the media used with respect to growth regulators (Manickavelu *et al*., 2006), carbon concentration (Al-Sane, 2005; Shibli and Ajlouni, 2000; Manickavelu *et al*., 2006) and amino acids (Sivakumar, *et al*., 2004).

The importance of plants for the production of pharmaceutical substances via tissue cultures is found to have potential as a supplement to traditional agriculture in the industrial production of bioactive plant metabolites (Gopi and Vatsala, 2006).

A number of plant species, commonly sought for their secondary metabolites, are native to very remote and sometimes politically unstable geographic areas of the world (Gopi and Vatsala, 2006). Plants that produce valuable secondary products are subjected to environmental instability with an increasing difficulty to acquire plant derived compounds, which promote scientists to develop new utilities for industrial applications (Gopi and Vatsala, 2006; Ogita, 2005).

The production of plant pharmaceuticals, using large-scale plant tissue cultures, provides an attractive alternative to the unstable and unreliable supply lines currently in place. Therefore, bioreactor facilities can be established at the processing site for mass production (Gopi and Vatsala, 2006).

Two major technical challenges need to be met in order to propel tissue culture production of secondary metabolites into commercialization (Frin and Jones, 2000; Hohe *et al*., 2002; Whitaker and Hashimoto, 1986). The first challenge is the ability to obtain tissue culture systems that produce the desirable secondary

metabolite and to screen and select for variant cultures that synthesize considerable levels of that compound. The second challenge is to design bioreactor systems that can be manipulated by scientists to exploit the genetic and biochemical capabilities of the plant cell culture to optimize production levels (Pan and Staden, 2000). Therefore, the use of bioreactor technology has been suggested to be successfully applied to plant mass propagation and other development processes defined by bioengineers, including the production of secondary metabolites (Frin and Jones, 2000; Hohe *et al*., 2002; Pan and Staden, 2000).

Several studies for improving the production of secondary metabolites, to increase the yields, have been discussed by many researchers (Al-Sane *et al*., 2005; Arafeh *et al*., 2006; Elfahmi, 2006; Gopi and Vatsala, 2006; Ogita, 2005; Sivakumar *et al*., 2004). In their studies, cell suspension culture systems, screening methods, regulation of secondary metabolic pathways, use of biotic agents, scale-up issues, immobilized cells, stability and preservation of high- yielding cell lines were discussed.

(-)-Colchicine Production in Cell Cultures

(-)-Colchicine production in cell cultures has been reported earlier. However, the amount of (-)-colchicine recorded in the tissues cultures of *Colchicum* and *Gloriosa* were 10–25 times lower than those found in plants growing *in vivo* (Hayashi et al., 1988; Finnie and Van Staden, 1991).

In cell cultures of *Colchicum*, feeding of some precursors; e.g. phenylalanine and tyrosine, had no effect on (-)-colchicine formation, however feeding with *p*-coumaric acid, tyramine and (-)-demecolcine increased (-)-colchicine formation in *Colchicum* cell cultures (Yoshida et al., 1988). *C. autumnale* is the first plant species exploited for the production of (-)-colchicine *in vitro*. Callus culture of *C. autumnale* was induced on Linsmaier-Skoog medium (Linsmaier and Skoog, 1965). Hayashi et al. (1988) published the first report on the production of (-)-colchicine by plant tissue culture.

Undifferentiated callus tissues were induced from flowering shoots of *C. autumnale* in the presence of 2,4-D and later cultured on liquid Murashige-Skoog medium (1962) containing indole butyric acid (IBA) and kinetin. Hayashi et al. (1988) also studied the effects of growth substances and nutritional factors on the formation of (-) colchicine. The only effective carbon source for growth and (-)-colchicine formation (~5 µg/g fr. wt.) in *C. autumnale* was found to be 3% sucrose. Although nitrate or ammonium as the sole nitrogen source inhibited the formation of (-)-colchicine, growth and (-)-colchicine accumulation *in vitro* were better (~6 µg/g fr. wt.) with 20 mM ammonium plus 40 mM nitrate. The addition of SO_4^{-2} markedly increased the accumulation of (-)-colchicine to ~40 µg/g fr. wt.

(-)-Colchicine was extracted recently *in vitro* from a Jordanian *Colchicum* species. It was identified in callus and in cell suspension of *C. hierosolymitanum* by means of HPLC (Daradkeh, 2007). Daradkeh (2007) found that (-)-colchicine content in *C. hierosolymitanum* was the highest (0.242 mg g-1DW) on callus grown on 0.2 M of sucrose. No (-)-colchicine was detected in callus grown on sucrose free media. Cell suspension had 0.012 $mg.g^{-1}$ DW (-)-colchicine from suspended cells grown under darkness.

