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Production of Colchicine by using Plant Cell Culture

**A thesis submitted to Dublin City University in fulfilment of
requirements for the degree of Doctor of Philosophy by**

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Under the supervision of Dr. Michael Parkinson B.S.c., Ph.D.

October 2005



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Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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This thesis is dedicated to my parents and my wife for all their encouragement and support over the last number of years.

Abstract

Plant cell and tissue culture is an established alternative to the harvest and extraction of whole plant material for the production of valuable secondary metabolites. Colchicine, a secondary metabolite of *Colchicum autumnale* and *Gloriosa superba* has anti-mitotic and anti-inflammatory properties, has been used for centuries in the treatment of gout and more recently for familial Mediterranean fever, and has been recognized for some time as an anti-tumour agent. This thesis investigated the biotechnological application of cell and tissue cultures of *Colchicum autumnale* and *Gloriosa superba*. Two analytical methods (HPLC and ELISA) have been developed and optimised for determination of the colchicine accumulated in liquid medium and in plant tissue. Callus tissue of *Colchicum autumnale* and callus and root tissue of *Gloriosa superba* has been established, growth kinetics and accumulation of colchicine examined, and culture optimized for accumulation of colchicine. Precursor feeding has been investigated and yields of colchicine have been increased in both plant cells and root cultures by the addition of relatively low levels of suitable precursors. Total Colchicine accumulated in *Colchicum autumnale* culture fed with 1mM coumaric acid was 180µg/g dry weight compared to 63µg/g dry weight for the control and the same treatment to the *Gloriosa superba* culture accumulated 85µg/g dry weight compared to 22µg/g dry weight for the control. Continuous extraction of colchicine using Amberlite resin as a solid phase extraction has been demonstrated to increase production. Combination of precursor feeding with *in situ* extraction led to significant enhancement of colchicine production and recovery using a continuous *in situ* extraction system. Combination of precursor feeding and adsorption on an Amberlite column significantly enhanced the total colchicine accumulation at the end of the batch culture by 3.5 and 3.7 fold in a culture supplemented with 0.5mM coumaric acid or 3-phenyl propionic acid respectively.

The growth rate of *Gloriosa superba* root culture in liquid medium can be estimated by simple, reliable and non-invasive methods by measuring the conductivity and the amount of soluble carbohydrate in the liquid medium.

A simple model for enhanced production of colchicine has been developed. In this study the two types of bioreactor, air-lift bioreactor and trickle column bioreactor systems were examined for growing *Gloriosa superba* root tissues on a pilot scale.

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Abbreviations

ATP	Adenosine Tri Phosphate
BAP	Benzylaminopurine
BSA	Bovine serum albumin
°C	Degree Celsius
Ci	Curie
cm	Centimetre
Conc.	Concentration
CV	Coefficients of variation
C4H	Cinnamic acid 4-hydrosylase
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
Ed(s)	Editor(s)
ELISA	Enzyme-Linked Immunosorbent Assay
<i>et al.</i>	And others
FDA	Fluorescein Diacetate
FW	Fresh weight
DW	Dry weight
2,4-D	2,4-Dichlorophenoxyacetic acid
<i>g</i>	gravity
<i>g</i>	Gram
h	Hour (s)
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
IBA	Indoyl Butric Acid
l	Litre
Log	Logarithm (common)
m	milli(10^{-3})
M	Molar
min	Minute
ml	Millilitre
MS	Murashige and Skoog Medium

μ	micro(10^{-6})
NAA	Naphthalene acetic acid
NaCl	Sodium chloride
NaOH	Sodium hydroxide
nm	Nanometer
OD	Optical Density
OPD	O- phenylenediamine Dihydrochloride
PAL	phenylalanine ammonia lyase
PBS	Phosphate buffered saline
psi	Pound per square inch
rpm	Revolutions per Minute
s	Second (s)
SAM	S-adenosylmethionine
SAH	S-adenosyl-homocysteine
SH	Schenk and Hildebrandt Medium
TDZ	Thidiazuron
TYD	Tyrosine decarboxylase
v/v	Volume per volume (concentration)
w/v	Weight per volume (concentration)

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Chapter One

GENERAL INTRODUCTION

1.1 Plant Natural products

Throughout history, there has been an essential intimate relationship between human and plants, principally for food, but also for shelter, clothing and treatment of sickness. The higher plants have been the key to human welfare from the earliest dawn of human existence. Food, materials, fragrances, dyes and medicine have been derived from higher plants. The plant kingdom has provided a wide variety of natural products with diverse chemical structures and a vast array of biological activities, many of which have found applications in the health sciences. Even today, the World Health Organisation estimates that up to 80% of people still rely mainly on traditional remedies such as herbs for their medicines (Tripathi and Tripathi, 2003). Higher plants synthesise a tremendous array of chemical structures, probably greater than any other group of living organisms. The number of chemical structures in plants is estimated to be nearly fourfold greater than in the microbial kingdom. World-wide, 121 clinically useful prescription drugs are derived from plants (Payne *et al.*, 1992). Pharmaceuticals, flavours, and fragrances are examples of high-value products that represent a multibillion dollar industry (Rajia *et al.*, 1993). Stafford *et al.*, (1986) reported that approximately one out of four prescribed medicines is recovered from plants. Many of these products are derived by direct extraction from harvested plants.

1.1.1 Secondary metabolites

Secondary plant metabolites, as the name implies, are not primary products of the biosynthetic pathways and have been defined as compounds not produced by all plants, whose function are unknown. However, they may play a crucial role in the survival of the plant as a whole and generally have considerable biological activity (Nickell, 1962). The biosynthetic pathways, function and biological activity of most classes of secondary metabolites of plants are described in a number of publications (Nakanishi, 1974; Evans, 1996; Dewick, 2001).

Plant secondary metabolites have been used for centuries in traditional medicine. Nowadays, they correspond to valuable compounds such as pharmaceuticals, cosmetics, fine chemicals, or more recently nutraceuticals. Recent surveys have established that in western countries, where chemistry is the backbone of the pharmaceutical industry, 25% of the molecules used are of natural plant origin (Payne *et al.*, 1992). Secondary metabolites are limited in distribution in the plant kingdom, typically being restricted to a particular family group. Secondary metabolites include alkaloids, terpenes, phenolic compounds, tannins and glucosides. Secondary metabolites have no apparent function in plant primary metabolism but may serve as chemical defences against microorganisms, insects, predators and even other plants, and for other reasons yet to be discovered. Many of these plant secondary metabolites are pharmacologically active, and have found use in traditional medicine over thousands of years. A good example of a widespread metabolite family is given by phenolics; because these molecules are involved in lignin synthesis, they are common to all higher plants. However, other compounds such as alkaloids are sparsely distributed in the plant kingdom and are much more specific to defined plant genus and species. Secondary metabolite compounds usually have very complicated structures.

There are currently two major approaches to the manufacture of such fine chemicals: Firstly through the harvesting and extraction of, typically purpose grown, whole plants, and secondly through, often sophisticated, synthetic organic chemistry. Where products are still obtained from the whole plant, this is usually because the substance is difficult, or impossible to synthesise chemically. Consequently, in many cases organic synthesis is not cost effective, and extraction from field-grown plants has been the major method used to economically produce these important secondary metabolites (Balandrin *et al.*, 1985)

1.1.1.1 Alkaloids

A group of organic nitrogenous compounds with complex chemical structures isolated from some plants are called alkaloids (Kutchan, 1995) and are found in approximately 20% of plant species (Facchini, 2001). There are very many of them, and they have varied structures. Benzyloquinoline alkaloids are a very large and diverse class of alkaloid. This group contains such varied physiologically active members as colchicine, emetine (an antiamoebic), morphine and codeine. Due to the complex structure of the benzyloquinoline alkaloids they are still isolated from plants for many years (Zenk *et al.*, 1988; Dewick, 2001). Now, many attempts have been made to produce alkaloids in plant tissue cultures of alkaloid-producing plants. The yields of alkaloids produced, if any, have been typically low compared to the parent plant and not cost effective by comparison with field grown plants..

1.2 Colchicine

Colchicine is the major alkaloid of autumn crocus (*Colchicum* species) and other members of the Liliaceae family such as the glory lily (*Gloriosa superba*), and is named after *Colchicum*, a plant containing colchicine.

Colchicine can be described, without doubt, as one of the most interesting alkaloids: inspection of colchicine structure (Figure 1.1) has shown no obvious relationship to any other known families of plant alkaloids, and it appeared to be the “odd men out” (McDonald *et al.*, 1998).

1.2.1 Colchicine presence in the plant kingdom

Most Colchicaceae contain colchicine. The Colchicaceae (Figure 1.1) is a Monocotyledonae family of flowering plants. and it includes approximately 225 species in 22 genera. The subclass family Liliaceae include *Burchardia*, *Colchicum*, *Gloriosa*, *Hexacyrtis*, *Iphigenia*, *Littonia*, *Neodregea*, *Onixotis*, *Ornithoglossum*, *Sandersonia*, *Wurmbea*. (Evans, 1996).

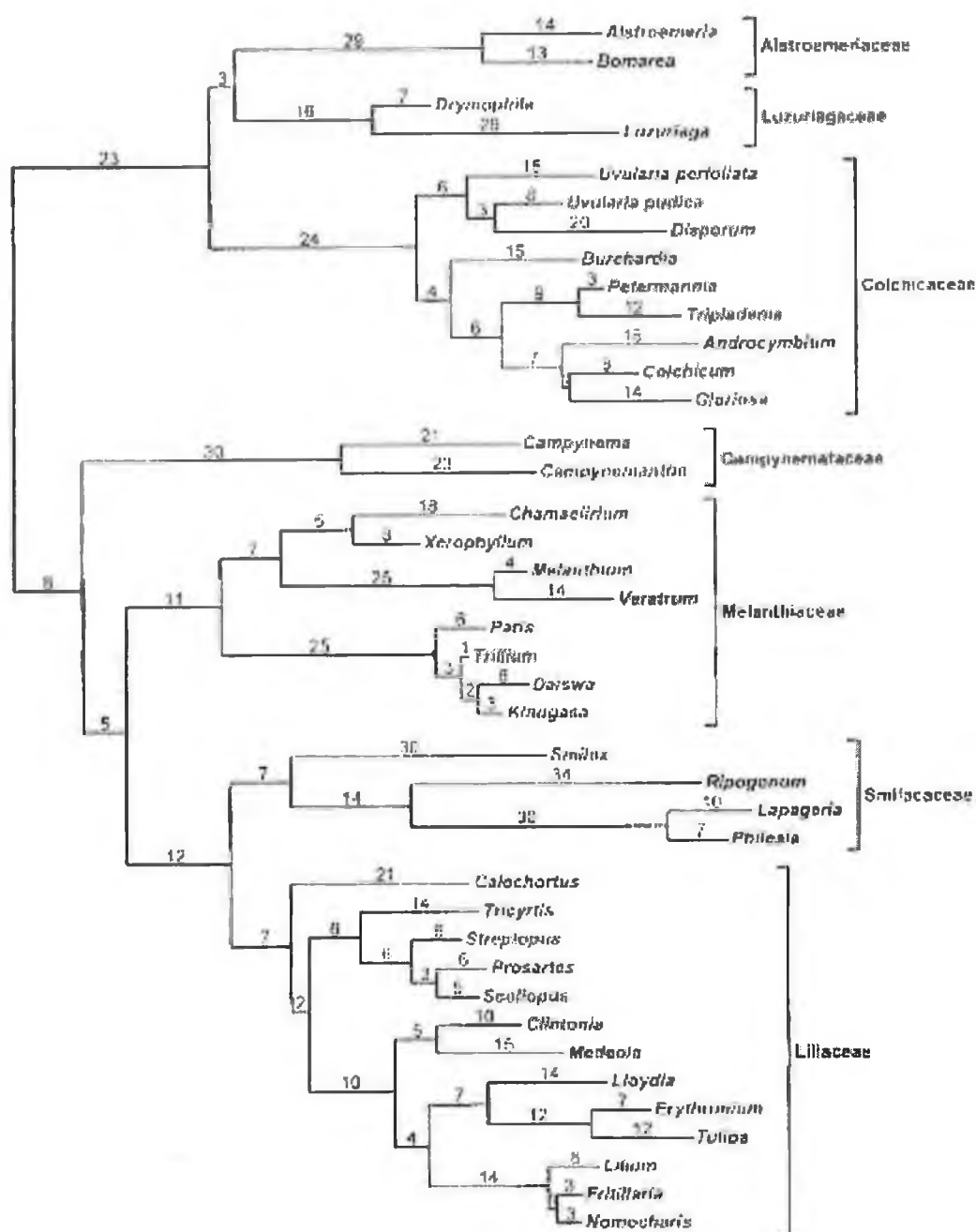


Figure 1.1: Phylogeny of Liliales family, adapted from Vinnersten and Bremer (2001).

Colchicine is the principle alkaloid of *Colchicum autumnale* and *Gloriosa superba*. Moreover colchicine was found in some other genera belonging to the Liliaceae family, namely *Androcymbium*, *Merendera*, *Sandersonia* and *Littoria* (Wildman, and Pursey 1968; Finnie and Vanstaden, 1991; Evans, 1996; Ellington, et al. 2003).

The main commercial source for colchicine is obtained from two members of the lily family (Liliacea), *Colchicum* species, and *Gloriosa superba*, as they have a higher colchicine content than the other species (Finnie and Vanstaden, 1991).

The plants of the genus *Colchicum* have been known for more than 3000 years for their marked biological effects. In various regions *Colchicum* is known as Naked-ladies, and the Autumn Crocus or Meadow Saffron. The bulbs (corms) produce green foliage in the spring. The leaves may be up to 10 to 15 inches long and 3 to 4 inches wide. And the foliage remains until summer. Flowers may be white, pink, or lavender. *Colchicum* species produce alkaloids which have been included in many pharmacopoeias. The genus *Colchicum* includes 42 species. Most of which are endemic in Southern Europe, northern Africa and the Middle East (Ondra *et al.*, 1995). Colchicine can also be extracted from *Gloriosa superba* (sometimes called the *Glory Lily*) from tropical Asia and Africa.

1.2.2 History of Colchicine

Despite the fact that it is poisonous, Colchicine has been used as a medical plant for more than 3000 years. Seeds of *Colchicum* plants are believed to have been used medicinally by ancient Egyptians due to the abundance of the plants in northern Africa. Its scientific name comes from the Greek Colchis, an ancient country on the easternmost coast bordering on the black Sea, where they were found for the first time (Frankova, *et al.* 2005). Autumn crocus was recommended for pain relief of the joints by Alexander of Tralliers in the 6th century, it was used to treat gout in the sixth century (Hartung, 1954), and in 11th century Persia it is being used by Avicenna (Silverman, 1992). *Colchicum* corms are mentioned in the 1618, 1627, 1632, 1639 editions of the London pharmacopoeias after which it was removed but it did reappear in the 1788 edition (Evans, 1996).

In 1780 the French army officer Nicolas Husson reintroduced treatment with *Colchicum autumnale* because of its particular efficacy in acute gout (Levy, *et*

al. 1991). *Colchicum* seeds were introduced as an effective treatment of gout in 1820 (Evans, 1996). And colchicine is still one of the drugs of choice for treatment of acute gout. Colchicine was first isolated in 1820 by Pelletier and Caventou, (Reviewed by Man Tat Yu 1995).

Clewer, *et al* (1915) isolated colchicine from dried tubers of *Gloriosa superba*. A number of researchers have isolated colchicine from *Gloriosa superba* (Sarin *et al.*, 1974; Thakur *et al*, 1975)

The richest natural source of Colchicine is found in the seeds and corms of two genres *Colchicum* and *Gloriosa*. *Colchicum autumnale* seeds contain the highest concentration in the plant, which is 0.6-1.2% of dry weight. The corm contains 0.6% colchicine (Evans, 1996). On other hand, Finnie and Vanstaden (1991) reported that *Gloriosa superba* seeds gave a yield of 0.61% of dry weight and tubers from the plants from which the seeds were obtained yielded 0.9% of dry weight. It is important to note that all parts of the plant contain colchicine and due to limited supplies of the seed and corm, efforts have been made to isolate it from the flowers and leaves (Evans, 1996).

1.2.3 Colchicine mechanism of action

The ability of colchicine to bind to microtubules was reported in 1960 (Frankova, *et al.* 2003). The mechanism of action of colchicine is by binding to tubulin, the protein subunit of microtubules, where it blocks microtubule polymerization and therefore inhibits cell processes that depend on microtubule function. Due to the inhibitory action of colchicine on microtubule polymerization, treated cells are arrested in mitosis at the metaphase stage as microtubules are essential for moving chromosomes during cell division. The transport of vesicles is also affected (Capraro and Brossi, 1984).

Modern medicine uses colchicine as a therapeutically active alkaloid, which is known to have cancerostatic, antirheumatic, anti-inflammatory, antimitotic and emetic effects (Boye and Brossi, 1992; Martin and Shearn, 1987).

1.2.4 Medical uses of colchicine

The curative properties of *Colchicum* were known to the ancient civilizations of Greece, Egypt and India (Leete, and Nemeth, 1960). Colchicine has many medicinal uses, as follows:

1.2.4.1 Gout

Colchicine is still one of the more effective treatments for the intensive pain associated with Gout attack (Brossi, 1990; Baselt, and Cravey, 1989). Gout (also called gouty arthritis) is often thought of as a disease of rich living style and associated with too much uric acid in the blood (Hyperurecemia) which turn into crystals of monosodium urate which are then deposited in the joints and causing the common symptoms of Gout inflammation (severe pain, redness and swelling of the affected joints) specially in the feet and ankles. It prevents or relieves gout attacks by reducing inflammation.

1.2.4.2 Familial Mediterranean fever (FMF)

Colchicine is also used to treat familial Mediterranean fever (a rare congenital condition) that is an ethnically restricted genetic disease and affects the people in the Mediterranean area. It is characterised by episodes of fever with abdominal, chest, scrotum and skin pain and arthritis (Brossi, 1990; Steuer, et. al., 1997). In 1972 it was suggested that daily administration of colchicine may prevent the painful and disabling febrile attacks of (FMF). Since then the drug has become the mainstay for therapy of the disease (Levy *et al.*, 1991). Colchicine can be used as a minor diagnostic tool in FMF (Livneh and Langevitz, 2000)

1.2.4.3 Behcet's Disease

Behcet's disease is a multi-systemic disorder affecting people from the far eastern countries, eastern mediterranean countries and Japan. The disease usually affects adults between the ages of 20 and 40. Its cause is unknown and it can be characterised by oral and genital ulceration, skin lesion, pulmonary and renal manifestations, arthritis and central nervous system disorders (Onder and Gurer, 1999). Colchicine seems to be a suitable treatment for oral, genital and articular manifestation of the disease at a dose of 1.5mg/day (Sullivan *et al.*, 1998).

1.2.4.4 Miscellaneous diseases

Due to colchicine's anti-fibrotic and anti-inflammatory activities it has been used in the treatment of liver diseases such as primary biliary cirrhosis (PBC) and alcoholic hepatitis. Colchicine has also been proposed as a treatment for chronic hepatitis B (Lin *et al.* 1996).

1.2.5 Non-medical uses of colchicine

Colchicine can be used to artificially induce polyploidy in plants. Due to its antimitotic properties it will prevent spindle formation, at metaphase, of dividing cells. The chromosomes in the cell continue to divide with no sister cell formation (Evans, 1996) leading to doubling in chromosome number. The advantages of increased chromosome number include production of large flowers or fruit, increased hardness, increase in size of various organs or change in season of maturity. Colchicine treatment led to a sixty fold increase in valepotriates in *Valeriana wallichii* cell suspension culture. This was suggested not to be due to higher ploidy level (Evans, 1996) suggested a role of colchicine as an elicitor of secondary product formation.

1.2.6 Colchicine physical and chemical properties

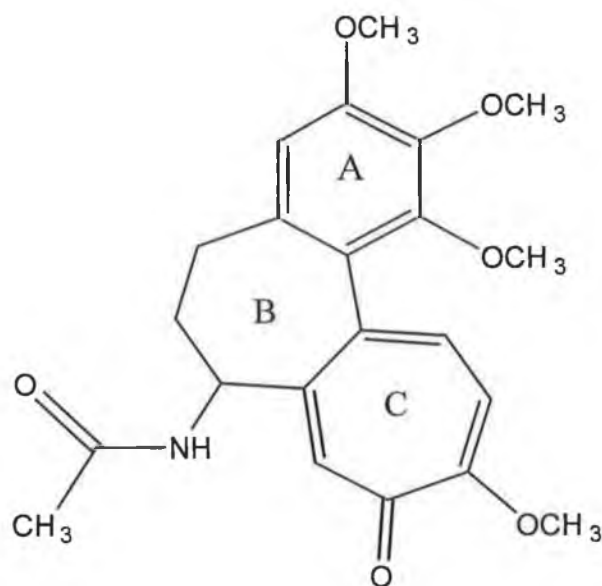


Figure 1.2: Chemical structure of Colchicine

Molecular Formula: C₂₂H₂₅NO₆

The chemical name for colchicine or Acetyltrimethylcolchicinic acid is:

(S)-N-(5,6,7,9-tetrahydro-1,2,3,10-tetramethoxy-9-oxobenzo (alpha) heptalen-7-yl) acetamide. (molecular weight 399.43). Colchicine consists of pale yellow scales or powder; it darkens on exposure to light. Colchicine is an alkaloid soluble in water, freely soluble in alcohol and in chloroform, and slightly soluble in ether.

1.2.7 Colchicine chemical synthesis

For years, synthetic chemists have been challenged with developing syntheses of plant natural products. However, often due to their structural complexity, the resulting multi-step syntheses have rarely found application in large scale production as required in pharmaceutical drug industry. For many years many attempts have been made to chemically synthesise colchicine. However the yield of colchicine is too low, and the production is not economically feasible compared to extraction from the plant. Table 1.5 illustrates the groups which have tried to synthesise colchicine.

Many compounds from plants have either defined chemical synthesis, or are far more expensive to produce by synthesis than by growing the plant. This is due to the stereo-specificity of the plant synthesis (Roberts, 1988).

Table1.1: Chemical synthesis of colchicine carried out by different groups.

Synthesis	Starting material	(±)-Desacetaminocolchicine	(±)-Colchicine
Eschenmoser	pyrogallol	17 steps, 0.234%	24 steps, 0.00243%
van Tamelen	pyrogallol	14 steps, 0.0425%	21 steps, 0.000442%
Nakamura	monomethylpyrogallol	N/A	22 steps, 0.0000675%
Woodward	aminocrotonate	N/A	24 steps, ???
Martel	trimethoxyphenylpropylchloride	15 steps, 0.298%	22 steps, 0.00310%
Scott	pyrogallol	8 steps, 0.000462%	15 steps, 0.0000481%
Boger	pyrogallol	11 steps, 3.66%	18 steps, 0.038%
Matsui	trimethoxyphenylacetaldehyde	7 steps, 2.36%	14 steps, 0.0245%
Kato	trimethoxyphenylpropanol	8 steps, 1.25%	15 steps, 0.013%
Tobinaga	bis(aryl)propane	6 steps, 34.4%	12 steps, 1.02%
Evans	3,4-dimethoxyphenol	7 steps, 23.6%	13 steps, 3.03%
Banwell	trimethoxybenzaldehyde	17 steps, 0.810%	14 steps, 1.1% (84% ee)

Adapted from http://daecr1.harvard.edu/pdf/smnr_1997-1998_Michael_Forrest.pdf

1.3 Alternative options for producing secondary metabolites

Currently, many secondary metabolites are produced solely from substantial quantities of whole plant parts. Often the plants cultivated are tropical or subtropical, and the cultivation periods between planting and harvesting make selection of high-yielding strains difficult, thus resulting in expensive drugs. In spite of these difficulties and costs, the extraction of medicines from wild or cultivated plants is still widely practiced because of the lack of credible alternatives (DiCosmo and Misawa, 1995). The levels of secondary metabolites in plants are affected by many factors, including climate change and pathogens and also may vary considerably depend on the plant species. Traditional agricultural methods often require months to years to obtain a crop. Cultivation may take decades such as for the taxol yielding, *Taxus brevifolia* trees (Kieran *et al.* 1997). Obtaining sufficient supplies of

appropriate plant materials for the isolation of secondary metabolites has become more difficult and expensive in recent years. This is because many of these species are endangered due to severe over collection. Therefore, in addition to the two routes of natural product synthesis described above, new routes for production of secondary products in large quantity which meet the new quality standards appear necessary. This has promoted industries, as well as scientists, to consider the possibilities of investigation into plant cell cultures as an alternative supply for the production of plant natural products. Indeed, the use of plant cell and tissue culture for production of secondary metabolites under controlled conditions, removed from the various environmental constraints of the plant's natural habitat, has many advantages. Plant cell and tissue culture could provide a means for increasing product yields far beyond those already obtained in the whole plant (Dicosmo and Misawa, 1995).

It has been considered for a long time that undifferentiated cells, such as callus or cell suspension cultures were not able to produce secondary compounds, unlike differentiated cells or specialized organs (Krikorian and Steward 1969). Zenk and co-workers experimentally demonstrated that this theory was wrong, as they could observe dedifferentiated cell cultures of *Morinda citrifolia* yielding one gram of anthraquinones per litre of medium (Zenk *et al.* 1975). This finding opened the gate to the *vitro* culturists who extensively studied the possible use of plant cultures for the production of secondary compounds of industrial interest (mainly pharmaceuticals and dyes). The cells of plant cultures are thought to be totipotent, that is they possess all the genetic information necessary for the functioning of the entire plant including secondary metabolism. This totipotency is readily demonstrated by their ability to regenerate many intact plants (e.g. carrot and tobacco) from cell culture lines (Grambow *et al.*, 1972). Thus under suitable conditions, cell cultures should be able to produce secondary metabolites either naturally or by the transformation of exogenously supplied precursors. Explants of an alkaloid producing plant species, cultured *in vitro*, has been found to retain the capacity

to synthesise alkaloids identical to that in the intact plant. Sometimes, a high yield of secondary metabolites are observed in tissues grown as callus masses produced during tissue differentiation (Benavides and Caso 1993)

1.4 Plant cell and Tissue culture

Plant cell culture is an established alternative to the harvest and extraction of whole plant material for the production of valuable secondary metabolites. Plant cell and tissue culture involves the removal of plant tissue (explant) from a plant species of interest under sterile conditions and placing it in a medium containing essential organic and inorganic compounds which facilitate the growth and division of the plant cells. Therefore, plant cell culture may offer a viable alternative to direct extraction of natural products from plants. Plant cells which maintain their *in vivo* biochemical pathways can be grown in liquid or semi-solid media using a variety of bioreactors ranging from simple shaker flasks to complex, well-controlled bioreactors. Suspension cultures of plant cells in liquid media are preferred over semi-solid media for production purposes because the cultures grow more quickly and are easier to manipulate (Dornenburg and Knorr, 1996B). There are a series of distinct advantages to producing valuable secondary metabolites in plant cell culture, rather than the whole crop plant. These include the following: 1- synthesis of bioactive secondary metabolites is run under a controlled environment, independent from climatic and soil conditions; 2- negative biological influences that affect secondary metabolites production in nature are eliminated (microorganisms and insects); 3- it is possible to select strains and cell line with higher production of secondary metabolites; 4- The process may be precisely controlled with easy manipulation for optimisation of growth and production; 5- The time needed for production may be significantly less than that necessary for agricultural production; 6- product recovery and purification may be simpler when product is secreted to the extracellular medium.

Plant cell culture is viewed as a potential means of producing useful plant products and may circumvent conventional agriculture, with all its attendant problems and variables. These problems include: environmental factors, disease, political and labour instabilities in the producing countries (often Third World countries), uncontrollable variations in the crop quality, inability of authorities to prevent crop adulteration, losses in storage, and handling. Plant cell and tissue culture is one of the growing areas of biotechnology because of its potential to generate improved crops and production of important natural products. Plant tissue culture has an important role to play in the production of secondary metabolites and in the manipulation of plants for improved agronomic performance. We should perhaps begin seriously to consider a large scale plant cell culture somewhat analogous to the production of chemicals and some proteins from microbial and animal cell cultures.

The use of plant cell culture systems to produce valuable metabolites is generally considered a feasible technology, although there has been little commercial success with only a few compounds produced in this manner (Tabata and Fujita 1985). The reasons that such processes are not successful include low product yields, lack of biosynthetic stability (DiCosmo and Misawa, 1995), and difficulties with scale-up (Zhang and Furusaki, 1999). Possibly the most promising application of plant tissue cultures may be their use in the study of secondary metabolism in the plant (Verpoorte et al. 1994).

1.4.1 Historical background

It is believed that Hildebrandt (1854-1945) was the pioneer and father of plant tissue culture. He was the first person to culture isolated, fully differentiated cells as early as 1898 (reviewed by Bhojwani and Razdan, 1983). He used palisade tissue of *Lamium purpureum* and *Eichornia crassipes*. In 1934 the first plant tissue culture was grown from the isolated roots of tomato seedlings by White (reviewed by Bhojwani and Razdan, 1983). Muir *et al.*, (1958), were the first to grow single cells of tobacco successfully. They placed single cells

on a filter paper which was laid on a mother callus. In 1957, Skoog and Miller demonstrated that tobacco pith tissue grown on a tissue culture medium (*In vitro*) could be made to form callus tissue. The production of plant metabolites by callus and cell suspension cultures have been carried out on an increasing scale since the end of the 1950's. Since the 1950's large scale plant cell culture has been seen as having major potential in the industrial production of plant secondary metabolites (Routier and Nickell, 1956); subsequently the large scale cultivation of tobacco and a variety of plant cells was examined by (Tulecke and Nickell, 1959).

Plant cell culture technologies were introduced at the end of 1960's as a possible tool for both studying and producing plant secondary metabolites (Vanisree, et al. 2004). Various workers have drawn attention to the possibility of culturing plant cells on a large scale for production of important compounds (Routier and Nickell 1956; Tulecke and Nickell, 1959; Nickell, and Tulecke 1960).

Since the first report in 1956 by Routien and Nickell of the production of secondary metabolites in liquid culture, many plant cell cultures have been established and their secondary metabolites investigated while research to date has succeeded in producing a wide range of valuable secondary products in callus and cell suspension cultures. The scheme of production of some important and bioactive plant secondary metabolites in cell cultures is presented in Table 1.2.

Table 1.2: Bioactive secondary metabolites from plant cell cultures

Plant name	Active ingredient	Culture type	Reference
<i>Agave amaniensis</i>	Saponins	Callus	Andriany et al., 1999.
<i>Ailanthus altissima</i>	Alkaloids	Suspension	Anderson et al., 1987.
<i>Ailanthus altissima</i>	Camphorone alkaloids	Suspension	Anderson et al., 1986.
<i>Allium sativum</i> L.	Alliin	Callus	Malpathak and David., 1986.
<i>Aloe saponaria</i>	Tetrahydroxyanthracene glucosides	Suspension	Yagi et al., 1983.
<i>Ambrosia tenuifolia</i>	Alkaloids	Callus	Goleniowski and Trippi., 1999.
<i>Anchusa officinalis</i>	Rosmarinic acid	Suspension	De-Eknamkul and Ellis, 1985.
<i>Brucea javanica</i> (L.) Merr.	Camphorone alkaloids	Suspension	Liu et al., 1990.
<i>Bupleurum falcatum</i>	Saccharonates	Callus	Wang and Huang, 1982.
<i>Camellia Sinensis</i>	Theanine, γ -glutamyl derivatives	Suspension	Onihara and Furuya, 1990.
<i>Canavalia ensiformis</i>	L-Canavanine	Callus	Ramirez et al., 1992.
<i>Capsicum annuum</i> L.	Capsaicin	Suspension	Johnson et al., 1990.
<i>Cassia acutifolia</i>	Anthraquinones	Suspension	Nazif et al., 2000.
<i>Catharanthus roseus</i>	Indole alkaloids	Suspension	Moreno et al., 1993.
<i>Catharanthus roseus</i>	Catharanthine	Suspension	Zhao et al., 2001.
<i>Choisya ternata</i>	Furoquinoline alkaloids	Suspension	Sejourne et al., 1981.
<i>Chrysanthemum cinerariaefolium</i>	Pyrethrins	Callus	Rajasekaran et al., 1991.
<i>Chrysanthemum cinerariaefolium</i>	Chrysanthemic acid and pyrethrins	Suspension	Kueh et al., 1985.
<i>Cinchona</i> L.	Alkaloids	Suspension	Koblitz et al., 1983.
<i>Cinchona robusta</i>	Robustaquinones	Suspension	Schripsema et al., 1999.
<i>Cinchona</i> spe.	Anthraquinones	Suspension	Wijnsma et al., 1985.
<i>Cinchona succirubra</i>	Anthraquinones	Suspension	Khouri et al., 1986.
<i>Citrus</i> sp.	Naringin, Limonin	Callus	Barthe et al., 1987.
<i>Coffea arabica</i> L.	Caffeine	Callus	Waller et al., 1983.
<i>Cornus kousa</i>	Polyphenols	Suspension	Ishimaru et al., 1993.
<i>Corydalis ophiocarpa</i>	Isoquinoline alkaloids	Callus	Iwasa and Takao, 1982.
<i>Croton subhyratus</i> Kurz	Platanol.	Callus	Morimoto and Murai, 1989.
<i>Cruciata glabra</i>	Anthraquinones	Suspension	Dornenburg and Knorr, 1996.
<i>Cryptolepis burchanani</i> Roem. & Schult	Cryptosin	Callus	Venkateswara et al., 1987.
<i>Digitalis purpurea</i> L.	Cardenolides	Suspension	Hagimori et al., 1982.
<i>Dioscorea deltoidea</i>	Diosgenin	Suspension	Heble and Straba, 1980.
<i>Dioscorea doryophora</i> Hance	Diosgenin	Suspension	Huang et al., 1993.
<i>Duboisia leichhardtii</i>	Tropate alkaloids	Callus	Yamada and Endo, 1984.
<i>Ephedra</i> spp.	L- Ephedrine D-pseudoephedrine	Suspension	O'Dowd et al., 1993.

continued ...

<i>Eriobanva japonica</i>	Triterpenes	Callus	Taniguchi et al., 2002.
<i>Eucalyptus tereticornis</i> SM.	Steroids and Phenolic compounds	Callus	Venkateswara et al., 1986.
<i>Eucommia ulmoides</i>	Chlorogenic acid	Suspension	Wang et al., 2003.
<i>Fumaria capreolata</i>	Isoquinoline alkaloids	Suspension	Tanahashi and Zeuk, 1985.
<i>Gentiana</i> sp.	Secoiridoid glycosides	Callus	Skrzypczak et al., 1993.
<i>Ginkgo biloba</i>	Ginkgolide A	Suspension	Carrier et al., 1991.
<i>Glehnia littoralis</i>	Flavonoxanthone	Suspension	Kitamura et al., 1998.
<i>Glycyrrhiza echinata</i>	Flavonoids	Callus	Ayabe et al., 1986.
<i>Glycyrrhiza glabra</i> var. <i>glandulifera</i>	Triterpenes	Callus	Ayabe et al., 1990.
<i>Hyoscyamus niger</i>	Tropane alkaloids	Callus	Yamada and Hashimoto, 1982.
<i>Isoplexis isabellina</i>	Anthraquinones	Suspension	Arrebola et al., 1999.
<i>Linum flavum</i> L.	5-Methoxyxanthophyllanthoxin	Suspension	Uden et al., 1990.
<i>Lithospermum erythrorhizon</i>	Stilbene derivatives	Suspension	Fujita et al., 1981.
<i>Lithospermum erythrorhizon</i>	Stilbene derivatives	Suspension	Fukui et al., 1990.
<i>Lycium chinense</i>	Cercoside	Suspension	Jang et al., 1998.
<i>Morinda citrifolia</i>	Anthraquinones	Suspension	Zenk et al., 1975.
<i>Morinda citrifolia</i>	Anthraquinones	Suspension	Bassetti et al., 1995.
<i>Mucuna pruriens</i>	L-DOPA	Suspension	Wichers et al., 1993.
<i>Mucuna pruriens</i>	L-DOPA	Callus	Brain K. R., 1976.
<i>Nandina domestica</i>	Alkaloids	Callus	Ikura and Irokawa, 1988.
<i>Nicotiana rustica</i>	Alkaloids	Callus	Tabata and Hiraoka, 1976.
<i>Nicotiana tabacum</i> L.	Nicotine	Suspension	Mantell et al., 1983.
<i>Nothapodytes foetida</i>	Campothecin	Callus	Thengane et al., 2003.
<i>Ophiorrhiza pumila</i>	Campothecin related alkaloids	Callus	Kitajima et al., 1998.
<i>Panax ginseng</i>	Saponins and Sapogenins	Callus	Furuya et al., 1973.
<i>Panax notoginseng</i>	Ginsenosides	Suspension	Zhong and Zhu, 1995.
<i>Papaver bracteatum</i>	Thebaine	Callus	Day et al., 1986.
<i>Papaver somniferum</i> L.	Alkaloids	Callus	Furuya et al., 1972.
<i>Papaver somniferum</i>	Morphine, Codeine	Suspension	Siah and Doran, 1991.
<i>Peganum harmala</i> L.	β -Carboline alkaloids	Suspension	Sasse et al., 1982.
<i>Phytolacca americana</i>	Betacyanin	Suspension	Sakuta et al., 1987.
<i>Picrasma quassioides</i> Bennett	Quassin	Suspension	Scragg and Allan, 1986.
<i>Podophyllum hexandrum</i> royle	Podophyllotoxin	Suspension	Uden et al., 1989. Chattopadhyay et al., 2002.
<i>Polygala amarella</i>	Saponins	Callus	Desbene et al., 1999.
<i>Polygonum hydropiper</i>	Flavonoids	Suspension	Nakao et al., 1999.
<i>Portulaca grandiflora</i>	Betacyanin	Callus	Schroder and Bohm, 1984.
<i>Ptelea trifoliata</i> L.	Dihydrofuro [2,3-b] quinoxaline alkaloids	Callus	Petit-Paly et al., 1987.
<i>Rauvolfia sellowii</i>	Alkaloids	Suspension	Rech et al., 1998.
<i>Rauvolfia serpentina</i> Benth.	Reserpine	Suspension	Yamamoto and Yamada, 1986.

continued ...

<i>Rauvolfia serpentina</i> x <i>Rhazya stricta</i> Hybrid plant	3-Oxo-rhazulinam	Callus	Gerasimenko et al., 2001.
<i>Ruta</i> sp.	Aeridene and Furoquinoline alkaloids and coumarins	Callus	Baumert et al., 1992.
<i>Salvia fruticosa</i>	Rosmarinic acid	Callus & suspension	Karam et al., 2003
<i>Salvia miltiorrhiza</i>	Lithospermic acid B and Rosmarinic acid	Callus	Morimoto et al., 1994.
<i>Salvia miltiorrhiza</i>	Cryptotanshinone	Suspension	Miyasaka et al., 1989.
<i>Sapium sebiferum</i>	Tannins	Callus & suspension	Neera and Ishimaru, 1992.
<i>Scopolia parviflora</i>	Alkaloids	Callus	Tabata et al., 1972.
<i>Scutellaria columnae</i>	Phenolics	Callus	Stojakowska and Kistel, 1999.
<i>Solanum chrysotrichum</i> (Schldl.)	Solanadene saponins	Suspension	Villareal et al., 1997.
<i>Solanum laciniatum</i> Ait	Solanodine	Suspension	Chandler and Dodds, 1983.
<i>Solanum paludosum</i>	Solanargine	Suspension	Badaoui et al., 1996.
<i>Stizolobium hassjoo</i>	1, DOPA	Suspension	Huang et al., 2002.
<i>Tabernaemontana divaricata</i>	Alkaloids	Suspension	Sierra et al., 1992.
<i>Taxus</i> spp.	Taxol	Suspension	Wu et al., 2001.
<i>Taxus baccata</i>	Taxol, baccatin III	Suspension	Cusido et al., 1999.
<i>Taxus cuspidata</i>	Taxoids	Suspension	Ketchum et al., 2003.
<i>Tecoma sambucifolium</i>	Phenylpropanoid glycosides	Callus	Pletsch et al., 1993
<i>Thalictrum minus</i>	Berberin	Suspension	Kobayashi et al., 1987.
<i>Thalictrum minus</i>	Berberin	Suspension	Nakagawa et al., 1986.
<i>Torreya nucifera</i> var. <i>radicans</i>	Diterpenoids	Suspension	Orihara et al., 2002.
<i>Trigonella foenum-graecum</i>	Saponins	Suspension	Brain and Williams, 1983.

Adapted from Mulabagal, and Tsay, (2004)

1.4.2 Application of large scale plant tissue culture

The ability to produce secondary metabolites in a predictable quantity would be a major benefit to many manufacturers. There has been considerable interest in plant cell cultures as a potential alternative to traditional agriculture for the industrial production of secondary plant metabolites (Butcher, 1977; Morris and Fowler, 1980; Sato and Yamada, 1984).

The production of secondary metabolites in a large scale bioreactor is therefore attractive alternative method for high value biochemicals production, with the possibility of controlling production according to demand (Schiel and Berlin, 1987; Kraus and Reinhard 1989). Thus, the production of useful and

valuable secondary metabolites in large bioreactors located in the consuming country is an attractive proposal.

The ability of the secondary metabolites to be released from the intracellular cell suspension should decrease the feedback inhibition and that could be used for large-scale plant cell and tissue cultures. The advantage of this method is that it can ultimately provide a continuous, reliable source of natural products. The basic technologies for obtaining high yields of specific secondary metabolites from large-scale cultures are still being developed (Street, 1977); Yeoman, *et al.*, 1980; Berlin, 1984; Shuler *et al.*, 1984).

Plant cell culture technology shows promise for the large-scale production of valuable plant products, but the commercial use of plant cell cultures is not routine because of difficulties in achieving acceptable, reproducible product levels in reasonable periods of time. However, this technology is still being developed and despite the advantages outlined above, there are a variety of problems to be overcome before it can be adopted on a wide scale for the production of useful plant secondary metabolites. Over 50 different secondary metabolites are synthesized in remarkable amounts by plant cell cultures (Stroble *et al.*, 1991). These are examples of compounds which are produced in proportions equal to or higher than in the intact plant from which the cells were originally obtained (Table 1.3).

The disadvantage of *In vitro* plant culture is that large-scale production is not as easy as microorganisms and yeasts, and the yield is economically insufficient and expensive in many cases.

Table 1.3: Secondary metabolites produced in high levels by plant cell cultures.

Compound	Plant Species	Yield (% dry wt)	
		Cell Culture	Whole Plant
Shikonin	<i>Lithospermum erythrorhizon</i>	20 (s*)	1.5
Ginsenoside	<i>Panax ginseng</i>	27 (c)	4.5
Anthraquinones	<i>Morinda citrifolia</i>	18 (s)	2.2
Ajmalicine	<i>Catharanthus roseus</i>	1.0 (s)	0.3
Rosmarinic acid	<i>Coleus blumei</i>	15 (s)	3
Ubiquinone-10	<i>Nicotiana tabacum</i>	0.036 (s)	0.0003
Diosgenin	<i>Dioscorea deltoidea</i>	2 (s)	2
Benzylisoquinoline alkaloids	<i>Coptis japonica</i>	11 (s)	5–10
Berberine	<i>Thalictrum minor</i>	10 (s)	0.01
Berberine	<i>Coptis japonica</i>	10 (s)	2–4
Anthraquinones	<i>Galium verum</i>	5.4 (s)	1.2
Anthraquinones	<i>Galium aparine</i>	3.8 (s)	0.2
Nicotine	<i>Nicotiana tabacum</i>	3.4 (c)	2.0
Bisoclaurine	<i>Stephania cepharantha</i>	2.3 (s)	0.8
Triptolide	<i>Tripterygium wilfordii</i>	0.05 (s)	0.001

*s = suspension; c = callus

Adapted from DiCosmo and Misawa (1995)

1.5 Approaches to increase cell culture productivity

Early attempts at growing plant cells in culture and isolating their secondary metabolites often failed, primarily due to the control and operation of the culture systems using the same techniques employed for microbial systems, where significant differences in cell structure and growth requirements between the two types of cells were not taken into account. Recent research has begun to develop strategies for overcoming these problems and some promising results have been obtained with optimizing the cultural conditions, selecting high-producing strains, and employing precursor feeding, transformation methods, hairy root cultures, immobilised cells and techniques designed to encourage the excretion of the derived products into the medium (DiCosmo and Misawa, 1995). For plant cell culture systems to become economically viable, it is important to develop methods that would allow for consistent generation of high yields of products from cultured cells (Berlin and

Sasse, 1985). Many factors influence the production of secondary metabolites by cultured plant cells. Cell line selection, product induction schemes such as production medium formulation (Barz *et al.* 1977; Kutney *et al.*, 1983); Stafford *et al.*, 1985; Morris, 1986), transformed and organ cultures (Rhodes *et al.*, 1986B), precursor feeding, and elicitation, with many elicitors used to increase natural product formation in plant cell cultures for biotechnological purposes (Eilert *et al.* 1985; Szabo *et al.*, 1999). Another means to enhance natural product accumulation is feeding plant cell cultures with commercially available or easily extractable metabolic precursors. Application of an early precursor, the amino acid L-phenylalanine, to cell cultures of *Linum flavum* resulted in a three to fivefold increase in the levels of 6-methoxypodophyllotoxin (Van Uden *et al.*, 1990). On-line product harvesting, to increase the rate of secondary metabolite synthesis, has been often used (Payne and Shuler, 1988), together with other product-enhancing steps such as elicitation or precursor feeding. Thus, a variety of approaches are being investigated by many researchers to increase productivity of useful plant metabolites in plant cell cultures.

Separation of the product for continuous cultivation and production of active secondary metabolites is another approach, and for isolation purposes, the metabolites have to be released into the culture medium rather than be retained within the cells (Evans, 1996).

1.6 Commercial application of plant cell culture

1.6.1 Pharmaceutical application of plant tissue culture

For the majority of world's population medicinal plants are the most exclusive source of life saving drugs. The dramatic decrease in plant resources, ecological problems and high labour costs for cultivation of plants may lead to the increasing use of plant cell cultures for the production of these high value substances, Table 1.4 Illustrates the important pharmaceutical drugs derived from plants.

Table 1.4: Essential drugs derived from a plant origin

Product	Use	Plant species	Cost (US\$ per kilogram)
Ajmalicine	Antihypertensive	<i>Cath. roseus</i>	37,000
Artemisinin	Antimalarial	<i>Artemisia annua</i>	400
Ajmaline	–	<i>Ra. serpentina</i>	75,000
Acematine	–	<i>Acotinum</i> spp.	n/a
Berberine	Intestinal ailment	<i>C. japonica</i>	3250
Camptothecin	Antitumour	<i>Camptotheca acuminata</i>	432,000
Capsaicin	Counterirritant	<i>Ca. frutescens</i>	750
Castanospermine	Glycoside inhibitor	<i>Castanospermum australe</i>	n/a
Codeine	Sedative	<i>P. somniferum</i>	17,000
Colechicine	Antitumour	<i>Colchium autumnale</i>	35,000
Digoxin	Heart stimulant	<i>Di. lanata</i>	3000
Diosgenin	Steroidal precursor	<i>Dioscorea deltoidea</i>	1000
Ellipticine	Antitumour	<i>Orchrosia elliptica</i>	240,000
Emetine	–	<i>Cephaelis ipecacuanha</i>	1500
Forskolin	Bronchial asthma	<i>Coleus forskolii</i>	n/a
Ginsenosides	Health tonic	<i>Panax ginseng</i>	n/a
Morphine	Sedative	<i>P. somniferum</i>	340,000
Podophyllotoxin	Antitumour	<i>Podophyllum petalum</i>	n/a
Quinine	Antimalarial	<i>Cinchon. ledgeriana</i>	500
Sanguinarine	Antiplatelet	<i>Sanguinaria canadensis</i>	4,800
Shikimin	Antibacterial	<i>L. erythrorhizon</i>	4500
Taxol	Anticancer	<i>Taxus brevifolia</i>	600,000
Vincristine	Antileukemic	<i>Cath. roseus</i>	2,000,000
Vinblastine	Antileukemic	<i>Cath. roseus</i>	1,000,000

Adapted from Ramachandra and Ravishankar (2002)

Plant cell suspension cultures has been suggested over the past 50 years, and are increasingly being seen as, a new source of existing and novel high-priced pharmaceuticals (Tom *et al.* 1991). *In vitro* culture of alkaloid producing plant has been found to retain the capacity to synthesise alkaloids identical to that in the intact plant. Sometimes, high yield of secondary metabolites is observed in tissues grown as callus (Benavides and Caso, 1993). A vast amount of work has been reported during the last decade and the majority of common medicinal plants have been subjected to cell culture investigation. Nevertheless, in the majority of cases, yields of the secondary metabolites have been commercially disappointing. Several patents have been obtained for the production of metabolites from cultures such as allergens, diosgenin, L-dopa, ginsenosides, and glycyrrhizin (Staba, 1980; Bajaj, 1988).

Only a few products of plant cell culture have reached the market place, perhaps because of the lengthy testing programmes required of drugs. The success of Mitsui Chemical Japan in producing shikonin from *Lithospermum erythrorhizon* and berberine from *Coptis japonica* is especially noteworthy (Guardiola *et al.* 1995). Scale-up of production through tissue culture has been developed for the production of Taxol by the company, Mitsui Chemical and Phyton Inc. Some of the pharmaceutical industry applications of plant cell culture are indicated in Table 1.4.

Table 1.4: Active pharmaceutical ingredients produced by plant cell culture in large-scale.

Product	Species	End-use	Manufacturer
Rosmarinic acid	<i>Coleus blumei</i>	Anti-inflammatory	Natterman (Germany)
Scopolamine	<i>Duborisia sp.</i>	Anticholinergic	Mitsui Chemicals (Japan)
Shikonin	<i>Lithospermum erythrorhizon</i>	Antibiotic, pigment	Mitsui Chemicals (Japan)
Taxol	<i>Taxus baccata</i> <i>Taxus media</i>	Anti-tumour	Mitsui Chemicals (Japan) Phyton (USA/Germany)

Adapted from <http://www.biohelsinkiconf.com/download/oksman-biotechelsink03.pdf>

1.6.2 Food and flavour ingredients

Plant cell and tissue culture has also been used to produce a wide range of food ingredients, including flavours, colourants, essential oils, sweeteners (Stafford, 1991; Dornenburg and Knorr, 1996A). The large-scale production of ginseng from plant cell culture has been accomplished by Nitto Denko Co. in Japan since 1988. The production is about 20g dry weight per litre by 20,000 and 25,000 litre bioreactor in a four week period (Hibino & Ushiyama, 1999).

1.7 Colchicine in plant tissue culture

Although colchicine has been known for many centuries, plant cell and tissue culture research on *Colchicum* and *Gloriosa* is relatively new and is limited to a small number of people: The first report of *In vitro* culture was by Hayashi *et al.* (1988A and 1988B) and Yoshida *et al.* (1988A and 1988B) who initiated callus culture of *Colchicum autumnale*. They were also the first workers to report the presence of alkaloids in *Colchicum autumnale* callus tissue and later successfully established a cell suspension culture. Poulev *et al.* (1994) initiated callus cultures from *Colchicum variegatum*. Ghosh *et al.* (2002) established root cultures of *Gloriosa superba* from callus cultures. Sivakumar and co-workers managed to regenerate plantlets and produce corms from *Gloriosa superba* (Sivakumar *et al.*, 2003A and 2003B). Also Sivakumar *et al.*, (2004) carried an *In vitro* precursor feeding study using phenylalanine and tyrosine on *Gloriosa superba* callus tissue.

1.8 Aims and objectives

The objective of the research presented in this thesis is to develop and optimise a process for the production of colchicine from plant cell culture. The research seeks to produce the valuable compound, colchicine through an alternative means, using cell and tissue cultures of *Colchicum autumnale* and *Gloriosa superba*.

In this work efforts are focused on the stimulation of colchicine biosynthetic activities, and a number of parameters were examined with a view to stimulate colchicine accumulation in cell cultures of *Colchicum autumnale* and *Gloriosa superba*.

To achieve this objective, the aim of the research and development was.

1- To initiate and develop callus tissue culture from *Colchicum autumnale* and *Gloriosa superba*.

2- To develop and optimise analytical methods for assaying colchicine in tissue and liquid medium.

3- To subject the tissue cultures to various treatments in an attempt to stimulate the accumulation of colchicine.

4- To study the biosynthetic capacity of these cultures by feeding potential precursors to the cultures.

5- To study the effect of continuous extraction on the accumulation of colchicine.

6- To develop an integrated process that rationally combines different enhancement strategies for further productivity increases in colchicine production.

Chapter Two

The establishment of plant tissue cultures

2.1 INTRODUCTION

Plant tissue cultures are a potential source for a huge range of useful chemical compounds. It is now widely being used as a model system to investigate the production of specific secondary products as they offer experimental advantages both to basic and applied research and to the development of models with scale-up potential (Buitelaar and Tramper, 1992; Chang and Sim, 1995).

The utilisation of biotechnology for the production of secondary metabolites in plant tissue is critically dependent on induction of sterile cultures from explants. *In vitro* plant cell and tissue culture involves the removal of plant tissue (explants) from a plant species of interest under sterile condition, and transfer to a medium containing essential organic and inorganic compounds which facilitate the growth and division of the plant cells (Pierik, 1987). Obtaining sterile plant tissue material can be very difficult, and even with suitable precautions taken, there may be a high level of contamination if the explant is not disinfected properly. The solutions used to sterilise explants must preserve the plant tissue but at the same time remove or destroy any bacterial or fungal contamination (Torres, 1989). The medium must provide all the nutritional requirements for sustained growth of excised plant tissue. The three major components are the carbon source, nitrogen source, and inorganic constituents. There is a degree of consistency in salt requirements of diverse plant cell and organ cultures. The macro- and micro-nutrients provided by these media match those required by the whole plant quite closely. Since tissue cultures are not autotrophic, a carbon source must be added to the medium, and sucrose usually gives good results. Vitamins are also usually included as they may be a critical factor for healthy growth, and may stimulate growth (Misawa, 1994; Al-Khayri, 2001). Plant cell culture therefore requires water, mineral salts, an organic source of carbon and various hormones and vitamins for sustained growth.

Plant cell and tissue cultures can be divided into two major groups, unorganised and organised. Unorganised cultures are typified by callus and cell

suspension cultures. Callus culture is a proliferation of an unorganized and undifferentiated outgrowth of cells on the wounds of differentiated tissues and organs, typically grown on agar-solidified medium. Callus forms naturally on plants in response to wounding, infestations, or at the graft union (Goff and Bottino, 1981). The initiation of callus formation is referred to as callus induction. It is possible with many different plant species to produce a callus and then to subculture this further on a new medium. Suspension cultures: cultures of cells or tissue in liquid medium may be initiated by transferring a piece of friable callus tissue into a liquid medium and agitating on a rotary shaker. It may consist of single cells, and aggregates of cells. Organised organ culture e.g Root, Shoot, and other plant organ cultures are also grown and may have advantage over unorganised cultures in terms of stability of production and spectrum of products formed.

This chapter deals with the initiation and growth of callus and root tissue from *Colchicum autumnale* and *Gloriosa superba*, and examines the effect of various growth regulatory hormones on the growth in culture of both species.

Research on the tissue culture of *Colchicum autumnale* and *Gloriosa superba* is fairly recent. One of the earliest reports of the culture of *Colchicum autumnale* was by Yoshida *et al.* (1988A and 1988B), who initiated callus from *Colchicum autumnale* flowering shoots in MS medium (Murashige and Skoog, 1962). They were also the first workers to report the presence of colchicine alkaloids in *Colchicum autumnale* callus tissue and later successfully established a cell suspension culture. This was followed by Poulev *et al.* (1994 and 1995), who initiated callus tissue from *Colchicum variegatum* and found they could be maintained by sub-culturing into fresh medium every 4 weeks for over nine years. Ghosh *et al.* (2002) and Sivakumar *et al.* (2003A) successfully initiated callus tissue from *Gloriosa superba* using SH medium.

2.2 MATERIALS AND METHODS

2.2.1 Material

2.2.1.1 Plant Materials

- 1- Corms of *Colchicum autumnale* was obtained from Taylors and sons bulbs Ltd., Holbeach-UK.
- 2- Seeds of *Colchicum autumnale* and *Colchicum byzantinum* were obtained from Bjorn malkmus (www.Rareplants.de).
- 3- Seeds of *Gloriosa superba* were the kind gift of Indena Company (6 Settala Milano - Italy).

2.2.1.2 Laboratory equipment

1. Orbital shaker SK-71 (JELO TECH)
2. Petri Dishes (9cm) (Sarstedt)
3. Glass beads sterilizer (K-Midt).
4. Fluorescence Microscope
5. Autoclave (TOMY SS-325).
6. Erlenmeyer flasks (10, 25, 50, 100, 250, 500, 1000 and 2000ml) Pyrex.
7. AcrodiscTM 32mm (0.21µm) (PALL Life Sciences).

2.2.1.3 Chemicals

Sucrose, myo-Inositol, Nicotinic acid, Glycine, Pyridoxine-HCL, Thiamine, 2,4-Dichlorophenoxyacetic acid (2,4-D), 3-Indolebutiric acid (IBA), Naphthalene acetic acid (NAA), 6-Furfurylaminopurine (Kinetin), Sulphuric acid, Ethanol, Phenol, Acetone, and Fluorescein Diacetate (FDA) were obtained from Sigma-Aldrich.

Murashige and Skoog medium (MS), Schenk and Hildebrandt (SH), Daishin agar, 6-benzylaminopurine (BAP), Zeatin riboside, and Thidiazuron (TDZ) were obtained from Duchefa Biochemia – Netherlands.

2.2.2 Solution preparation

2.2.2.1 Preparation of growth-regulator hormones

Some auxins and cytokinins (2,4-D, IBA, NAA, BAP and Kinetin) required a co-solvent to assist solubility. A concentration of 1mg/ml stock solution of each hormone was prepared individually by dissolving 50mg of each hormone in 0.5ml 1M KOH, and distilled water at approximately 45 °C was then added to make a final volume of 50ml stock solution of each hormone. Stock solutions were stored at 2-4 °C.

2.2.2.2 Preparation of Fluorescein Diacetate (FDA) solution

0.5g Fluorescein Diacetate was dissolved in 10ml acetone and stored in a glass bottle at -20 °C.

2.2.2.3 Preparation of H-Vitamins

The components of H-Vitamins are shown in Table 2.1. 100ml stock solution was prepared in ultra-pure distilled water and stored at 2-4 °C.

Table 2.1: H-Vitamin stock solution

Component	Concentration (mg/100ml)
Nicotinic acid	50
Glycine	20
Pyridoxine-HCl	5
Thiamine	5

2.2.3 Sterilisation procedure and aseptic manipulation

It is fundamentally important at all stages of the development of a tissue culture that it is kept, maintained and handled under aseptic conditions since cultures are grown on a medium rich in nutrients in which any microorganisms present will rapidly grow. Even if the contaminations do not impede the growth rate of plant tissue, their presence would invalidate any biochemical study.

2.2.3.1 Sterilisation of plant materials

During the initiation procedure, explants were sterilised using a commercially available chlorinated solution, DomestosTM (10% v/v) for 10 to 15 min. depending on the type of tissue (corm, flowering shoot, or seed) and washed thoroughly with sterile distilled water, then handled and transferred with a sterile forceps and scalpels to a sterile medium. All manipulations were carried out under aseptic condition.

2.2.3.2 Re-sterilisation of the equipment

Scalpels and forceps used in sterile operations were sterilised by immersing the tips of the instruments for 7sec. in the pot of a bead steriliser maintained at 230°C. The instruments were left to cool at the back of the laminar airflow cabinet prior to use.

2.2.3.3 Autoclaving process

The flasks containing culture medium or distilled water together with forceps, scalpels, and beakers were placed in the autoclave. Having ensured that the autoclave contained sufficient water, sterilisation was allowed to proceed at 103.4 KPa (121 °C) 15-30 minutes (depending on the volume of medium to be autoclaved). After autoclaving the medium was allowed to cool in a laminar airflow cabinet.

2.2.3.4 Maintenance of sterility

All culturing operations were carried out in a laminar airflow cabinet (Microflow). Prior to the beginning of any work in the cabinet, it was thoroughly swabbed with methylated spirit (IMS). The bead sterilizer was situated in the back of the laminar airflow cabinet.

2.2.3.5 Sterilisation and addition of growth-regulated compounds

Growth-regulates (hormones) were filter sterilised through a pre-sterilised 0.2µm membrane filter Acrodisc™ 32mm to ensure that medium containing agar did not set prior to pouring and that heat labile components were not degraded.

2.2.4 Media composition and preparation

The choice of medium used to produce callus cultures of *Colchicum autumnale* and *Gloriosa superba* was made based upon the previously reported *Colchicum autumnale* culture (Yoshida *et al.* (1988A and 1988B). In this work an initial medium of MS, supplemented with 2,4-D and Kinetin was used for initiation of callus tissue. SH was used to grow root cultures (Ghosh *et al.*, 2002).

2.2.4.1 Medium

The medium used in this work is based on two basal media (Murashige and Skoog, 1962); (Schenk and Hildebrandt, 1972) as a source of micro and macro elements. Their composition is shown in Table 2.2 and Table 2.3 respectively.

Table 2.2: Components of MS (Murashige and Skoog) basal salt mixture

Macro Elements	mg/litre	mM
CaCl ₂	332.02	2.99
KH ₂ PO ₄	170.00	1.25
KNO ₃	1900.00	18.79
MgSO ₄	180.54	1.50
NH ₄ NO ₃	1650.00	20.61

Micro Elements	mg/litre	μM
CoCl ₂ .6H ₂ O	0.025	0.11
CuSO ₄ .5H ₂ O	0.025	0.10
FeNaEDTA	36.70	100.00
H ₃ BO ₃	0.83	100.00
KI	0.83	5.00
MnSO ₄ .H ₂ O	16.90	100.00
Na ₂ MoO ₄ .2H ₂ O	0.25	1.03
ZnSO ₄ .7H ₂ O	8.60	29.91

Table 2.3: Components of SH (Schenk and Hildebrandt) basal salt mixture

Macro Elements	mg/litre	mM
CaCl ₂	151.00	1.36
KNO ₃	2500.00	24.73
MgSO ₄	195.05	1.62
(NH ₄)H ₂ PO ₄	300.00	2.61

Micro Elements	mg/litre	μM
CoCl ₂ .6H ₂ O	0.10	0.42
CuSO ₄ .5H ₂ O	0.20	0.80
FeNaEDTA	19.80	53.94
H ₃ BO ₃	5.00	80.86
KI	1.00	6.02
MnSO ₄ .H ₂ O	10.00	59.17
Na ₂ MoO ₄ .2H ₂ O	0.10	0.41
ZnSO ₄ .7H ₂ O	1.00	3.48

2.2.4.1.1 Callus Induction Medium

Callus tissues were initiated and maintained in MS medium. Table 2.4 outlines the ingredients.

Table 2.4: Callus induction medium

Component	Concentration (mg/litre)
Murashige and Skoog basal salt mixture	4400
Myo-inositol	1000
Sucrose	30000
2,4-D (Filter-sterilised)	2ml/l
Kinetin (Filter-sterilised)	1mg/l
H-Vitamins	10 ml/litre

2.2.4.1.2 Seed Germination Medium

Seeds were germinated in half strength MS medium. Table 2.5 outlines the ingredients.

Table 2.5: Seed germination medium.

Component	Concentration (mg/litre)
Murashige and Skoog basal salt mixture	2200
Sucrose	30000
H-Vitamins	10 ml/l

2.2.4.1.3 Root tissue Medium

Root tissues were initiated and maintained in two variants of SH medium shown in Table 2.6.

Table 2.6: Root induction and maintenance medium

Component	Concentration (mg/litre)	
	M1	M2
Schenk and Hildebrandt basal salt mixture	3200	3200
Sucrose	30000	30000
Myo inositol	1000	1000000
H-Vitamines	10ml/l	10ml/l
NAA	2 ml/l	2 ml/l
IBA	1ml/l -	
Kinetin	0.5 ml/l -	

2.2.4.2 Medium preparation

2.2.4.2.1 Suspension medium preparation

A batch of five litres of medium was prepared for each experiment to provide minimal medium differences between replicate flasks. Typically, for a litre of medium 800cm³ of ultra-pure water were placed in a 2000cm³ conical flask and the medium components were added. To ensure the MS media and sucrose were fully dissolved, a magnetic stirrer was used. Once all components dissolved, the media was made up to five litres with ultra-pure water and then the pH of the medium was adjusted to pH 5.7 with either 1M KOH or 1M HCL prior to autoclaving. Erlenmeyer flasks (10, 50, 100, 250ml) were used to contain the growing cell and tissue suspension. Prior to use, the flasks were washed thoroughly with hot water and wash-up liquid and rinsed five times with hot tap water, rinsed twice with distilled water then drained by inversion. Freshly prepared medium, in 50ml volumes was transferred to the

250ml flasks. The conical flasks were sealed with cotton wool bungs and capped with a double layer of aluminium foil to reduce evaporation and preserve sterility.

2.2.4.2.2 Solid medium preparation

The medium used for callus cultures was identical to that used in the culturing of the suspension culture but solidified with 8 g/litre of Daishin agar. Flasks were stoppered with a cotton bung and sealed with double layer of an aluminium foil. The flask was sterilised in the autoclave as before. After autoclaving, media was cooled down to 42°C in a water bath and the flask was taken to the laminar flow cabinet. Having removed the bung from the flask the neck was thoroughly flamed. The medium was then poured to approximately 20cm³ into sterile 9cm diameter Petri-dishes and left to solidify. If not used immediately, medium was stored for up to two weeks at 4°C. Plates stored for longer than two weeks showed signs of dehydration, and were always discarded.

2.2.5 Growth conditions

All plant tissue cultures were maintained at 25°C ± 1°C in a constant temperature growth room, under cool white fluorescent light using a 16 hour photoperiod with approximately 20 µmoles/m²/s of photosynthetically active radiation.

2.2.6 Subculturing and Maintenance of callus

To prevent exhaustion of nutrients, drying out of medium and resultant death of cultures, tissue was periodically transferred to freshly prepared medium. Callus tissue cultures of *Colchicum autumnale* and *Gloriosa Superba* were subcultured at six week intervals by breaking into 2mm to 1cm pieces and transferring these onto fresh medium.

2.2.7 Determination of the growth rate of plant cell cultures

Accurate and rapid measurements of cell tissue growth and assessment of growth rate are essential for examining the effect of factors on growth rate and for the development of plant cell and tissue. The most widely used methods of growth rate determination are the measurement of fresh weight and dry weight of cells (Sarkissian and Gray, 1990). In this study, determination of the tissue fresh weight was considered the best method that could be used for evaluation of the growth rate of callus and cell suspension cultures as it is simple, quick, reproducible and potentially non-destructive.

Tissue fresh weights were determined by transferring the tissue into a preweighed sterile microfuge tube or a sterile culture plate and the weight determined. The growth rate was expressed as Fresh weight increase (increment) = $[(W_t - W_0) / W_0] \times 100$ where W_0 is initial weight and W_t the weight after a defined period.

2.2.8 Initiation and maintenance of suspension cultures

Suspension cultures were initiated from friable callus tissue. Under sterile conditions, friable callus tissue was broken into pieces of 1-3mm diameter with a sterile scalpel, and transferred to fresh liquid suspension medium in Erlenmeyer flasks. The flask was transferred to an orbital shaker maintained at $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$ shaking at 120rpm. Suspension cultures were maintained by subculturing every 6 weeks. 50ml fresh medium was added to the 6 week-old culture in a 250ml Erlenmeyer flask, mixed, and divided equally between two 250ml sterile Erlenmeyer flasks.

2.2.9 Examination of cultures for contamination

Nutrient medium used for plant culture provides good growth conditions for bacteria and fungi. Inspection readily revealed contamination as a change in

the colour of the culture, turbidity of the culture or visible microbial growth. Routine examinations of cultures were carried on a weekly basis and at subculture. Liquid medium was checked for contamination by centrifugation of the medium and examination of the sediment under the microscope.

2.2.10 Microscopic examination of the culture

2.2.10.1 External examination

A stereomicroscope was used to examine callus tissue in the petri dish. An inverted microscope was used to examine suspension culture in Erlenmeyer flasks. Both methods provide a good technique for rapid monitoring of growth in culture and non-destructively and non-invasively without the need for sampling and slide preparation.

Suspension cultures were also observed directly by placing a few drops (20 μ l) of a cell suspension on a slide. A cover slip was placed over these cells which were immediately examined under a microscope.

2.2.10.2 Cell viability estimation

Cell viability tests were carried out routinely on suspension cultures. The Fluorescein diacetate (FDA) method (Aoyagi and Tanaka, 1994) was used to determine the proportion of living cells. The method is based on the fact that the enzymatic hydrolysis of FDA results in the accumulation of fluorescein inside living cells which exhibit a bright green fluorescence (Figure 2.1). In contrast to this, dead cells do not fluoresce green.

2.2.10.2.1 Fluorescein Diacetate Method

250 μ l cell suspension samples were placed in microcentrifuge tube and 5 μ l of 5% FDA in acetone added. The mixture was then left for five minutes at room temperature to allow for fluorescence to develop. The microcentrifuge tubes were then centrifuged for three minutes at 13,000rpm to pellet the cells. The supernatant was removed, 20 μ l water was added to resuspend the pellet and this was then placed on a slide and covered with a cover slip. The cells were viewed under a fluorescence microscope (20X), and for each test the cells in the 10 fields of view were enumerated. To determine the cell cultures viability, total cells were counted and the number of cells that are fluorescing will give accurate percentage viability.

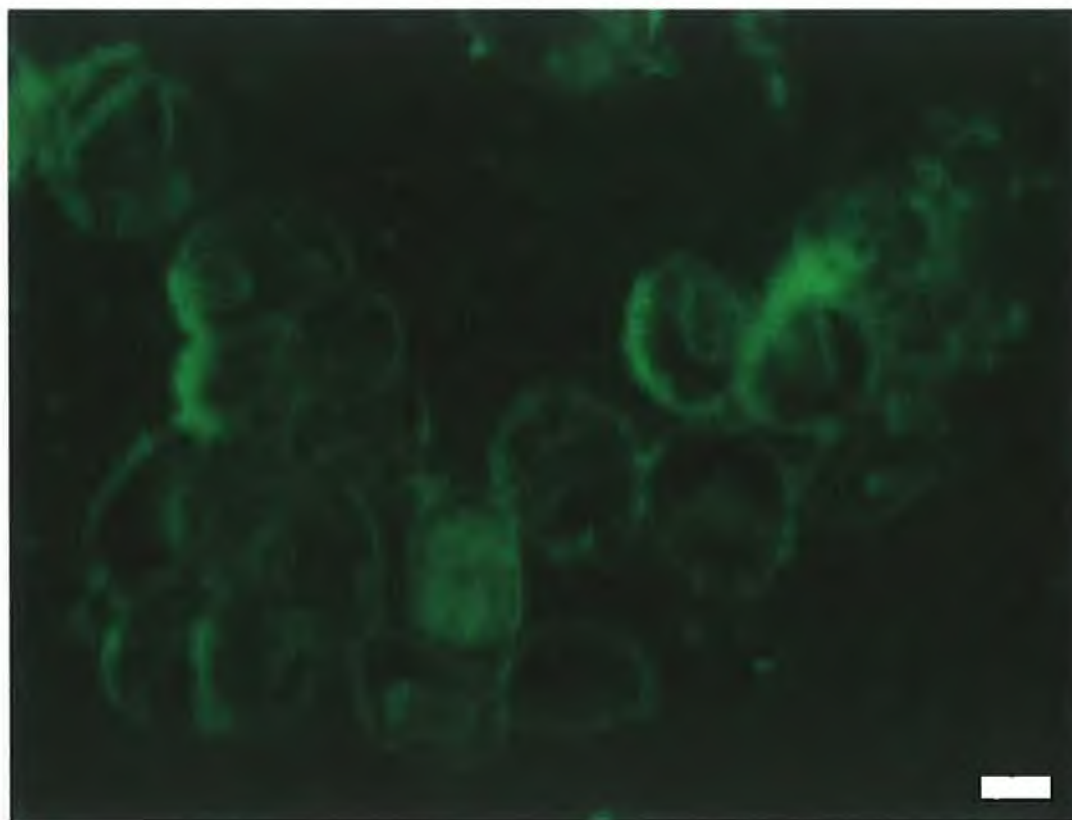


Figure 2.1: Cultured cells of *Gloriosa superba* stained with FDA and viewed under fluorescence microscope. Bar=20 μ M.

2.2.10.3 Histological examination of tissue cultures

Callus histology of tissue cultures of *Colchicum autumnale* and *Gloriosa Superba* was undertaken as described previously (Feder and O'Brien, 1968)

2.2.10.3.1 Dehydration and Fixing plant Tissue

Tissue was embedded in paraffin prior the sectioning on a microtome. In order to do this, the tissue was fixed then passed through the following series of solvents to effect dehydration and wax embedding:

- 1- Ethanol and Acetic acid (3:1 v/v) for 2 hr;
- 2- 90% Ethanol and water for 1 hr;
- 3- Absolute Ethanol 1/2hr for two times;
- 4- Absolute Ethanol and Xylol (1:1 v/v) for 1hr;
- 5- Xylol 1hr for two times;
- 6- Xylol and paraffin wax (1:1 v/v) for 1hr;
- 7- Paraffin wax in embedding oven (60 °C) for 1 day.

The tissue was then transferred to plastic moulds at 60 °C filled with molten paraffin wax, and the mould moved to an ice bath to ensure rapid solidification of wax. Sections of thickness 5, 10, and 15µm were cut with a microtome. The sections were then mounted by placing the section on a slide and gently warming the slide at 50°C after which the slide was left to dry overnight at room temperature.

2.2.10.3.2 Hydration and Fixation of plant Tissue

Hydration, which is necessary, prior to staining, was achieved by passing the slides which containing the section through a second series of solvents:

- 1- Xylol for 10 minutes;
- 2- Xylol and Ethanol (1:1 v/v) for 10 minutes;
- 3- Absolute Ethanol for 10 minutes;
- 4- 90% Ethanol for 10 minutes;
- 5- 80% Ethanol for 10 minutes;
- 6- 70% Ethanol for 10 minutes.

2.2.10.3 Staining of plant tissue sections

Sections were stained with Safranin O (1% w/v) in 50% ethanol and/or Methylene blue (1% w/v) in 50% ethanol

Staining procedure for section

- 1- Methylated spirits for 10 minutes;
- 2- Safranin O solution for 10 minutes;
- 3- Methylated spirits for 10 minutes;
- 4- Methylene blue for 10 minutes;
- 5- The slide was then washed with Methylated spirits and left to dry.

2.2.10.4 Image analysis

Determination of the cell size gives some idea about the difference in shape and size between the cells in callus tissue initiated from the same tissue. Image capture was carried out using a ZEISS microscope-mounted 3-CCD colour video camera (JVC) The images taken were analysed by using an image analysis program written by Dr. Donal OShea (DCU), which uses image analysis software (OPTIMAS version 6.5). It was necessary to calibrate the dimension of the picture, so that accurate measurements could be taken. This was done by having a 150µm scale image taken with the same camera and with the same magnification (X20). It was possible to determine the size of each individual pixel on the image. The cell wall was traced using the mouse and the software program used to accurately determine measurements such as length, breadth and area. These were then transferred from the image analysis program to a spreadsheet in Excel.

2.2.11 Tissue culture initiation

Attempts were made to initiate callus tissue cultures from *Colchicum autumnale*, *Colchicum byzantinum*, and *Gloriosa superba*.

Well developed shoot and root has been used as explants for induction of callus tissue (Chambers, *et al.* 1991). It was decided to establish tissue cultures from seedlings. Seeds are highly resistant structures and may be given a harsh sterilization treatment. Subsequent germination of seedlings provides highly juvenile material which is very suitable for culture.

2.2.11.1 Initiation of *Colchicum* callus tissue

2.2.11.1.1 Germination of *Colchicum* Seeds

Seeds of *Colchicum autumnale* and *Colchicum byzantinum* were kept 2hr under running water to soak.

The seeds were placed in a sterile 250ml beaker containing DomestosTM (10% v/v) for 15 min. The chlorinated solution was then poured off and the seeds washed three times for 5 min. each with sterile distilled water to remove the chlorinated solution. The solution containing seeds was swirled to remove air bubbles. The seeds were removed from the beaker to an empty sterile Petri dish prior to placing on to a germination medium. Sterilised seeds were aseptically placed onto 20cm³ of solidified One-half strength Murashige and Skoog medium (Table 2.5), at 10 -15 seeds per Petri dish and the Petri dishes were sealed with Parafilm. All manipulation was carried out in a laminar flow cabinet.

No germination occurred for 8 weeks for both *Colchicum* species. It is likely that the seeds had lost their capacity for germination.

2.2.11.1.2 Initiation of callus from corm and young flowering shoot tissue

Corm and young flowering shoots were used to initiate callus cultures of *Colchicum autumnale*. Corms were obtained in September and kept in the dark at room temperature until they started to form a young flowering shoot.

The young shoots and corm were washed under running water until all visible compost had been removed, then transferred to a sterile laminar flow cabinet. Young shoots, corm buds and corm tissue were then dissected out from the corm, trimmed to 3-5cm long pieces using a sterile scalpel and immersed in a sterile 500ml beaker containing an aqueous solution of commercial hypochlorite DomestosTM (10% v/v) for 10 min. during which gentle agitation was applied. The plant materials were then washed with sterile water so as to remove the chlorinated solution by three sequential rinses for 5min in sterile distilled water.

The plant material was removed from the water, transferred to an empty sterile Petri dish, then cut with a sterile scalpel into 2-3mm strips prior to placing on to a callus induction medium (Table 2.4), after which the Petri dishes were sealed with Parafilm.

2.2.11. 2 Initiation of *Gloriosa superba* callus tissue

2.2.11.2.1 Germination of *Gloriosa superba* Seeds

Preliminary experiments showed that the husk was a major source of contamination. Seeds of *Gloriosa superba* were kept overnight under running water to soak the husks attached to the seeds, then they were removed to a mesh nylon sheet and the softened husks removed by abrading the seeds against each other and the mesh. Seeds were then washed under running water to remove the husk.

The dehusked seeds were sterilised as described in section 2.2.11.1.1.

2.2.11.2.2 Callus initiation from germinated *Gloriosa superba* Seeds

Seeds were allowed to germinate for 4-6 weeks, and approximately 10% of the seeds germinated. Shoot and root explants were then dissected out, cut into small strips, and placed on callus induction medium plates and incubated at 25 ± 1 °C.

2.2.11.3 Initiation of *Gloriosa superba* root tissue

The callus tissues cultured in MS medium containing no growth-regulatory hormone started to differentiate to malformed root like structures culture, which was highly mucilaginous and grew as a mass.

Gloriosa superba callus tissues were induced to differentiate and form roots by manipulating the composition of the medium. The advantage of using root tissue cultures is that they grow relatively rapidly, and are relatively easy to prepare and maintain. The root can be grown indefinitely if they are periodically subcultured into fresh medium.

Two root initiation media were prepared in SH liquid medium (M1) supplemented with 2mg/l NAA, 1mg/l IBA and 0.5mg/l kinetin, and M2 supplemented with 2mg/l NAA.

2.3 Results

2.3.1 Callus tissue initiation

In the preparation of callus tissue, the plant materials used as explants were initially prepared from young shoot tissues from the corm or germinated seeds. Callus tissues were induced from isolated parts (explants) of *Colchicum autumnale* and *Gloriosa superba*.

2.3.1.1 Initiation of *Colchicum autumnale* callus culture

Typically 60% of the initial shoot explants especially from corm origin, became infected within four weeks of culturing but thereafter the infection rate was low and the remaining sterile pieces gave rise to callus cultures. Callus initiation from *Colchicum autumnale* shoots, flowering shoots and corm tissues was not evident during the first 4 weeks and callus-like structures become visible only between 4-6 weeks. The explants were extremely slow to grow; callus took from 4-6 weeks to appear and it took some 10-12 weeks before sufficient callus could be subcultured (Figure 2.2). The explant initially produced cream to pale yellow callus, and after the third subculture, the callus cells began to darken. The callus mass formed only on the cut edge of the explant, and after 4 months the callus started to form a globular shape of cell aggregates sized 1-2mm in diameter (Figure 2.3). Although callus growth on the initial explants was good, excision and transfer of this callus to fresh medium rarely resulted in the further production of large quantities of callus tissue. Corm tissue did not respond in the same way as shoot tissue in callus formation. Less than 10% of the corm tissue formed callus tissue, and it grew even more slowly than callus initiated from shoot tissue. The callus tissue growth rate was very slow and it only increased by approximately 15% in four weeks. On the removal of the callus tissue to medium lacking 2,4-D, multiple malformed roots occurred within 4-6 weeks (Figure 2.4). The malformed root tissues grew even more slowly than the callus tissue.



Figure 2.2: Callus tissue initiated from *Colchicum autumnale*. Bar 0.5cm



Figure 2.3: Formation of a globular shape of cells aggregate from *Colchicum autumnale* callus tissue. Bar 2mm

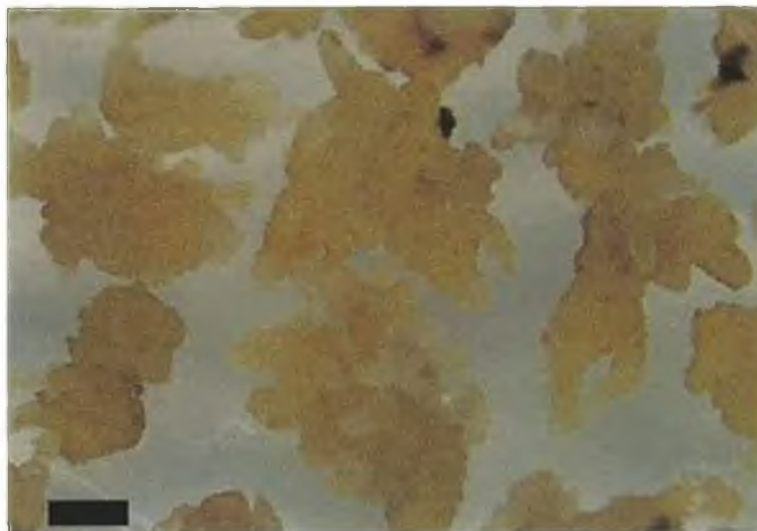


Figure 2.4: Development of multiple multiple malformed roots on callus of *Colchicum autumnale* callus on MS medium without 2,4-D. Bar 1cm

2.3.1.2 Initiation of *Gloriosa superba* callus culture

The comparative freedom from infection of the explant material ensured a very high success rate for this induction process, with typically more than 95% of explants producing viable callus tissue. Some explants began forming callus within 2-3 weeks and almost all had produced well-developed and prolific callus within 4-6 weeks. The callus growth on the initial explants was good, the callus mass formed on the cut edge of the explant and in the centre as well. Calluses were maintained by subcultures onto a fresh callus medium. The explant initially produced yellow to pale yellow callus cells (Figure 2.5). After the third subculture the callus tissue began to darken and the callus started to exude a clear viscous substance on the interface between the medium and callus tissue (Figure 2.6). On the removal of the callus tissue to medium without 2,4-D, multiple malformed roots occurred within 2-3 weeks (Figure 2.7).

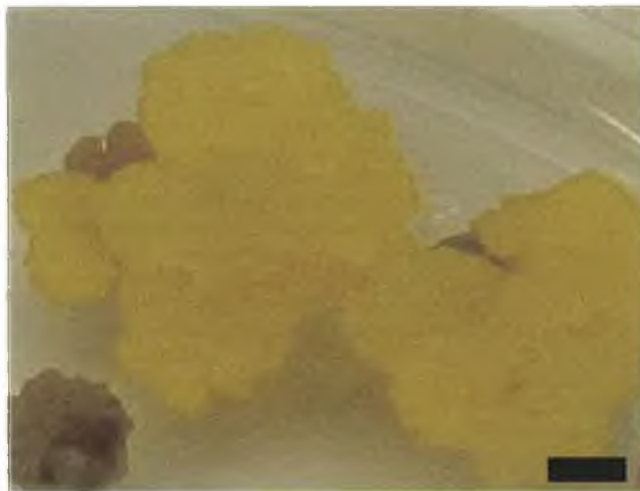


Figure 2.5: Callus initiation from *Gloriosa superba* shoots. Bar 0.25cm

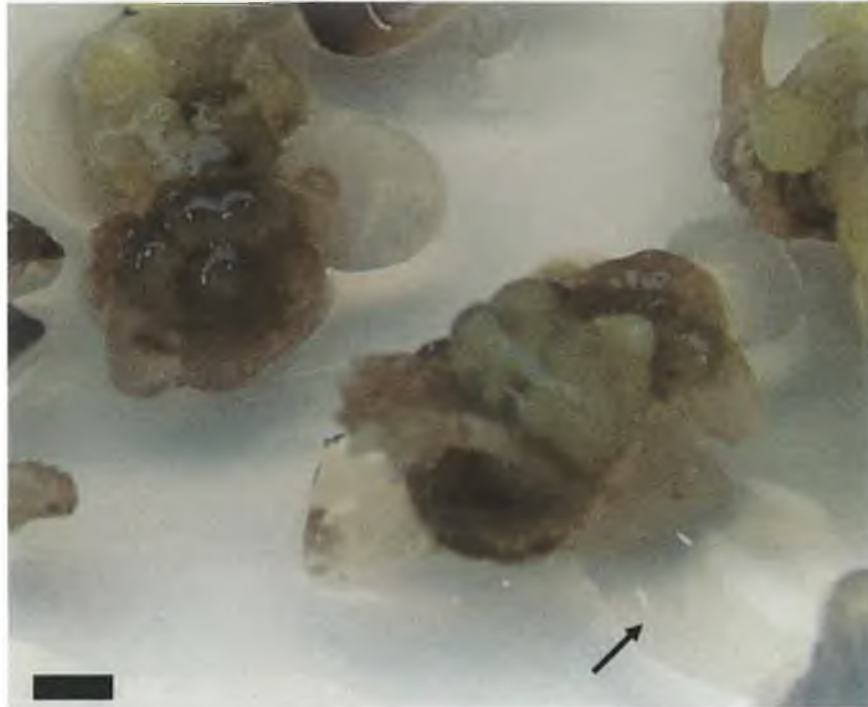


Figure 2.6: *Gloriosa superba* callus started to exude a clear viscous and mucilaginous substance on the interface between the medium and callus tissue (arrow indicates exudates at the medium interface). Bar 0.25cm

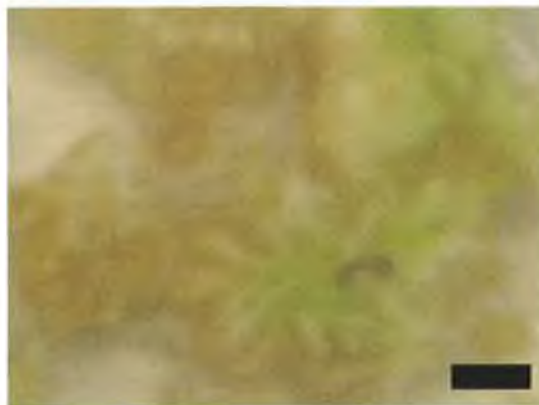


Figure 2.7: Development of multiple multiple malformed roots on callus of *Gloriosa superba* callus on MS medium without 2,4-D. Bar 1cm

2.3.2 The effects of plant growth regulators hormones on callus growth

In an effort to increase the growth rate of callus biomass and to examine the possibility of regeneration of plants from callus tissues originated from *Colchicum autumnale* and *Gloriosa superba*, studies were conducted to examine the effect of auxins and cytokinins on the development of callus tissue in concentrations both lower and higher than the concentration used for initiation of callus tissue. The tissue fresh weight was determined by transferring the callus tissues onto a preweighed plate containing solid medium, then the plate was weighed again and the fresh weight of the tissue obtained (inoculation weight). After incubating for four weeks (28days) at 25°C, the tissue was subcultured onto a new preweighed plate containing fresh solid medium, then the plate was weighed again and the weight of tissue determined (final weight) to determine the net fresh weight increase. Each treatment was replicated five times.

2.3.2.1 The effect of auxins on callus growth

The effect of different auxins upon *Colchicum autumnale* and *Gloriosa superba* callus growth rate was examined. The three auxins were; 2,4-D, IBA, and NAA.

Callus tissue were subcultured in media containing 0.5mg/l Kinetin and supplemented with one of the three different auxins (2,4-D, IBA, and NAA) at one of four concentrations (0, 1, 3, and 5mg/l). After four weeks the weight of callus tissue was determined. Figure 2.8 and Figure 2.9 show the percentage growth rate of *Colchicum autumnale* and *Gloriosa superba* respectively.

In terms of callus quality, the lowest concentration of 2,4-D (1mg/l) produced pale-yellow friable callus with *Colchicum autumnale* and produced compact yellow callus with *Gloriosa superba*. Increasing the 2,4-D concentration to (5mg/l) produced a hard and globular shaped callus in *Colchicum autumnale*. *Colchicum autumnale* callus tissue cultured on medium lacking auxins grew slowly and started to form malformed rootlets like structure (Figure 2.11).

Colchicum autumnale callus grew slowly on a medium supplemented with IBA or NAA (1mg/l) and produced dark-yellow callus and started to differentiate into malformed rootlets (Figure 2.10). *Gloriosa superba* callus produced on IBA or NAA was initially compact and yellow, and gradually started to change to a pale-green and at the end of culturing period some callus tissue started to proliferate into rootlets (Figure 2.12). Rooting occurred at a higher concentration of IBA and NAA (3 and 5mg/l).

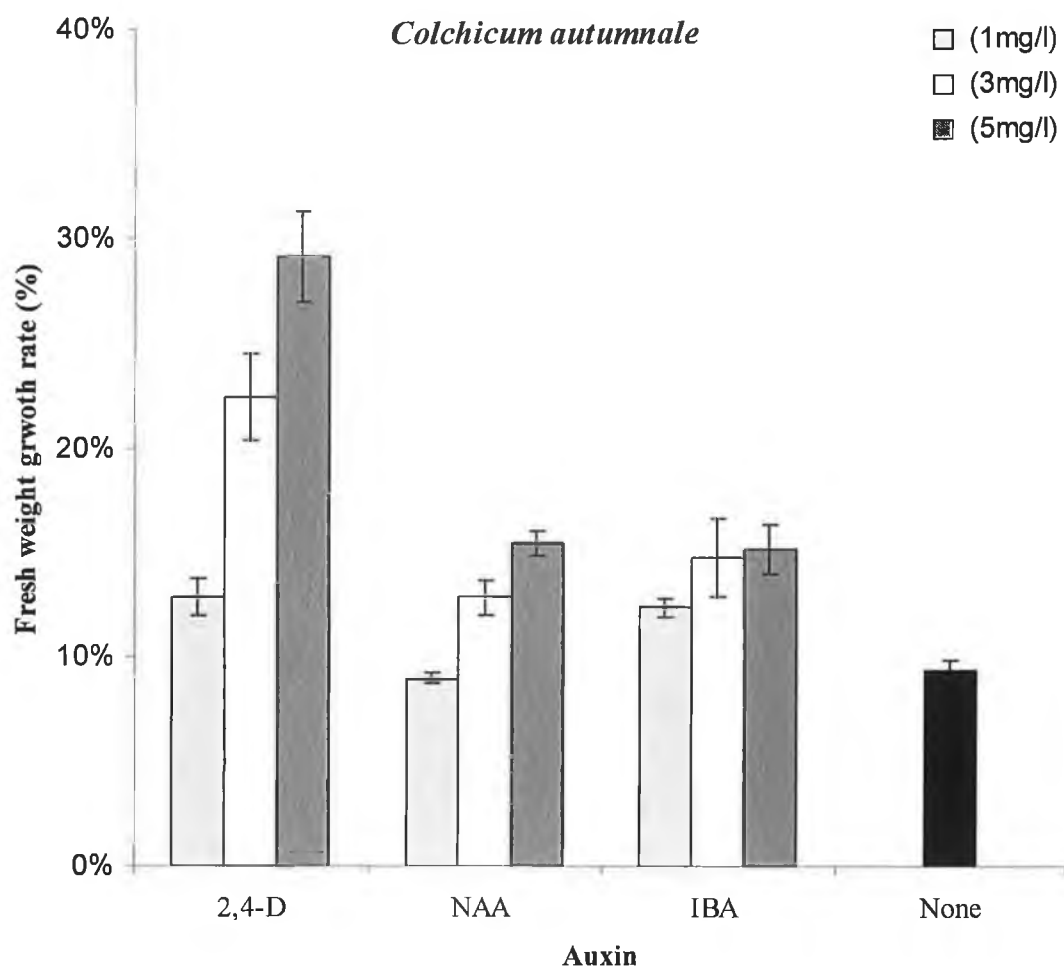


Figure 2.8: Growth rate (%) of *Colchicum autumnale* callus tissue cultures with three individual auxins at three different concentrations and the control grown in auxin free medium for four weeks. Results are the average of 5 plates and the standard errors are included.

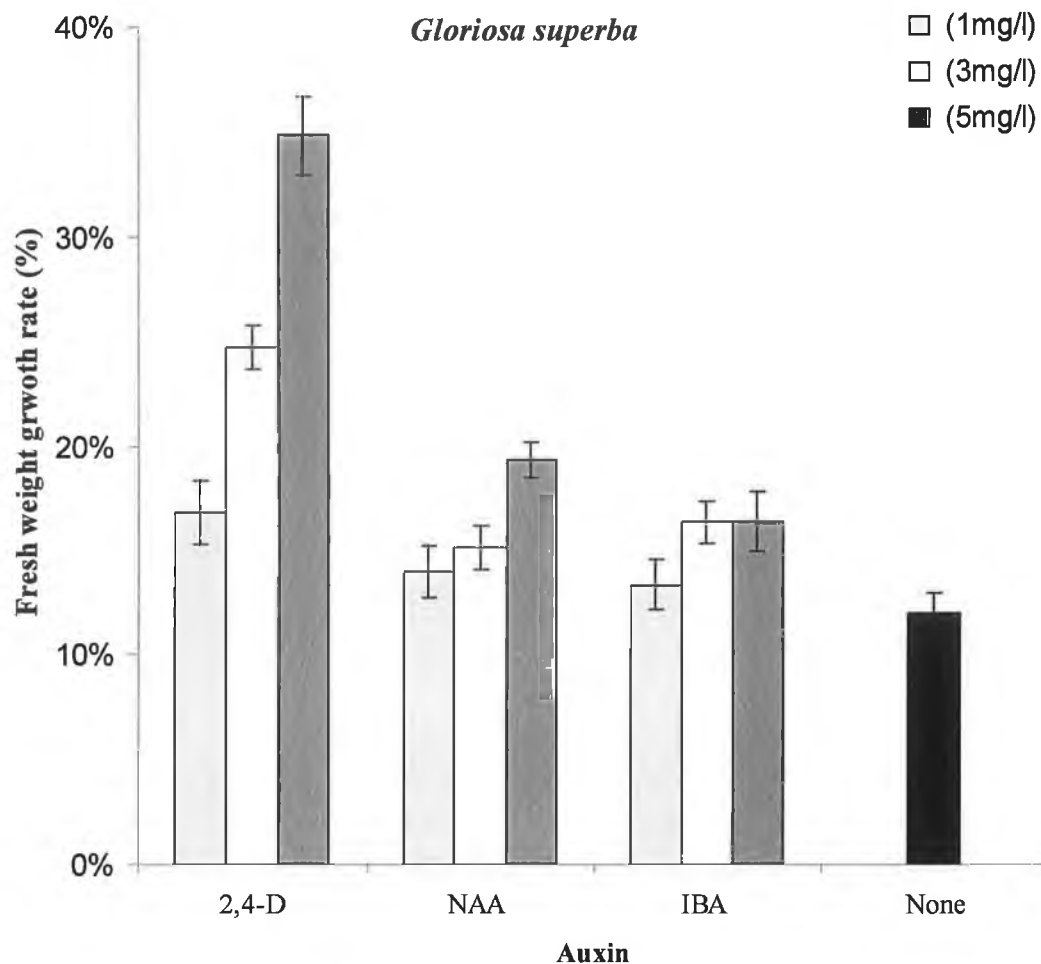


Figure 2.9: Growth rate (%) of *Gloriosa superba* callus tissue cultures with three individual auxins at three different concentrations and the control which grown in auxin free medium for four weeks. Results are the average of 5 plates and the standard errors are included.



Figure 2.10: *Colchicum autumnale* callus differentiated into multiple malformed roots cultured in a medium containing IBA (1mg/l). Bar 0.5cm

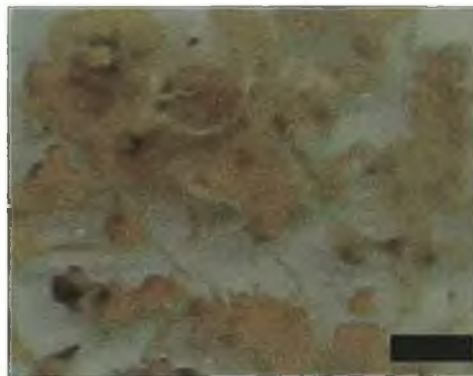


Figure 2.11: *Colchicum autumnale* callus differentiated to multiple malformed roots culture in a medium lacking auxins. Bar 1cm



Figure 2.12: Callus tissue initiated from *Gloriosa superba* callus tissue differentiated to multiple malformed roots culture in a medium containing IBA (1mg/l). Bar 1cm

2.3.2.2 Effect of cytokinins on callus tissue growth rate

The effect of two different cytokinins on the growth rate of callus tissues of *Colchicum autumnale* and *Gloriosa superba* was investigated. Callus tissues were subcultured on media supplemented with one of the two cytokinins (Kinetin and BAP) at three various concentrations (0.5, 1, and 2mg/l). All media were supplemented with 2,4-D (1mg/l). After four weeks the weight of callus tissue determined.

(Figure 2.13) shows the percentage growth rate of *Colchicum autumnale* and *Gloriosa superba* respectively.

In terms of callus quality, lower concentrations of kinetin (0.5-1mg/l) produced pale-yellow friable callus for *Colchicum autumnale* and compact yellow callus for *Gloriosa superba*. On the higher kinetin concentration (2mg/l) the callus tissues become hard and yellow for *Colchicum autumnale* and compact and dark yellow for *Gloriosa superba*. *Colchicum autumnale* callus grew slowly on medium containing BAP and produced dark-yellow callus (Figure 2.14). With the higher concentration (2mg/l) the callus tissue started to become brown at the end of culturing period (Figure 2.15). *Gloriosa superba* callus tissue grown on a medium containing BAP developed a lumpy yellow callus (Figure 2.16).