The *in vitro*-generated tubers of Gloriosa (Liliaceae) were found to accumulate 0.03% (-)-colchicine when analyzed after 6 months in culture (Ghosh and Jha,

2005). The effect of precursor feeding on (-)-colchicine production in root cultures of *G. superba* was studied (Ghosh et al., 2002). Also, treatments with *p*-coumaric acid and tyramine (each at 20 mg/l) increased (-)-colchicine content in root cultures of *G. superba* to 1.9 mg/g cell dry wt. (Ghosh et al., 2002). It was concluded that (-)-colchicine content of different *in vitro* cultures revealed a close correlation between root differentiation and (-)-colchicine accumulation (Ghosh and Jha, 2005). Levels of (-)-colchicine, extracted from *Gloriosa* callus, malformed roots and entire plantlets showed an increase that can be directly related to the amount of differentiation in culture (Finnie and Van Staden, 1994). In Sandersonja callus, the levels of (-)-colchicine produced in suspension were 10-25 times lower than those found within normal *in vivo* plants (Finnie and Van Staden, 1996).

(-)-Colchicine Biosynthesis

Biosynthesis of alkaloids, although controlled genetically, could be affected by different environmental factors, such as light, high temperature, stress and nutrients (Poutaraud and Girardin, 2004).

Numerous studies have shown the significant influence of mineral nutrition on alkaloid synthesis. For example, in *C. hierosolymitanum*, nitrogen, phosphorus and potassium improved morphological characteristics and alkaloid content on soil with a good supply (Al-Fayyad, 2002). In *C. autumanale* (Poutaraud and Girardin, 2004), a significant relation was shown between cobalt supply, cobalt content into the plant and synthesis and accumulation of (-)-colchicine. Mineral element uptake by plant is a complex process governed by numerous factors influencing each other: plant species, genotype, availability and mobility of the minerals in the soil and soil properties such as pH, organic matter… etc (Radanovic *et al*., 2002).

Alkaloids are biosynthesized from amino acids (Sivakumar *et al*., 2004). Therefore, the cloning of the genetic sequences for a particular plant specific indole alkaloid would not only require the splicing and expression of unknown genes, but would also require a major modification in the genetic regulation of the pathway (Whitaker and Hashimoto, 1986).

Yoshida *et al*. (1988) found that (-)-colchicine is biosynthesized from L-phenylalanine, L-tyrosine and L-methionine. Androcymbine is known to be a key intermediate in the pathway to (-)-colchicine. (-)-Demecolcine is a closer precursor of (-)-colchicine. They concluded that the trigger for (-)-colchicine formation occurs in biosynthetic pathway between the amino acids and (-)-demecolcine. Hayashi *et al*. (1988) reported that (-)-demecolcine accumulation may control the biosynthetic pathway between (-)-demecolcine and (-)- colchicine. However, (-)-demecolcine occurs in *Colchicum* corms in amounts equivalent to those of (-)-colchicine, accumulation induces the formation of (-)-demecolcine or metabolic levels of (-)-colchicine cells (Yoshida *et al*., 1988). The use of biotechnological approaches to improve the production of secondary metabolites with plant cells, including the biotransformation of suitable precursors and the modification of biosynthetic pathways, is considered to be suitable and economically attractive (Elfahmi, 2006; Sivakumar *et al*., 2004) .

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إنتاج الكولشيسين من نبات اللحلاح ودور زراعة الأنسجة

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ملخص

يعتبر إنتاج المواد الطبية التي تنتج طبيعياً كمنتجات ثانوية من النباتات الطبية باستخدام زراعة الأنسجة من خلال الكالوس أو محلول الخلايا المعلقة من أهم المشاريع الصناعية الواعدة. ويمتاز إنتاج المواد الطبية عبر الزراعة النسيجية بالعديد من المزايا: أ) توافر المادة النباتية طوال العام من أجل إنتاج المواد الطبية، ب) توفير ظروف أفضل لتصنيع المواد الثانوية واستخراجها، ج) إمكانية حدوث تفاعلات كيميائية تؤدي الى ظهور منتجات طبيعية ثانوية أخرى مهمة، د) التخلص من الضغوط السياسية والحواجز الحدودية والجغرافية في عملية إنتاج المحاصيل. ويعتبر الكولشيسين أحد أهم المواد الطبية الطبيعية التي ينتجها النبات كمنتج ثانوي. يوجد الكولشيسين عادة في جنس نبات اللحلاح *Colchicum*. ومازال الكولشيسين يستخدم عاملاً دوائياً مهماً ومادة مختبرية مهمة. وبسبب صعوبة الإنبات لنبات اللحلاح، تستخدم الكورمات أو الأبصال الفتية لإكثار الأصناف المختلفة من جنس نبات اللحلاح. وقد تم إنتاج الكولشيسين عن طريق الزراعة النسيجية من الكالوس ومن خلايا النبات من الأصناف المختلفة لجنسي اللحلاح *Colchicum* و الغلوريوسا *Gloriosa*.

الكلمات الدالة: الكولشيسين، زراعة الأنسجة، الكالوس، محلول الخلايا المعلقة.

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