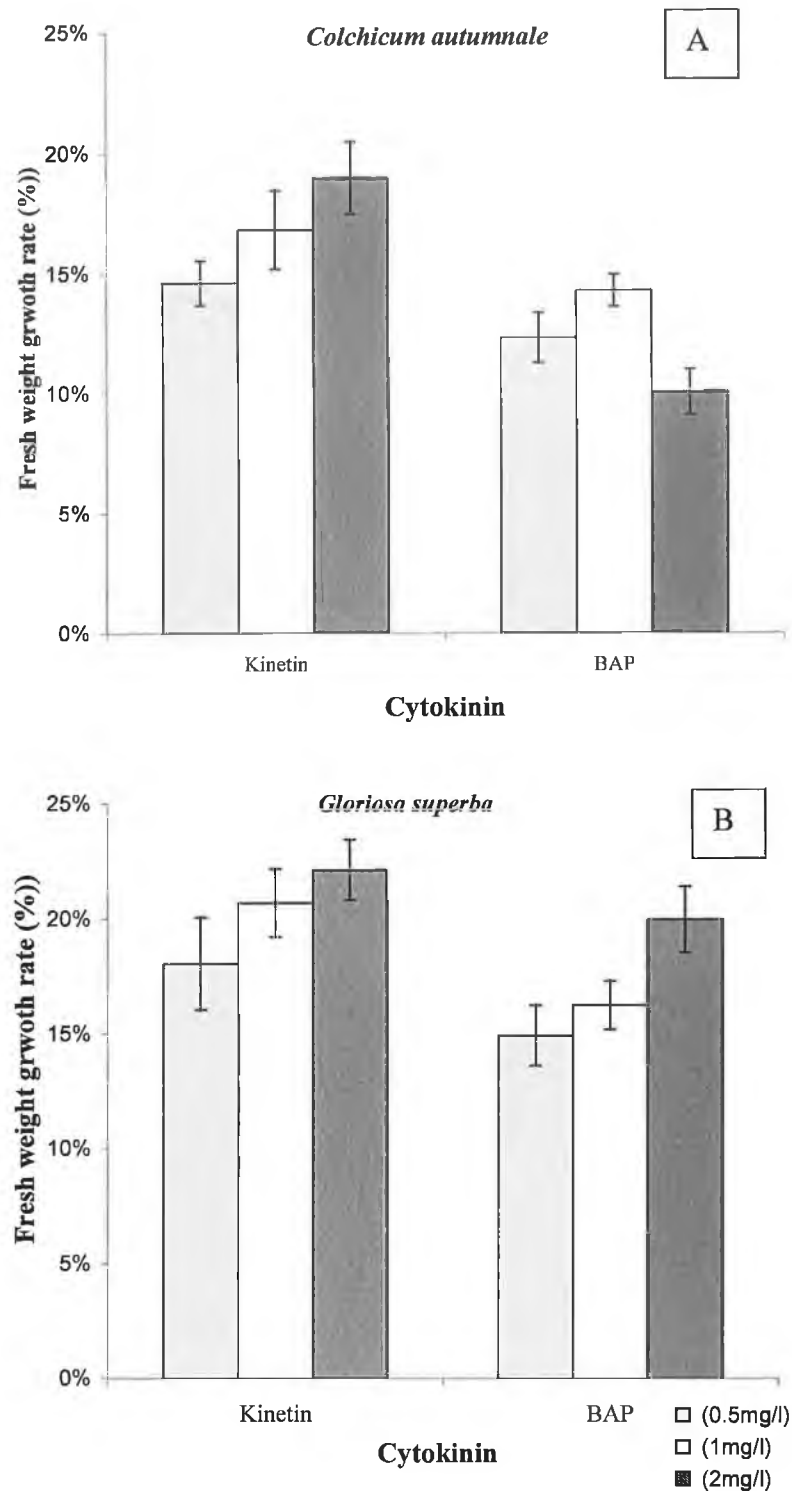


Figure 2.13: Growth rate of callus tissue cultures with either Kinetin or BAP at three different concentrations for four weeks, A= *Colchicum autumnale* and B= *Gloriosa superba*. Results are the average of 5 plates and the standard errors are included.

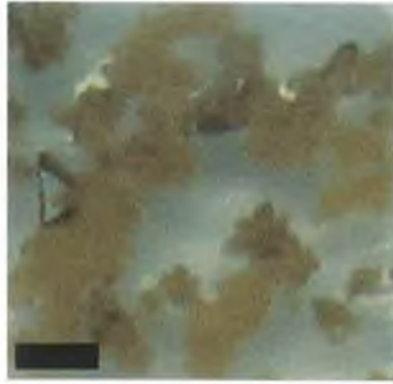


Figure 2.14: *Colchicum autumnale* callus tissue in BAP (1mg/l). Bar 1cm

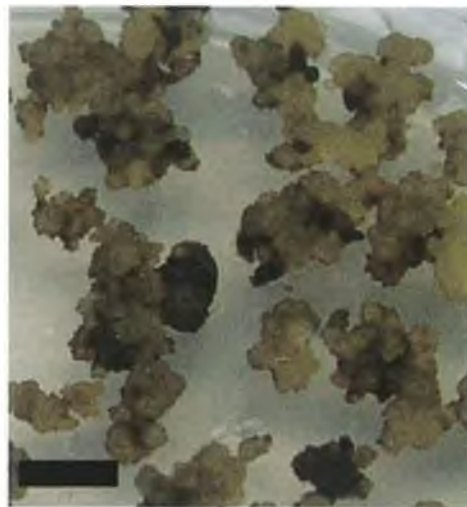


Figure 2.15: *Colchicum autumnale* callus tissue in BAP (2mg/l) (Bar 0.5cm).

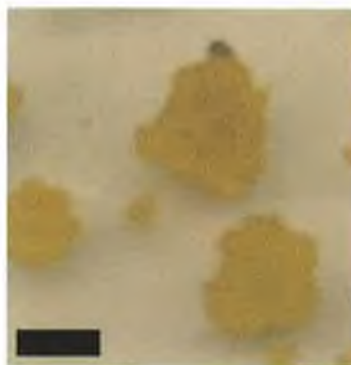


Figure 2.16: Callus tissue initiated from *Gloriosa superba* in BAP (1mg/l). Bar 1cm.

2.3.2.3 The effect of 2,4-D on cell suspension culture viability and growth rate

To examine the effect of different concentration of 2,4-D on the growth rate of *Colchicum autumnale* and *Gloriosa superba* cell suspension, three different concentrations (1, 3, and 5mg/l) of 2,4-D with (0.5mg/l) Kinetin were incorporated into MS liquid medium. Suspension cultures were established by transferring known weights of callus tissue into 10ml flasks containing 5ml liquid medium, after which the cell suspensions were incubated at 25 °C on a orbital shaker at 120rpm for four weeks. Initial cell viability was determined by taking 50µl sample from each cell suspension after overnight incubation. After four weeks incubation, another 50µl sample was taken from each flask for determination of the cell viability at the end of incubation period. The cell viability at the start and the end of the experiment was estimated by using the FDA method (Section 2.2.9.2). The cell suspensions were then transferred to a universal tube and centrifuged at 3500rpm for 10 min, the supernatant removed, the cells weighed and the growth rate calculated. Figure 2.17 shows the percentage growth rate and cell viability of *Colchicum autumnale* and *Gloriosa superba* respectively.

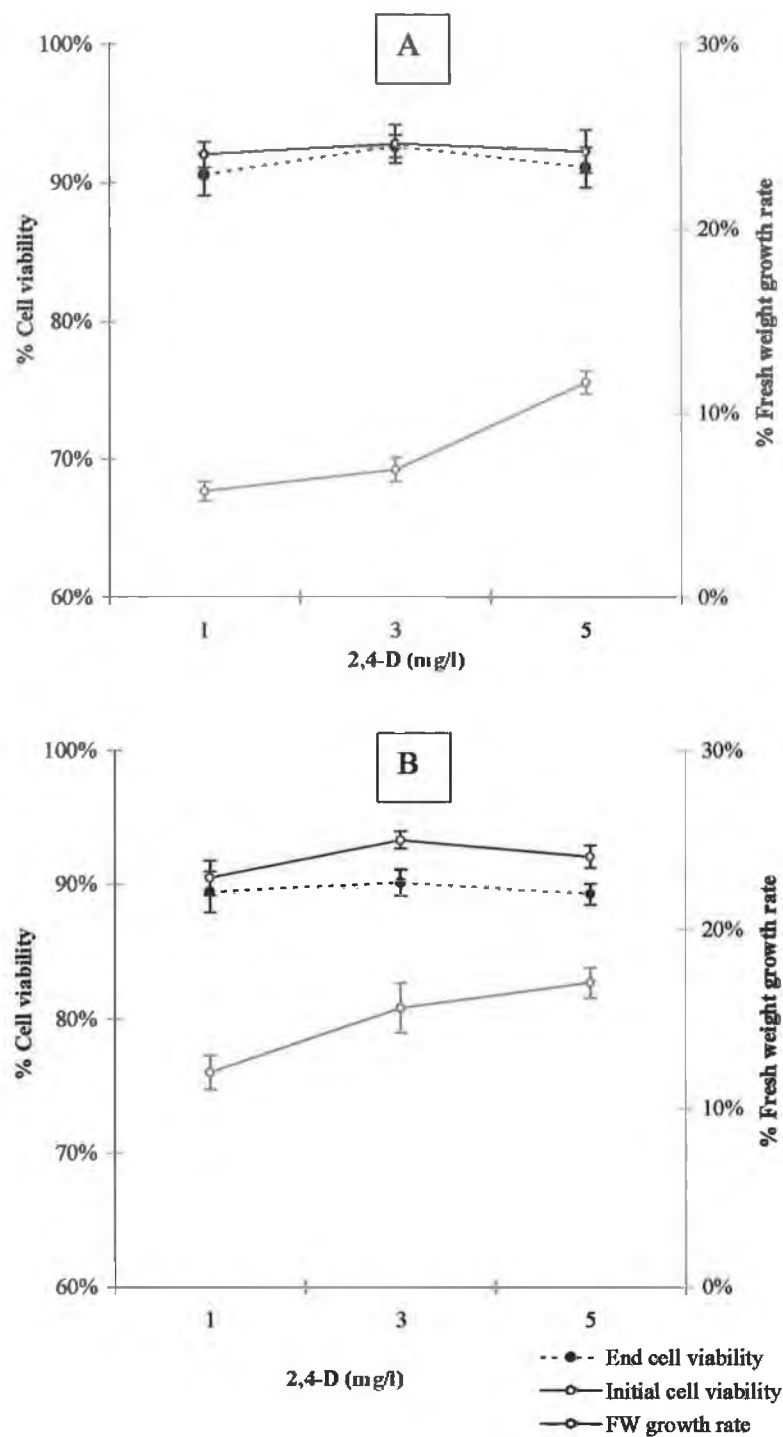


Figure 2.17: Growth rate (%) of cell suspension cultures with different concentrations of 2,4-D. A= *Colchicum autumnale* and B= *Gloriosa superba* for four weeks. Results are the average of five flasks and the standard errors are included.

2.3.3 Histological examination of callus tissue

The cell suspension culture and sections of callus tissue were examined under the microscope.

Sectioned and stained clumps of callus tissue (Figure 2.18) show some cellular organisation within the clumps. There is a cluster of cells in the centre which appear more densely stained than those of the outermost region. Applying image analysis described in (Section 2.2.10) for determination of the cell area, length and breadth, there was a difference in cell size and shape, the cell sizes were smaller in the centre compared to larger cells in the outer region. The broad band of cells near the centre of the clump of callus tissue, were small, densely cytoplasmic and without obvious vacuole perhaps indicating that the clump has a specific meristematic region.

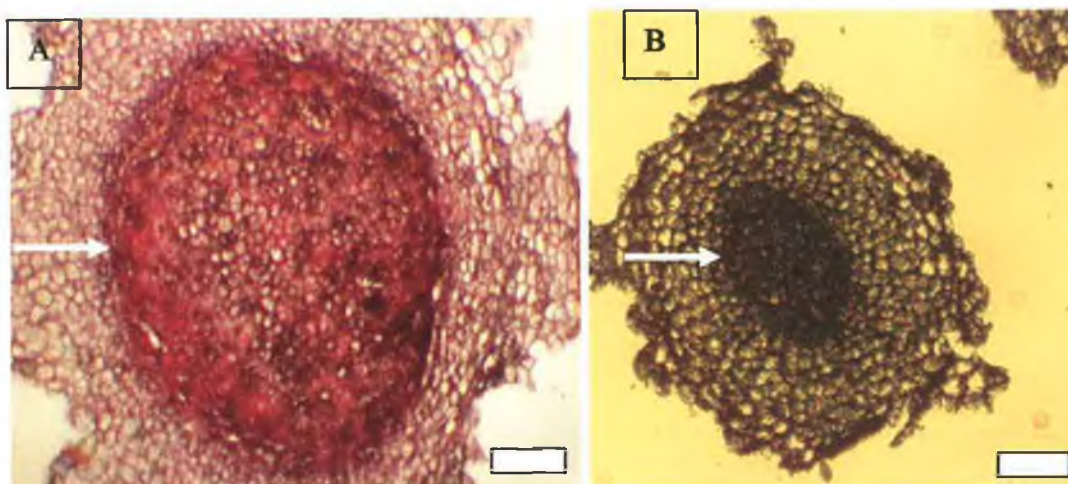


Figure 2.18: Section of callus tissue A= *Colchicum autumnale* and B= *Gloriosa superba* (arrow indicates density cytoplasmic cells). Bar = 100 μ m.

Figures 2.19 and 2.20 shows a putative proembryos like organization. The proembryogenic masses are groups of cells showing organized growth, with small, densely cytoplasmic cells of isodiametric shape.

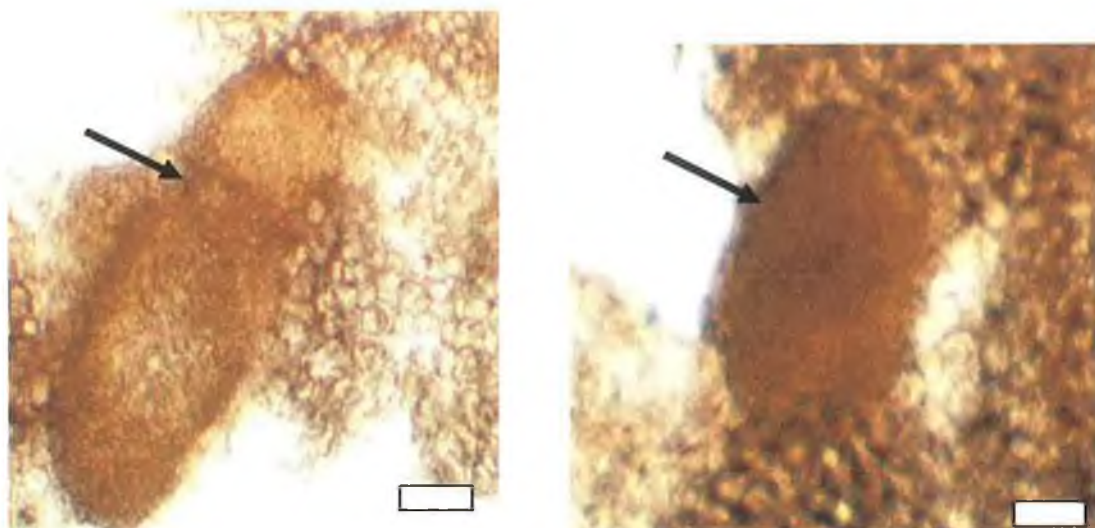


Figure 2.19: Section of *Colchicum autumnale* callus tissue show lump of highly density cells (Putative proembryos) (arrow indicates density cytoplasmic cells). Bar = 100 μ m.

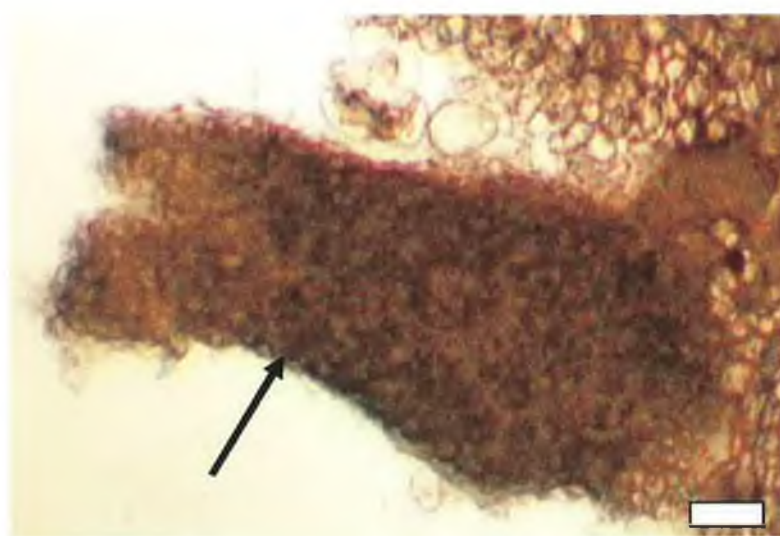


Figure 2.20: Section of *Gloriosa superba* callus tissue show lump of highly density cells (Putative proembryos) (arrow indicates density cytoplasmic cells). Bar = 100 μ m.

2.3.3.1 Cell size and shape

There was a difference in cell shape and size between the two species, as *Colchicum autumnale* cells were more spherical and slightly smaller than *Gloriosa superba* cells which have a more elongated shape.

Also there was dissimilarity in cell sizes within the same plant. The highly density cytoplasmic cells were smaller than the low density cytoplasmic cells. Examining sections of callus tissue, the central part of the callus formed from small and highly densite cytoplasmic cells and the cell in the external part were larger and low in cytoplasm. Figure 2.21 shows the relationship between cell breadth and cell area of *Colchicum autumnale* and *Gloriosa superba* respectively.

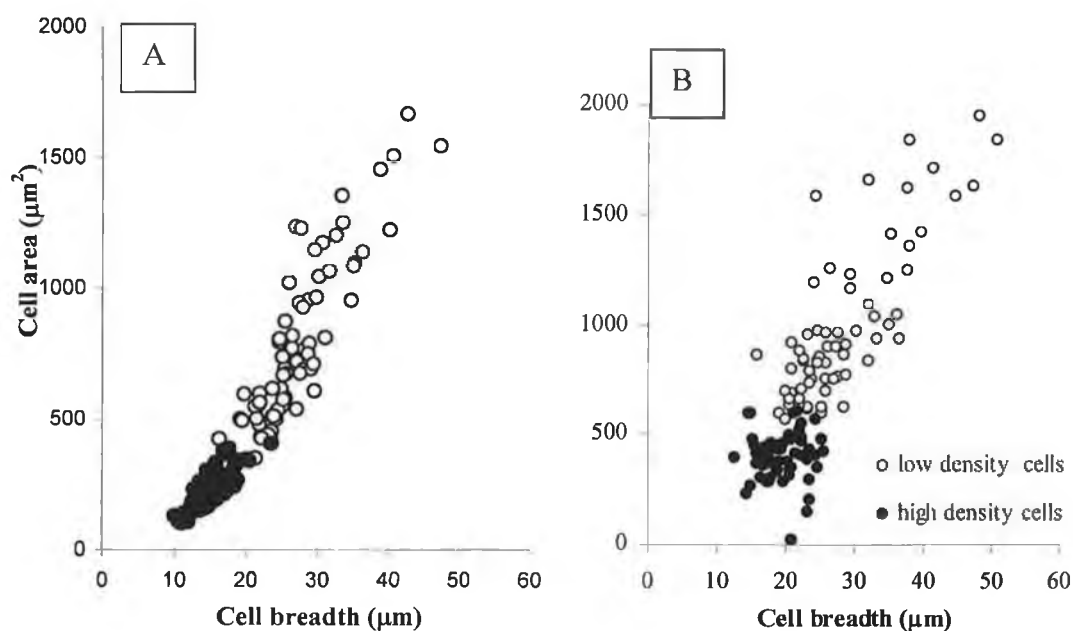


Figure 2.21: Cell size distribution between the densely cytoplasmic cells and non-densely cytoplasmic cells of A= *Colchicum autumnale* and B= *Gloriosa superba*..

2.3.4 Investigation of the possibility of plant regeneration

Some cultured plant cells possess totipotency, i.e., whole plants can be regenerated from single cells by modulating culture condition (Huang and Liu 2002). In exceptional conditions, and sometimes spontaneously, the regeneration of adventitious organs and /or embryos can occur from a callus tissue (Pierik, 1987).

Somatic embryogenesis or asexual embryogenesis is the process of embryo initiation and development from cells that are not products of gametic fusion.

2.3.4.1 Examination of the effect of cytokinins on shoot initiation from callus tissue

Induction of organogenesis was attempted using callus derived from shoot tissue of *Colchicum autumnale* and *Gloriosa superba*.

The effect of different cytokinins on the development of shoot tissue from *Colchicum autumnale* and *Gloriosa superba* callus tissue was examined. Four different naturally occurring and synthetic cytokinins were incorporated into the solid medium either individually, or in a mixture of two, at three different concentrations (0.5, 1, and 3mg/l). Four cytokinins were used Kinetin, BAP, Zeatin, and TDZ. In this study NAA (0.5mg/l) was included in all the treatments. Immediately prior to the experiment, the callus tissues used were maintained on a hormone-free medium for two weeks, to minimise the effect of any hormones used previously.

The medium supplemented with various cytokinins did not induce shoot formation from callus culture of *Colchicum autumnale* or *Gloriosa superba* at any given level of cytokinins combination.

The various concentrations used for shoot induction is presented in table 2.7.

Table 2.7: The various concentrations and combination of cytokinins used for shoot induction from callus tissue.

Medium	Cytokinins (mg/l)			
	Kinetin	BAP	TDZ	Zeatin
MS-1	1	1	-	-
MS-2	3	3	-	-
MS-3	1	-	0.5	-
MS-4	3	5	-	-
MS-5	-	1	0.5	-
MS-6	-	1	1	-
MS-7	-	3	3	-
MS-8	-	1	-	0.5
MS-9	-	3	-	1
MS-10	-	-	1	0.5
MS-11	-	-	1	1
MS-12	-	-	3	1

2.3.5 Induction of root tissues in *Colchicum autumnale* and *Gloriosa*

superba callus tissue

Induction of root tissue was attempted since callus tissue of *Colchicum autumnale* and *Gloriosa superba* started to differentiate into rootlet like structures in a medium containing NAA or IBA or lack of hormones.

Ghosh, *et al.* (2002) reported the initiation of root tissue from *Gloriosa superba* callus tissue by using SH medium supplemented with NAA (2mg/l), IBA (1mg/l), and Kinetin (0.5mg/l).

In an effort to initiate root tissue culture the above medium was introduced as a starting point for induction of root tissue from callus tissues. Callus tissue from *Colchicum autumnale* and *Gloriosa superba* was inoculated in a solid SH (M1) medium supplemented with NAA (2mg/l), IBA (1mg/l), and Kinetin (0.5mg/l). *Gloriosa superba* callus tissue started to differentiate into white rootlets by week three and the root tissue started to green by the end of the four weeks incubation period (Figure 2.22)

Colchicum autumnale callus differentiated to malformed rootlets but failed to form sustained root growth in the above medium (Figure 2.23). The growth rate of those malformed rootlets was extremely low compared to rootlets differentiated from *Gloriosa superba* callus tissue in both solid and liquid medium.

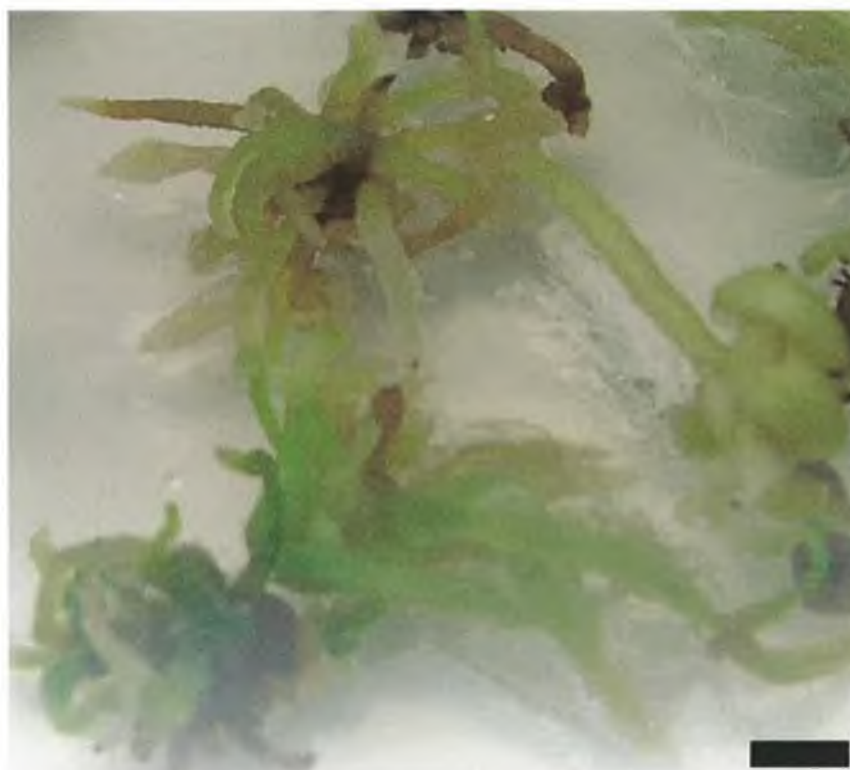


Figure 2.22: *Gloriosa superba* callus differentiated to form multiple rootlets in a SH medium supplemented with NAA (2mg/l), IBA (1mg/l), and Kinetin (0.5mg/l). Bar 1cm

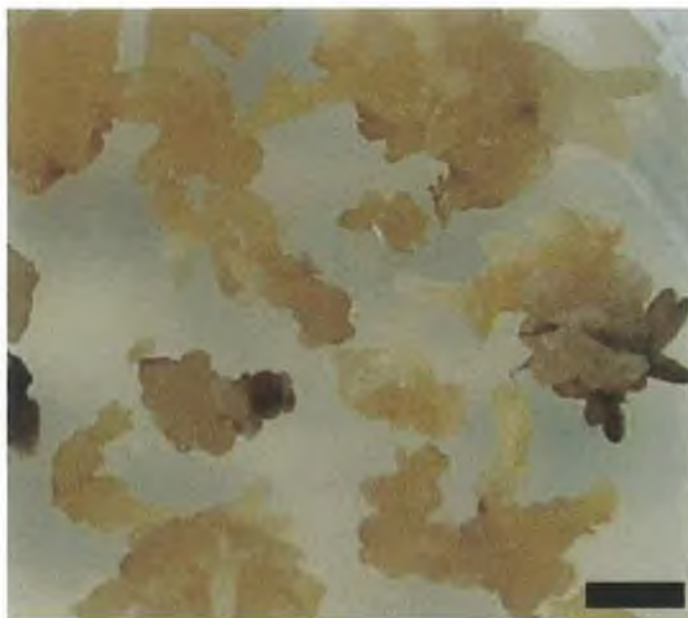


Figure 2.23: *Colchicum autumnale* callus differentiated to form multiple malformed rootlets tissue in a medium supplemented with NAA (2mg/l), IBA (1mg/l), and Kinetin (0.5mg/l). Bar 0.5cm.

2.3.5.1 Determination of the growth rate of malformed root tissue

The growth rate of malformed root tissue in medium lacking hormones was investigated. A known weight of malformed rootlets was transferred into solid or liquid SH medium free from growth hormones, and incubated for four weeks. After four weeks the fresh weight of the tissue was determined and the growth rate were obtained. Results are shown in Figure 2.24.

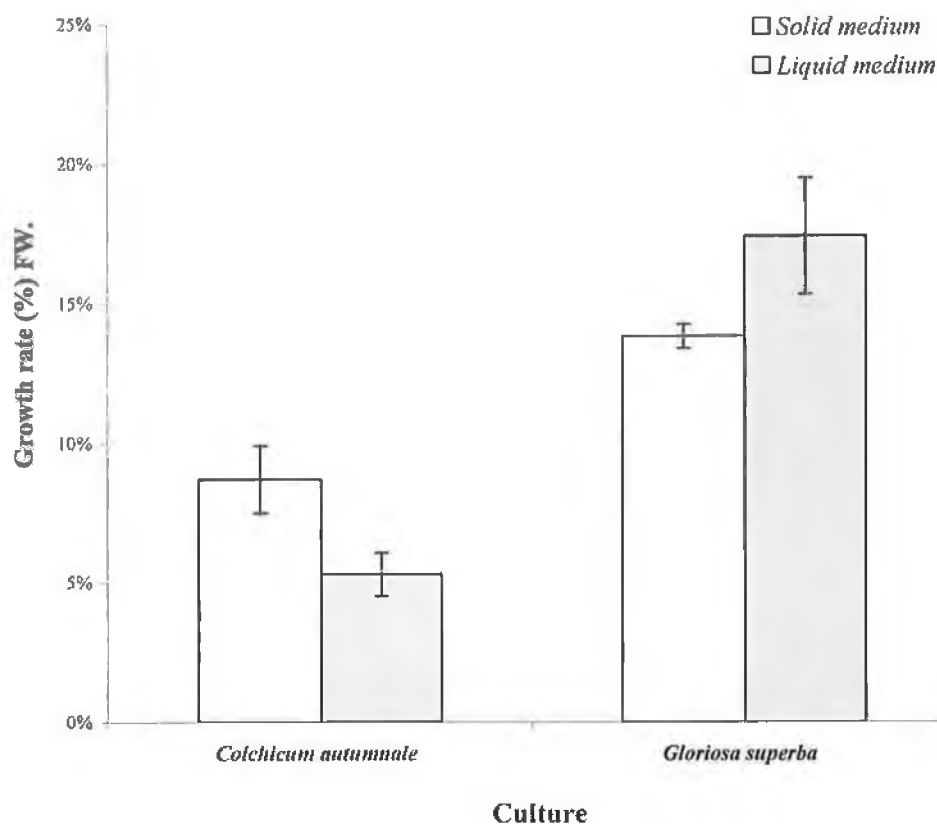


Figure 2.24: Fresh weight growth rate of malformed root culture from *Colchicum autumnale* and *Gloriosa superba* in medium without plant growth regulatory hormones over four weeks.

The growth rate of malformed rootlets differentiated from *Gloriosa superba* and *Colchicum autumnale* callus tissue in medium without plant growth regulatory hormones was low. *Gloriosa superba* grew only by 14% and 17.4% in solid and liquid medium respectively. And *Colchicum autumnale* grew only by 8.7% and 5.3% in solid and liquid medium respectively.

2.3.5.2 Initiation and maintenance of *Gloriosa superba* root tissues in liquid medium

Gloriosa superba callus tissues were induced to differentiate and form roots by manipulating the composition of the medium.

Two root culture lines were established (Figure 2.25) from the callus tissue in SH liquid medium M1 was supplemented with (2mg/l NAA, 1mg/l IBA and 0.5mg/l kinetin) and M2 was supplemented with (2mg/l NAA).

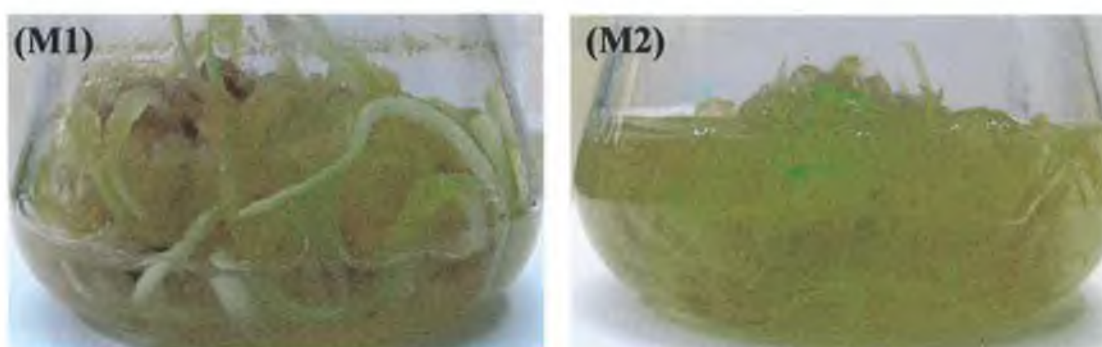


Figure 2.25: Root culture of *Gloriosa superba* grown in a 250ml flask containing SH liquid medium supplemented with (M1) [2mg/l NAA, 1mg/l IBA and 0.5mg/l kinetin] and (M2) 2mg/l NAA



Figure 2.26: Morphology of *Gloriosa superba* root culture in SH liquid medium (M1), (M2). Bar 1cm.

Gloriosa superba root tissues in M1 medium were relatively wide (1.5-2.5mm in diameter) roots without lateral roots. In the other hand root tissue in M2 medium were relatively thin (0.5-1.5mm in diameter) roots with laterals.

2.3.5.2.1 Determination of the growth rate of root tissue

A study was conducted to examine the growth rate of *Gloriosa superba* root tissue in liquid and solid media. Two SH media were used in this study, M1 supplemented with NAA (2mg/l), IBA (1mg/l), and Kinetin (0.5mg/l), and M2 supplemented with NAA (2mg/l).

The root tissues was transferred into solid or liquid SH medium (M1 and M2). Erlenmeyer 100ml flasks were pre-weighed, inoculated with root tissue and the flask reweighed again. 50ml of medium was then added and the flask incubated at 25°C on an orbital shaker (100rpm). After four weeks incubation the fresh weight of the tissue was determined and the growth rates were obtained.

The root tissue fresh weight was determined by transferring the tissues onto a preweighed plate containing solid medium, then the plate was weighed again and the fresh weight of the tissue was obtained (inoculation weight). After incubating for four weeks at 25 °C, the tissue was subcultured onto a new preweighed plate containing fresh solid medium, then the plate was weighed again and the weight of tissue determined (final weight) to determine the net fresh weight increase. There were six replicates of each treatment.

Figure 2.27 shows the growth rate of root tissue in M1 and M2 media. The growth rate of root cultures differentiated from *Gloriosa superba* callus tissue was extremely good in comparison to the growth of callus tissue increasing by three times in liquid medium and by 1.3 times in solid medium after four weeks. In the liquid medium used for *Gloriosa superba* root culture, a mucilaginous substance was produced making the medium thick and viscous.

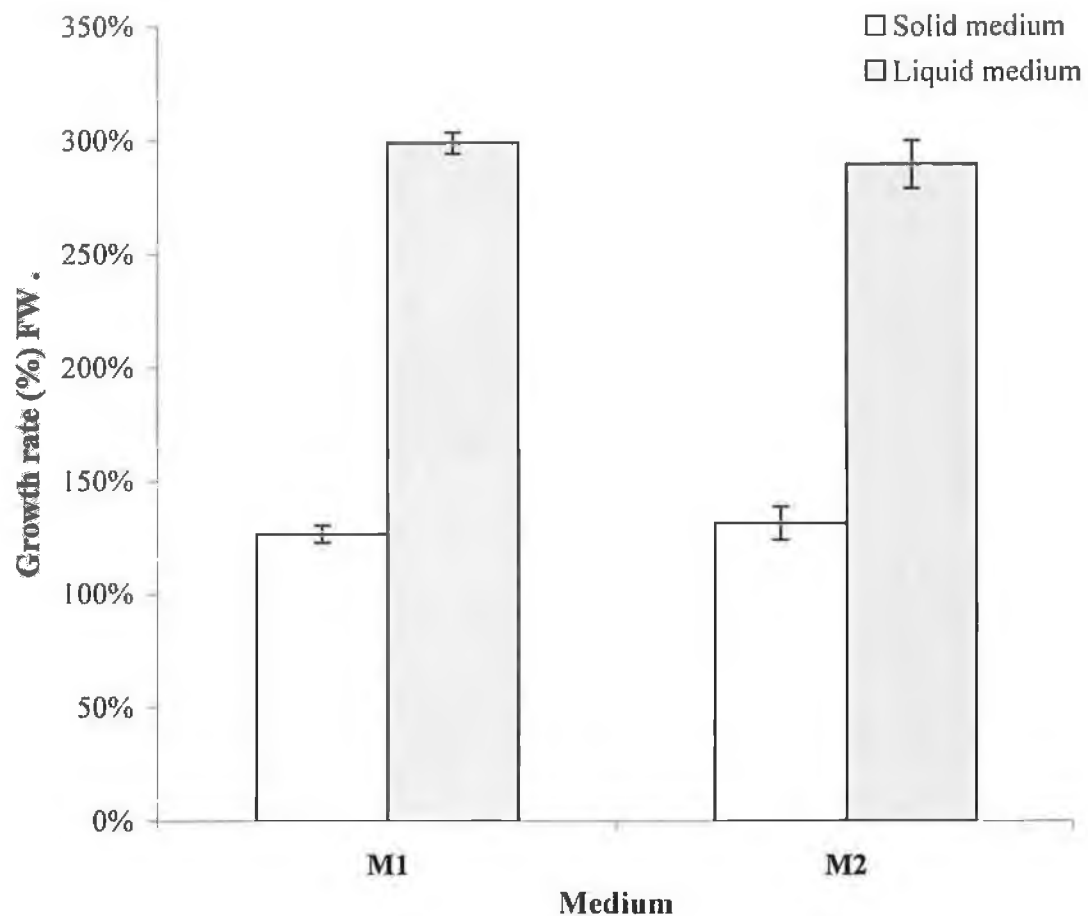


Figure 2.27: Growth rate of *Gloriosa superba* root cultures using M1 and M2 on solid and liquid medium over four weeks. Results are the average of six plates and the standard errors are included.

2.4 Discussion

A marked difference in the *In vitro* seed germination of the three species was observed (0% for *Colchicum autumnale* and *Colchicum byzantinum*) and (10% for *Gloriosa superba*). The surface sterilisation of explant was a critical and challenging problem and contamination provided the greatest problem; although great care was taken in sterilisation, the sources of contamination are so numerous that this problem can never be eliminated. Exposing the explant to longer time treatment and/or higher concentrated sterilising solution resulted in greater tissue necrosis and death. Moreover, shorter times and/or lower concentration of sterilising solution resulting in unsterilised and contaminated tissue. The callus culture successfully initiated from juvenile explants taken from *Colchicum autumnale* and *Gloriosa superba* did grow but with a very slow growth rate. Initially a particular problem associated with *Colchicum autumnale* callus culture was the relatively long doubling time for cells of 12-20 weeks, with consequent long duration of growth to achieve high callus tissue biomass. Callus tissue initiated from *Gloriosa superba* grew slightly more quickly than *Colchicum autumnale* callus tissue. The *Colchicum autumnale* callus tissue growth rates over four weeks were enhanced by 12.8, 22.4, and 29% on MS solid medium supplemented with 2,4-D in concentrations of (1, 3, and 5mg/l) respectively. Callus tissue initiated from *Gloriosa superba* callus tissue grew more quickly than *Colchicum* callus tissue and the growth rate of fresh weight in four weeks time were 16.8, 24.7, and 34.8% on MS solid medium containing 2,4-D concentration of (1, 3, 5mg/l) respectively.

Various concentrations (1, 3, 5mg/l) of three auxins (2,4-D, NAA, and IBA) in combination with (1mg/l) kinetin were tested for their effect on growth rates of callus tissue. It appeared to be that there was a direct relationship between 2,4-D concentration and callus fresh weight yield. The average fresh weight growth rate was promoted by increased 2,4-D concentration. The highest concentration of 2,4-D (5mg/l) showed relatively good callus tissue fresh weight growth rate by 29% and 34.8% for *Colchicum autumnale* and *Gloriosa*

superba respectively. It was found that using IBA and NAA resulted in a low growth rate callus fresh weight, and some callus tissues started to differentiate to multiple malformed roots at the end of the four weeks. *Colchicum autumnale* showed a lower response to the auxin treatments compared to *Gloriosa superba*.

Msitkita and Skirivin (1989) used 2,4-D and NAA in combination with BAP in various concentration (0, 0.25, 2.5, 5 mg/l) in MS medium for callus formation in *Gloriosa superba*, and they suggested that the maximum callus proliferation and doubling in size (every 12 days) was achieved with a medium supplemented with 2,4-D (1mg/l) and Kinetin (1mg/l). Sivakumar *et al.* (2003A) used 2,4-D in SH medium for initiation of embryogenic calli formation in leaf explants of *Gloriosa superba*. They used BAP (0.5mg/l) and IBA (0.25mg/l) for plantlet regeneration.

Hayashi *et al.* (1988A) had initiated callus using MS medium supplemented with 2,4-D (2mg/l) and they suggested that the cell growth rate was inhibited by 2,4-D, so they transferred the callus into MS medium supplemented with IBA (1mg/l) and Kinetin (1mg/l) where they attained higher callus tissue growth. The medium used by Hayashi *et al.* (1988A) proved ineffective in initiation and maintaining callus tissue in *Colchicum autumnale* and *Gloriosa superba*, even though the same explants (shoot tissue) were used in initiation of the callus from *Colchicum autumnale*. There is evidence that the growth of callus tissue is predominantly controlled by the genotypes of the plant used to initiate the callus (Ozgen *et al.*, 1996) and it has been reported that genotypes of certain monocotyledonous species may respond differently in culture (Pierik, 1976).

Varying the kinetin concentration incorporated into the medium had no significant effect on *Colchicum autumnale* callus fresh weight yield, with a moderate increase on *Gloriosa superba* callus fresh weight yield. *Colchicum autumnale* callus fresh weight yield were declined with BAP especially at the

higher concentration (2mg/l), and the tissue started to brown and growth rate declined.

The most effective plant growth regulatory hormones for the initiation and growth of callus tissue therefore appeared to be 2,4-D.

Due to the relatively good yield, friability and lack of organogenesis, medium supplemented with 2,4-D (3mg/l) and Kinetin (1mg/l) was chosen as the optimal growth hormone level for further callus growth and maintenance of *Colchicum autumnale* and *Gloriosa superba*.

Colchicum autumnale callus tissue grew moderately during the first 3-4 subcultures on medium supplemented with 2,4-D and Kinetin but eventually reached a steady growth rate with a doubling time of approximately 10-12 weeks.

Colchicum autumnale cell suspension cultures grow even more slowly than the callus tissues on solid medium with a growth rate by 5.8, 7, 11.7% with 2,4-D concentration (1, 3, and 5 mg/l) respectively. *Gloriosa superba* cell suspension cultures grow more quickly than *Colchicum autumnale* cell suspension cultures with growth rate by 12, 15.6, and 17% with 2,4-D concentration of 1, 3, and 5 mg/l respectively. No significant change in the cell viability was observed between the beginning and the end of incubation period, and this suggests 2,4-D had no adverse effect on cell viability even with higher concentration (5mg/l).

It is unclear why the callus tissue had an extended doubling time compared with other plant species like *Tobacco* callus, for example, which has a very high growth rate, with doubling in fresh weight from 4 to 5 times over 3 weeks (Helgeson *et al.* 1969).

The level of difficulty in establishing a culture varies greatly from species to species. In the case of many monocotyledonous plants, the process is often more protracted and difficult (Yeoman 1986). Poutaraud, and Girardin (2002) suggested that *Colchicum autumnale* has a rather unusual biology that does not favour cultivation. The natural seeds germination percent is low and the

delay between sowing and first flowering is reported to vary from three to five years.

Culture in simple medium supplemented with a strong auxin, such as 2,4-D, is generally sufficient to induce the formation of embryogenic callus in most species (Indra and Vimla 1991). According to Sharp *et al.* (1980) somatic embryogenesis development requires some prior callus proliferation, and embryos originate from induced embryogenic cells within the callus. In previous reports, 2,4-D was an essential hormone for the induction of somatic embryogenesis in many plants (Davidonis and Hamilton, 1983; McKersie and Brown, 1996; Zhang, 2000).

In a survey by Evans and Sharp (1986) on somatic embryogenesis in crop plants it was found that 70% of the explants were cultured and maintained on MS medium.

The application of embryogenic culture can be utilized for the production of artificial somatic embryos, the selection of somatic mutation and regeneration of transgenic plants (Jelaska, 1994). Embryogenic culture tissue from most higher plants is very similar in morphology with aggregates of embryogenic tissue being typically small, isodiametric, and densely cytoplasmic with small vacuoles (Finer, 1994). Proembryonic cells can be defined as cell clumps able to produce somatic embryos and consisting of small, dense, highly cytoplasmic cells, and having a meristem-like morphology (Williams and Maheswaran, 1986). Differentiation of somatic embryos is initiated in regions of densely cytoplasmic, meristematic cells. However, the content of cytoplasm was shown to be a major factor determining the embryogenic competence of single cells (Toonen *et al.* 1994). In the present study, it was predicted that the cells in the proembryogenic clusters may have developed from these types of small, densely cytoplasmic cells (Figures 2.19 and 2.20).

Although many studies related to micro-propagation of medicinal plants via somatic embryogenesis have been reported, in the literature there is no report of callus culture of *Colchicum autumnale* via somatic embryogenesis.

Sivakumar *et al* (2003A) managed to regenerate plantlets from *Gloriosa superba* callus tissue initiated from leaf explants on SH medium supplemented with 2,4-D. However, although different combinations of growth regulator hormones were assayed to induce plant regeneration in both species, none of the callus tissue cultures showed regeneration with any of the different concentration of auxins and cytokinins treatments. Lack of regeneration may possibly be explained by Aparan and Rashid (2003), who suggested that using high concentration of 2,4-D for a longer period is inhibitory to regeneration. Each developing embryoid passes through sequential stages of embryo formation, globular, heart shape, and torpedo shape. Although a given culture may differentiate these embryogenic cells, their further development may be blocked by an imbalance of chemicals in the culture medium.

The plant genotype can have a major impact on differentiation of plants from callus culture. Although many genotypes will proliferate to callus tissue when placed into culture medium, many of these genotypes will not produce plants. This trait (the ability to produce plants from tissue culture) has been shown to be under genetic control. A desirable genotype is expected to possess high callus induction and plant regeneration capacity. However, many studies have shown the absence of such a relationship between callus induction and plant regeneration capacity and, thus, the independence of these characters from each other (Sears and Deckard, 1982; Quesenberry and Smith, 1993; Ozgen, *et al.* 1998).

Callus tissue was transferred to a medium that contains components that stimulate shoot formation and could result into the formation of plantlets. Early studies (Skoog and Miller 1957) showed that the rate of shoot formation from callus was found to be dependant on the ratio of auxin to cytokinin in the culture medium.

Different natural and synthetic cytokinins [kinetin, BAP, Zeatin, and TDZ] were used either individually or in a combination of two at different

concentration (0.5, 1, 3mg/l) and after two subculturing in the same medium failed to induce or develop any shoots from callus tissue of *Colchicum autumnale* and *Gloriosa superba* even with a very strong cytokinins like TDZ and Zeatin. TDZ and Zeatin are two of the most active cytokinins and they induce *In vitro* shoot proliferation in many plant species. Several unsuccessful attempts were made in order to generate plants from callus tissue. The results indicated that failure in shoot formation is a major and limiting factor in obtaining whole plants from *Colchicum autumnale* and *Gloriosa superba*.

The medium supplemented with various cytokinins did not induce shoot formation from callus culture of *Colchicum autumnale* and *Gloriosa superba* at any given level of cytokinins combination.

Murashige, (1980) suggested that higher ratio auxin/cytokinins (over ten times) favours root development, and a lower ratio favours the development of shoots (under four times). *In vitro* induction of root tissue from *Gloriosa superba* callus tissue has been achieved in M1 and M2 medium. Two root cultures lines were established from callus tissue in two SH liquid media, M1 (supplemented with 2mg/l NAA, 1mg/l IBA and 0.5mg/l kinetin), and M2 (supplemented with 2mg/l NAA). Roots formed in M1 were thickened and unbranched while those in M2 were thinner and branched.

Colchicum autumnale failed to develop a good root tissue in both media and only malformed roots were grown from a callus tissue. Root developed from *Gloriosa superba* appears to respond in a similar manner in both root initiation media M1 and M2.

According to reports in the literature, non-transformed root cultures rarely grow very fast and often have a tendency to gradually turn into callus. An average growth cycle in such cultures is 60–70 days (Wysokińska and Chmiel, 1997).

The advantage of using *Gloriosa superba* root tissue cultures is that they grow rapidly, and are relatively easy to prepare and maintain. The root can be grown indefinitely if they are periodically subcultured into fresh medium.

Some root tissue cultures can produce alkaloids at higher levels than cell cultures or even parent plants (Woo et. al., 1995) and thus may provide an efficient means for the production of colchicine.

The growth rate of roots on solid medium was lower than the growth rate in liquid medium. This may be due to the fact that the root tissue were immersed in the liquid medium, and hence the roots have more contact with the nutrients in the medium, while on the solid medium the root tissue have only one contact side with the medium which was at the base of the tissue.

It was observed that the roots of *Gloriosa superba* were characterised by relatively fast growth and significant production of biomass (four times the mass of inoculum) in four weeks.

2.5 Conclusion:

Callus culture successfully initiated from juvenile explants taken from *Colchicum autumnale* and *Gloriosa superba*. 2,4-D had a significant affect on the growth rate of the callus culture of both species.

Colchicum autumnale and *Gloriosa superba* failed to develop a plantlets in a medium supplemented with various combination of growth regulatory hormones.

Two root cultures lines were established from *Gloriosa superba* callus tissue which was characterised by relatively fast growth rate and it seem practical for cultivation in bioreactors.

Chapter Three

Development and optimization of an analytical assay for colchicine

Section one: HPLC

3.1.1 Introduction

HPLC (High performance liquid chromatography) assay is the most widely employed technique in pharmaceutical analysis and Reverse phase HPLC is the most usually used HPLC method. It is estimated that 70-90% of separations take place on reverse phase columns (Poole and Schutte, 1984).

Highly sensitive and fast methods are required to detect low levels of colchicine in cells and tissue culture. A variety of HPLC methods have been described for the determination of colchicine in biological fluid (serum and urine) and in plants (Klein and Davis, 1980; Ondra *et al.*, 1995; Rosso and Zuccaro, 1998).

While excellent separation of colchicine has been achieved by HPLC, sensitivity depends upon the detection method. Tracqui and Kintz, (1996) had developed a very sensitive method, using HPLC coupled to ion spray mass spectrometry for the measurement of colchicine in biological fluids that gave a linear working range for colchicine of between 5-200ng/ml and a lower detection limit of 0.6ng/ml. This HPLC method appears to be more sensitive than a HPLC method coupled to UV detection. Thus HPLC may be an accurate, sensitive, and reproducible way of determining the colchicine concentration in an unknown sample.

The HPLC method developed in this study was applied for the detection determination of colchicine, demecolcine and colchicoside (Figure 3.1) in plant cell and tissue cultures and in the liquid medium. The method was then compared with the ELISA assay method using the same samples.

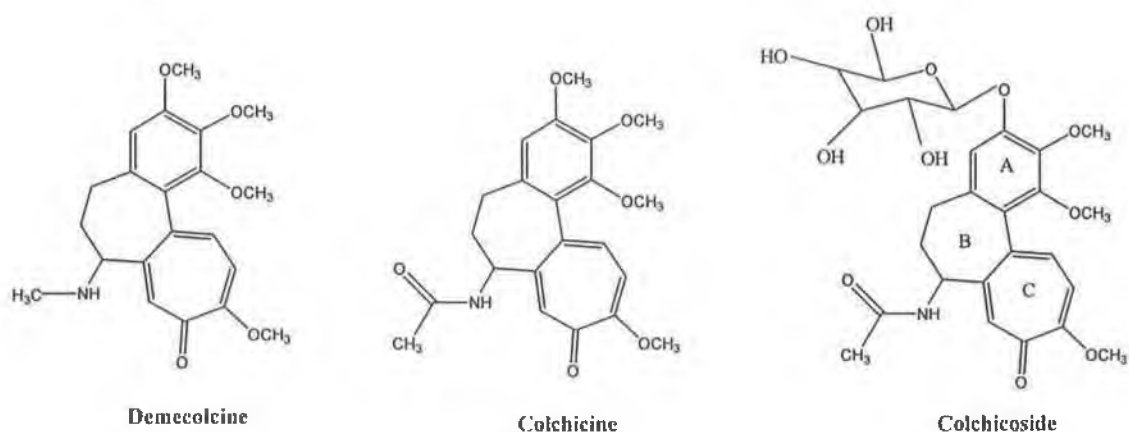


Figure 3.1: Chemical structure of colchicoside, Colchicine and Demecolcine.

3.1.2 Aim

To develop and refine a HPLC method to isolate, identify and quantify colchicine, demecolcine and colchicoside in plant cell culture. The method developed in this study was applied to determine colchicine in tissue and medium of *Colchicum autumnale* and *Gloriosa superba* plant tissue culture.

3.1.3 Materials and methods

3.1.3.1 Reagents

Colchicine, Demecolcine, and the solvents (Methanol, acetonitrile, chloroform, ethyl acetate) used in the chromatographic separation, extraction procedures and preparations of standard solutions were all HPLC grade and supplied by Sigma-Aldrich. Colchicoside was obtained from Indena, Italy.

Milli-Q water used to prepare Phosphate buffer was obtained by passing distilled water through a Milli-Q water purification system. All glassware was washed and rinsed with Milli-Q water.

3.1.3.2 Apparatus

The spectrophotometer spectrum for colchicine, demecolcine and colchicoside were carried out using UV-Visible Recording Spectrophotometer, (SHIMADZU).

The HPLC system used in this study was obtained from Varian system. The system consists of a Varian Inert 5012 pump. The injection system used was an automatic sample injector AI-200 RAININ with 100 μ l fixed loop. The detection system consisted of a Varian UV-Vis detector (9050).

3.1.3.3 Column system

The chromatography separations were performed using a LiChroCART^R RP-18 (5 μ m) column (250mm X 4mm) and the analytical column was protected by a guard column LiChroCART^R RP-18 (5 μ m) column (4mm X 4mm) purchased from Merck. All chromatograms and data was collected, processed and printed using a Star Varian LC workstation system control.

3.1.3.4 Chromatographic conditions

Analysis was performed isocratically using a mobile phase prepared with HPLC grade acetonitrile and methanol and phosphate buffer. The flow rate of the mobile phase was 1ml/min and the injection volume was 50 μ l. The column was kept and maintained at room temperature.

3.1.3.5 Preparation of solutions

3.1.3.5.1 Phosphate Buffer

Monobasic and dibasic sodium phosphate stock solutions were prepared NaH_2PO_4 (7.1 g/l) and Na_2HPO_4 (6 g/l).

50mM phosphate buffer solution was prepared by mixing 680ml of NaH_2PO_4 solution with 320ml of Na_2HPO_4 solution.

3.1.3.5.2 Mobile phase

The mobile phase was prepared by Acetonitrile, Methanol and phosphate buffer 50mM pH 6.2 (1:1:2 v/v).

All mobile phase solutions were filtered through a 0.45 micron membrane filter (Millipore), and degassed for 20min in an ultrasonic bath. The mobile phase was prepared fresh each day.

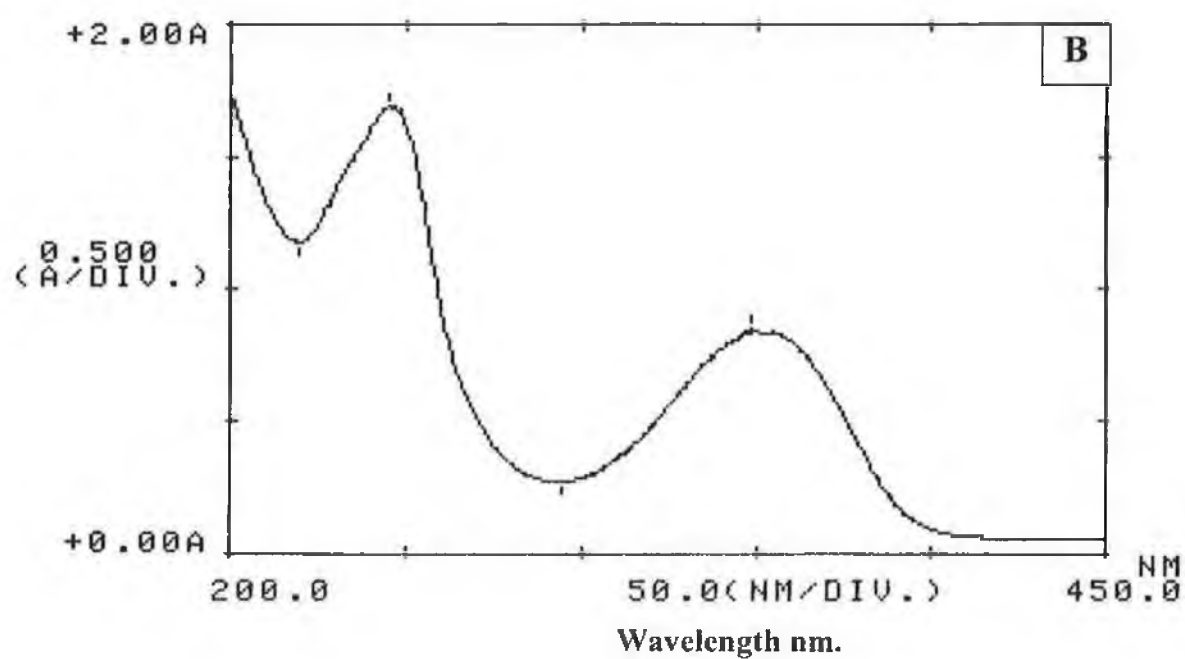
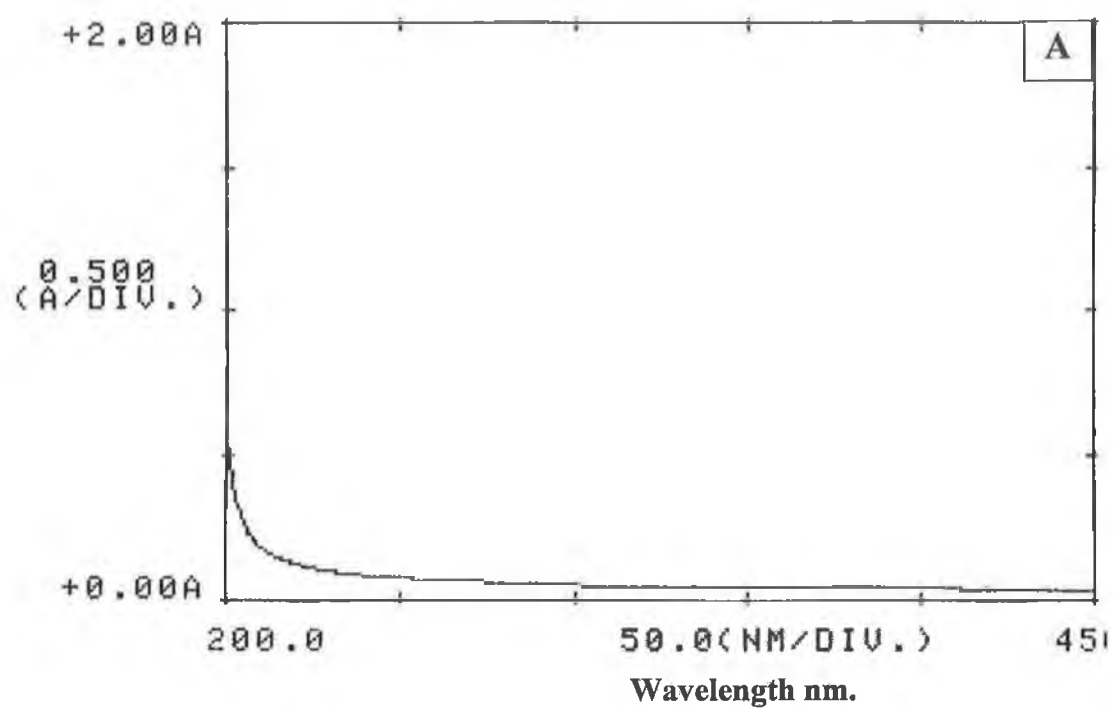
3.1.3.5.3 Standard solution

All standard stock solutions were prepared in HPLC mobile phase, and stored in the dark at 2-4 $^{\circ}$ C.

3.1.3.6 Chromatography analysis

3.1.3.6.1 Optimisation of the detection wavelengths

The principle of spectrophotometric detection is based on the simultaneous recording of signals at individual wavelengths. In order to establish the optimal wavelength for the UV-visible detector connected to the HPLC, standard solutions of the three compounds colchicine, demecolcine and colchicoside were prepared in mobile phase and scanned in the 200-450nm range. The UV-visible spectra of the three compounds exhibit two strong absorbance wavelengths at 245nm and 350nm, and absorbance minima at 220nm and 294nm. Absorbance at 245nm was sharper and stronger than that at 350nm. However a wide range of compounds absorb at 245nm and these could interfere with the assay whereas absorbance at 350nm showed less interference and was therefore chosen to optimise and validate the method. The mobile phase possesses no UV absorption between 200-450nm, and the change in the ratio of acetonitrile, methanol and phosphate buffer do not cause difference in absorbance (Figure 3.2). The peak homogeneity was viewed on screen in a two dimensional contour plot and the contour lines were found to be perfectly symmetrical (Figure 3.3).



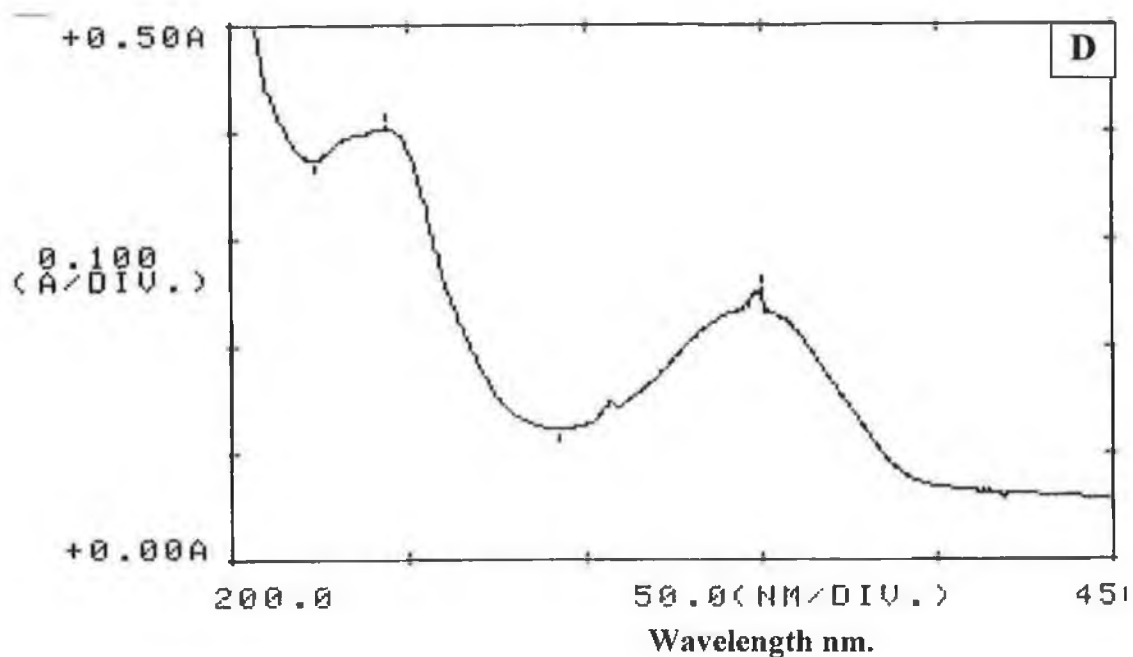
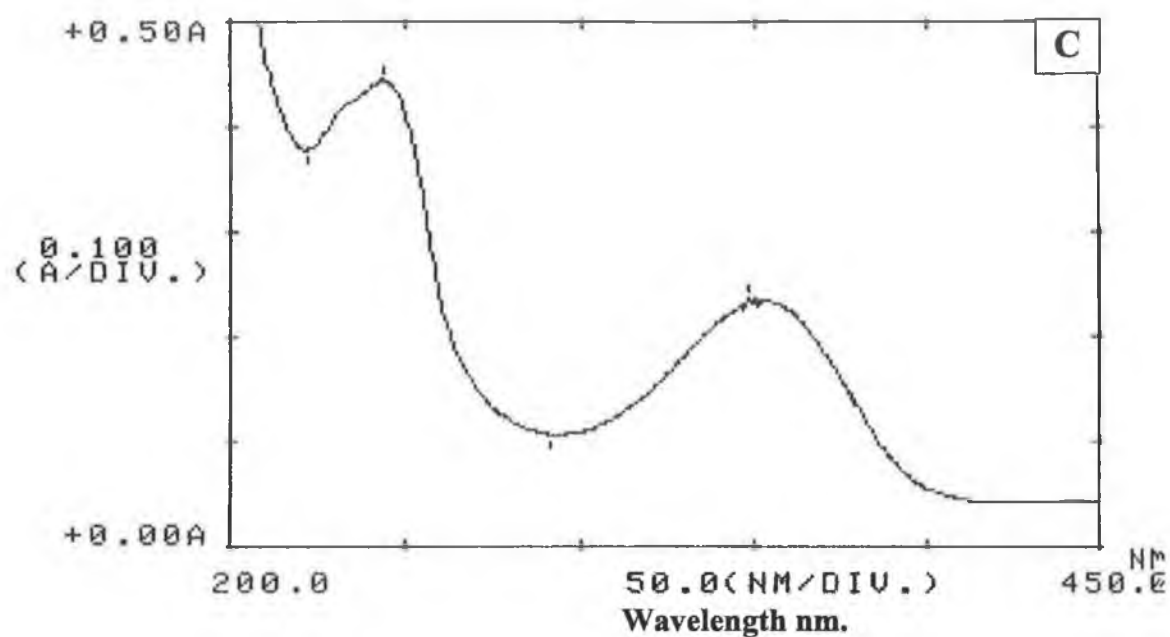


Figure 3.2: UV absorbance spectrum scans of A= Mobile phase, B= Colchicine, C= Demecolcine, and D= colchicoside.

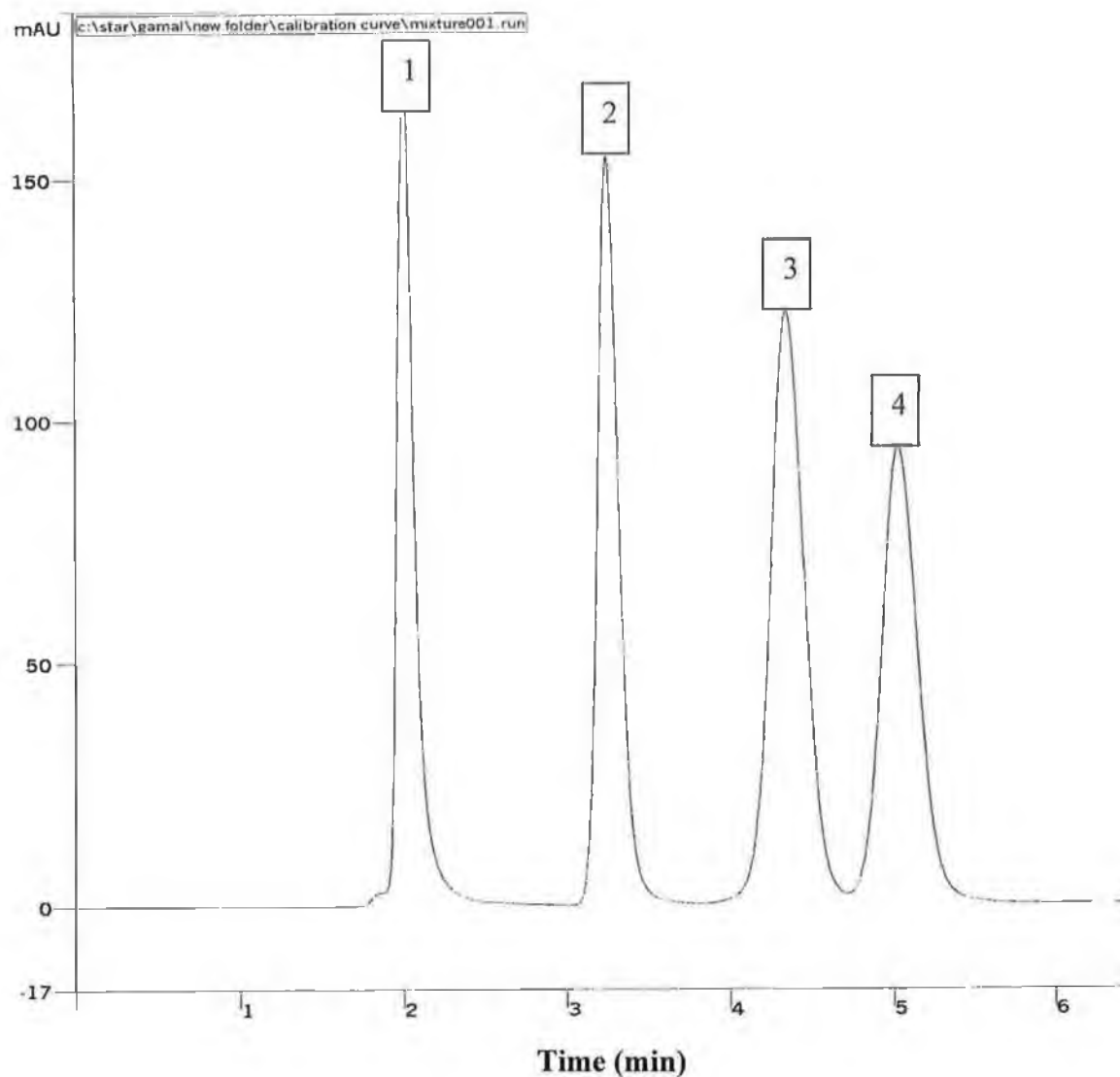


Figure 3.3: HPLC chromatogram standard mixture solution of Peak 1 = Colchicoside (20 μ g/ml), Peak 2 = (Internal standard) Acetosyringone (100 μ g/ml), Peak 3 = Colchicine (20 μ g/ml), and Peak 4 = Demecolcine (20 μ g/ml).

3.1.3.6.2 Calibration and quantification

A range of different concentrations of colchicine, demecolcine and colchicoside standards prepared in mobile phase was used to calibrate the drugs concentration. The slope and intercept of the calibration curves were obtained by linear regression of peak area ratios of the area unit of drug/area unit of internal standard versus drug concentration.

3.1.3.7 Culture extraction procedure

The extraction of colchicine, demecolcine and colchicoside from plant tissues was developed to give the following final procedure.

Three solvents (HPLC grade) Methanol, Chloroform and Ethyl acetate were investigated.

1ml of ultra-pure water was added to glass tubes containing 50mg of freeze dried *Gloriosa superba* seed powder, and 5ml of one of the solvents was added. The mixture was vortexed for 30sec and transferred to a mortar containing 50mg acid washed sand and blended by a pestle. The mixture was returned back to the original tube, centrifuged at 4,472Xg for 10min and the supernatant transferred to a clean tube and lyophilised until dry. The residue was reconstituted with 2ml mobile phase, centrifuged at 13,000rpm for 30min and the supernatant assayed. By comparing the peak area of colchicine, it was found that the recovery of alkaloid using ethyl acetate was only 60% of that achieved with methanol and chloroform. Furthermore, there were a higher number of interfering compounds in the ethyl acetate extract especially around the retention time of colchicoside. Given that ethyl acetate showed lower recovery compared to methanol and chloroform and also resulted in the extraction of undesirable interference compounds it was eliminated as an extraction solvent.

3.1.3.7.1 Optimisation of extraction procedure

5ml solvent was added to 50mg fresh weight tissue in 1ml ultra-pure water, and the mixture was transferred to a mortar containing 50mg acid washed sand and blended by a pestle. The mixture was transferred back to the original tube and centrifuged for 30min at 4,472Xg. The organic phase was then transferred to a clean tube and lyophilised dry. The residue were reconstituted with 1ml mobile phase and transferred to a microcentrifuge tube and centrifuged at 13,000 rpm for 30min and the supernatant assayed and this referred as single extraction.

This extraction step was repeated for extraction of the plant materials and the two extracts were mixed together and assayed. The results obtained from the two combined extraction steps were assumed to provide 100% alkaloids recovery.

A single extraction using chloroform or methanol produced an extract with a highly comparable recovery of 87% and 89% respectively, without extraction interfering compounds. It was therefore decided to use methanol as a solvent to extract colchicine from plant tissue as it can be injected directly into the HPLC system and does not need a drying step.

3.1.3.7.2 Modified extraction procedure

In order to extract alkaloids from a small amount of plant tissue (20-50mg fresh weight) the above method was modified. 1ml methanol containing the internal standard acetosyringone (100µg/ml) was added to residual plant tissue and vortexed for 30 seconds. The mixture was transferred to a mortar containing 20mg acid washed sand and blended by a pestle. The mixture was transferred back to the original microcentrifuge tube, and centrifuged at 13,000 rpm for 30min. The supernatant was transferred to an auto sampler 1.5ml vial, capped and stored at -20 °C prior to assay.

3.1.3.7.3 Extraction of colchicine from liquid medium

The samples were collected from liquid medium, and 1ml transferred into microcentrifuge tube and centrifuged at 13,000rpm for 30min. The supernatant was transferred to an auto sampler vial for assay.

3.1.3.7.3 Extraction of colchicine from solid medium

After four weeks of culturing, when the plant tissues were subcultured to fresh medium the used agar medium was cut into small pieces with a spatula, transferred to centrifuge tubes, and centrifuged at 26,891Xg for 30min at 4 °C. The volume of the resultant supernatant was determined and these were then assayed for colchicine concentration.

3.1.3.8 The effect of freeze drying on colchicine

Five concentrations of colchicine solution ranging from (0.5-2µg/ml) were prepared in mobile phase in duplicate; one set were frozen at -80 °C for 3h then lyophilised overnight. The dry samples were resuspended in mobile phase and assayed by HPLC simultaneously with the second set of non freeze dried samples that had been stored overnight at room temperature.

3.1.3.9 Data Analysis

The concentration of colchicine was expressed in µg/g Dry weight, based on the mean value of multiple analyses (3-4 samples) carried out in triplicate. Mean values with standard error were calculated and displayed in graphs using Microsoft Excel for windows.

3.1.4 Results

The HPLC method (Klein and Davis, 1980; Yoshida *et al.* (1988A and 1988B); Ondra *et al.*, 1995) involved the use of a Lichrosorb RP-18 column, and a mobile phase consisting of (7:1:12) v/v acetonitrile, methanol and phosphate buffer pH=6.2. This method was optimised for different ratios of phosphate buffer, methanol and acetonitrile. Different ratios of organic and phosphate buffer were used in order to establish the mobile phase composition which will give the optimum resolution and retention time of the various compounds. It was observed that when the organic content of the mobile phase was low (< than 25%) there was poor separation between colchicoside and the internal standard; likewise when the organic content was (> 50%) there was a tailing in the colchicine peak and this was not completely resolved from the peak of demecolcine. It was observed that decreasing the amount of phosphate buffer and increasing the ratio of methanol to equal amount of acetonitrile (1:1:2) v/v gave a good separation of all components (Figure 3.3) with high resolution peaks for each compound and no interfering of impurities in samples of plant tissue extract. The peak symmetry was also improved.

In the development of this method, there was a need to have an internal standard to correct for the variable recovery of analyte following sample preparation or differences in volume of sample injection. In this study, acetosyringone was found to be a useful internal standard. Acetosyringone eluted shortly after colchicoside and its peak was completely resolved from other peaks in the chromatogram.

The retention times of colchicoside, colchicine, and demecolcine were 2.01, 4.35 and 5.03min respectively. As seen in Figure 3.3 the method completely separated colchicoside, colchicine, and demecolcine. Furthermore, the peaks were completely resolved with no interfering peaks identified in tissue extract. The extraction efficiency was found to be 89%.

The relationship between the area ratio of colchicine to internal standard versus concentration was linear ($r^2 = 0.999$) at both low and high range

calibration curves. Figure 3.4 shows the colchicine calibration curve in a concentration ranging from (25ng/ml – 180µg/ml).

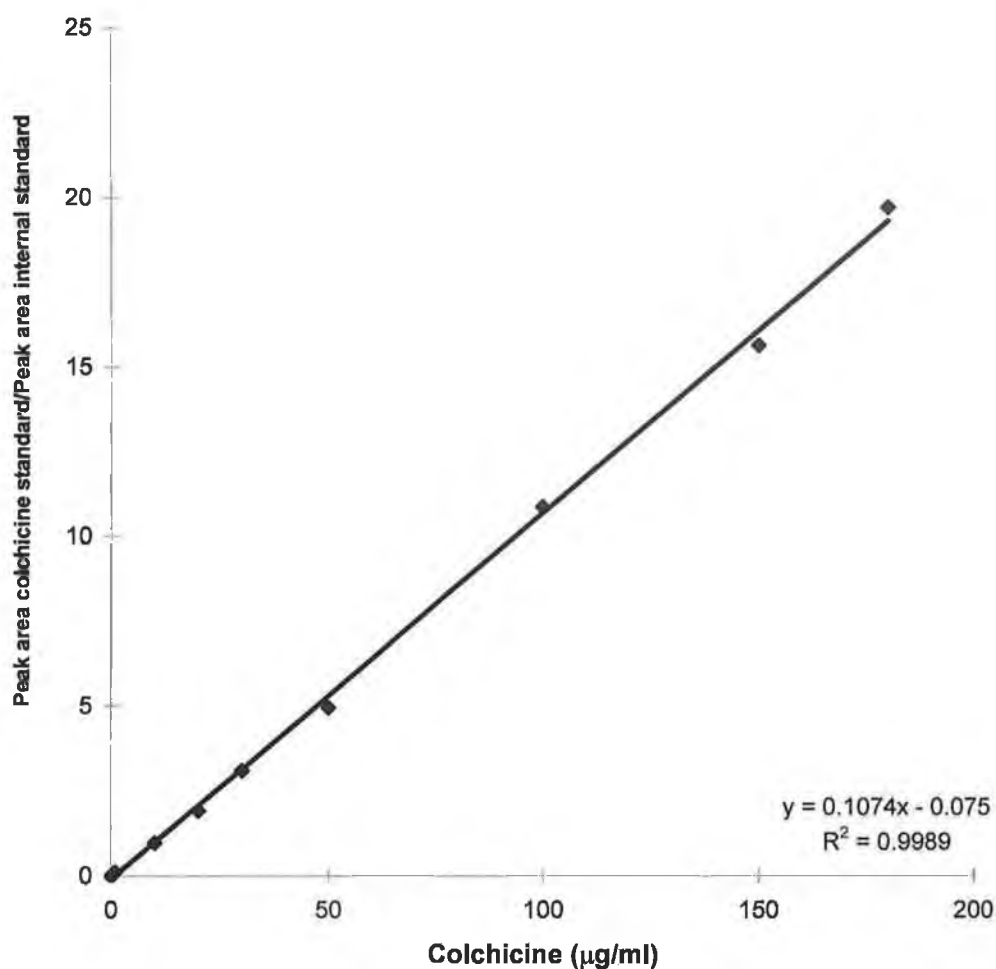


Figure 3.4: HPLC calibration curve for colchicine standards

3.1.4.1 Method validation

The validation of a method is the procedure used to prove that this method is sufficiently accurate and reliable to provide confidence in the data produced. The main criteria used for validating HPLC method include precision and accuracy, linearity, specificity, and limit of detection.

3.1.4.1.1 Precision and accuracy

Precision is the general term which expresses the variability between repeated determinations and can be divided into repeatability and reproducibility. Repeatability refers to replicate analyses of homogenous samples during one day (intra-assay precision). Reproducibility refers to replicate analyses over several days (inter-assay precision).

Both the intra-assay (within-day) and inter-assay (between-days) variability of this method were assessed. Each standard was prepared in duplicate in a mobile phase and assayed. Inter-assay variability was assessed by analysing each standard in quadruplicate on the same day. The mean values of the peak area ratio (peak area of standard/area peak of internal standard) were obtained, and linear regression analysis performed by plotting mean values of the peak ratios versus the amount added. The regression equation was then used to calculate the amount found for each individual standard, and the mean, standard deviation and coefficient of variation (%CV) was determined. Precision was expressed as percent coefficient of variation $[(\text{standard deviation} / \text{Expected return}) \times 100]$. Inter-assay variability was assessed by analysing with the second sets of standard, samples prepared previously on four separate days. Linear regression analysis was carried out for each day of calibration, and the amounts found for all standards. The mean, SD and %CV level was determined.

Table 3.1 shows the intra-assay precision for colchicine. Table 3.2 shows the inter-assay precision for colchicine. The mean coefficients of variation (CV) for intra and inter assay for colchicine were respectively (2.46 and 2.6). The results are shown in (Tables: 3.1 and 3.2).

Table 3.1: Intra-assay precision for colchicine

Conc. µg/ml	Average amount found (\pm SD)	%CV
0.05	0.046 (0.0025)	5.44
0.1	0.093 (0.0029)	3.12
0.5	0.480 (0.0192)	4.02
1	1.001 (0.012)	1.17
10	10.23 (0.109)	1.07
100	103.99(1.241)	1.19
150	147.87 (1.712)	1.19

Mean CV%= 2.46

Table 3.2: Inter-assay precision for colchicine

Conc. (µg/ml)	Day1	Day2	Day3	Day4	Average (\pm SD)	%CV
0.05	0.045	0.045	0.047	0.048	0.046 (0.0013)	2.74
0.1	0.092	0.092	0.094	0.100	0.095 (0.0038)	4.05
0.5	0.469	0.484	0.515	0.472	0.485 (0.021)	4.32
1	0.99	0.970	1.027	0.968	0.99 (0.027)	2.76
10	10.32	10.00	10.53	10.48	10.33 (0.234)	2.27
100	101.96	104.15	102.76	102.09	102.74 (1.007)	0.98
150	147.49	148.79	151.12	147.50	148.72 (1.712)	1.15

Mean CV%= 2.6

3.1.4.1.2 Accuracy

The accuracy, in terms of the difference between the amount of colchicine added and the amount found, can be seen from the intra- and inter-assay precision data and the regression equation of the four days assays (Table 3.3). The correlation coefficients were close to unity, and all intercepts closely go through the origin.

Table 3.3: Regression equation and correlation coefficient from the four days HPLC assay data.

Assay (day)	Regression equation	r^2
Day1	$y=0.0976X + 0.026$	0.9996
Day2	$y=0.0988X + 0.0234$	0.9993
Day3	$y=0.0995X + 0.0197$	0.9999
Day4	$y=0.0976X + 0.029$	0.9996

3.1.4.1.3 Linearity

Linearity of the method is its ability to produce a response which is proportional to the concentration of the standard within a given range. The response of different concentrations of standard was subjected to linear least squares regression analysis, and the correlation coefficient is used as an estimate of correlation. Linearity was assessed from the inter-assay precision and linear regression equations are shown in (Table 3.3). In this test, the linearity of the relationship between ratio of peak area and concentration for all calibration curves showed a linear response in the concentration range (25ng/ml – 180µg/ml) (Figure 3.4).

3.1.4.1.4 Alkaloid stability

Standard solutions of colchicine, demecolcine, colchicoside and the internal standard were periodically analysed to test their stability. Both stock solution and diluted samples were examined for possible decomposition under different conditions. No variation in the concentration of the tested compound was found when stock solutions in methanol and in mobile phase were stored at either room temperature or at 4°C for three weeks. Extracted samples were found to be stable at 4°C for at least 2-3 weeks.

3.1.4.1.5 Limit of detection

The limit of detection is the minimum concentration of analyte which gives a difference in response for analyte over the blank (Mobile phase). This is defined as a signal-to-noise ratio of 3, the blank measured peak was found to be 45µA. The limit of detection was that concentration which produces a signal of 150µA was reached with a 10ng/ml sample of colchicine i.e. 0.5ng colchicine injected samples (50µl injection volume).

Limit of quantification is the minimum levels of analyte which can be reliably determined to yield a peak area and were found to be (25, 30 and 30ng/ml) for colchicine, demecolcine and colchicoside respectively.

3.1.4.1.6 Specificity

The specificity of the method is its ability to resolve the analyte from potential interfering compounds. The question of whether a chromatographic peak is due to a single chemical entity or not, must be addressed during validation. The specificity of the method was determined by comparing the chromatograms obtained from the samples of plant extract with those obtained from standard samples. Several samples were examined for the presence of endogenous components which might interfere with the colchicine assay system.

In an effort to increase the confidence in specificity of the method, three samples of plant extract and liquid medium of each species were injected twice and each time were monitored at two different wavelengths, 245nm and 350nm, and the ratio of the area under the peak of the two readings determined and compared with standard colchicine samples. A comparison between the ratios of the peaks obtained with the two wavelength (245 and 350nm), showed that the average ratio of the plant samples were found to be comparable with that of colchicine standard solution (0.524, SD = ± 0.0334) and (0.542, SD = ± 0.0131) respectively. This indicates that the peak of colchicine from the plant samples were in agreement with the peaks of the colchicine standards (Figure 3.5).

The figure also shows that the standard deviations ranged approximately in the same interval of the average ratio of the colchicine standard samples. A oneway ANOVA test showed non significant variation. This could mean that the peaks of the plant extract and liquid medium were for colchicine which has a strong absorbance at those two individual wavelengths, evidencing another positive aspect of the specificity of the HPLC method.

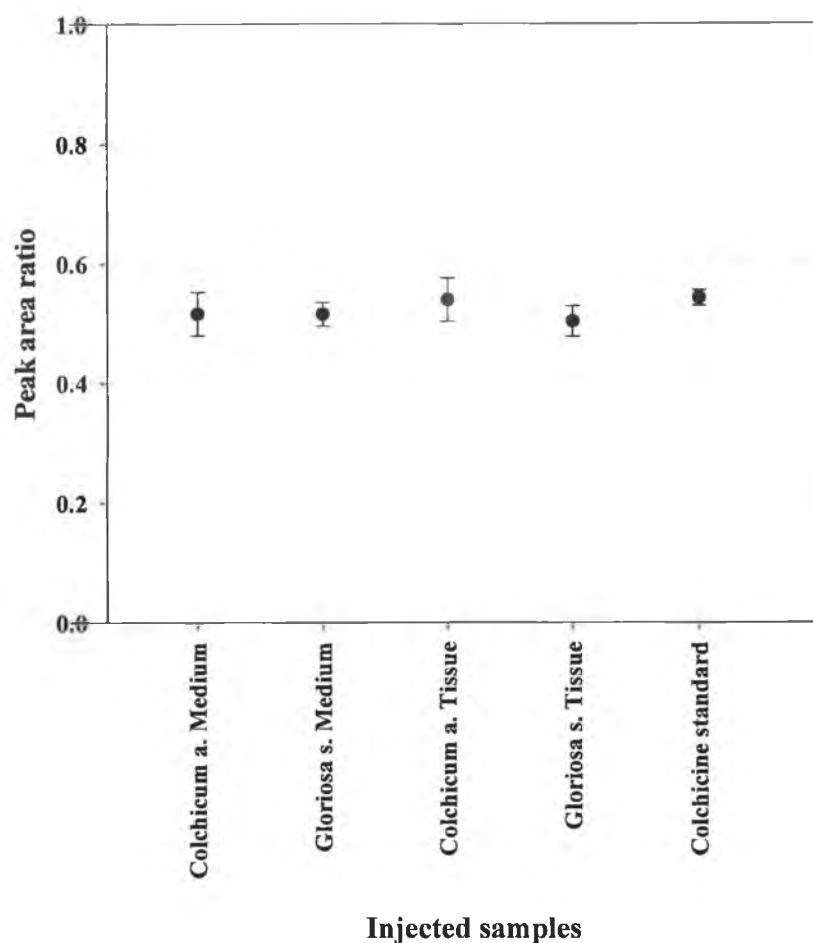


Figure 3.5: Average peak area ratio at 245 and 350nm of the plant samples and colchicine standard solution.

3.1.4.2 The effect of freeze drying on colchicine stability

The freeze dried samples were resuspended using mobile phase and assayed by HPLC simultaneously with the second set of non freeze dried samples, and the area under the peak was plotted against colchicine concentration (Figure 3.6). The graph shows overlapping of the regression lines of the both samples, and this indicates that freezing and lyophilisation has no effect on colchicine stability.

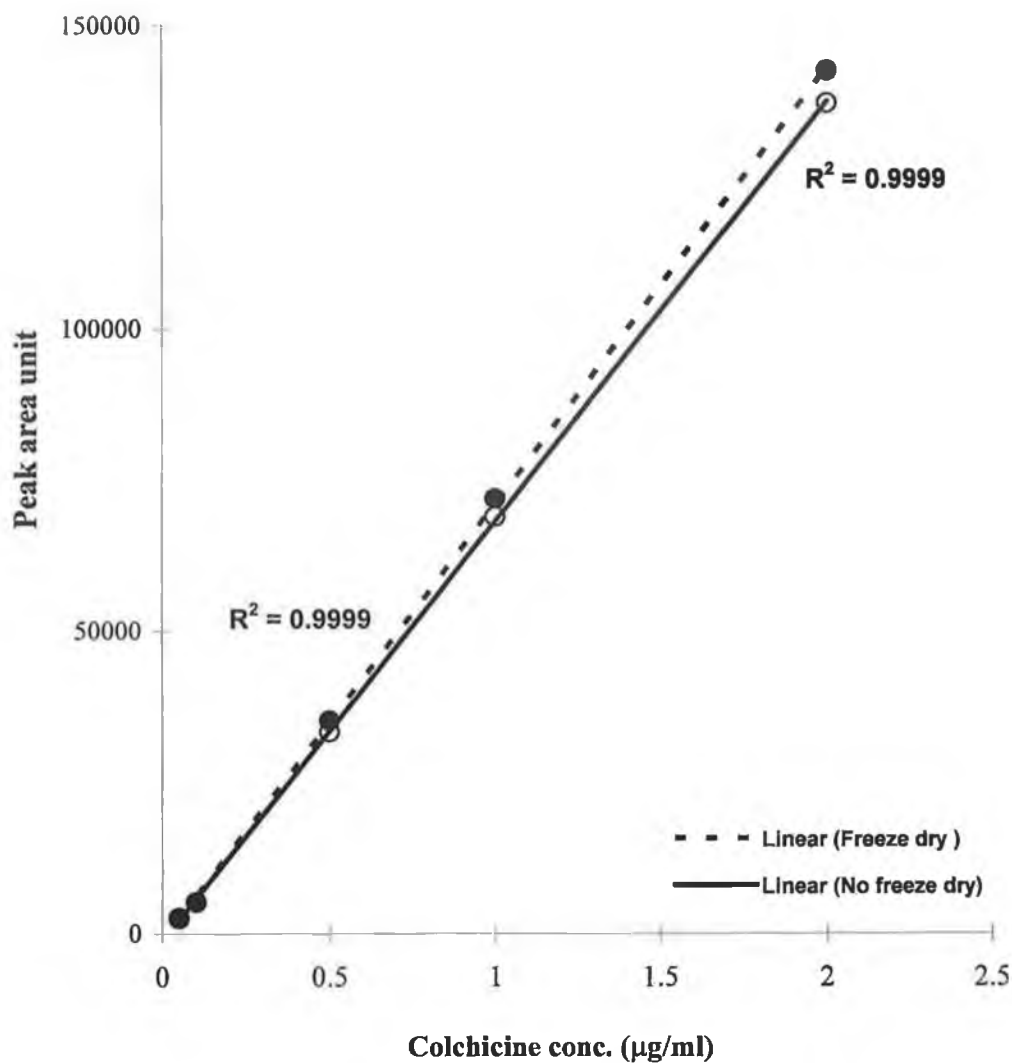


Figure 3.6: The regression lines obtained from two set of samples, the first one (closed circles) freeze dried and the second one (open circles) non dried samples.

3.1.4.3 Application of the HPLC assay method

The HPLC method was applied for determination of the colchicine concentration in medium and plant tissue.

3.1.4.3.1 Determination of the colchicine concentration in callus tissue

Callus tissue samples were collected at the subculturing time from different plates containing MS medium. The media were supplemented with various hormone treatments, as follows: Callus 2,4-D = callus tissue maintained on solid medium supplemented with 2,4-D (3mg/l) and Kinetin (1mg/l); Callus IBA = callus tissue maintained on solid medium supplemented with IBA (1mg/l) and Kinetin (1mg/l); Callus -H = callus tissue maintained on solid medium without hormones; Cell suspension = callus tissue maintained in liquid medium supplemented with 2,4-D (1mg/l) and Kinetin (0.5mg/l). Samples were collected randomly from five different plates of each hormone treatments.

The callus tissues were frozen at -80 °C for 3h, and then lyophilized overnight. The tissue was weighed, and then extracted using the method described in (Section 3.3.7.2), and assayed.

Figure 3.7 shows the concentration of colchicine in *Colchicum autumnale* callus culture maintained in different hormone regimes and in comparison with floral shoot and corm tissue taken from the intact plant.

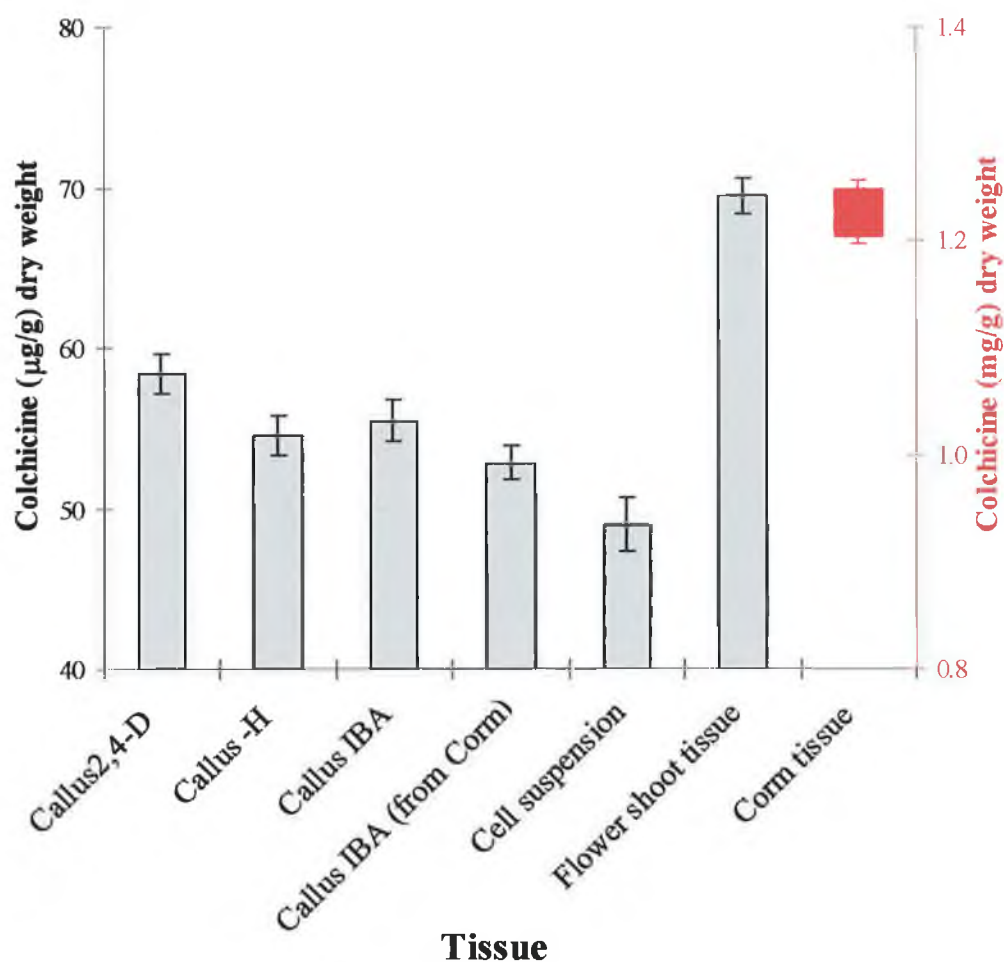


Figure 3.7: The concentration of colchicine in *Colchicum autumnale* callus tissue, and floral shoot. Samples were collected from five plates with the same hormone treatments. The red square is the concentration (mg/g) of colchicine in *Colchicum autumnale* corm tissue. Results are the average of tissue samples collected and the standard errors are included.

Figure 3.8 shows the amount of colchicine accumulated in *Gloriosa superba* cultures maintained in different hormone regimes, and in comparison with seed and shoot tissue taken from the intact plant.

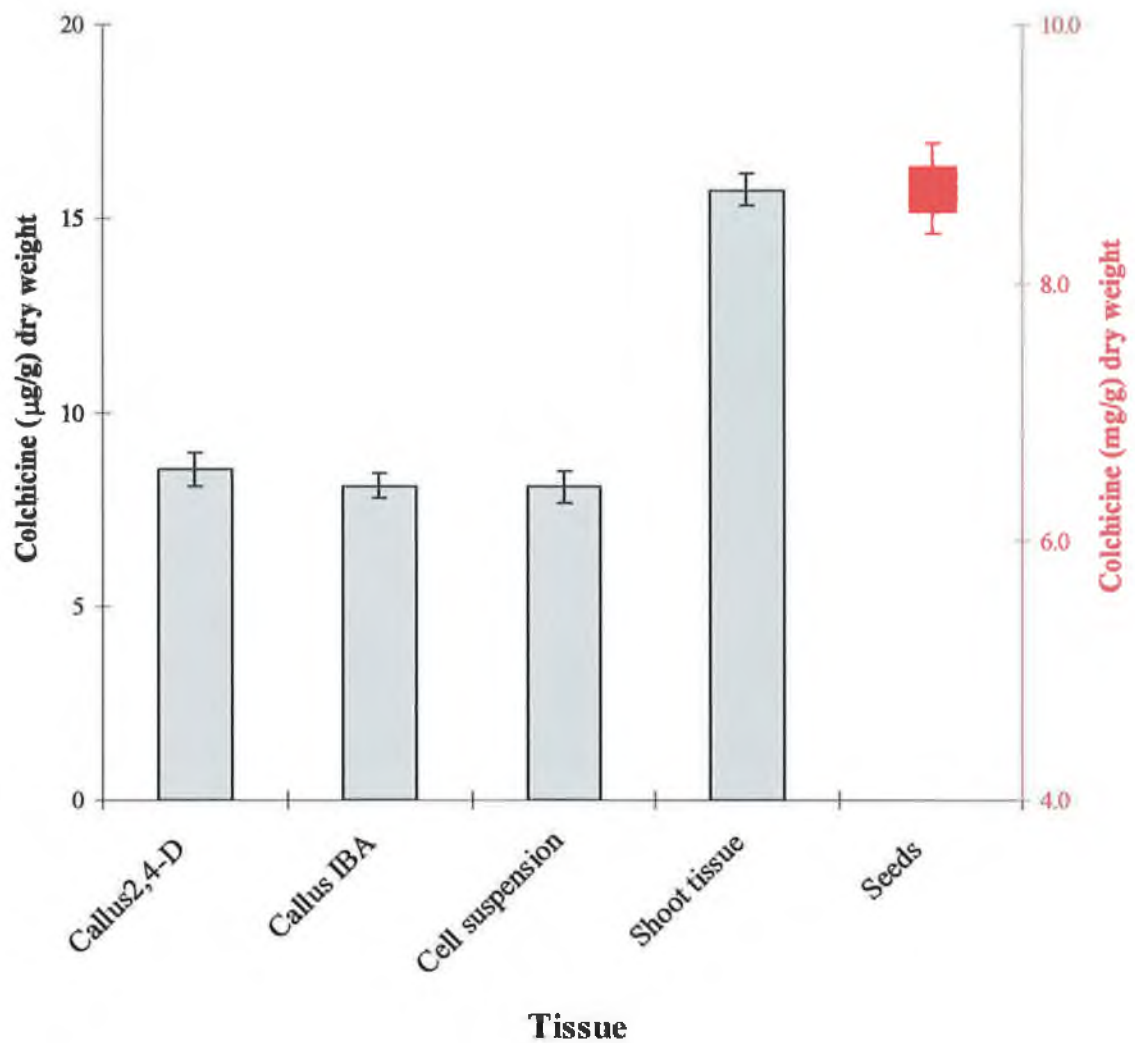


Figure 3.8: The concentration of colchicine in *Gloriosa superba* callus tissue and shoot tissue. Samples were collected from five plates with the same hormone treatment. The red square is the concentration (mg/g) of colchicine in *Gloriosa superba* seeds. Results are the average of tissue samples collected and the standard errors are included.

3.1.4.3.2 Determination of the colchicine concentration in solid medium

Following four weeks of culturing the callus tissues were subcultured in fresh medium, and the used solid agar media were collected using the method described (section 3.1.3.7.3). Figure 3.8 shows the concentration of colchicine in the solid medium.

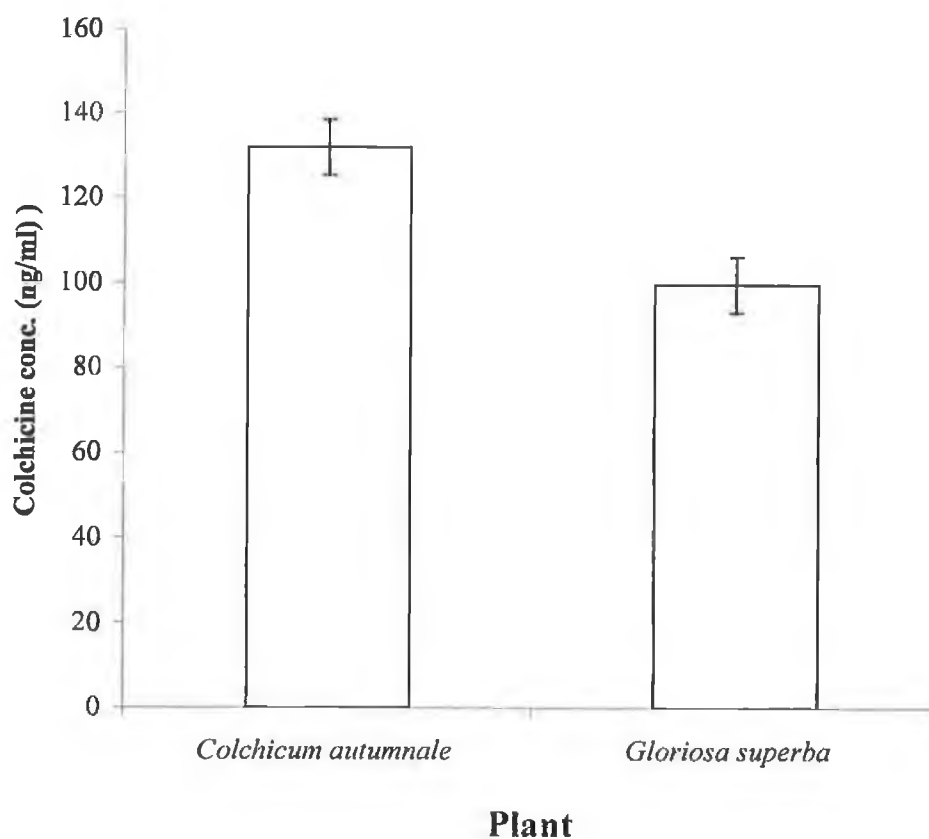


Figure 3.9: Colchicine concentration ($\mu\text{g/ml}$) found in solid medium following four weeks culturing of callus tissue *C. autumnale* and *G. superba*. Results are the average of media collected from six different plates and the standard errors are included.

3.1.4.3.3 Determination of the colchicine concentration in Liquid medium

Following four weeks culturing of the cell suspension culture 1ml samples were collected from six different flasks. Figure 3.9 shows the concentration of colchicine in the liquid medium.

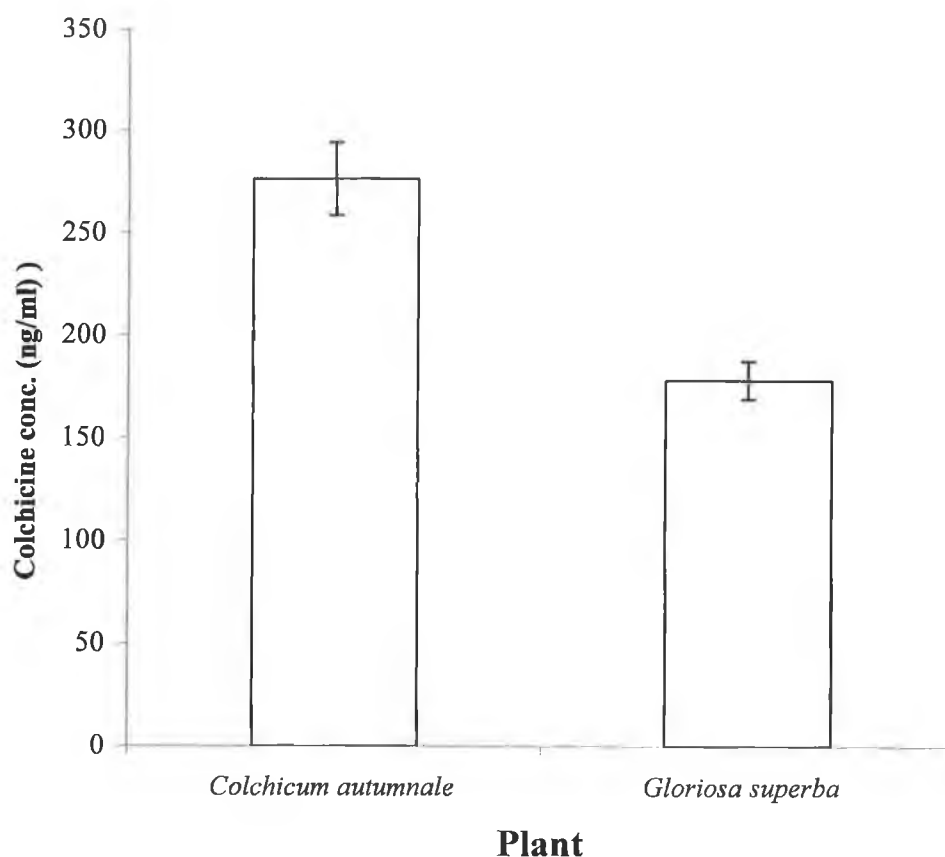


Figure 3.10: Colchicine concentration found in the liquid medium following four weeks culturing of *C. autumnale* and *G. superba* cell suspension culture. Results are the average of 1ml sample liquid medium collected from six different flasks. Standard errors are included.

3.1.5. Discussion

We have looked at the optimisation of detection and of chromatographic conditions and extraction procedures. Method development initially involved optimisation of the mobile phase. The key approach was to find the most appropriate mobile phase in the HPLC system. Various closely related isocratic mixtures were evaluated for a satisfactory resolution of colchicine, demecolcine and colchicoside in order to obtain a potentially successful mobile phase. The most satisfactory separation was obtained with Acetonitrile- Methanol- Phosphate buffer (50mM), pH 6.2 (1:1:2). The major factor examined was the ratio of organic component (acetonitrile and methanol) to the phosphate buffer and they were adjusted until a maximum resolution and symmetrical peak was achieved.

The development of the extraction procedure concentrated on using a solvent which gave a clean extract, little interference with chromatography, high percentage of extraction recovery and minimised the number of extraction steps. Methanol and chloroform gave a very good recovery for colchicine from plant tissue. Ethyl acetate was abandoned at early stage of extraction procedure, because it has a low recovery rate compared to chloroform and methanol. Chloroform was also eliminated as extraction solvent, because it is harmful. It also can not be injected directly into the HPLC system and needs an extra drying step. Methanol was selected as extraction solvent, as it gave an over all higher recovery for colchicine with few extraction steps and short analysis time.

Studies on intra-assay (within day) and inter-assay (between days) variability were carried out. Intra- and inter-day variability did not exceed 10% with less error throughout the examined range. There was an excellent linear relationship and the correlation coefficient of the regression lines generated from intra-assay (n=4) and inter-assay (n=4) experiments were near unity ($r^2=0.999$) with the slopes relatively constant over a concentration range of 25ng/ml – 180µg/ml. The method has excellent precision over a wide range of the calibration standard curve with the average CV value for intra-assay and

inter-day precision of 2.46% and 2.6% respectively. The present assay is sensitive, reproducible and convenient. This method provides a sensitivity of at least 25ng/ml colchicine concentration based on 0.5-1ml sample volume, which is suitable for studies using a small amount of plant tissue. The assay sensitivity was comparable with the assay reported by Tracqui et. al 1996 (5ng/ml) which used highly sophisticated equipment, and more sensitive than other reported methods, of Klein and Davis, (1980) and Fang and Zhang, (1999) who reported a method with linear working concentration in the range 10-200µg/ml and 40-240µg/ml respectively.

Nonetheless, new calibration curves were performed at the beginning of each chromatographic session to ensure accuracy.

It had been considered for a long time that undifferentiated cells, such as callus tissue or cell suspension cultures were not able to produce secondary metabolite products (Krikorian and Steward, 1969). Figure 3.7 and figure 3.8 shows that the callus tissues initiated from *Colchicum autumnale* and *Gloriosa superba* were indeed able to accumulate colchicine, although at a lower level compared to the amount of colchicine accumulated in corm or seeds. Colchicine levels in callus tissue of *Colchicum autumnale* were low, ranging from 49-58 µg/g dry weight in comparison with the 30-40 µg/g fresh weight reported by Hayashi *et al.*, (1988A). No significant difference in colchicine accumulation was found between callus tissues grown on solid medium supplemented with different auxins, but there was a slight decrease in colchicine accumulation in tissue grown in liquid medium. This may be due to the fact that colchicine was released from tissue into the surrounding liquid medium. The amount of colchicine in intact plant shoot tissue (69 µg/g dry weight) was comparable with the amount of colchicine in the callus tissue. The amount of colchicine found in corm tissue (1.23 mg/g dry weight) was about twenty times higher than the amount in the callus tissue culture.

Colchicine levels in callus tissue of *Gloriosa superba* was significantly lower (8 µg/g dry weight) than that of *Colchicum autumnale* callus tissue (49-58

µg/g dry weight). The amount of colchicine in shoot tissue was nearly double the amount in callus tissue. There were no significant changes in colchicine accumulation in callus tissue grown on solid medium supplemented with different auxins, or in liquid medium.

In contrast to other alkaloids such as sanguinarine produced by cell suspensions of *Papaver somniferum* (Archambault *et al.* 1996) and capsaicin produced by *Capsicum frutescens* cells, which released capsaicin into the medium (Lindsey, *et al.*, 1983), colchicine has never, until now been detected in the culture medium (Ghosh, *et al.*, 2002).

Colchicine was detected in both the solid and liquid medium used for callus tissue culture of *Colchicum autumnale* and *Gloriosa superba*.

The concentration of colchicine accumulated in the solid medium was 132 ng/ml (SD±16) and 99 ng/ml (SD±16) for *Colchicum autumnale* and *Gloriosa superba* respectively.

The concentration of colchicine accumulated in the liquid medium were 276 ng/ml (SD±44) and 178 ng/ml (SD±23) for *Colchicum autumnale* and *Gloriosa superba* respectively.

There was a significant difference between the amount of colchicine released into solid and liquid medium. That might be due to the fact that the cells were suspended in liquid medium, while callus tissue grown on solid medium has only one contact side with the medium.

No demecolcine or colchicoside were detected in tissue culture or in the medium. Colchicoside was only detected in seed extracts of *Gloriosa superba* where a high concentration of colchicine was also found. There is evidence that the glucosylated alkaloid is accumulated in the matured bulbs and seeds. The alkaloid-specific glucosylation probably occurs in the tissues during maturation (Yoshida, *et al.* 1988; Poutaraud and Girardin, 2003) as the authors did not detect colchicoside either in leaves or capsules of *Colchicum*

autumnale, and that colchicine content decrease as the colchicoside content increases with seed maturation.

3.1.6 Conclusions

A convenient and sensitive HPLC method for quantifying colchicine, demecolcine and colchicoside in culture medium and plant tissue was developed. The assay method was simple, precise and accurate for the determination of colchicine in medium and tissue culture and enabled comparatively easy processing of large number of samples.

This method was used to demonstrate: A- That colchicine was present in the intact plant in anticipated concentration. B- That cell cultures accumulate colchicine. C- That colchicine was detected in the media of callus and cell suspension cultures.

Sensitivity is a major criterion for every method, and in order to quantify colchicine in a small quantity of plant tissue which may contain low colchicine concentration, the limit of quantification of this assay at sensitivity 25ng/ml of colchicine may be insufficiently sensitive. There is therefore a need for a more sensitive method.

The HPLC method developed in this study was applied for detection and determination of colchicine, demecolcine and colchicoside in plant cell and tissue cultures and in the liquid medium. The method was then compared with the ELISA assay method using the same samples.

Section II: ELISA assay

3.2.1 Introduction

HPLC is sufficiently sensitive for detection of many alkaloids from large scale tissue cultures. However, where alkaloids are produced in low concentrations or in small scale cultures, it can be difficult to reliably measure alkaloid concentration. It is also a lengthy and costly procedure. Robust and sensitive method for detection and quantification of colchicine in very low concentration was critical in order to investigate whether a particular precursor fed to a culture could stimulate its production. An alternative and a complementary analytical technique were therefore required. Enzyme-Linked Immunosorbent Assay (ELISA) has become one of the most popular methods for quantification of analytes in samples because, in addition to their sensitivity and specificity, they are simple and less costly than most other analysis methods. ELISA is a useful and powerful method for estimating ng/ml to pg/ml concentrations of analytes in solution, such as serum, urine and tissue culture supernatant and extracts. Immunoassay is a bio-analytical method that relies on the specific binding of an antibody to an antigen. Although various types of immunoassay have been developed and frequently used for the detection of a variety of substances (Harlow, and Lane 1988), Enzyme-linked immunosorbent assay (ELISA) is the most widely accepted format in drug immunoassays.

3.2.1.1 Assay principle

Direct competitive ELISA and indirect competitive ELISA are the most prevalent types of ELISA for the analysis of small molecules. The indirect, two-step method uses a labelled secondary antibody for detection (Figure 3.11). This was first described by Weller and Coons, (1954) and is still a popular method. A disadvantage with this method is that an extra incubation step is required in the procedure.

In the indirect competitive ELISA system, which was used in this work, the antigen (antigen-protein conjugate) is immobilized and bound to the surface of the ELISA plate usually on 96-well micro plate (Figure 3.11) and unoccupied sites blocked by an excess of protein. Washing step is carried out between each step to remove the unbind materials. A fixed amount of primary antibody is added, along with the free antigen the substance whose presence is to be determined, which in this case is colchicine. There is competition between the free and bound antigen for binding to the primary antibody. A 2nd enzyme-labeled antibody is added which binds to the primary antibody, bound to the immobilised antigen. This enzyme is responsible for converting a colourless substrate to a coloured product. The detection occurs with the addition of a substrate (urea peroxide) which is utilized by the bound enzyme-labeled antibody to produce a yellow colour upon reaction with the enzyme. The activity of the enzyme is determined by optical density reading of absorbance of the coloured product using a spectrophotometric micro-well reader. The resulting absorbance values are inversely proportional to the colchicine concentration in the sample: high concentrations of antigen in the assay will compete successfully for the free primary antibody and leave most of it in solution leaving little bound antibody for the secondary antibody to bind to and hence low absorbance reading. With low concentrations of antigen, high levels of primary antibody will bind to the antigen-protein conjugate leading to high absorbance values.

Quantitative determination is generally performed by progressively diluting the sample. The absorbance readings from these samples are then plotted against the log concentration, and the calibration curve generated from these experiments can be used to determine unknown concentrations of the substance.

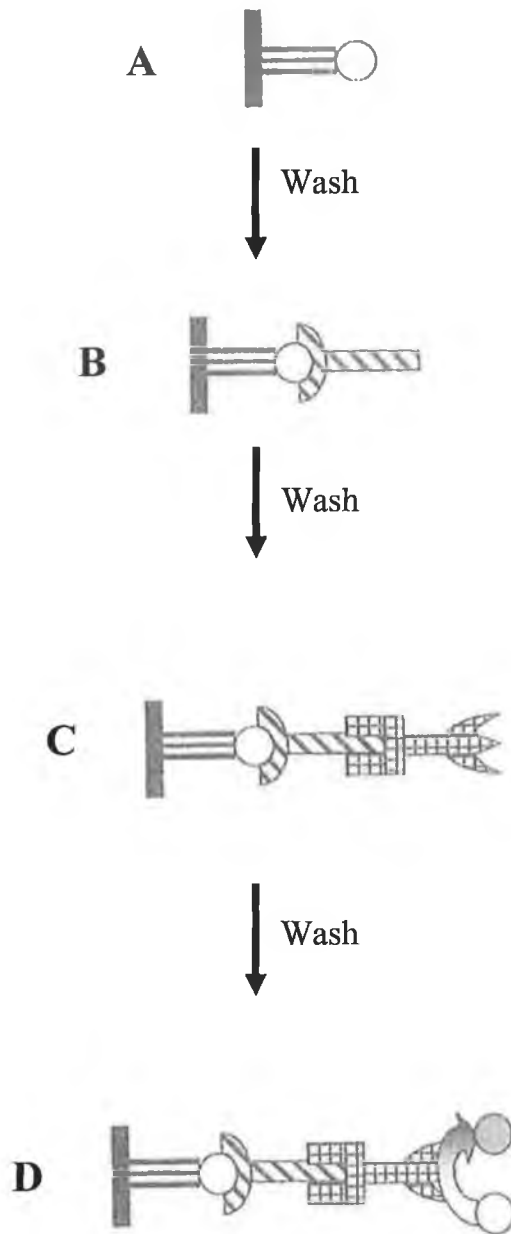


Figure 3.11: Schematic presentation of the indirect ELISA: A= Antigen and antigen protein conjugate are bonded to the plates, B= primary antibody is introduced, and the free antigen competes with the bound antigen for binding to the first antibody, C= secondary, labelled-enzyme is bound to the primary antibody, D= enzyme-substrate, linked to the secondary antibody, produces a colour product. The resulting absorbance values are inversely proportional to the colchicine concentration in the sample. Washing step is carried out between each step to remove the unbind materials.

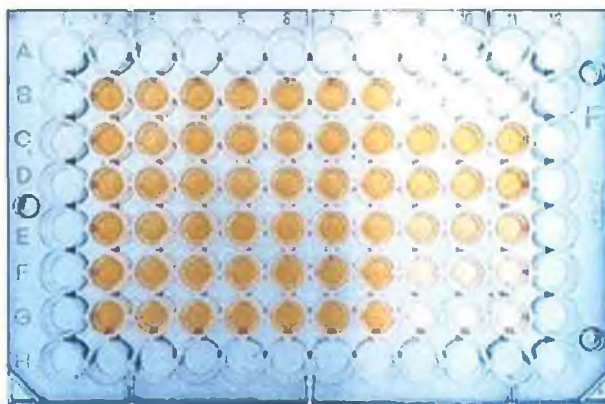


Figure 3.12: Nunc-Immuno flat bottom plate used in development and optimisation of the ELISA assay.

3.2.2. Aim

Development and evaluation of an ELISA strategy that could be employed for determination the quantity of colchicine in culture liquid medium and plant tissue extract.

3.2.3 Materials and Methods

3.2.3.1 Equipment

96-Well microplate for ELISA (Nunc Immuno TM plates Maxi Sorp surface)

Obtained from Lennox company, Dublin.

8 channel micropipette 50-350 μ l (BRAND).

Multichannel spectrophotometer, Titertek Multiskan plus (ICN flow).

Dialysis tubing cellulose membrane (Sigma)

3.2.3.2 Chemicals and Immunochemical

Ethanol, Sodium Periodate (NaIO_4), Glycerine, Bovine serum albumin, Ovalbumin (Albumin from chicken egg), Potassium carbonate (K_2CO_3), Sodium borohydride (NaBH_4), Formic acid (CH_2O_2), Goat Anti-rabbit IgG (Whole molecule) with peroxidase conjugate, Tween-20, O-phenylenediamine Dihydrochloride (OPD) tablet set, colchicine and demecolcine were obtained from Sigma. Colchicoside was donated from Indena, Company Italy. Phosphate buffered saline (PBS) tablets were obtained from OXIDA. Marvel - dried milk powder was obtained locally.

3.2.3.3 Extraction procedure

Plant material was extracted as described in section 3.1.3.7.3, the methanol extract was lyophilised and the residue resuspended in 1ml PBS.

3.2.3.4 Solution preparation

3.2.3.4.1 PBS solution

The PBS (0.1M, pH 7.4) solution was prepared by dissolving 10 tablets in 1l of ultra pure water.

3.2.3.4.2 Tween – PBS 0.05% solution

Tween 20 – PBS solution was prepared by adding 0.5ml to a 1l of PBS.

3.2.3.4.3 Blocking solution

5% Marvel solution was prepared by dissolving 5g of marvel milk in 100ml of PBS solution.

3.2.3.5 Development of an immunoassay

The first procedure in the development of an immunoassay is the production of the antibodies. The steps include selection of hapten, synthesis of hapten, immunization and purification of the antibody. The next step involves the development and optimisation of the assay.

3.2.3.5.1 Preparation of antigen (colchicoside-BSA conjugate)

It is well known that under ordinary circumstances, low molecular weight compounds (below 1000 g/mole) are generally not immunogenic (Kutney, *et al.* 1980).

Colchicine is such a small molecule (M.wt = 399) and hence not capable of triggering an immune response in an animal. In order to elicit an immune response, small molecules such as colchicine must first be covalently linked to a larger protein called a carrier protein, such as bovine serum albumin (BSA), to make them immunogenic prior to being introduced into the host organism. To ensure production of specific antibody, the protein conjugate must resemble as closely as possible the structure of the original molecule. As colchicine does not possess a suitably reactive chemical group for the preparation of a hapten, we used colchicoside, which has the same structure as colchicine but with an attached sugar group which can be used for conjugation to a protein. We followed the method described by Poulev, *et al.* (1994). A solution of 10mg colchicoside in 0.5ml of 67% ethanol was prepared and 2.5ml of periodate (NaIO₄) (6.25mg/ml) was added dropwise over a period of 30min with stirring at room temperature. Two drops of 50% aqueous glycerine was added to neutralise the excess of NaIO₄, followed by a further 30 min of stirring, then the precipitate was removed by filtration. The filtrate was added to a 10ml solution of bovine serum albumin (BSA) (2mg/ml) in water dropwise with stirring at room temperature. The pH was kept at 9.2-9.5 by adding 5% aqueous K₂CO₃. After an additional 90min, 10ml of fresh solution

of NaBH_4 (1mg/ml) in water was added dropwise and the whole mixture stirred for 3h. Two drops of 1M formic acid (HCOOH) was added to neutralize any excess NaBH_4 followed by 1h of stirring. The pH of the reaction mixture was adjusted to 8.5 with 1M KOH. The mixture was then transferred into a dialysis cellulose tube and dialysed 3 times for 24h each against PBS at 4 °C. The conjugate was split into 1ml aliquots and stored at -20 °C.

3.2.3.5.2 Antibody production

Rabbits are the most popular species used to generate antisera, and the most commonly used adjuvant for antibody production is complete Freund's adjuvant, followed by 2 or 3 subsequent booster injections (immunogen emulsified with incomplete Freund's adjuvant at 2-4 weeks intervals). The immunisation conjugate used was Albumin-colchicoside conjugate prepared just described (Section 3.2.3.5.1). 1ml of Albumin-colchicoside conjugate (1mg/ml solution) was emulsified in an equal volume of complete Freund's adjuvant. 1ml of the emulsion was inoculated subcutaneously at multiple sites into a New Zealand female white rabbit. A blood sample was collected by ear vein puncture before the immunisation and used as control blood (pre bleed serum). After two weeks a booster immunisation was prepared by emulsifying colchicoside conjugate 1mg/ml with an equal volume of Freund's incomplete adjuvant. The mixture was inoculated subcutaneously in different sites to the same rabbit. After another two weeks a second booster immunisation was applied again to the same rabbit.

After 14 days of the second booster immunisation, a blood sample was collected and tested for antiserum titre. A reasonable amount of antibody against colchicine was detected in the sera. 25ml of blood was collected from the rabbit. The serum was separated by centrifugation at 4472Xg for 10 min. Serum was dialysed overnight at 2-8 °C against 1 litre PBS solution and this step was repeated twice, and the serum stored at -20 °C. Serum stored at -20 °C could be kept without detectable loss of activity over periods of months.

3.2.3.5.3 Standard protocol for ELISA

Titre and characterization of antiserum were performed by competitive ELISA following standard method (Harlow, and Lane, 1988).

- 1) All the wells on the plate were coated with a 100µl of colchicoside-BSA. The dilution was made up from a 1mg/ml stock solution in PBS.
- 2) The plate was covered in parafilm and incubated at 37 °C for 1 hour.
- 3) The plate was then washed three times with freshly made PBS-Tween solution and one time with PBS then tapped dry on tissue paper.
- 4) The plate was blocked with a 5% Marvel solution in PBS-Tween, with 350µl being added to each well.
- 5) The plate was covered in parafilm and incubated overnight at 4 °C.
- 6) Washing was again carried out three times using PBS-Tween solution and once with PBS, then tapped dry on tissue paper.
- 7) Dilutions of colchicine standards and the unknown samples were made up in PBS and 50µl was added to the wells.
- 8) The polyclonal antiserum (1st antiserum) and pre-bleed serum sample were diluted in PBS-Tween and 50µl was added per well.
- 9) The plate was covered in a parafilm and incubated at 37 °C for 1 hour.
- 10) After incubation the plates were washed three times with PBS-Tween solution and once with PBS then tapped dry on tissue paper.
- 11) The 2nd antibody anti-rabbit IgG was made up in PBS-Tween with 1% Marvel to a dilution of 1:5000. 100 µl was added to each well then the plate was covered in parafilm and incubated at 37 °C for 1 hour.
- 12) The plates were washed three times with PBS-Tween solution and once with PBS then tapped dry on tissue paper.
- 13) OPD substrate was made up by dissolving urea buffer (gold tablet) in 20 ml of distilled water, then the (silver tablet) OPD dissolved in the urea buffered solution, and 100µl was added to each well.
- 14) The plate was covered in tinfoil and incubated at 37 °C for 30min.

15) The absorbance was read at 450nm using a Titertek Multiskan Plate reader every 10min.

3.2.4 Results

Developing the ELISA methodology involve validation and optimisation of the assay components in order to obtain a good sensitivity and a reliable assay.

3.2.4.1 Analysis of the conjugate

The conjugate mixture was dialysed three times in order to make sure that the non-conjugated colchicoside was cleared from the mixture. The dialysed colchicoside conjugate solution was scanned by spectrophotometer between 200-400nm. Figure 3.13 shows the scan.

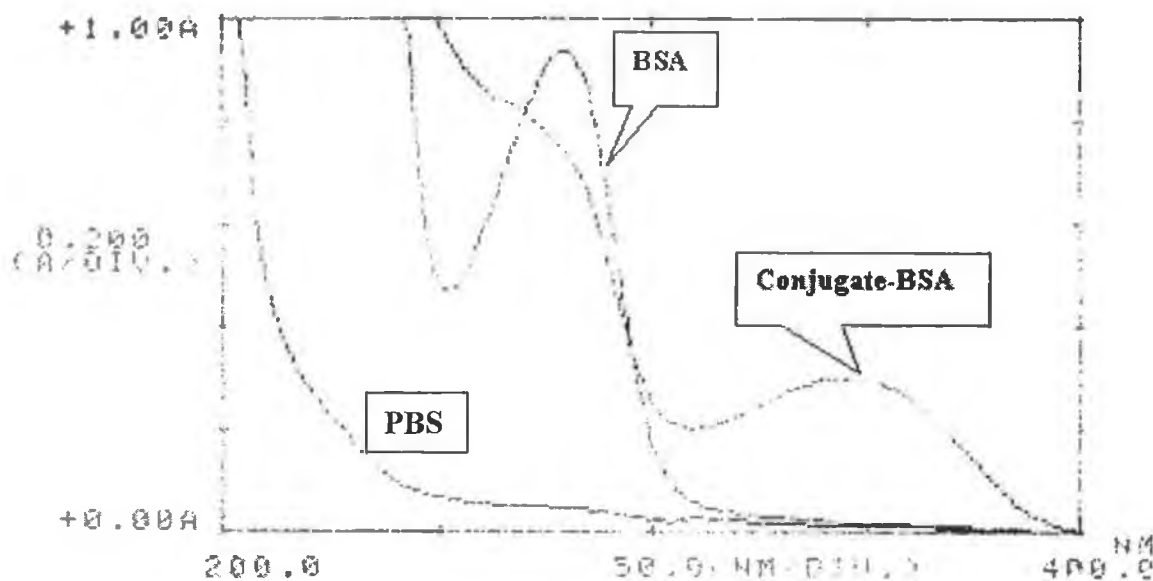


Figure 3.13: Spectrophotometer scans of the three solutions PBS, conjugate-BSA and BSA.

3.2.4.2 Antiserum titre

To determine the range of the 1st antiserum concentration and if there was any non-specific antibody binding to the plate, a series of ELISAs were carried out as per the standard protocol (section 3.2.3.5.3). The concentration of colchicoside-BSA used to coat the plate was 10µg/ml. The dilutions of the 1st antiserum and pre-bleed serum were made in PBS from frozen stock solution at a concentration which ranged from 1:100 to 1:204800. 100µl of the 1st antiserum, pre-bleed or PBS was added to the wells. First antiserum dilutions were added to wells in rows A-E, the pre-bleed serum dilutions were added to wells in rows F,G and the PBS was added to wells in row H.

There was a need to consider the potential background absorbance reading of the pre-bleed serum on the assay. The results are shown in Figure 3.14.

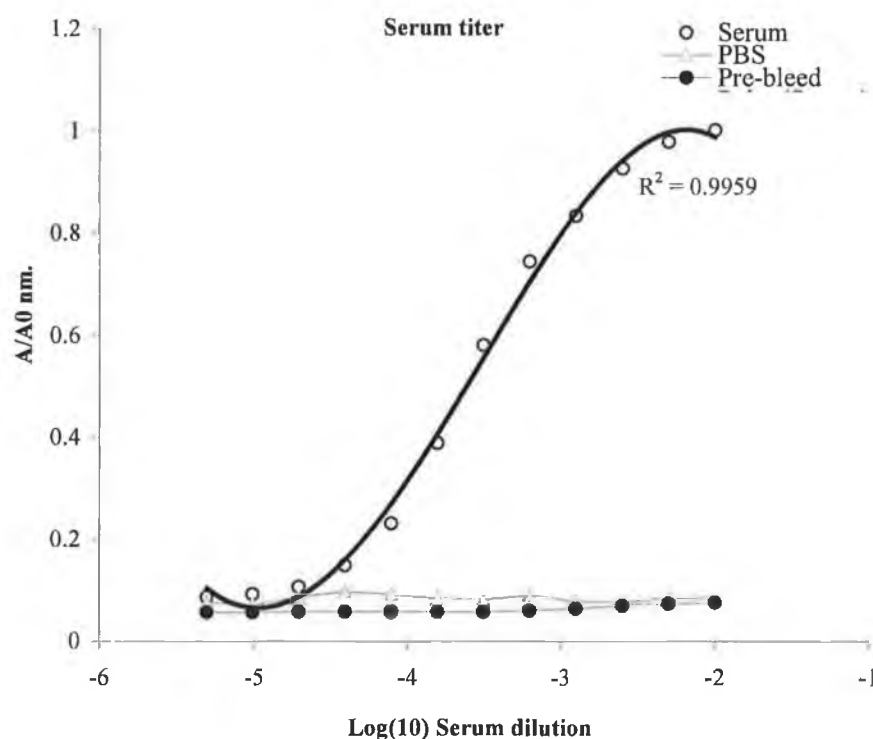


Figure 3.14: Titre serum curve for the 1st antiserum (open circle), Pre-bleed serum (solid circle) and PBS as negative control (triangle). Each point on the curve is the mean of three replicates.

There is a smooth linear decrease in absorbance with log decrease in the 1st antiserum concentration.

3.2.4.3 ELISA Checkerboard titrations

ELISA requires that all the concentration of the reagents used in the ELISA be optimised. In order to determine the optimal working concentration, each component of the ELISA system must be assessed by a checkerboard ELISA. The process of Checkerboard titrations involves the dilution of two reagents against each other to examine the activities inherent at all the resulting combinations.

Conduction of checkerboard titration is useful to estimate the titre of antibody or to determine the optimum dilution of antibody/antigen for coating or competition in ELISA performance. Careful selection of antibody/antigen dilution significantly reduces background signal, improving assay sensitivity.

3.2.4.3.1 Determination the optimum dilution of 1st antiserum

The aim is to determine the linear working range of the assay at different 1st antiserum dilutions.

The ELISA standard protocol was used (section 3.2.3.5.3) except that a serial dilution of colchicine was used between 1×10^{-5} and 1×10^{-10} g/ml and 50 μ l was added per well in column 1 to 12 before addition of the 1st antiserum and pre bleed serum dilution. The 1st antiserum dilution was added to wells in rows A-G and pre-bleed serum added to wells in rows H, dilutions were made up in PBS and ranged between (1:1000 - 1:64000) and were prepared by serial dilution. Results are shown in Figure 3.15.

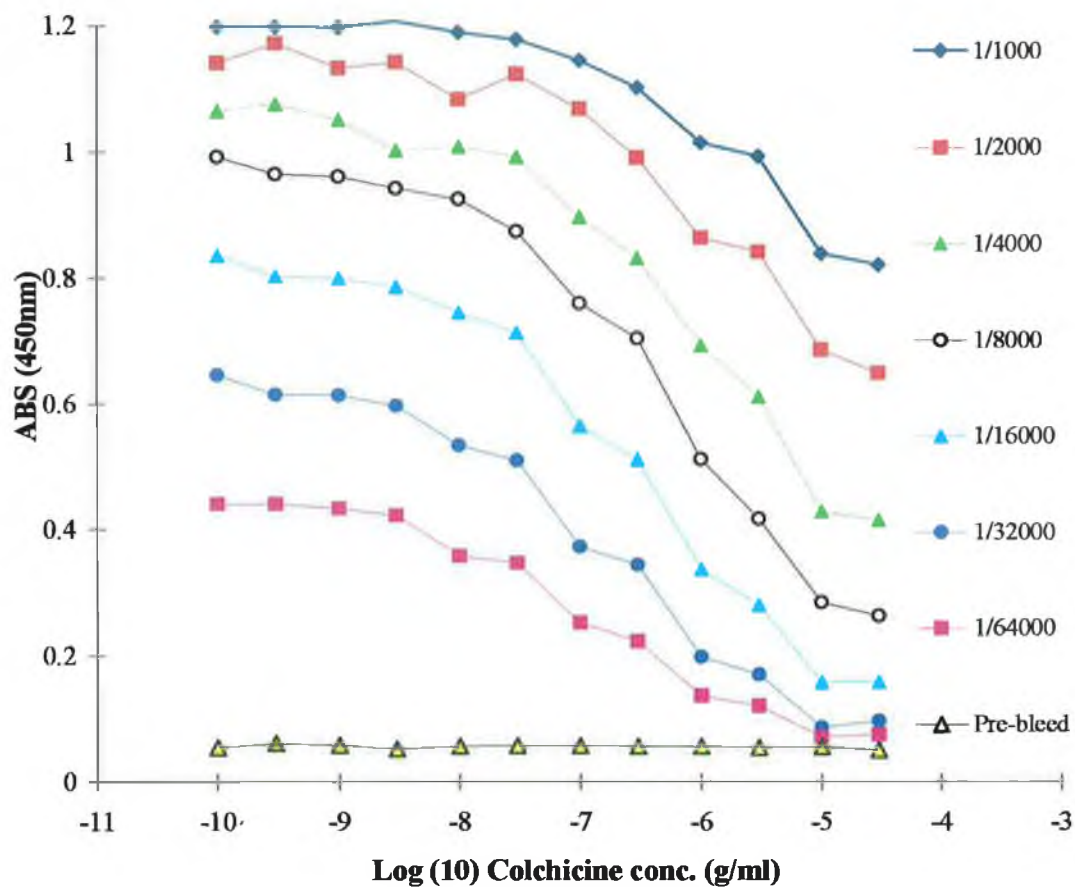


Figure 3.15: ELISA Checkerboard plot of absorbance values obtained relating different colchicine concentration to different dilutions of antiserum.

The optimal antiserum dilution, which gave the greatest change in absorbance per change in colchicine concentration, was determined to be 1:8000.

3.2.4.3.2 Determination of the optimum coating concentration of colchicoside-BSA

It was possible to estimate the colchicoside-BSA dilution that gives a good colour development as a result of binding with antibodies. This prevents wasting antigen from excess colchicoside-BSA added to the plate, which is then washed away after the coating phase. It was possible to select the smallest possible amount of antigen which gives a good titration curve for the antiserum.

The ELISA standard protocol (section 3.2.3.5.3) was used except that a plate was coated with 100µl of various concentrations of colchicoside-BSA varying between 5 - 100µg/ml made in PBS and added to well in rows A-G, no competing colchicine were used in this experiment. The 1st antiserum was added per well in column 1 to 10 and pre-bleed serum added in column 11. The dilutions were made up in PBS ranging from (1:1000 - 1: 204800) and prepared by serial dilution. PBS added in column 12 was used as a negative control. Results are shown in (Figure 3.16).

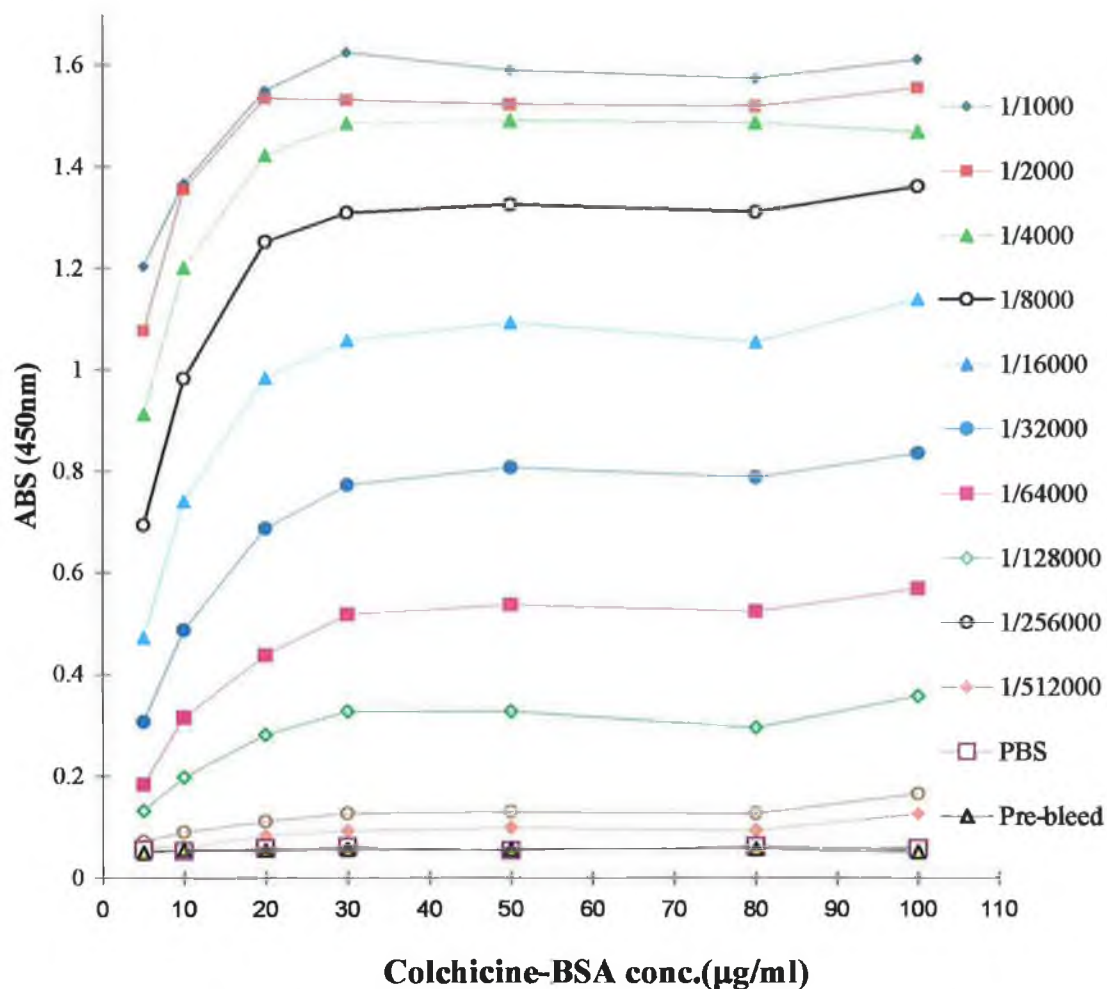


Figure 3.16: Absorption relationship values for different colchicoside-BSA concentration against series dilution of antiserum in Checkerboard.

Colchicoside-BSA at 30µg/ml was found to be the optimum dilution of coating and was used in all subsequent experiments. Beyond 30µg/ml there was no change in response and this was therefore selected as the optimal concentration of colchicoside-BSA to use.

3.2.4.4 Calibration curve

ELISA was carried out as described in the standard protocol in (Section 3.2.3.5.3) except that the concentration of colchicoside-BSA used to coat the plate was 30µg/ml and the dilution of the 1st antiserum used was 1:8000.

Wide-range calibration standard solutions were prepared ranging from 10⁻⁵ to 10⁻¹⁰ g/ml colchicine; the individual standards were prepared by performing log serial dilutions. The standard curves were constructed by plotting log(10) of the standard concentration against absorbance reading from the standard concentration. The calibration curve was fitted to a four-parameter regression equation which describes a sigmoidal relationship between the measured response and the logarithm of concentration.

Figure 3.17 shows the ELISA calibration curve for colchicine. The concentration of colchicine in the samples can be calculated by interpolation from the calibration curve using the four-parameter equation.

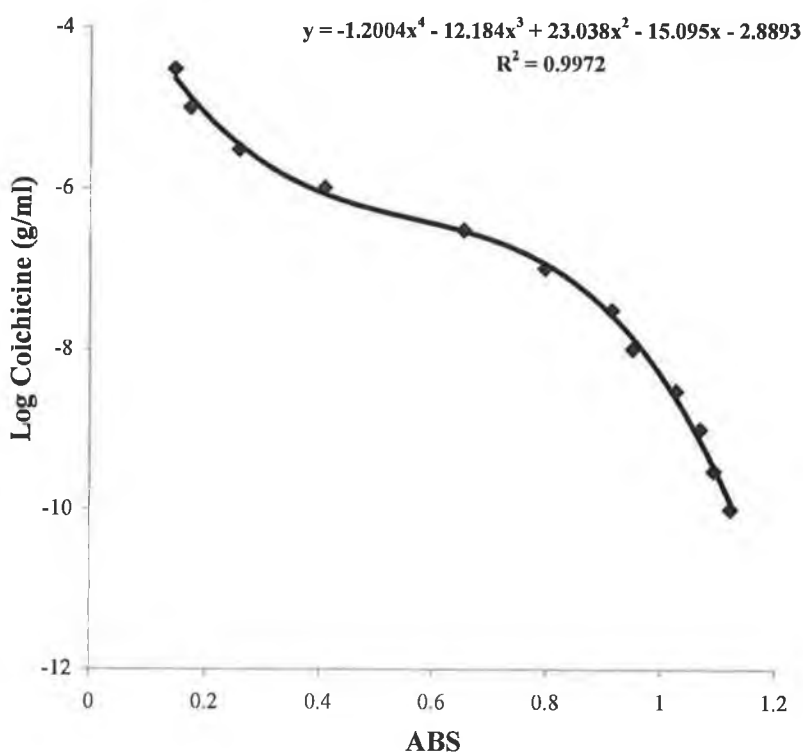


Figure 3.17: Standard colchicine calibration curve fitted with a polynomial four-parameter equation.

3.2.4.5 Assay specificity

Specificity is the ability to measure the analyte unequivocally in the presence of other components, either exogenous or endogenous. Since the assay was designed for application to crude plant extracts and liquid medium, checks of assay specificity were carried out using colchicine-like structures of potential cross reactivity with the colchicine antibody.

3.2.4.5.1 Cross reactivity study

Cross reactivity may be defined as a measure of the antibody response to structurally related compounds, as a result of shared epitopes. In the development of an ELISA assay, an antibody for analyte to be detected is produced. In order for the antibody-combining site to bind with the epitope on the antigen, they must have complementary shapes to 'fit into' one another. However, sometimes the antibody binds with substances other than the analyte of interest, due to a similar antigen epitope occurring on another substance. Consequently false positive results for colchicine may be generated. An experiment was therefore designed to investigate whether any of the structurally related compounds (colchicoside, demecolcine or fed precursors) or the components of MS medium may cross react in the colchicine ELISA and possibly generate erroneous results. Competitive binding assays were performed with serial dilutions of each of the above compounds in parallel with similar dilutions of colchicine. The cross reactivities of structurally or physiologically related compounds were determined by comparing the concentration required to yield reduction in signal recorded in the presence of analyte to 50% of the value obtained for colchicine.

To determine cross reactivity the antibody was mixed with the test compound. ELISA was performed as described in (section 3.2.3.5.3) except that the colchicine standards were replaced with decreasing concentrations of the potential cross reactive compound. Results are shown in Figure 3.18 and 3.19.

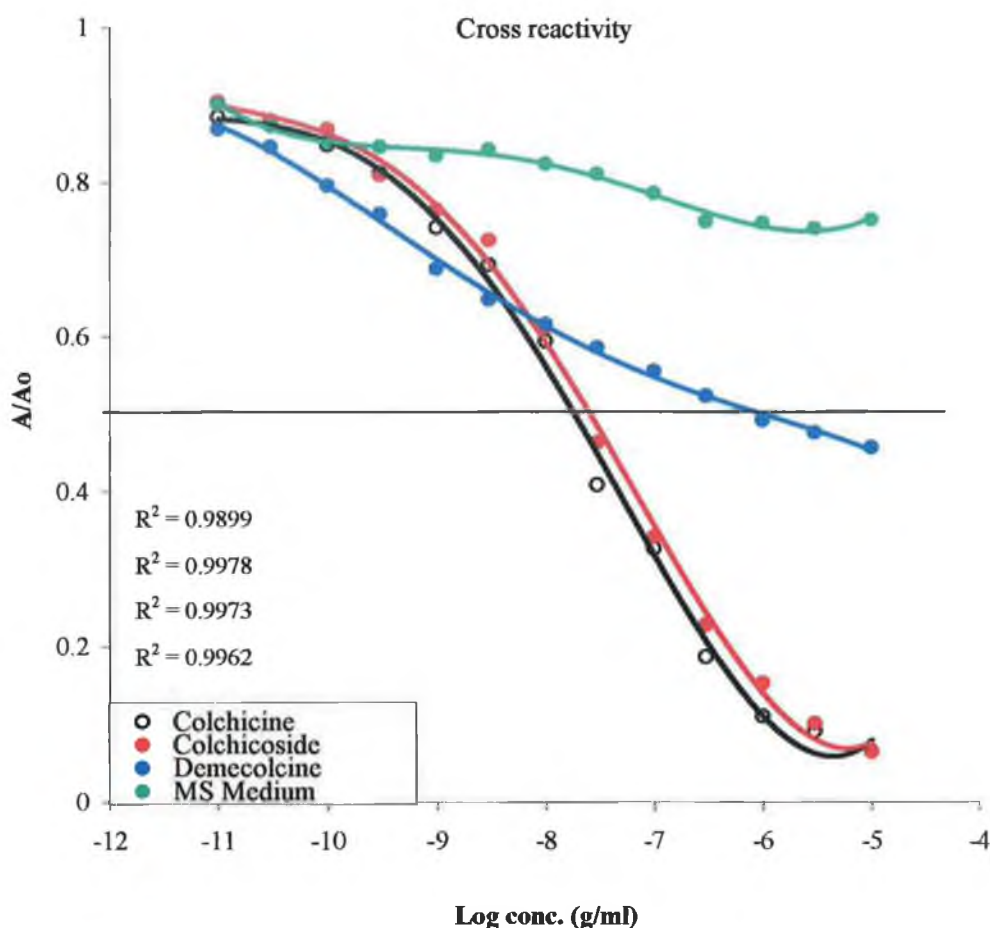


Figure 3.18: Overlay plot showing the cross reactivity of the antibody with colchicoside, demecolcine, and MS medium.

Table 3.4: cross reactivity of colchicoside and demecolcine

Substance	% Cross reactivity	Conc. (g/ml) at 50% absorbance
Colchicoside	72.94	2.4×10^{-8}
Demecolcine	2.30	7.7×10^{-7}

The percentage cross reactivity is calculated using the ratio of concentrations of cross reactant to the colchicine.

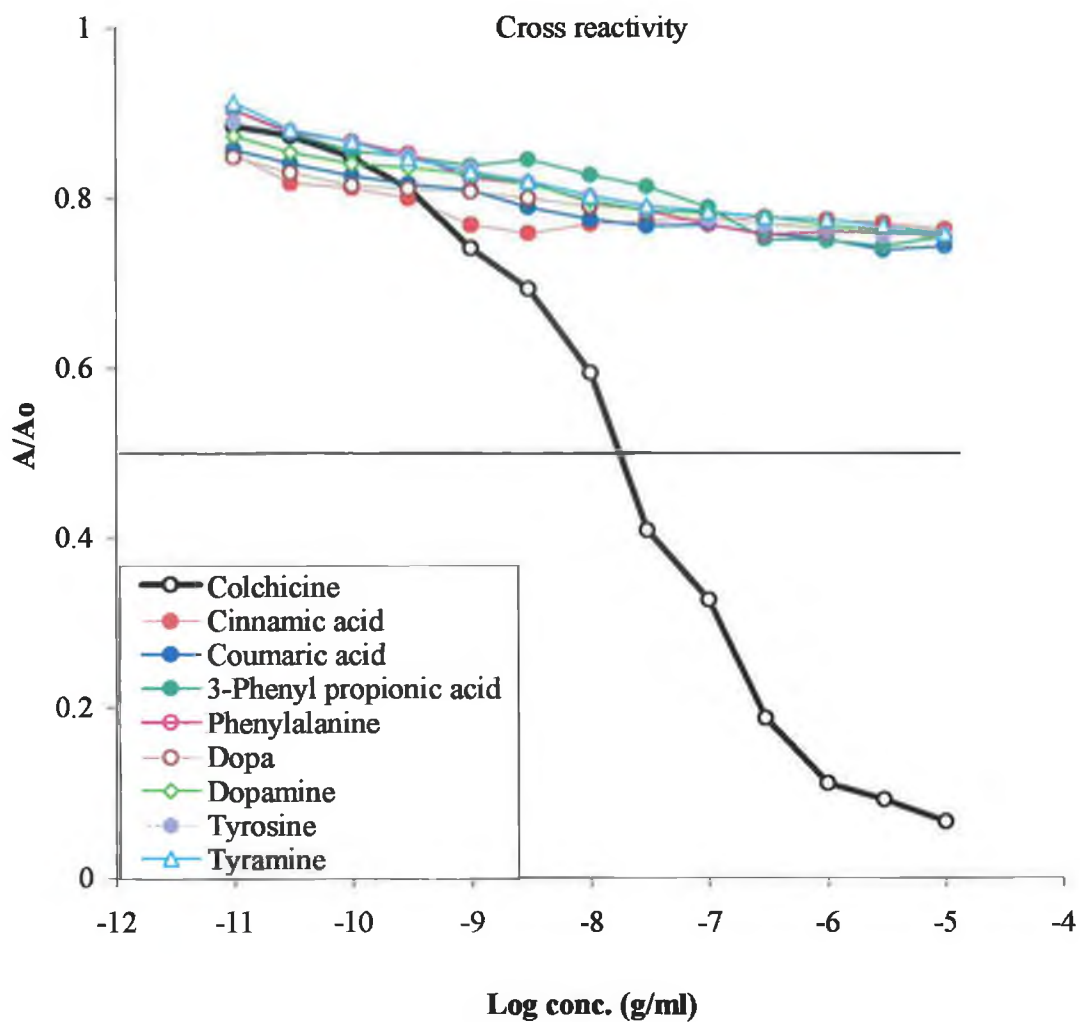


Figure 3.19: Overlay plot showing the cross reactivity of the antibody with various precursors.

3.2.4.8 Assay Linearity

A good four-parameter fit and the linearity of the ELISA assay was established by plotting the A/A₀ absorbance against log colchicine concentration and Logit A/A₀ against log colchicine concentration respectively. Results are shown in Figure 3.20 and 3.21.

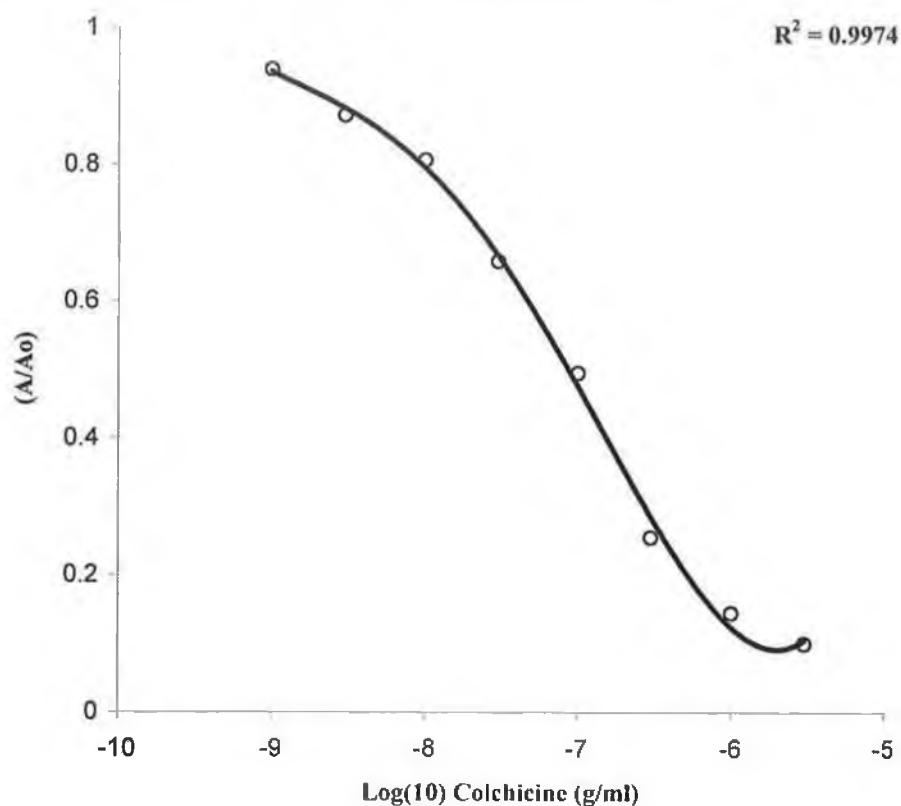


Figure 3.20: Colchicine standard curves obtained by plotting A/A₀ against colchicine concentration.

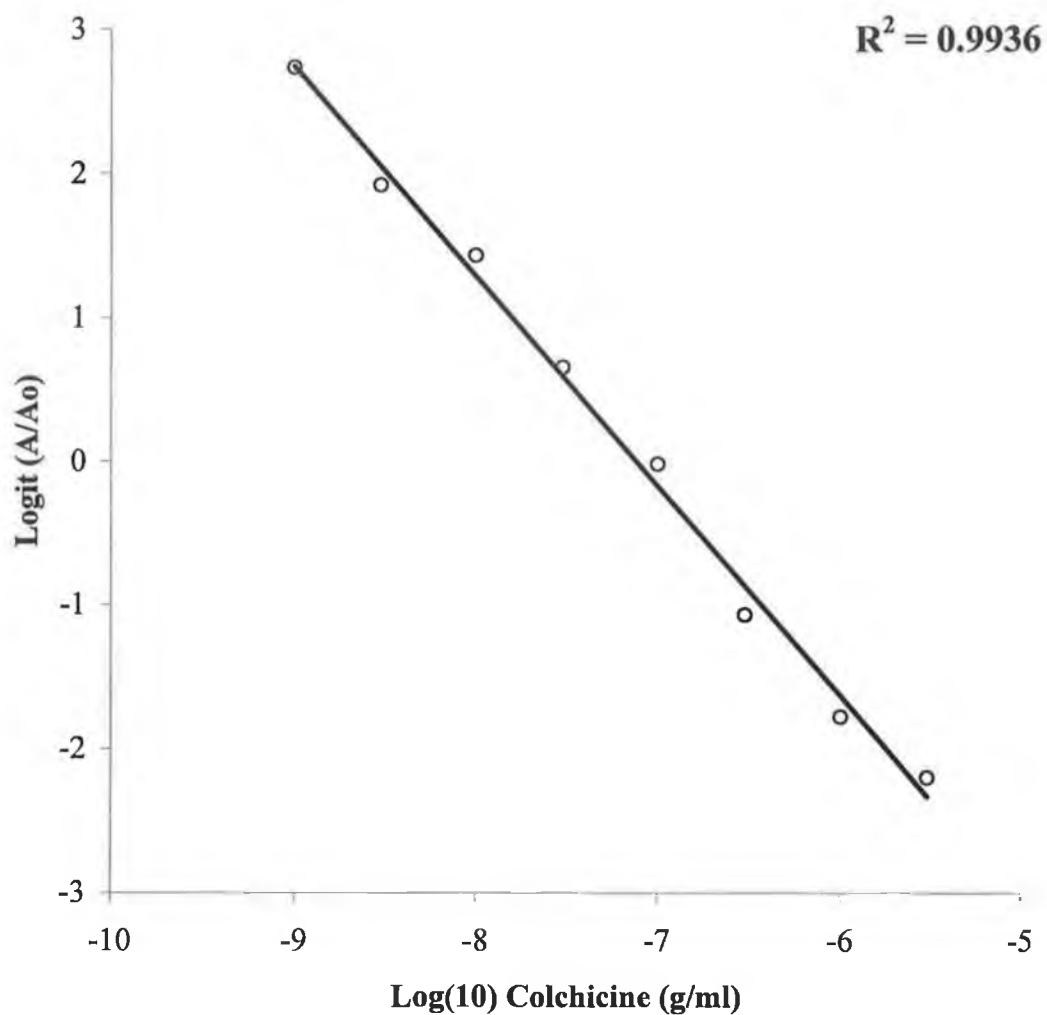


Figure 3.21: Linear colchicine standard curves obtained by plotting Logit A/Ao absorbance against log(10) colchicine concentration.

3.2.4.6 Assay sensitivity

In order to measure the precision of the ELISA assay, intra-day assay variability and inter-day assay variability were tested. Eight sets of standards were assayed. The coefficients of variation (CV) were determined to estimate the accuracy of the ELISA method, expressing standard deviation as a percent function of the mean. The CV values were found to range from 5.3 – 15.9 % with average CV of 11.13% throughout the measuring range for intra-assay. The coefficient of variability inter-day assay were determined and ranged from 3.6 – 12.7% and average variability was 9%. It can be seen therefore that there was a low degree of variation within the assay with CV% obtained well below the 20% suggested by Findlay *et al* (2000). The results obtained are shown in (Table 3.5).

Table 3.5: Intra-assay and inter-assay coefficients of variation (CV %) and percentage accuracies for the ELISA assay of colchicine.

Colchicine (ng/ml)	Intra-assay		Inter-assay	
	CV %	Accuracies	CV %	Accuracies
20	10.64%	101.71%	10.94%	100.63%
40	5.25%	97.03%	6.27%	98.72%
100	5.59%	99.17%	3.63%	99.94%
200	12.65%	99.49%	8.46%	99.97%
400	14.04%	101.44%	7.28%	101.96%
1000	14.91%	100.96%	11.09%	102.03%
2000	10.12%	103.99%	11.27%	102.44%
3000	15.85%	104.76%	12.66%	101.81%
Average	11.13%	101.07%	8.95%	100.94%

3.2.4.7 Correlation of ELISA with the HPLC method

The correlation of results obtained using HPLC and ELISA assay was examined. The correlation between the concentrations of colchicine determined by HPLC and ELISA are shown in (Figure 3.22).

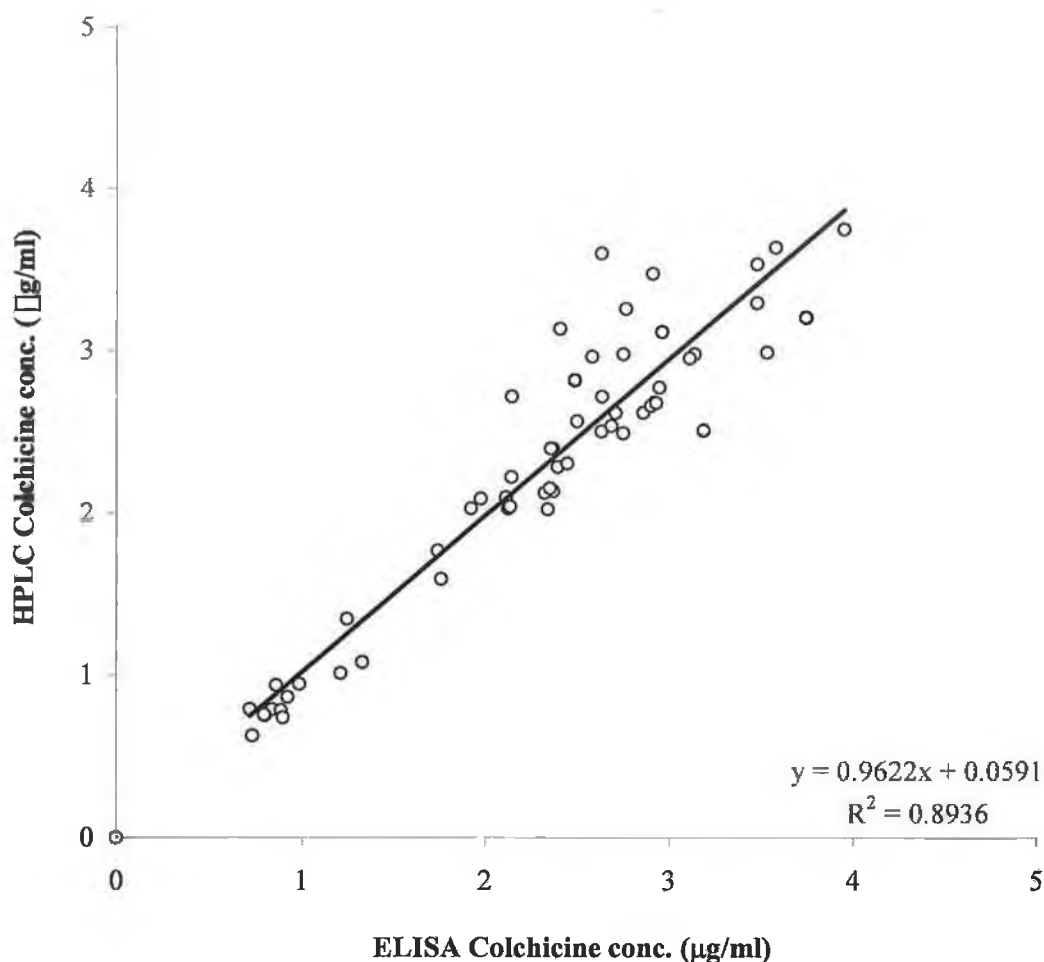


Figure 3.22: Relationship between concentrations of colchicine (µg/ml) assayed and determined by HPLC and ELISA.

The value of the correlation coefficient showed a good correlation between the two sets of data.

3.2.5. Discussion

The goal of this study was to develop and validate an Enzyme-Linked Immunosorbent Assay (ELISA) for detection and quantification of colchicine in the medium and plant tissue culture. Colchicine determination using ELISA is limited to one publication (Poulev, *et al.*, 1994), where the ELISA procedure is described without details of the assay performance. A different technique was used in that study which was a direct competitive ELISA.

The lack of a commercially available antibody against colchicine required us to generate the polyclonal antibody ourselves, and that involved the production of colchicoside-BSA conjugate and rabbit immunisation. The colchicoside-BSA produced was cleaned by membrane dialysis and the spectrophotometer scan showed that the conjugate has an absorption spectrum characteristic of both BSA and colchicine demonstrating the residues of colchicine were bound to the BSA (Figure 3.13).

It is clear that the Pre-bleed serum sample did not interfere with the absorbance reading as readings are all at the same level as PBS which is a negative control (Figure 3.14).

Antiserum dilutions of 1:1000 and 1:2000 gave similar results showing a plateau from 10^{-7} to 10^{-10} g/ml colchicine, and this indicates that antiserum was in excess. The optimal linear working range of the assay between 10^{-5} to 10^{-10} g/ml colchicine was generated at antiserum dilutions of 1:4000 to 1:16000. Lower antiserum dilutions do not have any useable linear range (Figure 3.15).

The antibody raised against colchicoside-BSA showed a high specificity towards colchicine. The cross-reactivity of the colchicine was investigated with structurally similar substances and some precursors. The level of cross reactivity observed with antibody was determined against a number of potential cross reactive compounds. With the exception of colchicoside which shares all the functional groups of colchicine, there was no significant cross-

reactivity with demecolcine or other components of the culture medium and precursors (Figure 3.19 and 3.20).

The high cross reactivity of the assay with colchicoside, should not affect the specificity of the assay as no colchicoside was detected in plant samples assayed by HPLC.

The logit conversion of the A/A₀ absorbance against log colchicine concentration produced a linear relation and the measuring range of this assay was found to range from 0.05 – 150 ng colchicine, in the 50µl sample assayed. The slope of the interpolation line revealed that the quantifications made using HPLC were approximately 96% of those made using the ELISA.

The optimum colchicoside-BSA conjugate concentration for coating the plate was found to be 30µg/ml. Coating the plate with a colchicoside-BSA concentration higher than 30µg/ml will have no impact on the assay (Figure 3.16). The wells coated with PBS and pre-bleed gave the same mean absorbance (A_{450nm} = 0.0066), these low absorbances indicating low levels of non-specific binding to the plate.

The ELISA developed in this work meets the requirements of an assay which is sensitive and easy to perform and proved to be a suitable assay for quantification of very low concentrations of colchicine in plant tissue culture. The detection limit of the ELISA was 50pg, and the optimised ELISA procedure developed in this study was approximately 25 times more sensitive than the HPLC method developed previously.

The sensitivity of this ELISA method is comparable with that described by Poulev *et al.* (1994) who reported a sensitive ELISA method for determination of colchicine in plant tissue with concentration ranging from 0.05-350ng/ml and with a detection limit of 10pg. The added advantage of this method over Poulev *et al.* (1994) is that there is no need for antibody purification. It also eliminates the requirement of the preparation of enzyme-labelled colchicoside.

The results of sample analysis by ELISA and HPLC were highly positively correlated (Figure 3.23) even though the two methods used very different preparations and techniques. Considering the possible sources of variation involved, a correlation coefficient of 0.8936 was considered a good correlation.

3.2.6 Conclusions:

Results obtained from the ELISA were easily reproducible, consistent and suitably sensitive in its detection and determination of colchicine in liquid medium and plant tissue extracts, and that indicates that this ELISA may be a useful tool for detection and determination of colchicine in tissue and culture medium of the plant culture.

This therefore provides a fast, cheaper and versatile method for the detection of colchicine in liquid medium and plant tissues.

Two effective and complementary methods HPLC and ELISA have been developed for determination of colchicine in the plant tissue and culture medium.

Chapter Four

**Approaches for stimulating the colchicine biosynthetic pathway in
plant cell and tissue culture**

4.1 Introduction

Plants produce an immense and diverse array of organic compounds called secondary metabolites. These metabolites are often differentially distributed among limited taxonomic groups within the plant kingdom and participate in interesting biological activities that can have high therapeutic value. Indeed, many highly interesting secondary metabolites are produced by very rare and exotic plant species. Individual secondary metabolites are often unique to a certain plants, evolving for the particular needs of that species. These products are formed following a series of biochemical reactions which convert primary metabolites such as amino acids into secondary metabolites such as alkaloids. Secondary metabolites and their intermediates are derived from the primary metabolic pathways, the two metabolic pathways being interconnected.

Secondary metabolites are characterized by enormous chemical diversity and every plant has its own characteristic set of secondary metabolites which exhibit multiple steps with numerous branch points (Oksman-Caldentey, and Inze 2004). The biosynthesis pathways for most of the secondary metabolites are very long and complicated, requiring several steps and key enzymes before the desired end product (secondary metabolite) is formed (Oksman-Caldentey and Hiltunen, 1996).

4.1.1 Colchicine biosynthesis

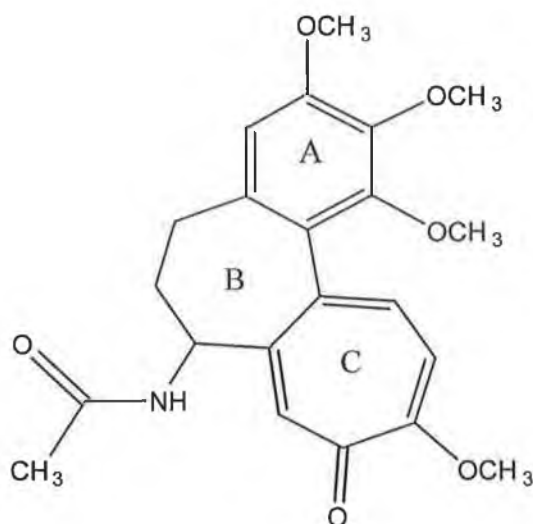


Figure 4.1: Chemical structure of colchicine

The chemical structure of colchicine is unique (Figure 4.1), with a tropolone ring (Ring C). Occurrence of the ring is rare in higher plants (Evans, 1996) and the existence of the ring in the structure meant that it had little likeness with other alkaloids; this meant that at one time it was viewed as “*sui generis* and no structure relationship with other alkaloids can be recognised” (Herbert, *et al.* 1990).

Ring (A) is thought to be derived from Tyrosine, and ring C from phenylalanine, with methionine providing the attached methoxyl groups, this being extrapolated from tracer experiments (Yoshida, *et al.* 1988B). There has been a relatively small amount of research directed at the production of colchicine by plant cell cultures compared with the production of other alkaloids in *Catharanthus roseus* or *Taxus* cultures. The pathway of colchicine biosynthesis is a complex one, with many steps, and was inadequately understood until recently. Leete and Nemeth, (1960) conducted the first precursor feeding experiment on colchicine biosynthesis in *Colchicum byzantinum*, demonstrating the incorporation of 3-C¹⁴ phenylalanine (0.06%)

into colchicine and summarizing previous biogenetic hypotheses. These experiments were rapidly followed by Battersby, and Reynolds (1960) indicating the incorporation of labelled tyrosine, acetate and methionine (0.23; 0.13, and 0.03% respectively) into this tropolonic alkaloid colchicine in growing *Colchicum autumnale*. The major surprise arose, however, when it was shown that colchicine is a modified isoquinoline alkaloid with over 10% incorporation of isoquinoline into colchicine (Battersby 1967). The major break-through in the biosynthesis of colchicine came from the demonstration that autumnaline and O-methylandrocymbine are intermediates with extraordinarily high incorporation rates into the target alkaloid colchicine (9.6% and 15.2% respectively) from labelled precursor feeding experiments on *Colchicum autumnale* (Battersby, *et al.* 1972). The early and intermediate stages of colchicine biosynthesis were more recently investigated by Herbert, (1990) and co-workers, who demonstrated that the early stage of biosynthesis in the major pathway to colchicine involved the conversion of L-phenylalanine to cinnamic acid by phenylalanine ammonia-lyase. This compound is then in turn reduced to cinnamaldehyde, subsequently to dihydrocinnamaldehyde, which, after hydroxylation in the 4 position yields the aldehyde that condenses stereoselectively with dopamine to a trihydroxylated phenethylisoquinoline.

The isoquinoline alkaloids have tyrosine as a precursor, which is modified to dopamine which then condenses with 3,4-dihydroxy-phenylacetaldehyde to form isoquinoline alkaloids (Figure 4.1).

The biosynthetic pathway to colchicine can be scrutinized into two parts; the first leads from primary metabolites to phenyl-isoquinoline, and the second part leads from this branch point intermediate to colchicine. The phenyl-isoquinoline is the first alkaloidal intermediate in the biosynthetic pathway for isoquinoline alkaloids (Herbert *et al.*, 1990; Kattah, 1990).

An aromatic L-amino acid decarboxylase catalyzes the decarboxylation of aromatic amino acids in animals and plants. In plants, L-tyrosine/L-Dopa decarboxylase (TYDC) has attracted considerable attention due to its involvement in the biosynthesis of several important groups of pharmaceutical

alkaloids (Facchini *et al.*, 2000). Conversion of the trihydroxylated phenethyl isoquinoline involves a number of methylations, each of which may be stimulated by S-adenosylmethionine (SAM), or L-methionine. A representation of the principal steps of the earliest part in the proposed colchicine biosynthetic pathway the biosynthesis is shown in below in (Figure 4.2)

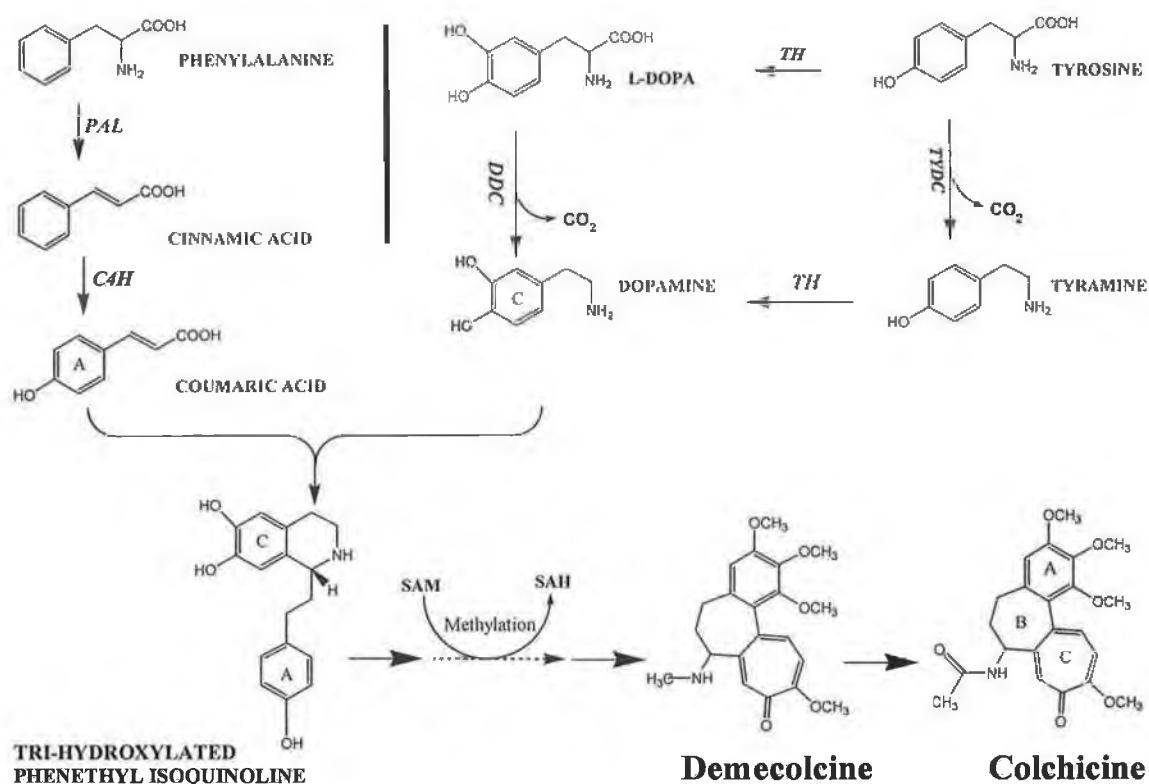


Figure 4.2: The early part of colchicine biosynthetic pathway. DDC= Dopa decarboxylase, TH= Tyrosine hydroxylase, TYDC= Tyrosine decarboxylase, PAL= Phenylalanine ammonia lyase, C4H= Cinnamic acid 4 hydroxylase. (Yoshida, *et al.* 1988B; Herbert *et al.*, 1990; Kattah, 1990; Nasreen, *et al.*, 1997).

4.1.2 Manipulation of secondary metabolism

Producing amounts of commercially viable secondary metabolites may be difficult without manipulation and stimulation of the metabolic pathways. There are a range of different ways for manipulation of biosynthetic pathways, two of which have been investigated in this chapter.

4.1.2.1 Precursor feeding

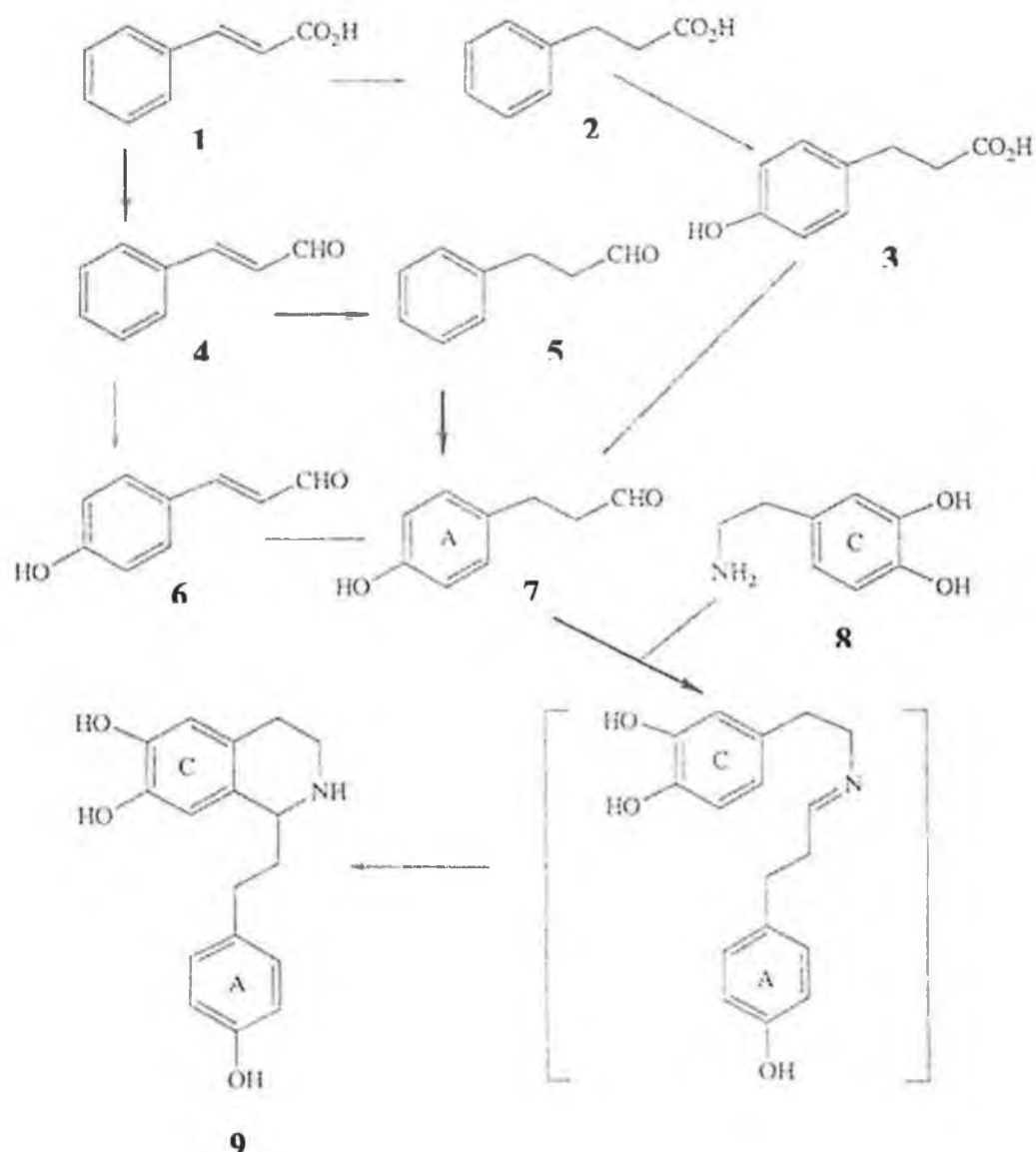
In order to determine which compounds are utilised in a secondary metabolite pathway to produce a compound of interest, a method known as precursor feeding is used. This involves the addition of assumed precursors to the culture and investigation of which result in increased production of the desired secondary metabolites and overcome a rate-limiting step in the pathway. The idea behind precursor feeding is supplying the culture with an identified rate-limiting compound in the biosynthetic pathway. Substances occurring early in the pathway are known as precursors, and those occurring close to the end product are identified as intermediates. Precursor feeding has been an obvious and popular approach to increase secondary metabolite production in plant cell cultures. The addition of biosynthetic precursors to the medium may improve alkaloid production where the productivity is limited by lack of that particular precursor(s) (Whitmer, *et al.*, 2002).

Thus under suitable conditions, cell cultures should be able to produce secondary metabolites either *de novo* or by the biotransformation of exogenously supplied precursors or intermediates (Poots, 1979). The precursor of a secondary metabolite may also be a precursor for primary metabolite production (Mann, 1987).

It is likely that the precursors may be either incorporated directly into the product, or precursors may enter a specific product indirectly through degradative metabolism and entry into interrelated pathways (DiCosmo and

Misawa, 1995). The high incorporation of a precursor into a target compound indicates that that precursor supply is limiting and should be increased. Attempts to induce or increase the production of plant secondary metabolites, by supplying precursor or intermediate compounds, have been effective in many cases. The addition of phenylalanine as a precursor led to improvement in rosmarinic acid yield in *Coleus blumei* cell cultures (Ibrahim, 1987). Addition of phenylalanine to *Salvia officinalis* suspension cultures stimulated the production of rosmarinic acid and also decreased the production time (Ellis and Towers, 1970). Feeding of ferulic acid to cultures of *Vanilla planifolia* resulted in increased vanillin accumulation (Romagnoli, and Knorr, 1988). Amino acids have been added to cell suspension culture media for production of tropane alkaloids, indole alkaloids and other products. A stimulatory effect on alkaloid biosynthesis in *Datura* cell cultures is caused by feeding of phenylalanine, tyrosine and Phenylpyruvate. (Hiraoka *et al* 1973), and in *Catharanthus roseus* by feeding Tryptophan (Zenk *et al* 1977). Feeding phenylalanine, tyrosine and other potential phenylisoquinoline precursors may therefore stimulate production of isoquinoline alkaloids.

A trace experiment for colchicine production was carried out by Herbert, *et al.* (1990) and the early biosynthetic pathway is illustrated below.



The above scheme is the early colchicine biosynthetic pathway adapted from Herbert, *et al.* (1990). 1= Cinnamic acid, 2= 3-Phenyl-propionic acid, 3= Phloretic acid, 4= Cinnamaldehyde, 5= 3-Phenyl-propionaldehyde, 6= 3-(4-Hydroxy-phenyl)-propenal, 7= 3-(4-Hydroxy-phenyl)-propionaldehyde, 8= Dopamine, 9= Tri-hydroxylated phenethyl isoquinoline.

4.1.2.1 Elicitation

Elicitation is one of the manipulative techniques available to promote the productivity of secondary metabolites from plant cell cultures. Elicitation is the induction of secondary metabolites production by molecules or treatments. Elicitation of secondary metabolites in various plant cell and tissue culture systems with both biotic and abiotic elicitors enhanced the formation of secondary metabolites (Funk, and Brodelius, 1990; Corry, *et al.* 1993). Some chemicals in the plant environment are especially potent inducers of certain parts of the plant metabolism. Jasmonic acid and its derivatives are considered to be compounds involved in part of a signal transduction system which induces particular enzymes catalyzing biosynthetic reactions to form defense compounds such as polyphenols or alkaloids (Hahlbrock, and Scheel, 1989).

The effect of elicitors is dependent on a number of factors which may interact and include the specificity of elicitor, its concentration, the duration of treatment and stage of growth of the culture when applied (Holden, *et al.*, 1988; Yeoman, *et al.* 1990).

It was shown that methyl jasmonate induced the accumulation of a wide range of secondary metabolites in many plant tissue cultures (Dittrich *et al.*, 1992). Methyl jasmonate has been reported to induce the activities of some enzymes (Laskaris, *et al.*, 1999; Dong, and Zhong, 2001). In the past few years, jasmonate and methyl jasmonate have been shown to be inexpensive and effective elicitors of secondary metabolite production to increase natural product formation in plant cell cultures for biotechnological purposes, for example paclitaxel in *Taxus* suspension cultures (Yukimune *et al.*, 1996; Ketchum *et al.*, 1997) and rosmarinic acid in suspension cultures of *Lithospermum erythrorhizon* and *Coleus blumei* (Mizukami *et al.*, 1993; Roberts and Shuler, 1997). Mizukami *et al.*, (1992) reported that the level of rosmarinic acid was increased 2.5 times and the activity of the enzyme phenylalanine ammonia lyase (PAL) in the cells rapidly increased by the addition of yeast extract into the liquid growth media. A yeast polysaccharide preparation induced L-tyrosine decarboxylase in suspension cultures of

Thalictrum rugosum and *Eschscholtzia californica* (Marques, and Brodelius, 1992).

4.2 Aim

To study the effect of elicitation and precursor feeding on colchicine accumulation in cell and tissue cultures of *Colchicum autumnale* and *Gloriosa superba*.

Plant tissue and cell culture offer the advantages of providing naked (no epidermis) aseptic tissue, enabling consistent inoculation of cells at any time, and production of satisfactory amounts of material for biochemical study. This work was designed to focus on the earliest part of the colchicine biosynthetic pathway. Exploring the steps in the colchicine biosynthesis pathway has identified a number of precursors that have the potential to increase productivity when applied to cell or tissue suspension culture. Since all these biogenetic schemes involve two amino acids (phenylalanine and tyrosine), or their metabolites, it seemed rational to feed these two amino acids, and their analogues to investigate their effect on colchicine accumulation in cell and tissue culture of *Colchicum autumnale* and *Gloriosa superba*. In order to examine which compounds stimulate the production of metabolites, precursor feeding with, a range of phenylalanine and tyrosine analogues shown in (Figures 4.2 and 4.3) was applied.

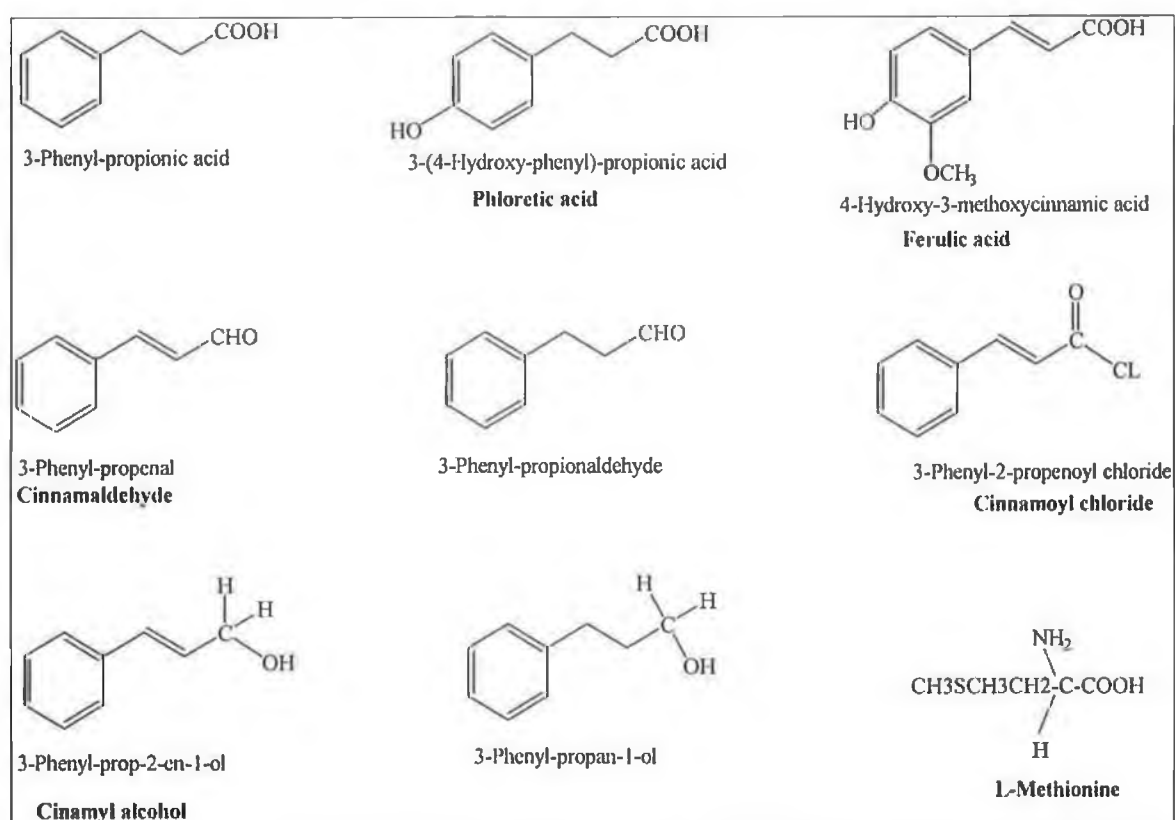


Figure 4.3: Range of different phenylalanine and tyrosine analogues used as precursors for stimulating colchicine biosynthetic pathway.

4.3 Materials and Methods:

4.3.1 Materials and Instruments

Ferulic acid, cinnamic acid, coumaric acid, tyrosine, tyramine, L-dopa, dopamine, phenylalanine, L-methionine, S-adenosylmethionine (SAM), and methyl Jasmonate were obtained from Sigma-Aldrich. Yeast extract was obtained from Oxoid.

The rest of precursors used in this study were obtained from Lancaster UK.

Bio-Rad and Costar 24 well cell culture sterile plates were obtained from Lennox Ireland.

Freeze-drier system Labconco, Sonicator (Model Vibra cellTM 502, Sonocs and Materials Inc., USA).

4.3.2 Preparation of the Precursors

Each of the precursors was made up individually to a 1, 0.5, or 0.1mM concentration in MS medium supplemented with 3% sucrose and with no growth hormones. The required amount was weighed and pre-dissolved in 2-3 drops of 1mM KOH and made up to 20ml in a universal tube. The solutions were sonicated in a sonicator water bath for 10min to ensure that the compounds were dissolved. The pH of the precursor solutions were adjusted using 1mM or 1M HCL to pH=5.7. The precursors were then sterilised by filtration using a 0.22 μ m membrane filter.

4.3.4 Yeast extracts

The carbohydrate fraction isolated from the yeast extract was prepared by ethanol precipitation as described by Hahn and Albersheim, (1978). 10g of yeast extract was dissolved in 50ml distilled water in a 500ml flask and 200ml ethanol added. Thereafter the flasks were sealed with cotton wool bungs and capped with a double layer of aluminium foil and placed on a shaker at 4 °C overnight. The mixture was then allowed to settle overnight at 4 °C. The

supernatant was removed and discarded, the gummy precipitate dissolved in 50ml distilled water and the ethanol precipitation repeated again. The precipitate was dissolved in 10ml distilled water and used as a stock solution stored at 4 °C. The solution was diluted down to 1/100, 1/1000, 1/5000, and 1/10000 in MS medium supplemented with 3% sucrose and with no growth hormones. The diluted yeast extract solutions were sterilised by autoclaving.

4.3.5 Feeding technique

Plant tissue culture (approximately 25-50mg of callus FW) was placed in a multi-well plate, and 1ml of precursor added individually to each well. The plates were sealed with parafilm and covered with aluminium foil and incubated at 25 °C on an orbital shaker at 120rpm for 72h. All treatments were performed in triplicate. Following three days of incubation, the cell suspensions were collected in microcentrifuge tubes, centrifuged at 13000rpm for 20min and the supernatant transferred to a clean tube for assay. The residue was frozen at -80 °C for 2h then lyophilised overnight and the dried residue weighed, and extracted (using the method described in section 3.1.3.7.2). The level of colchicine in the medium and tissue was then assayed.

4.3.5 Macroscopic observations

Macroscopic examinations were performed to examine the effect of precursors on the tissue. The changes in the colour of tissue culture after incubation with precursors were noted.

4.3.6 Data analysis

Concentrations of colchicine were expressed in (µg/g) tissue dry weights, based on mean value of analyses carried out in triplicate. The mean value with standard error was calculated and plotted in graph using Microsoft Excel for windows.

4.4 Results

4.4.1 Effect of precursor feeding on colchicine accumulation in cell suspension culture

The effect of precursor feeding on colchicine accumulation in cell suspension cultures was examined. Precursors were prepared in MS medium, added individually to plant cell cultures in three different concentrations (1, 0.5, or 0.1mM), and incubated for 72h at 25°C on an orbital shaker (120rpm). The level of colchicine in the incubation medium and tissue was assayed.

Coumaric acid and 3-phenyl propionic acid significantly increased colchicine accumulation in both *Colchicum autumnale* and *Gloriosa superba* cell suspension cultures. Also colchicine accumulation in *Gloriosa superba* root cultures was significantly stimulated by coumaric acid and 3-phenyl propionic acid. Other precursors were largely ineffective.

Colchicine accumulation was related to the concentration of precursor applied.

4.4.1.1 *Colchicum autumnale* cell suspension culture

The level of total colchicine accumulation in cell suspension cultures of *Colchicum autumnale* was significantly increased by feeding cinnamic acid, coumaric acid or 3-Phenyl propionic acid, with a more than three-fold increase in accumulation with 1mM coumaric acid. 3-Phenyl propionic acid significantly increased colchicine accumulation with up to a two-fold increase. Cinnamic acid increased colchicine accumulation by nearly 30% compared to the control. The rest of the precursors did not stimulate colchicine accumulation relative to the control. All the potential precursors fed significantly increased the amount of colchicine released into the liquid medium. Figure 4.4, 4.5, and 4.6 shows the results of this study for *Colchicum autumnale* culture fed with precursors in different concentrations (1, 0.5 or 0.1mM) respectively.

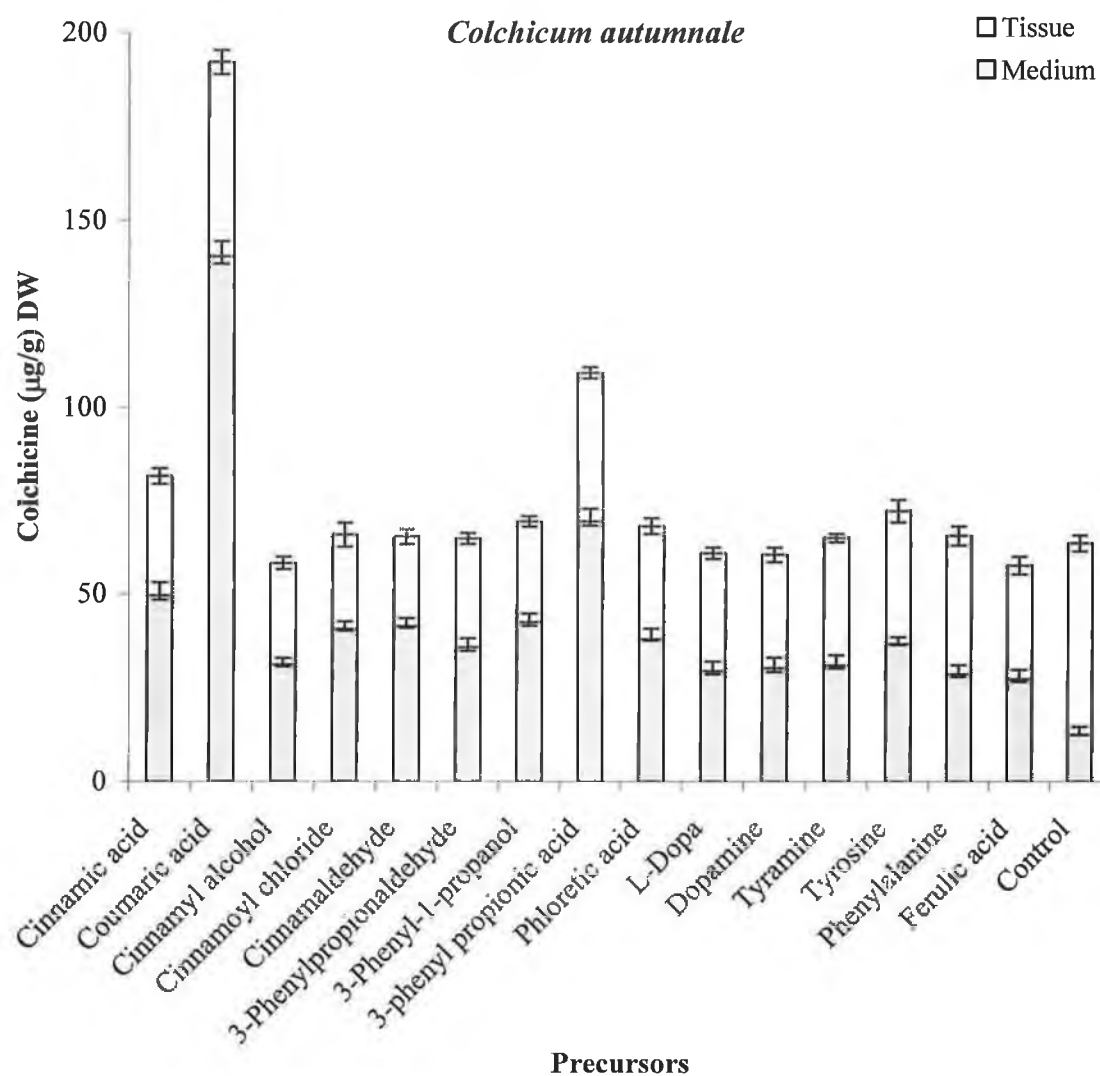


Figure 4.4: The level of colchicine accumulation in *Colchicum autumnale* cell suspension culture fed with 1mM precursors, the grey and white area in the bar are the amount of colchicine ($\mu\text{g/g}$) dry weight in the medium and tissue respectively. Results are the average of three replicates and are shown with the standard error of the mean.

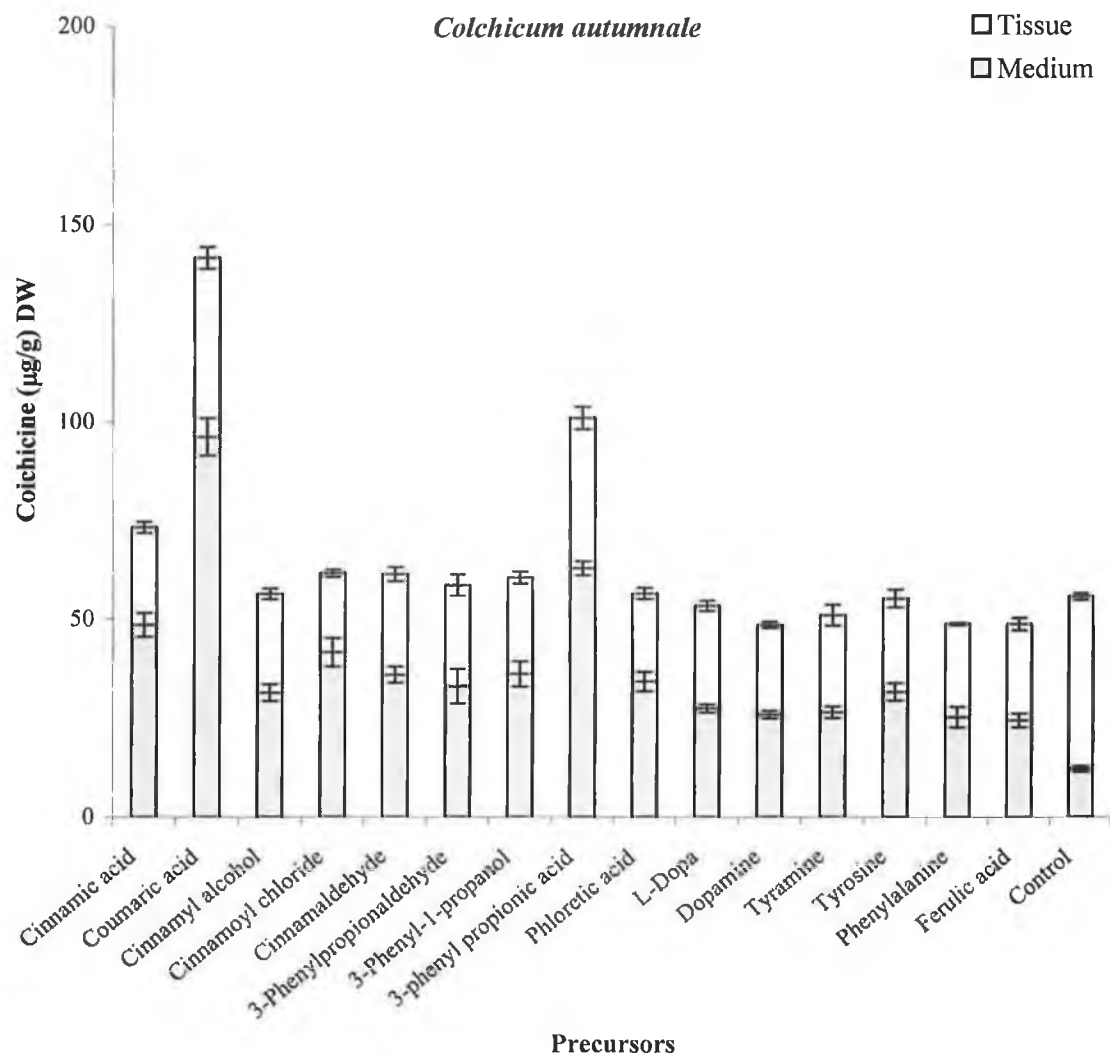


Figure 4.5: The level of colchicine accumulation in *Colchicum autumnale* cell suspension culture fed with 0.5mM precursors, the grey and white area in the bar are the amount of colchicine (µg/g) dry weight in the medium and tissue respectively. Results are the average of three replicates and are shown with the standard error of the mean.

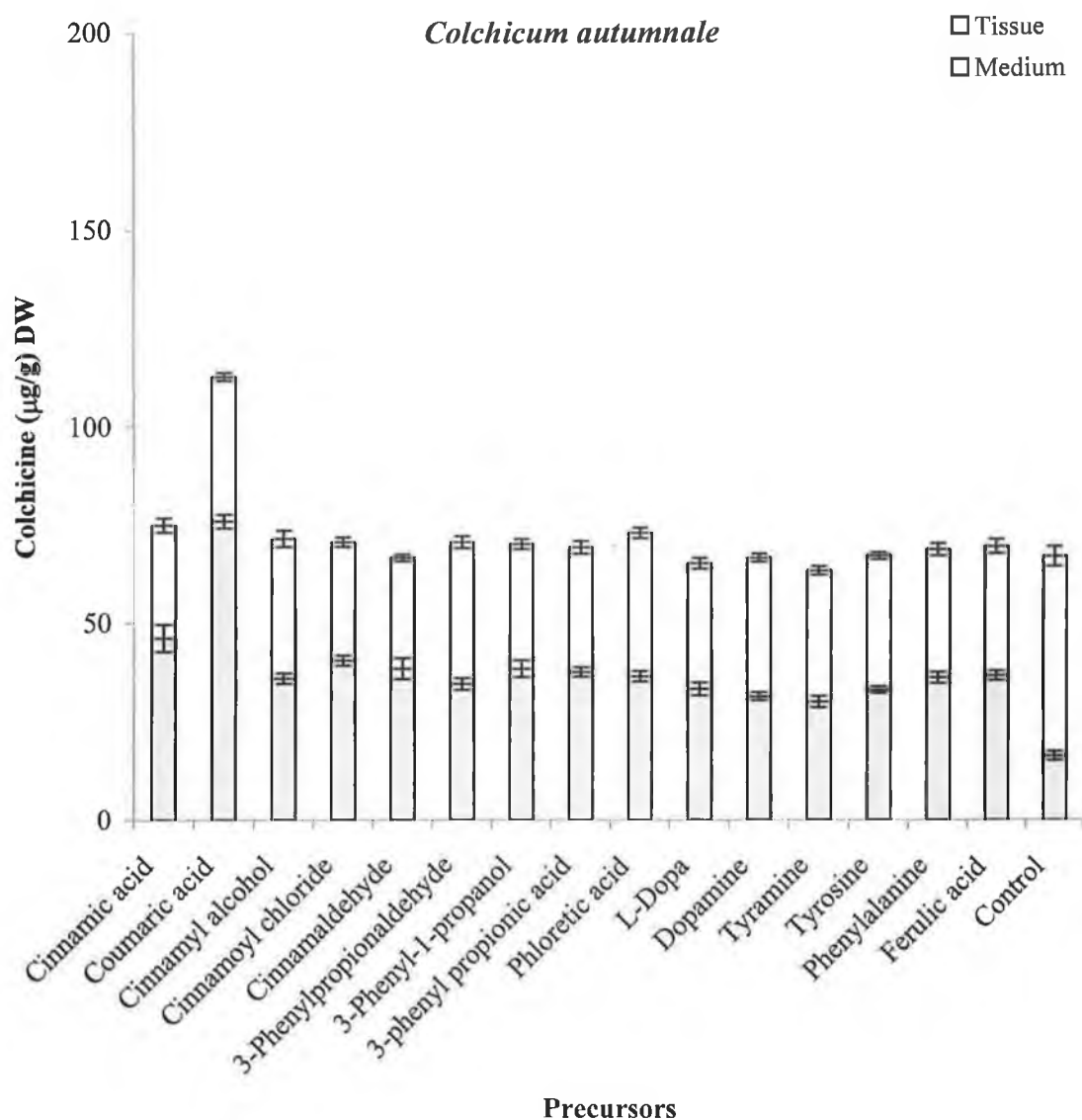


Figure 4.6: The level of colchicine accumulation in *Colchicum autumnale* cell suspension culture fed with 0.1mM precursors, the grey and white area in the bar are the amount of colchicine (µg/g) dry weight in the medium and tissue respectively. Results are the average of three replicates and are shown with the standard error of the mean.

4.4.1.2 *Gloriosa superba* cell suspension culture

Coumaric acid and 3-Phenyl propionic acid significantly increased the level of total colchicine accumulation in *Gloriosa superba* cell suspension culture, with an over three-fold increase in colchicine accumulated by feeding 1mM coumaric acid or 3-Phenyl propionic acid. Cultures fed with 0.5mM coumaric acid or 3-phenyl propionic acid accumulated almost the same amount of colchicine as with 1mM however cultures fed 0.1mM concentrations had a much reduced increases in the total colchicine accumulated compared to the culture fed with 1 or 0.5mM (Figure 4.8). The rest of precursors did not stimulate colchicine accumulation with 1, 0.5, or 0.1mM concentration. Figures 4.7, 4.8, and 4.9 shows the results of this study for *Gloriosa superba* culture fed with precursors in different concentration (1, 0.5 or 0.1mM) respectively.

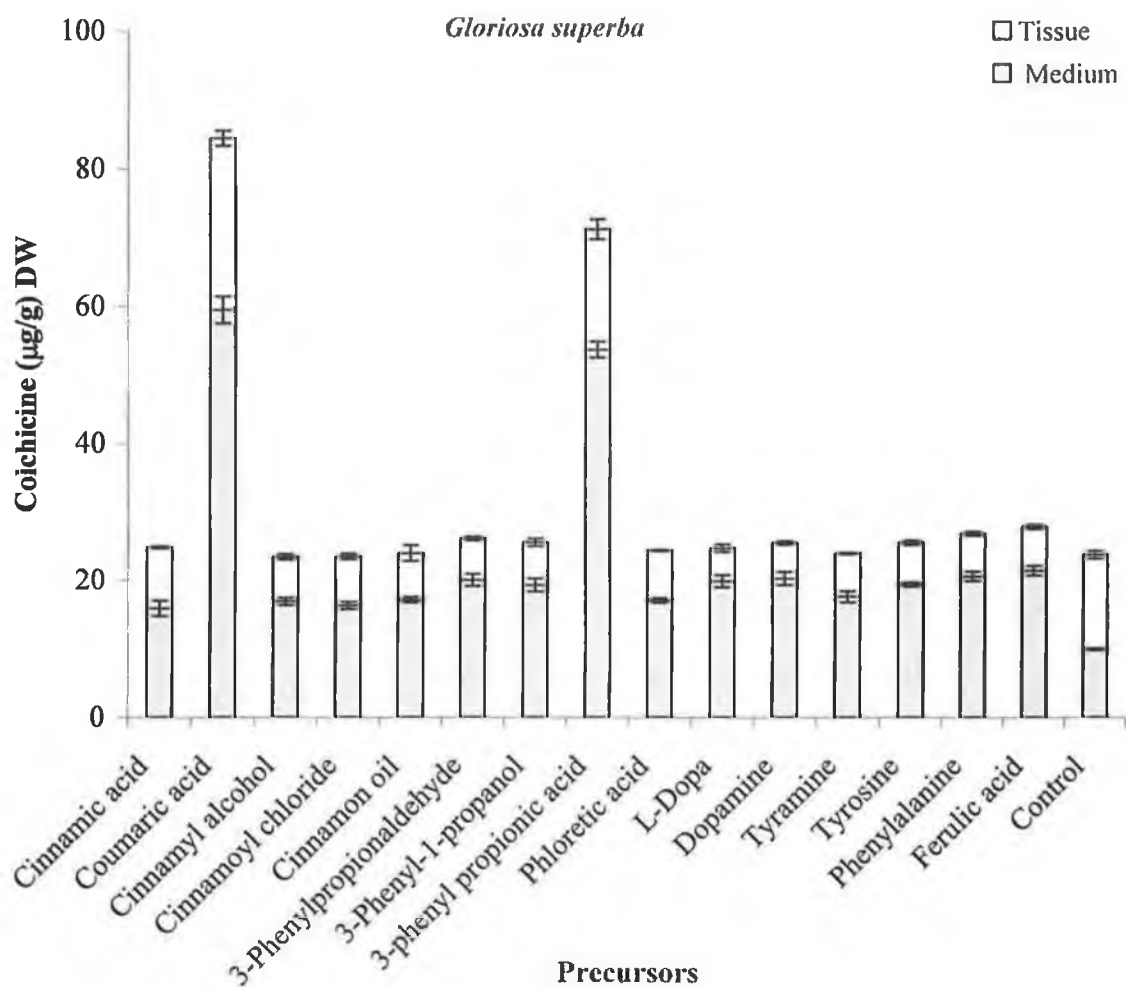


Figure 4.7: The level of colchicine accumulation in *Gloriosa superba* cell suspension culture fed with 1mM precursors, the grey and white area in the bar are the amount of colchicine ($\mu\text{g/g}$) dry weight in the medium and tissue respectively. Results are the average of three replicates and are shown with the standard error of the mean.

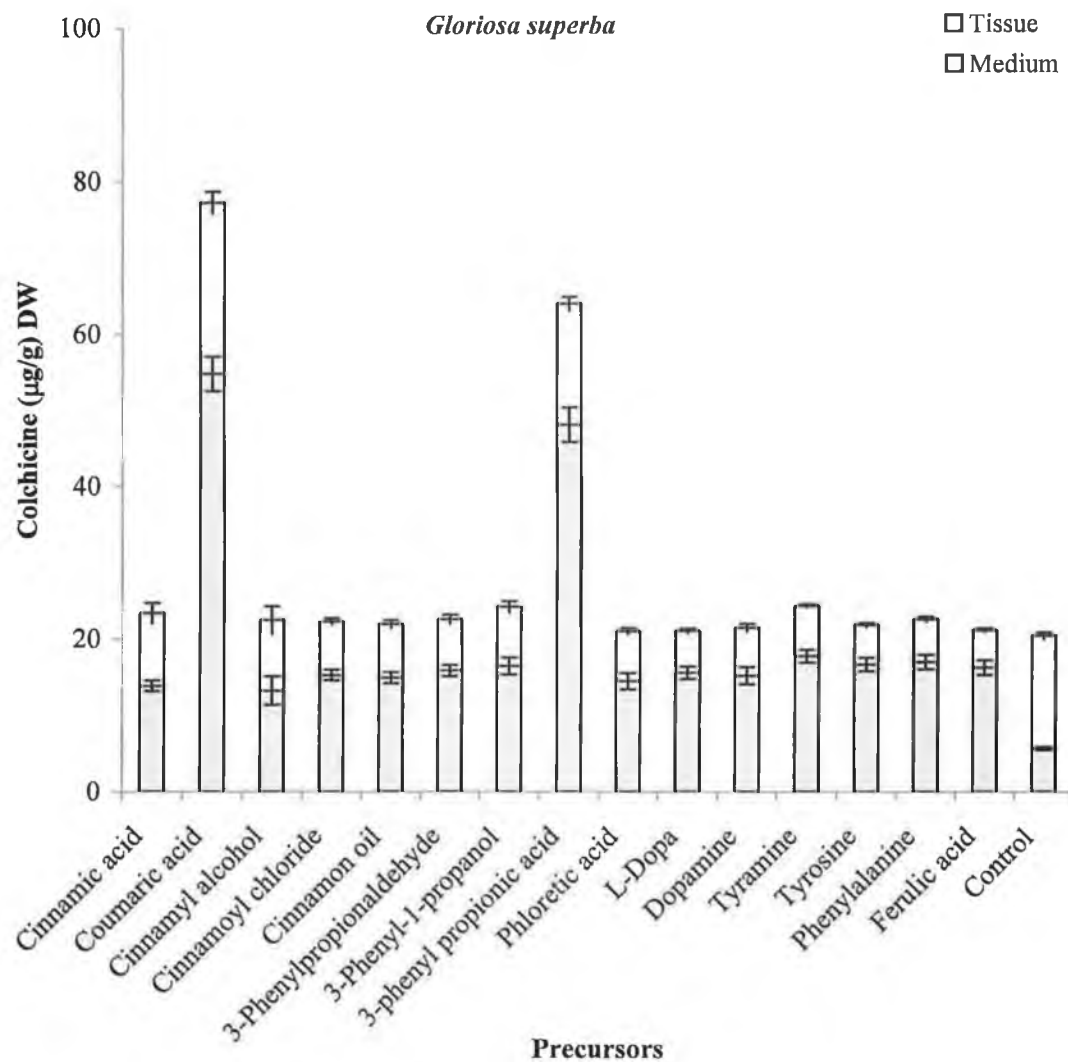


Figure 4.8: The level of colchicine accumulation in *Gloriosa superba* cell suspension culture fed with 0.5mM precursors, the grey and white area in the bar are the amount of colchicine ($\mu\text{g/g}$) dry weight in the medium and tissue respectively. Results are the average of three replicates and are shown with the standard error of the mean.

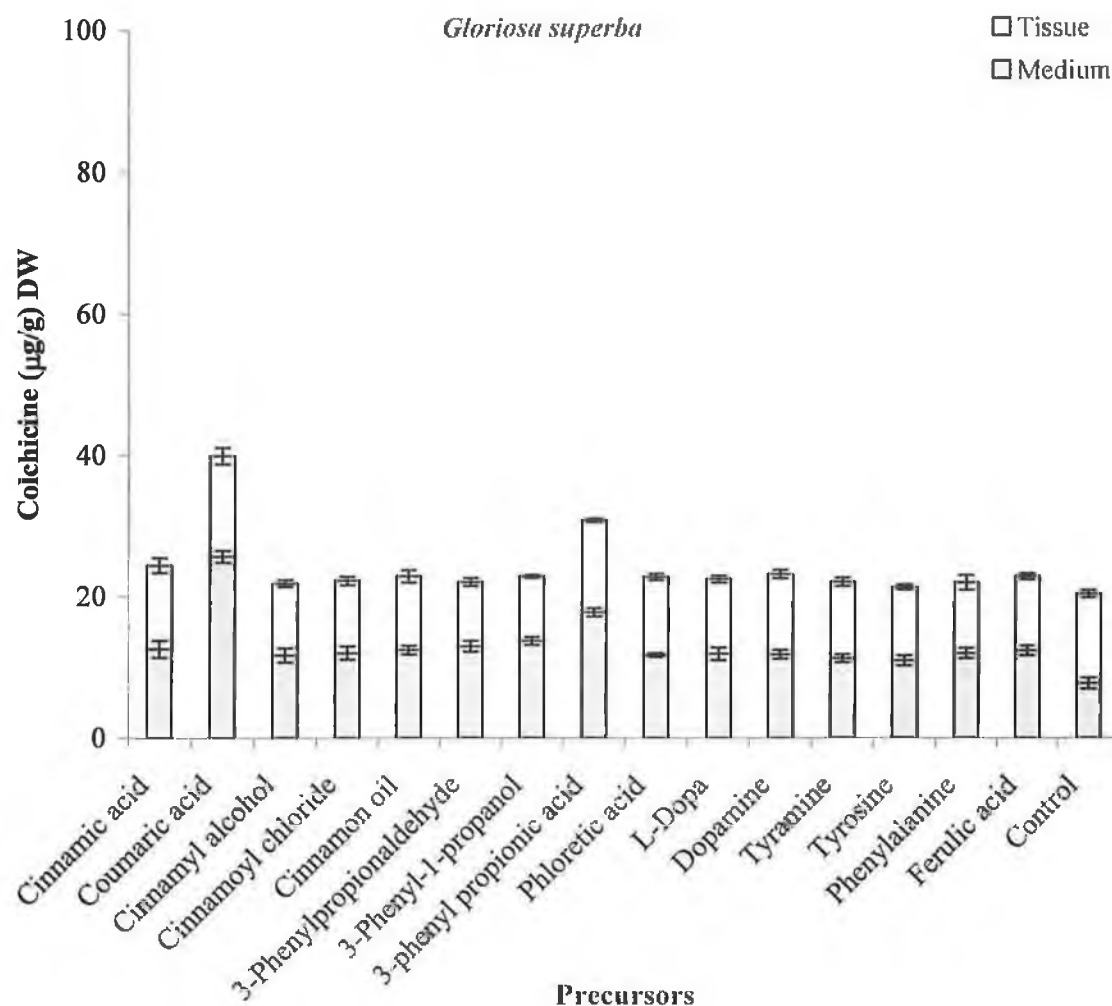


Figure 4.9: The level of colchicine accumulation in *Gloriosa superba* cell suspension culture fed with 0.1mM precursors, the grey and white area in the bar are the amount of colchicine ($\mu\text{g/g}$) dry weight in the medium and tissue respectively. Results are the average of three replicates and are shown with the standard error of the mean.

4.4.2 Effect of precursor feeding on colchicine accumulation in *Gloriosa superba* root tissue

The capability of feeding precursors to stimulate colchicine accumulation in *Gloriosa superba* M1 and M2 root tissue was examined. Root tissue culture accumulated relatively lower amounts of colchicine than the cell suspension, and M2 root culture accumulated 25% more colchicine than M1 root culture. Coumaric acid or 3-Phenyl propionic acid significantly increased the level of total colchicine accumulation in both *Gloriosa superba* M1 and M2 root tissue cultures, with over two-fold increase in colchicine accumulated by feeding 1mM coumaric acid or 3-Phenyl propionic acid compared to the control. The rest of the precursors did not stimulate colchicine accumulation in both *Gloriosa superba* M1 and M2 root culture. Although feeding 0.5mM of coumaric acid or 3-phenyl propionic acid showed relatively lower colchicine accumulated in comparison to 1mM.

4.4.2.1 M1 root tissue culture

Figures 4.10, and 4.11 shows the results of this study for *Gloriosa superba* M1 root culture fed with precursors at different concentration (1 or 0.5 mM) respectively.

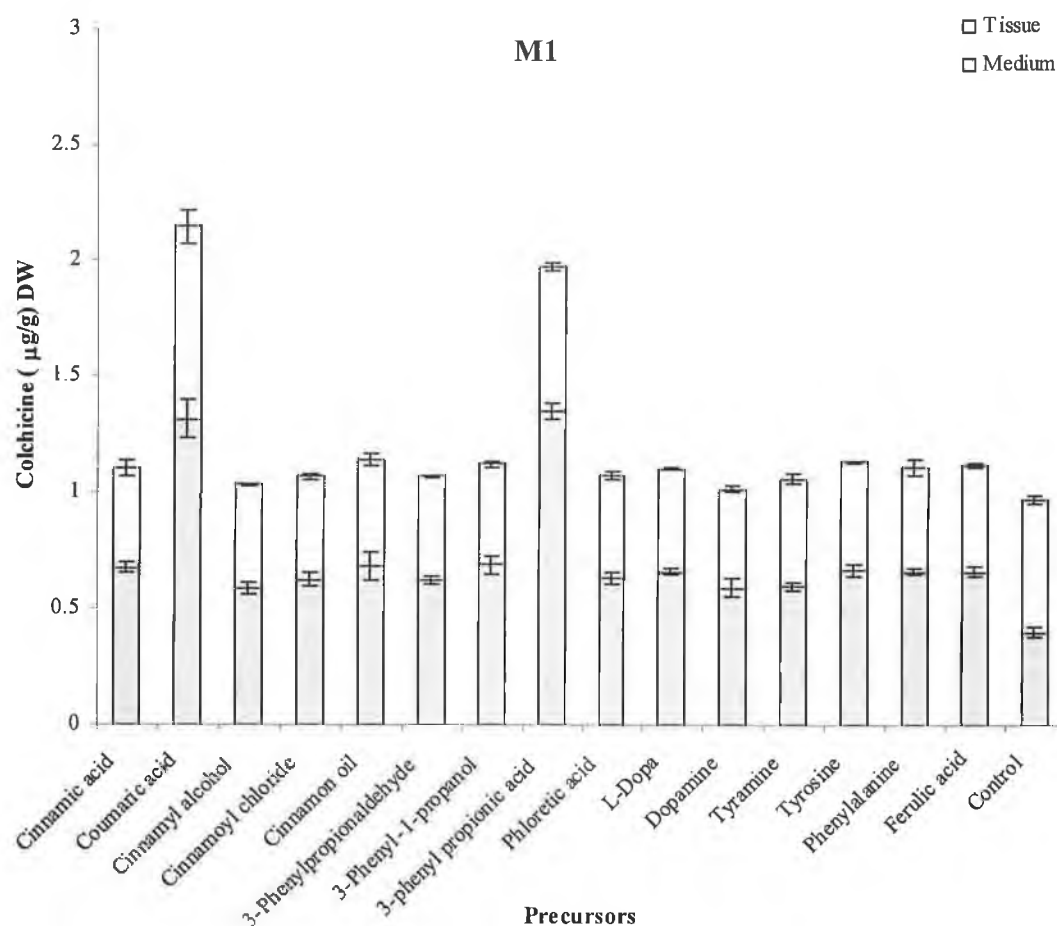


Figure 4.10: The level of colchicine accumulation in *Gloriosa superba* M1 root culture fed with 1mM precursors, the grey and white area in the bar are the amount of colchicine (µg/g) dry weight in the medium and tissue respectively. Results are the average of three replicates and are shown with the standard error of the mean.

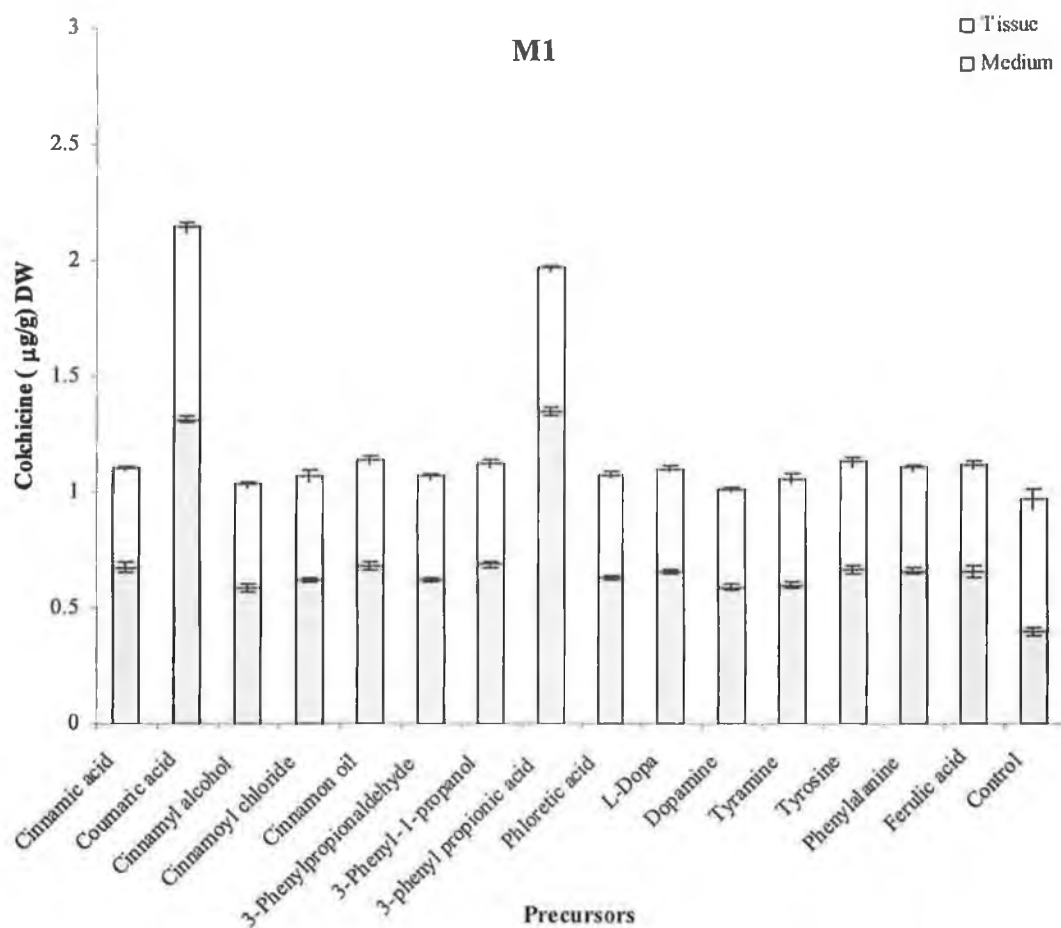


Figure 4.11: The level of colchicine accumulation in *Gloriosa superba* M1 root culture fed with 0.5mM precursors, the grey and white area in the bar are the amount of colchicine ($\mu\text{g/g}$) dry weight in the medium and tissue respectively. Results are the average of three replicates and are shown with the standard error of the mean.

4.4.1.4 M2 root tissue culture

Figures 4.12, and 4.13 shows the results of this study for *Gloriosa superba* M2 root culture fed with precursors in different concentration (1 or 0.5 mM) respectively.

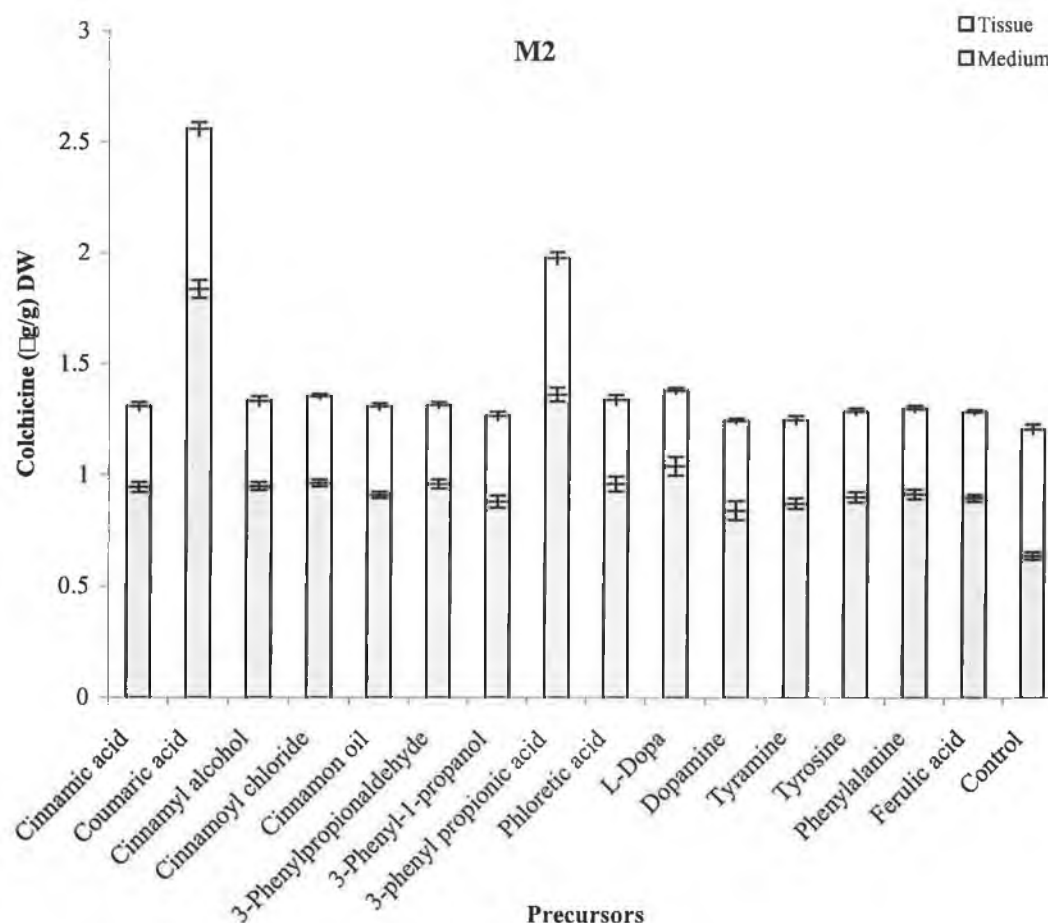


Figure 4.12: The level of colchicine accumulation in *Gloriosa superba* M2 root culture fed with 1mM precursors, the grey and white area in the bar are the amount of colchicine (µg/g) dry weight in the medium and tissue respectively. Results are the average of three replicates and are shown with the standard error of the mean.

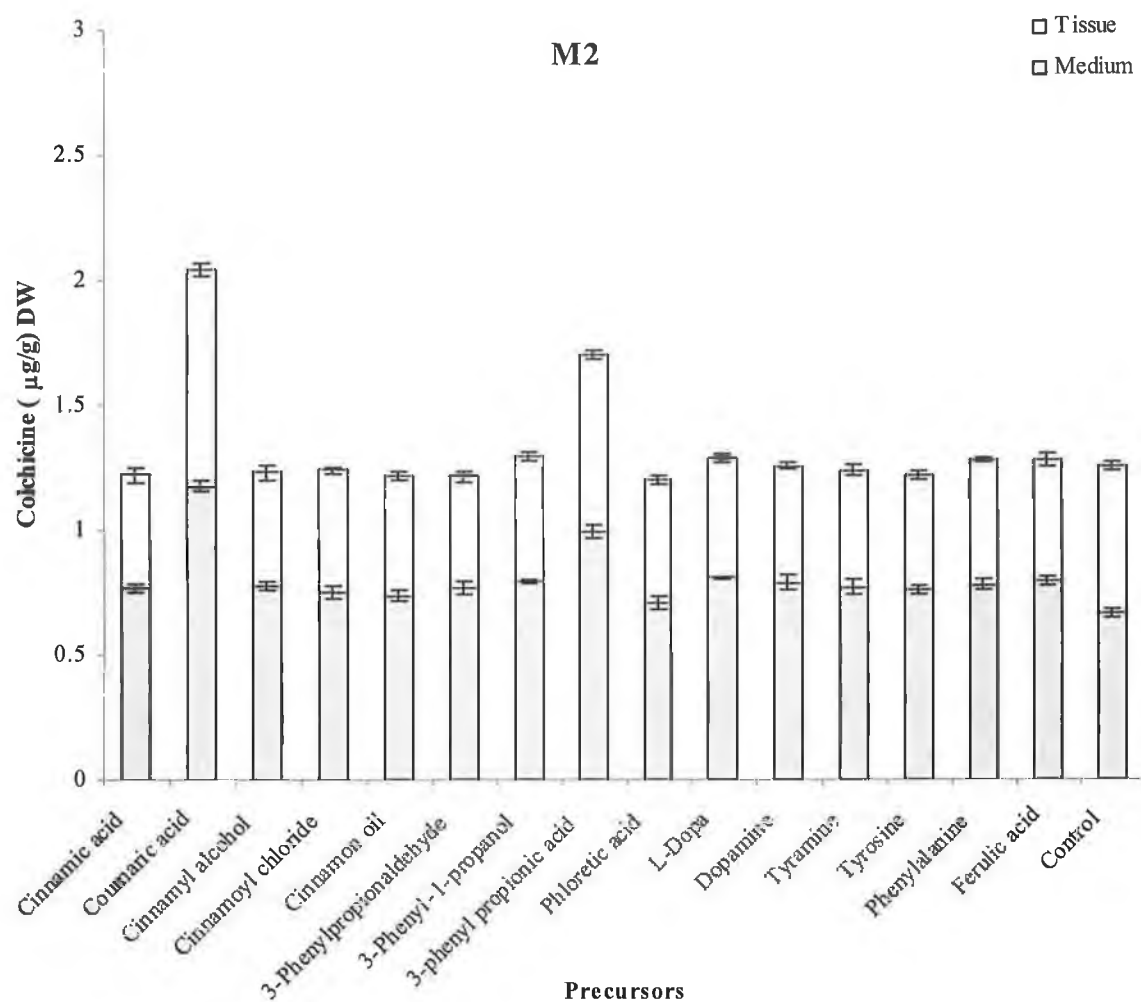


Figure 4.13: The level of colchicine accumulation in *Gloriosa superba* M2 root culture fed with 0.5mM precursors, the grey and white area in the bar are the amount of colchicine ($\mu\text{g/g}$) dry weight in the medium and tissue respectively. Results are the average of three replicates and are shown with the standard error of the mean.

4.4.2 Investigation of the possible synergistic effect of the precursors

The purpose of this experiment was to investigate whether concurrent application of two precursors can increase colchicine production to levels of the same or greater than the sum of the productivities of adding them individually. The previous experiment concluded that coumaric acid, and 3-phenyl propionic acid stimulated colchicine accumulation. It was decided therefore to combine coumaric acid or 3-phenyl propionic acid with one of the following potential precursors: cinnamic acid, dopamine, tyramine, tyrosine, or phenylalanine which was added in a final concentration of 0.5mM.

Combining cinnamic acid or 3-phenyl propionic acid with other precursors did not lead to increased accumulation of colchicine.

4.4.2.1 *Colchicum autumnale* cell suspension culture

Figure 4.14 shows the results of this study for *Colchicum autumnale* cell suspension culture

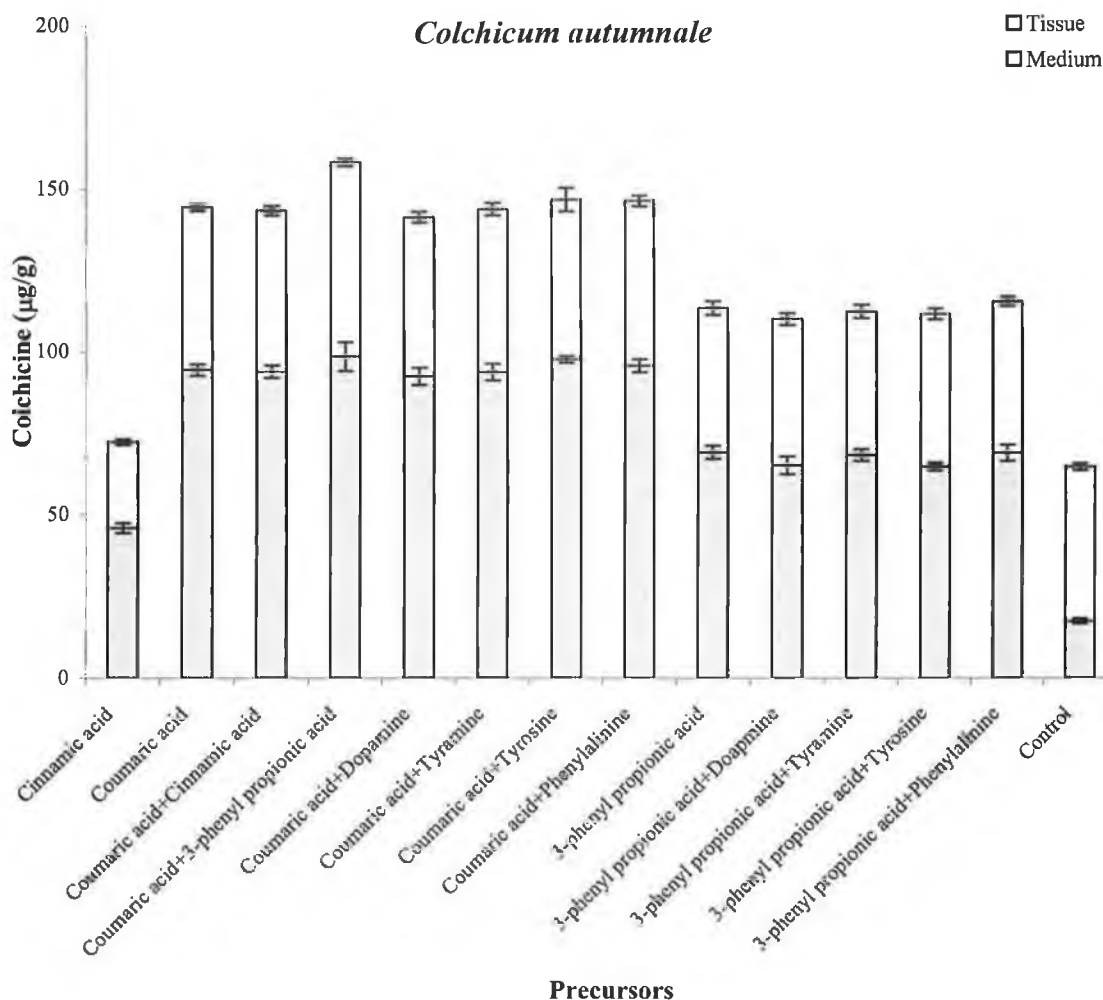


Figure 4.14: The level of colchicine accumulation in *Colchicum autumnale* cell suspension culture fed with 0.5mM individual precursors or a combination of two precursors, the grey and white area in the bar are the amount of colchicine (µg/g) dry weight in the medium and tissue respectively. Results are the average of three replicates and are shown with the standard error of the mean.

4.4.2.1 *Gloriosa superba* cell suspension culture

Figure 4.15 shows the results of this study for *Gloriosa superba* cell suspension culture

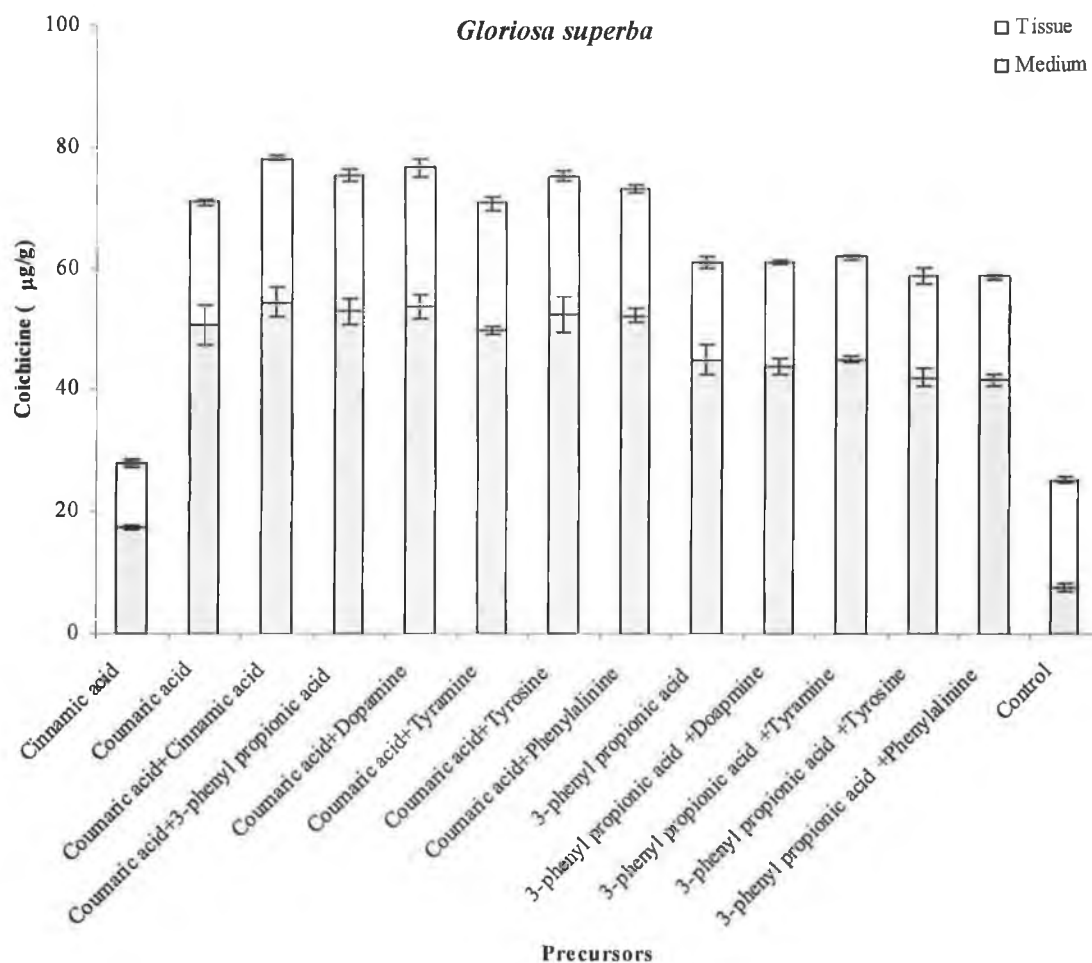


Figure 4.15: The level of colchicine accumulation in *Gloriosa superba* cell suspension culture fed with 0.5mM individual precursors or a combination of two precursors, the grey and white area in the bar are the amount of colchicine (µg/g) dry weight in the medium and tissue respectively. Results are the average of three replicates and are shown with the standard error of the mean.

4.4.2.1 *Gloriosa superba* root tissue

Figure 4.16 and 4.17 shows the level and distribution of colchicine accumulated in liquid medium and tissue of *Gloriosa superba* M1 and M2 root culture fed with 0.5mM individual precursors or a combination of two precursors, respectively.

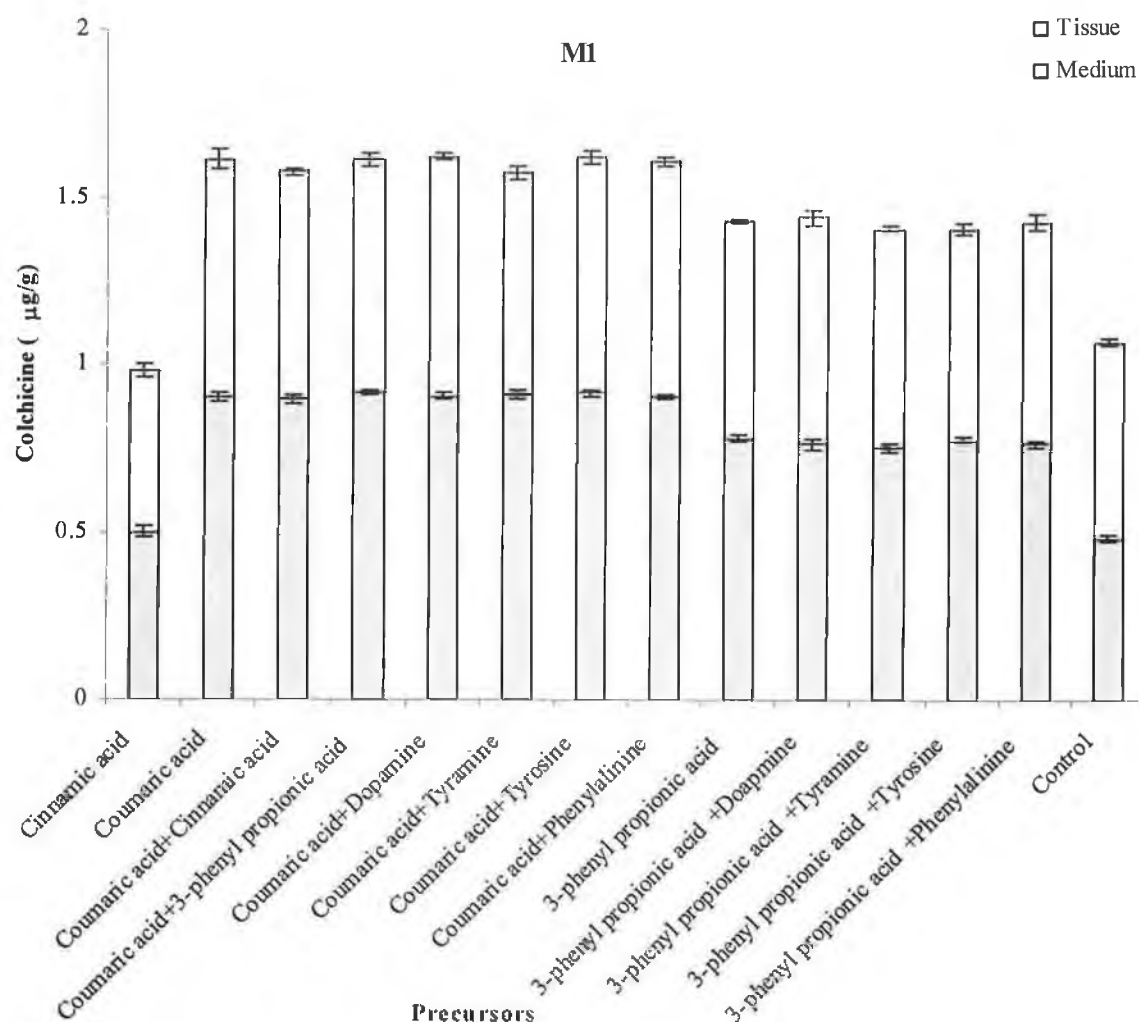


Figure 4.16: The level of colchicine accumulation in *Gloriosa superba* M1 root culture fed with 0.5mM individual precursors or a combination of two precursors, the grey and white area in the bar are the amount of colchicine ($\mu\text{g/g}$) dry weight in the medium and tissue respectively. Results are the average of three replicates and are shown with the standard error of the mean.

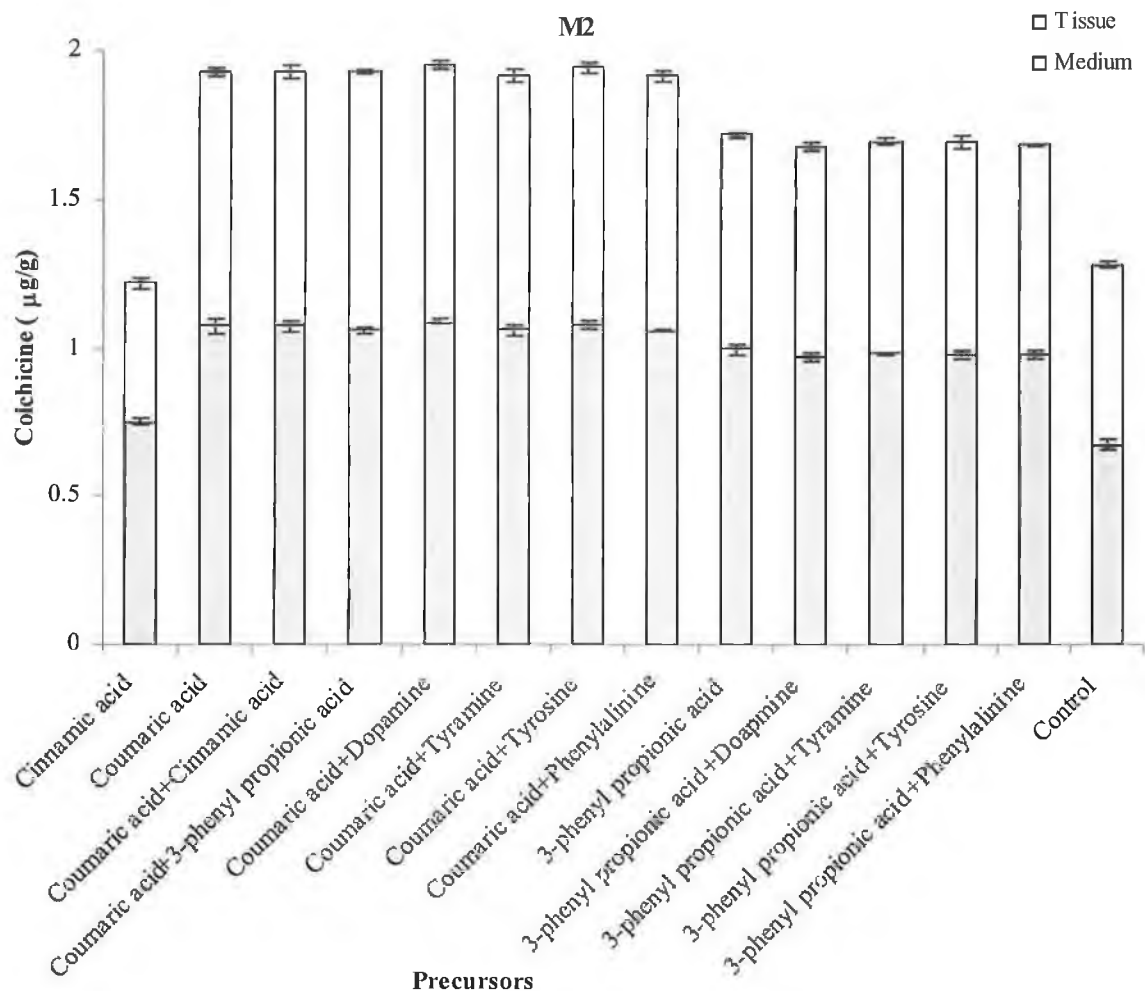


Figure 4.17: The level of colchicine accumulation in *Gloriosa superba* M2 root culture fed with 0.5mM individual precursors or a combination of two precursors, the grey and white area in the bar are the amount of colchicine ($\mu\text{g/g}$) dry weight in the medium and tissue respectively. Results are the average of three replicates and are shown with the standard error of the mean.

Gloriosa superba M1 and M2 root tissue culture show that no significant changes in total colchicine accumulation occurred by adding a combination of two precursors in contrast to addition either coumaric acid or 3-phenyl propionic acid individually.

4.4.3 Study of colchicine accumulation in tissue culture using solid medium supplemented with some precursors.

A concurrent precursor feeding experiment was performed, by incorporation of precursors into solid medium. The tissue was incubated on a medium supplemented with 0.5mM of individual precursors. The culture was incubated at 25 °C for four weeks. After four weeks the tissue was collected, and frozen at -80 °C for three hours then lyophilized overnight in the vacume freeze-dryer at -40 °C and 133×10^{-3} mbar. The dry tissue weighed. 1ml of PBS was added to the dried tissue culture and the samples were then sonicated on ice bath using a sonicator probe to lyse the cells and tissue at output 3 and 2 seconds pulse cycle for 1min.

4.4.3.1 Estimation protein concentration in tissue treated with precursors

The Bio-Rad Protein assay was used to measure the total amount of soluble protein in a crude extract. The Bio-Rad protein assay is a dye-binding assay based on the method of Bradford (1976). It is a simple and accurate procedure for determining concentration of solublised protein. It involves the addition of an acid dye to protein solution, and subsequent measurement by spectrophotometry of the differential colour change of the dye in response to various concentrations of protein.

A Micro-plate assay was used for the determination and measurement of protein content in callus and root tissue cultures. The estimated protein was based on a standard curve made using bovine serum albumin (BSA).

Ten microlitres of the extract was added to micro-plate well, 200 µl Bio-Rad solution was added, and the plate incubated at room temperature for 5min. Absorbance was measured at 620nm.

4.4.3.2 Determination of colchicine concentration in tissue

The sonicated mixture was centrifuged at 13,000rpm for 20min and the supernatant assayed for colchicine as described before.

Colchicum autumnale callus culture accumulated 21%; 49.7%, and 22.8% more colchicine with a medium supplemented with cinnamic acid, coumaric acid and 3-phenyl propionic acid respectively over the control.

Callus and root tissue were incubated for four weeks on a solid medium supplemented individually with 0.5mM precursors have shown a significant accumulation of colchicine. Figure 4.18 and 4.19 Show the results of this study for *Colchicum autumnale* and *Gloriosa superba* callus culture, respectively.

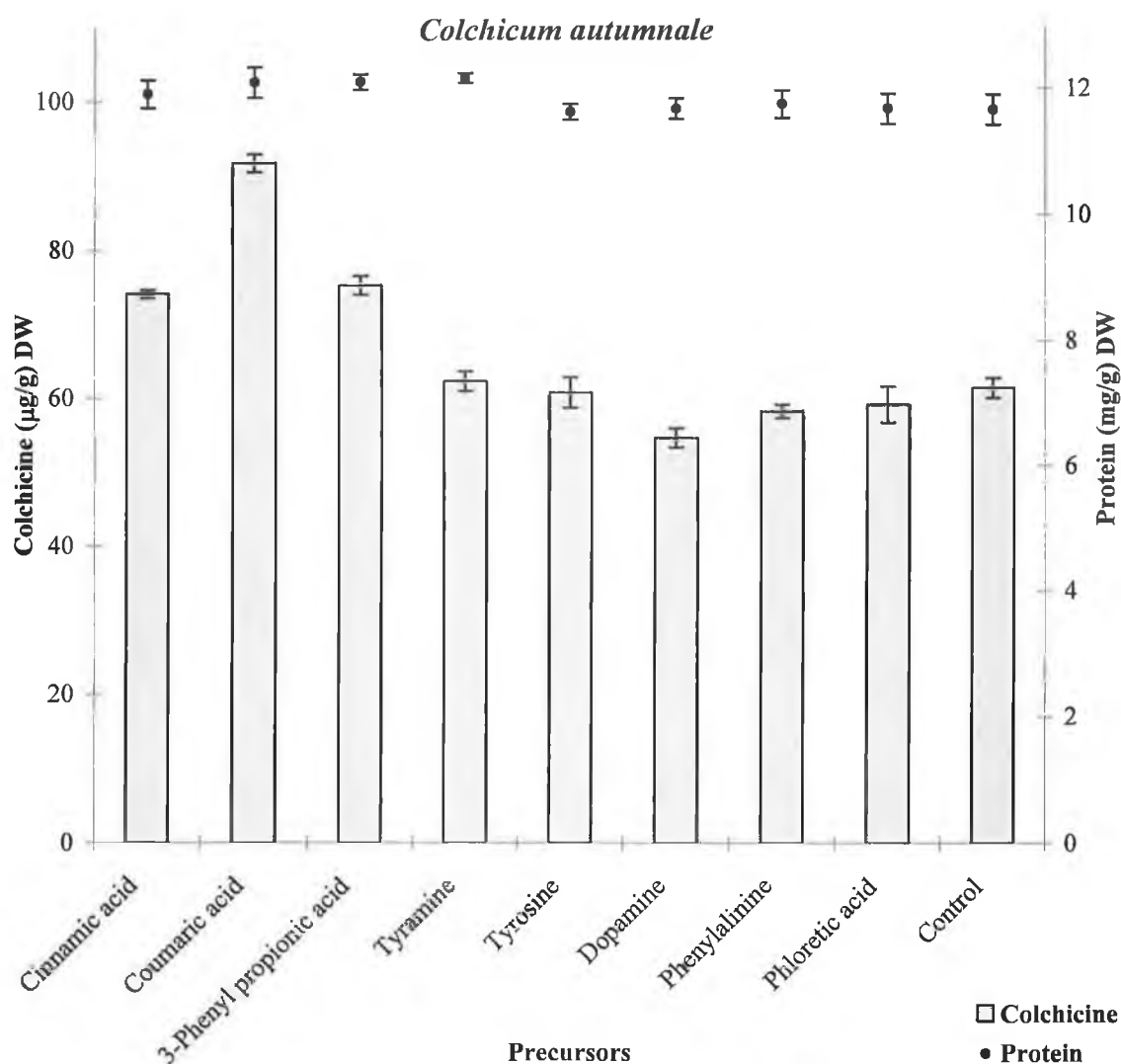


Figure 4.18: The level of colchicine accumulation and protein content in *Colchicum autumnale* callus culture on a solid medium supplemented with 0.5mM individual precursors, bars show the amount of colchicine ($\mu\text{g/g}$) dry weight. The points are the protein contents in callus tissue (mg/g) dry weight. Results are the average of three replicates and are shown with the standard error of the mean.

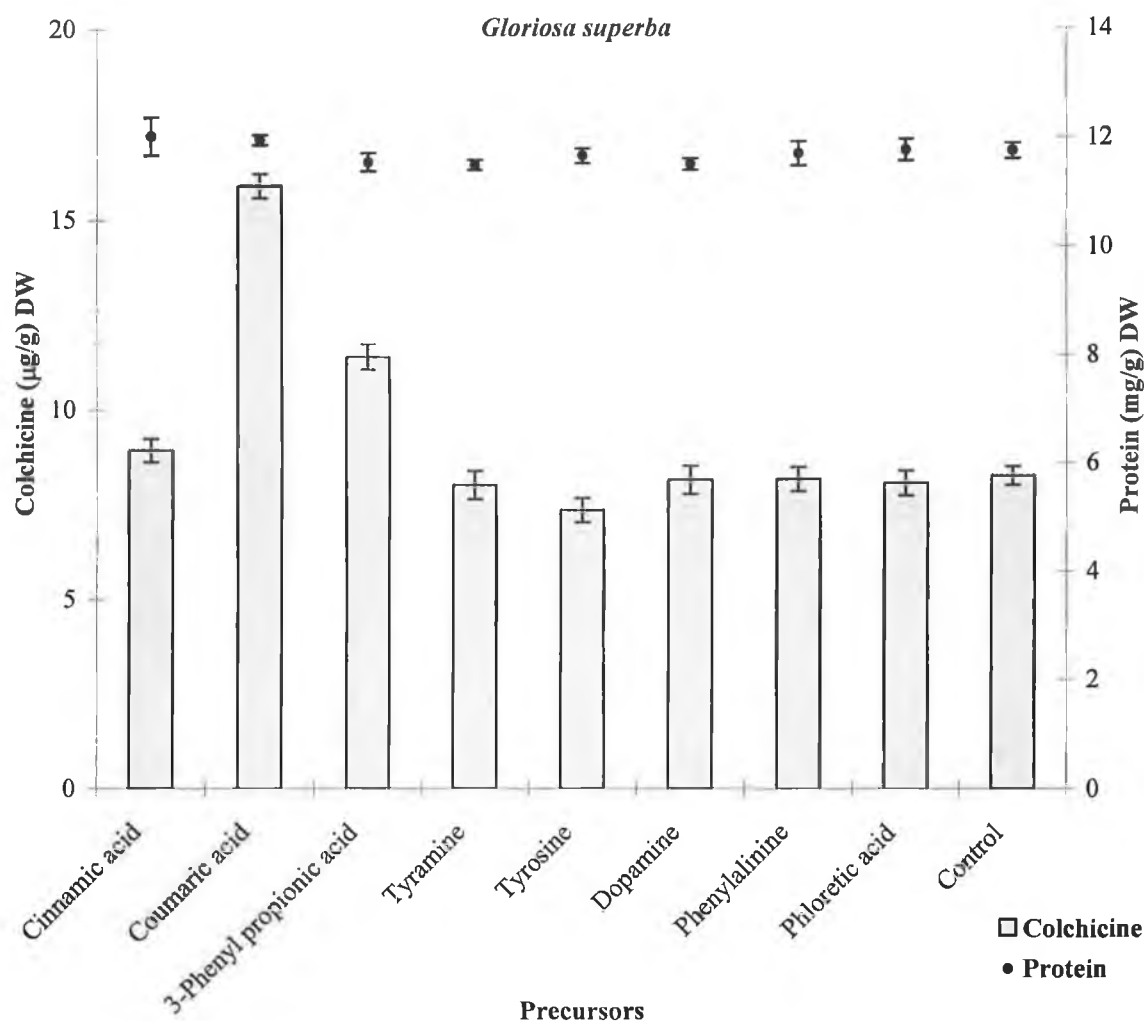


Figure 4.19: The level of colchicine accumulation and protein content in *Gloriosa superba* callus culture on a solid medium supplemented with 0.5mM individual precursors, bars show the amount of colchicine ($\mu\text{g/g}$) dry weight. The points are the protein contents in callus tissue (mg/g) dry weight. Results are the average of three replicates and are shown with the standard error of the mean.

Gloriosa superba callus culture accumulated 92% and 38% on a medium supplemented with coumaric acid and 3-phenyl propionic acid respectively over the control.

Figure 4.20 and 4.21 Show the results of this study for *Gloriosa superba* M1 and M2 root cultures, respectively.

Gloriosa superba M1 root tissue culture accumulated 18%; 62% and 45% over the control on a medium supplemented with cinnamic acid, coumaric acid and 3-phenyl propionic acid respectively.

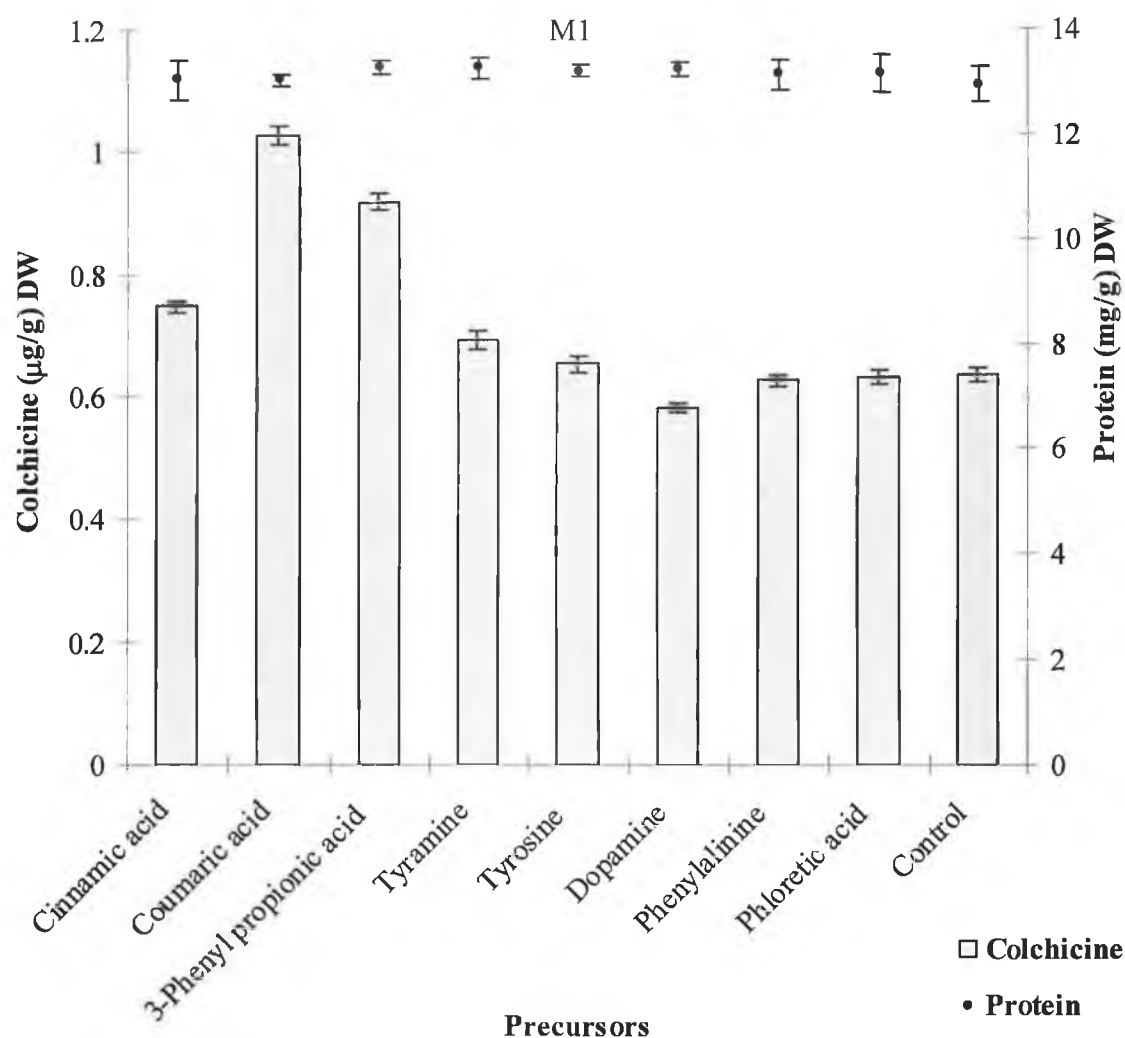


Figure 4.20: The level of colchicine accumulation and protein content in *Gloriosa superba* M1 root culture on a solid medium supplemented with 0.5mM individual precursors, bars show the amount of colchicine ($\mu\text{g/g}$) dry weight. The points are the protein contents in callus tissue (mg/g) dry weight. Results are the average of three replicates and are shown with the standard error of the mean.

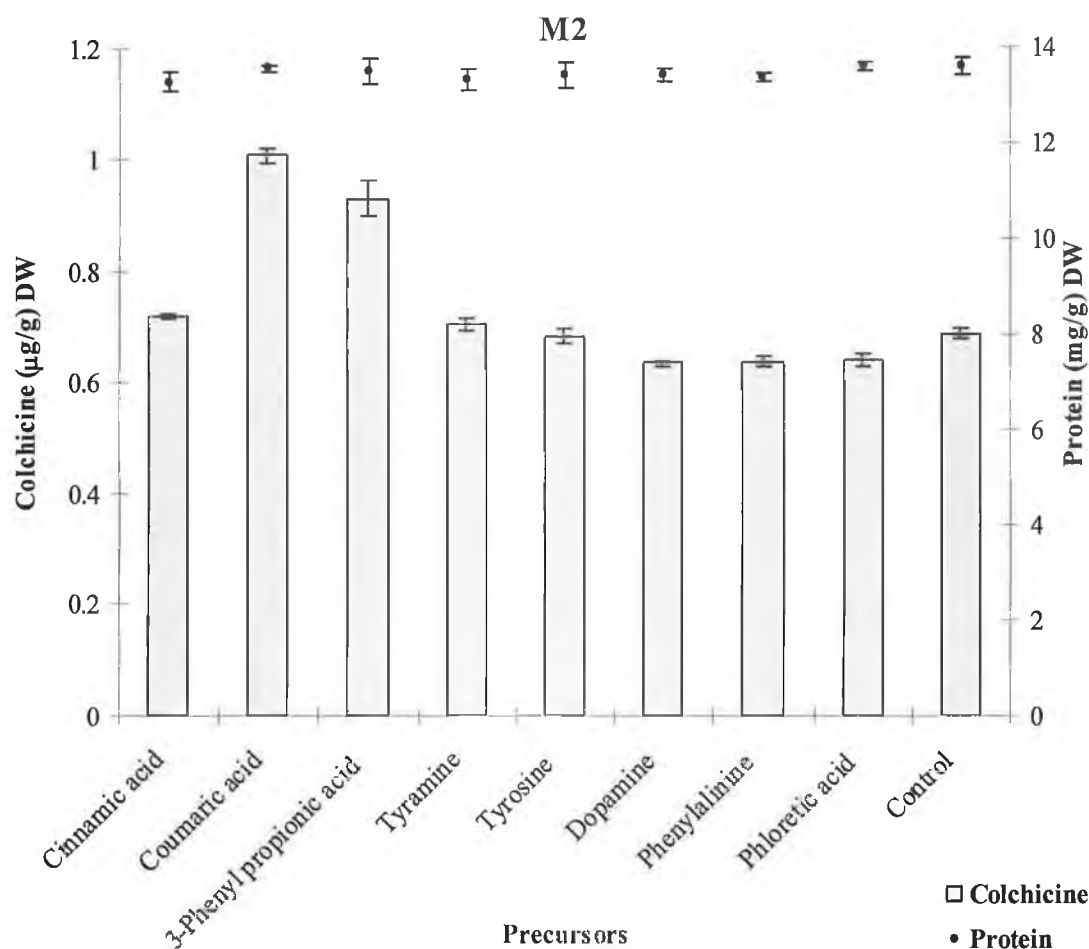


Figure 4.21: The level of colchicine accumulation and protein content in *Gloriosa superba* M2 root culture on a solid medium supplemented with 0.5mM individual precursors, bars show the amount of colchicine (µg/g) dry weight. The points are the protein contents in callus tissue (mg/g) dry weight. Results are the average of three replicates and are shown with the standard error of the mean.

Gloriosa superba M2 root tissue culture accumulated 46.9% and 35.5% more over the control on a medium supplemented with coumaric acid, and 3-phenyl propionic acid respectively.

4.4.4 Assessment of the toxicity of precursors to cultured cells

Macroscopic observations of the culture after three days incubation with some precursors showed a change in the colour of the culture as the tissue started to brown or blacken, which may indicate that the tissue had started to become necrotic (Table 4.1 and 4.2), which may indicate that some precursors are toxic to the tissue.

Table 4.1: Macroscopic colour changes of the cell suspension culture after three days incubation with precursors at three different concentrations.

Precursor	Precursor concentration (mM)					
	1.0		0.5		0.1	
	<i>C.a.</i>	<i>G.s.</i>	<i>C.a.</i>	<i>G.s.</i>	<i>C.a.</i>	<i>G.s.</i>
Cinnamic acid	++	++	-	+	-	-
Coumaric acid	+	+	-	-	-	-
Cinnamyl alcohol	-	-	-	-	-	-
Cinnamoyl chloride	+	+	+	-	-	-
Cinnamaldehyde	+	+	-	-	-	-
3-Phenylpropionaldehyde	+	+	-	-	-	-
3-Phenyl-1-propanol	+	+	-	-	-	-
3-phenyl propionic acid	-	-	-	-	-	-
Phloretic acid	+	+	-	-	-	-
L-Dopa	++++	++++	+++	++++	+++	+++
Dopamine	++++	++++	+++	+++	+	++
Tyramine	++++	++++	+++	+++	+	+
Tyrosine	++++	++++	+++	+++	+	+
Phenylalanine	-	-	-	-	-	-
Ferulic acid	+	+	-	-	-	-
Control	-					

Where *C.a.* = *Colchicum autumnale* and *G.s.* = *Gloriosa superba*

- = No change,
- + = Slight brown,
- ++ = Brown,
- +++ = Dark brown,
- ++++ = Blackened

Table 4.2: Macroscopic colour changes of the *Gloriosa superba* root culture after three days incubation with precursors at three different concentrations.

Precursor	Precursors concentration (mM)			
	1.0		0.5	
	M1	M2	M1	M2
Cinnamic acid	++	++	+	+
Coumaric acid	+	+	-	-
Cinnamyl alcohol	-	-	-	-
Cinnamoyl chloride	-	-	-	-
Cinnamaldehyde	+	+	+	+
3-Phenylpropionaldehyde	+	+	+	+
3-Phenyl-1-propanol	+	+	-	-
3-phenyl propionic acid	-	-	-	-
Phloretic acid	+	+	-	-
L-Dopa	++++	++++	++++	+++
Dopamine	++++	++++	+++	+++
Tyramine	++++	++++	+++	+++
Tyrosine	++++	++++	+++	+++
Phenylalanine	-	-	-	-
Ferulic acid	+	+	-	-
Control	-	-	-	-

Where - = No change,
 + = Slight brown,
 ++ = Brown,
 +++ = Dark brown,
 ++++ = Blackened

Culture browning occurred at high (1mM) of cinnamic acid and with L-dopa, dopamine, tyramine, and tyrosine at all concentrations. Culture browning was found associated with a drop in cell viability.

Cell viability tests were performed using the method described in section (2.2.10.2.1). In this study suspension cultures were treated with different concentrations of precursors to determine the toxicity level. Figure 4.22 and 4.23 show the percentage cell viability after 1, 2, and three days treatment with 1mM precursors for *Colchicum autumnale* and *Gloriosa superba* cell suspension culture, respectively.

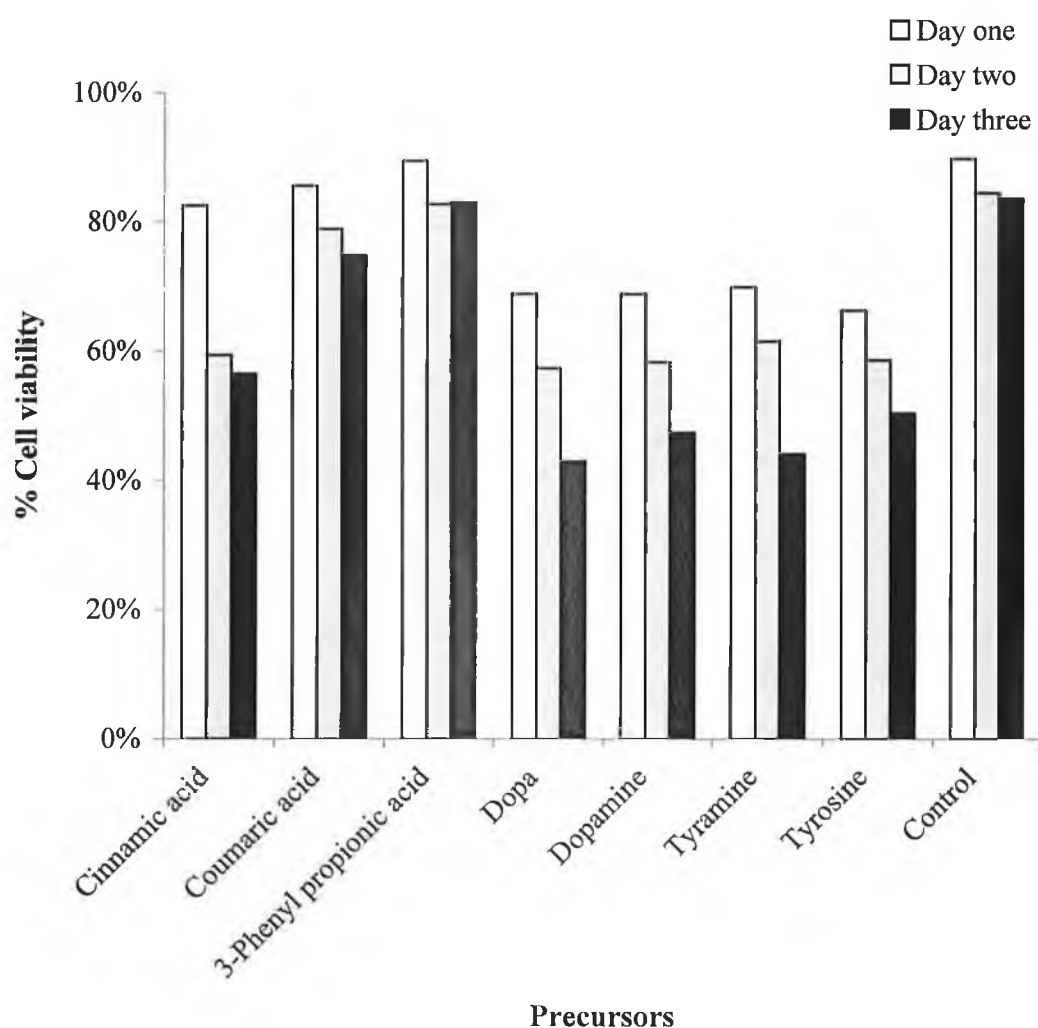


Figure 4.22: Cell viability of the *Colchicum autumnale* cell suspension treated with 1mM precursors.

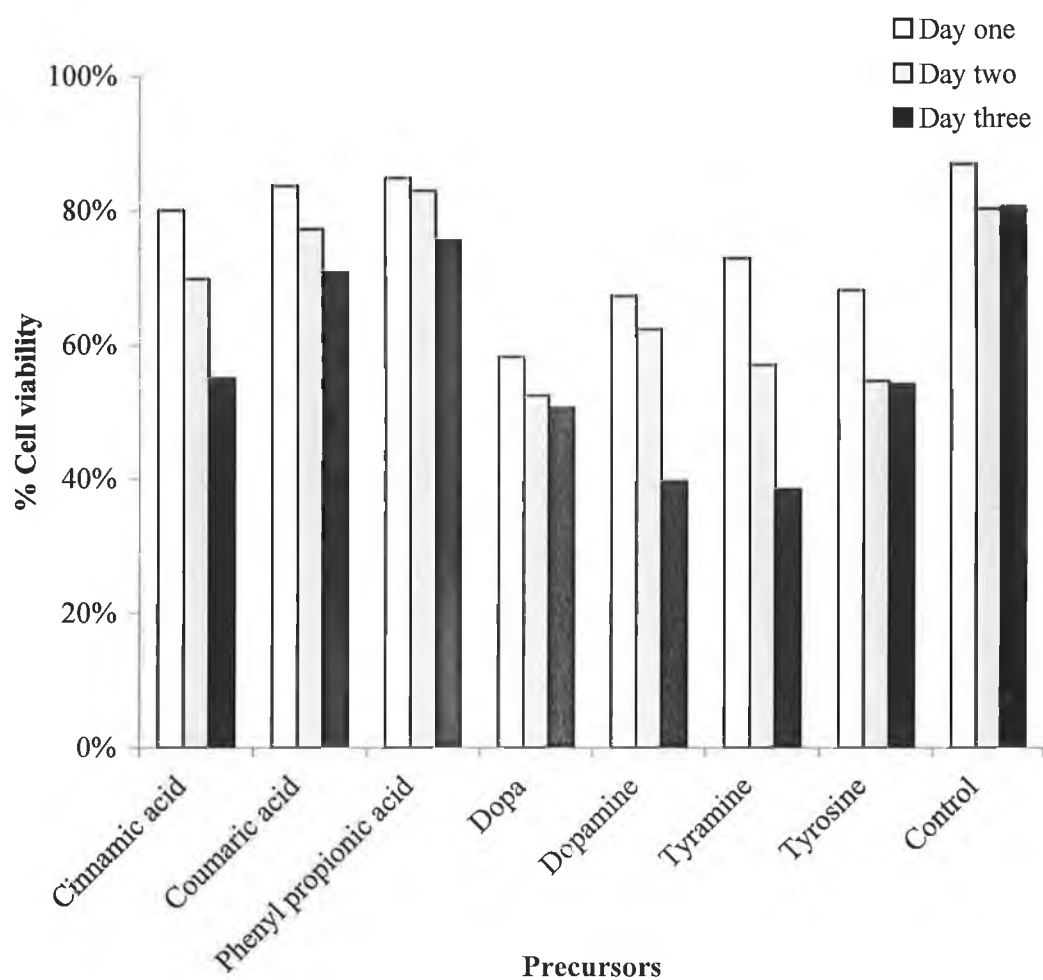


Figure 4.23: Cell viability of the *Gloriosa superba* cell suspension treated with 1mM precursors.

Figure 4.24 show the percentage cell viability after 1, 2, and three days treatment with cinnamic acid for *Colchicum autumnale* and *Gloriosa superba* cell suspension culture, respectively.

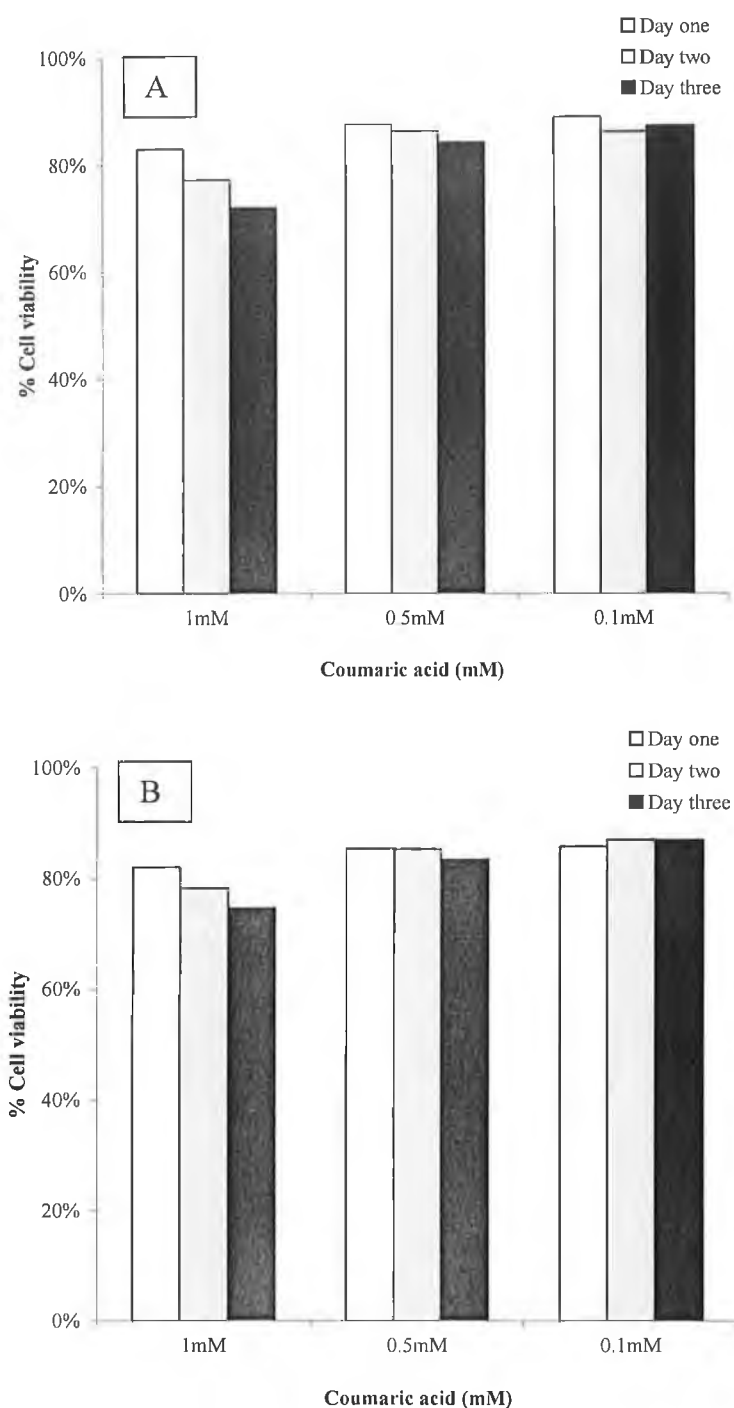


Figure 4.24: Cell viability of the cell suspension treated with (1, 0.5, or 0.1mM) cinnamic acid. A= *Colchicum autumnale*, B= *Gloriosa superba*.

Figure 4.25 show the percentage cell viability after 1, 2, and three days treatment with 3-phenyl-propionic acid for *Colchicum autumnale* and *Gloriosa superba* cell suspension culture, respectively.

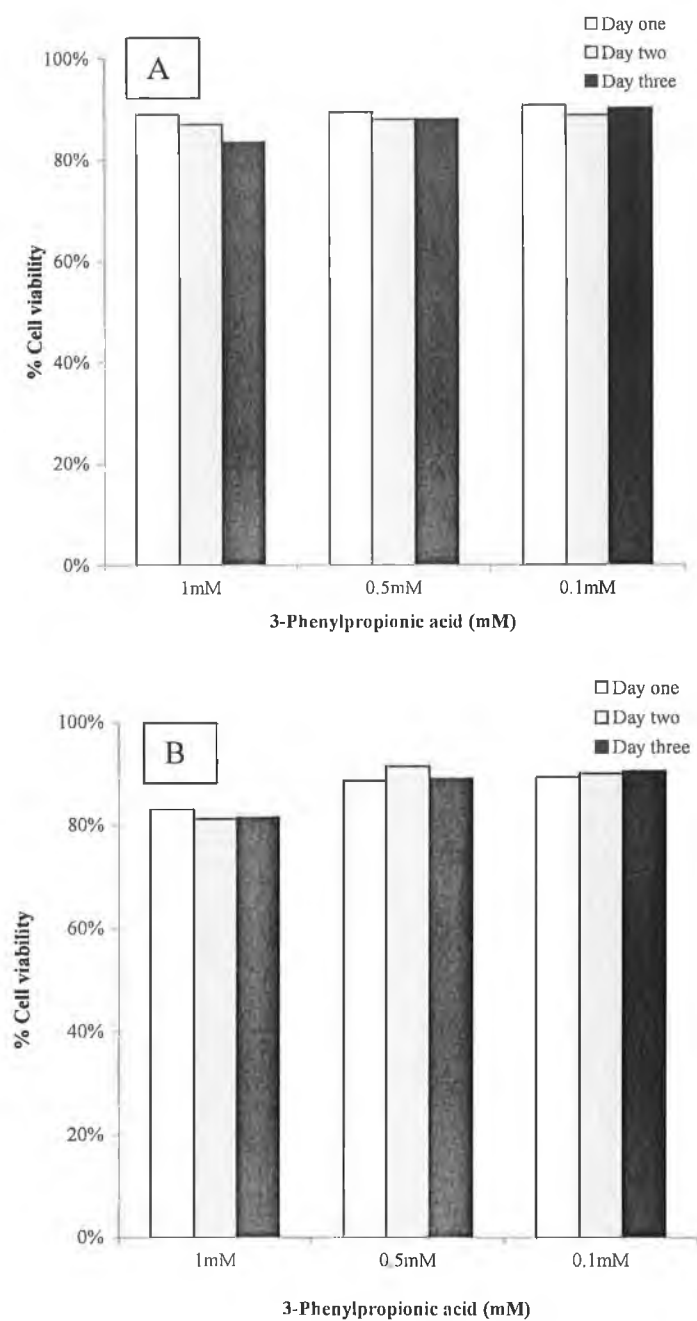


Figure 4.25: Cell viability of the cell suspension treated with (1, 0.5, or 0.1mM) 3-phenyl-propionic acid. A= *Colchicum autumnale*, B= *Gloriosa superba*.

4.4.4 Assessment of the toxicity of colchicine to cultured cells

The effect of colchicine on the viability of cell suspension culture was measured. *Colchicum autumnale* and *Gloriosa superba* cell suspension cultures were treated individually with a wide range of colchicine concentrations for three days. The results are shown in (Figure 4.26)

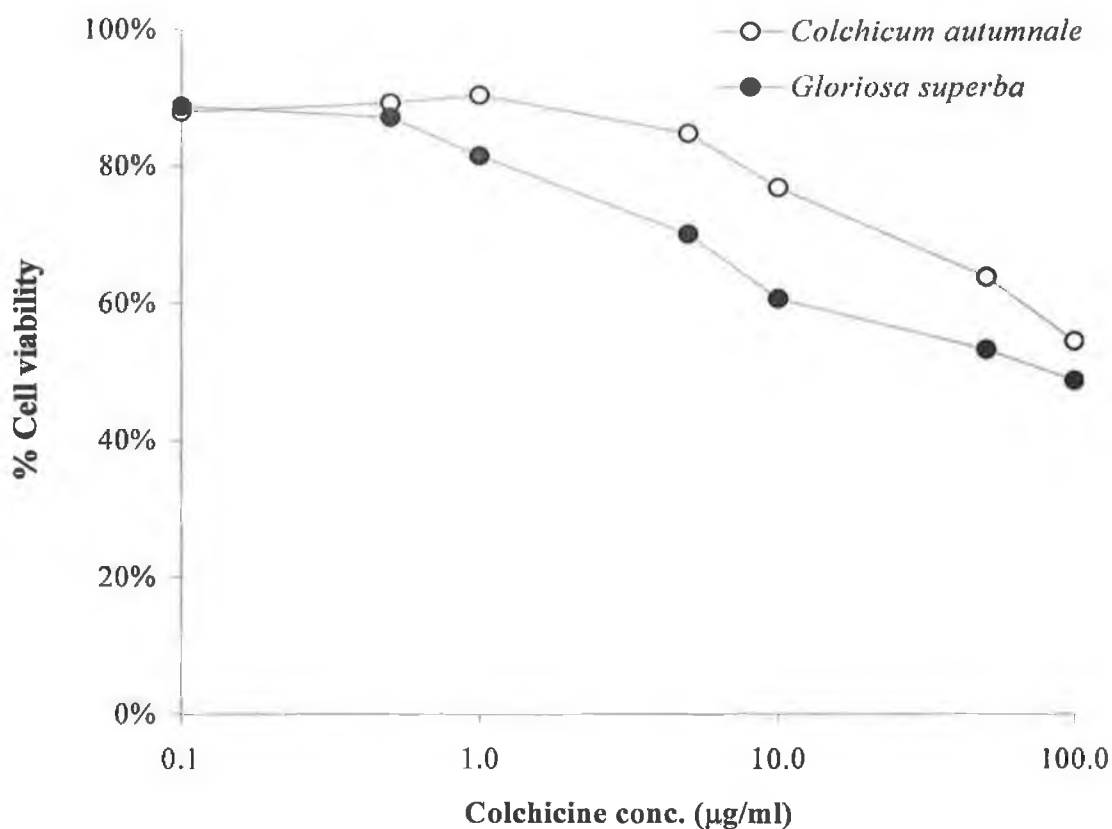
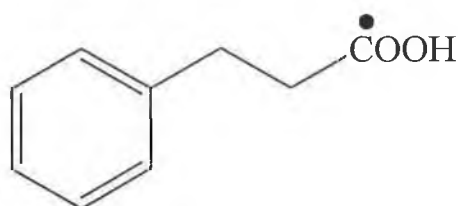


Figure 4.26: Cell viability of *Colchicum autumnale* and *Gloriosa superba* cell suspension cultures treated with different concentrations of colchicine for three days.

Cell culture viability was inversely related to colchicine concentration.

4.4.5 Tracer study by feeding radio-labeled 1-C¹⁴ 3-phenyl propionic acid

Radioactively labeled precursors may be used to establish the level of incorporation into more complex metabolites. The accumulation of colchicine after feeding C¹⁴ 3-phenyl propionic acid to cell suspension culture of *Colchicum autumnale* and *Gloriosa superba* was investigated.



1-C¹⁴ 3-Phenyl propionic acid

Labelled 1-C¹⁴ 3-phenyl propionic acid (2μCi) (Specific activity = 55mCi/mMol) (obtained from ARC, Inc USA) was added to plant cell suspension culture in a multi-well plate, and incubated for 72h at 25°C on an orbital shaker (120rpm). The cell suspensions were collected in microcentrifuge tubes, centrifuged for 20min at 13000rpm and the supernatant transferred to an auto-sampler vial for HPLC assay. The residue was lyophilised and the dried residue weighed, then extracted as described in (section 3.1.3.7.2). The peak of colchicine was collected in a scintillation vial and 10ml of scintillation fluid BCS (Amersham, USA) were added to each vial and counted using a LS 6500 Multi-purpose scintillation counter. The percentages of incorporation are shown in (Table 4.3).

Table 4.3: The (%) of incorporation of radio-labeled C¹⁴ 3-phenyl propionic acid in to colchicine

Plant	% of C ¹⁴ 3-phenyl propionic acid incorporation into colchicine	% of colchicine	
		Medium	Tissue
<i>Colchicum autumnale</i>	0.1	69.6	30.4
<i>Gloriosa superba</i>	0.07	78.4	21.6

4.4.1 Effect of SAM and L-methionine feeding on colchicine accumulation in callus tissue

It was known that numerous secondary metabolites possess a methyl group involving SAM at some point in their biosynthetic pathway. SAM serves as a cofactor in a variety of reactions and it is important in the biosynthesis of certain alkaloids where a methyl-group donor is necessary for the transmethylation reaction in the biosynthesis pathway (Belbahri, et al. 2000). The biosynthesis of SAM, using ATP and methionine is catalysed by S-adenosyl-L-methionine synthetase.

SAM and L-methionine were prepared with different concentrations (1, 0.5, 0.1, or 0.05mM) in MS medium supplemented with 3% sucrose and with no growth hormones and were added individually to cell suspension cultures in a multi-well plate with incubation for 72h at 25 °C on an orbital shaker (120rpm). All treatment was performed in triplicate. Thereafter the level of colchicine in the medium and tissue was assayed. Results are shown in (Figure 4.27 and 4.28) for *Colchicum autumnale* and *Gloriosa superba* cell suspension culture, respectively.

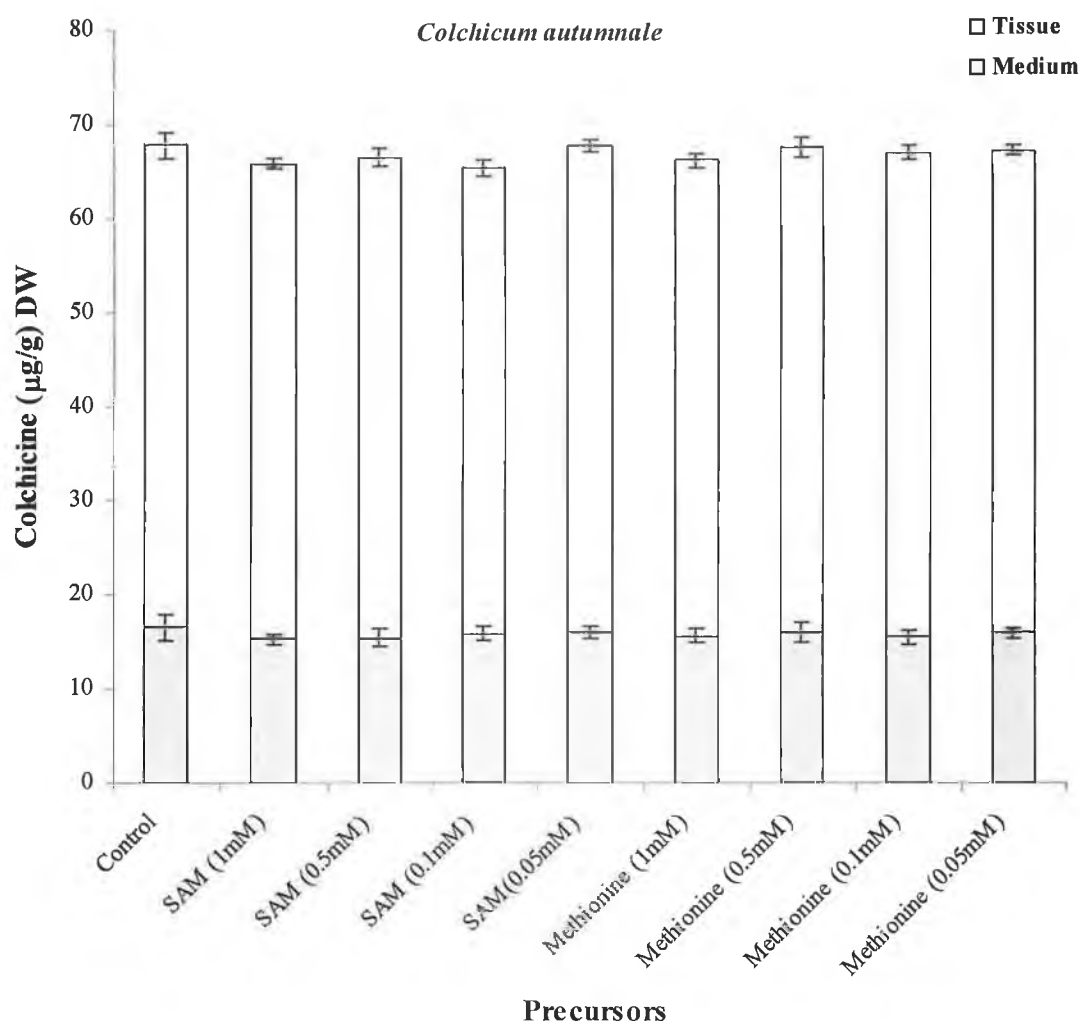


Figure 4.27: The level of colchicine accumulation in *Colchicum autumnale* suspension culture treated with different concentration of SAM or L-methionine, the grey and white area in the bar are the amount of colchicine (µg/g) dry weight in the medium and tissue respectively. Results are the average of three replicates and are shown with the standard error of the mean.

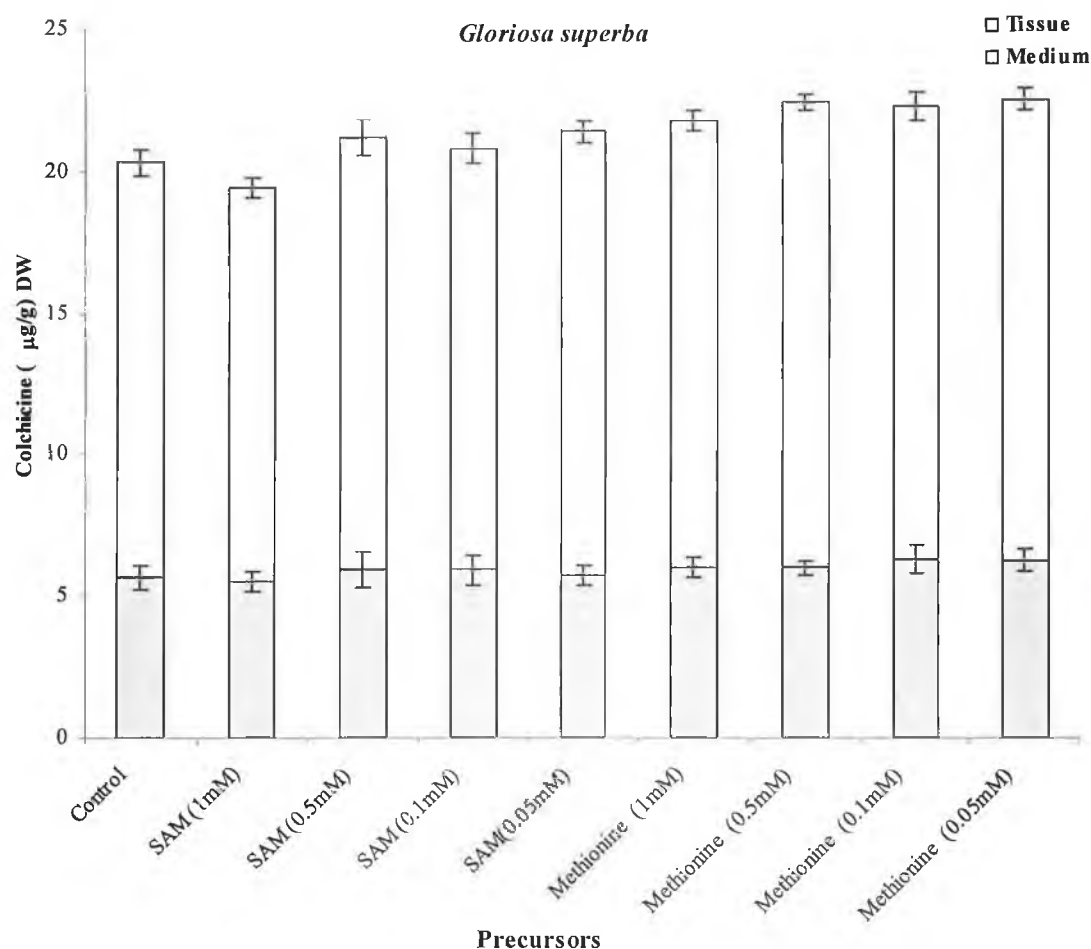


Figure 4.28: The level of colchicine accumulation in *Gloriosa superba* suspension culture treated with different concentration of SAM or L-methionine, the grey and white area in the bar are the amount of colchicine ($\mu\text{g/g}$) dry weight in the medium and tissue respectively. Results are the average of three replicates and are shown with the standard error of the mean.

The biosynthesis of colchicine involves a series of SAM dependent reactions; however, different concentrations of SAM and methionine (1; 0.5; 0.1, or 0.05mM) have proven that they are ineffective in the stimulation of colchicine accumulation.

4.4.6 Elicitation of colchicine accumulation

The effect of Yeast extract or methyl jasmonate addition on colchicine accumulation in callus tissue examined. Suspension cell cultures were separately treated with methyl jasmonate, in three different concentrations (1, 0.5, 0.1, or 0.05mM), and yeast extract (diluted down to 1/100, 1/1000, 1/5000, or 1/10000). Both elicitors were prepared in MS medium supplemented with 3% sucrose and with no growth hormones. The elicitors were added individually to cell suspension cultures in a multi-well plate and incubation for 72h at 25 °C on an orbital shaker (120rpm). All treatments were performed in triplicate. Thereafter the level of colchicine in the medium and tissue was assayed. Figure 4.29 and 4.30 show the level and distribution of the accumulated colchicine in the liquid medium and tissue culture treated individually with different concentrations (1, 0.5, 0.1, or 0.05mM) of methyl jasmonate or (1/100, 1/1000, 1/5000, or 1/10000) dilution of yeast extract for *Colchicum autumnale* and *Gloriosa superba* cell suspension culture, respectively.

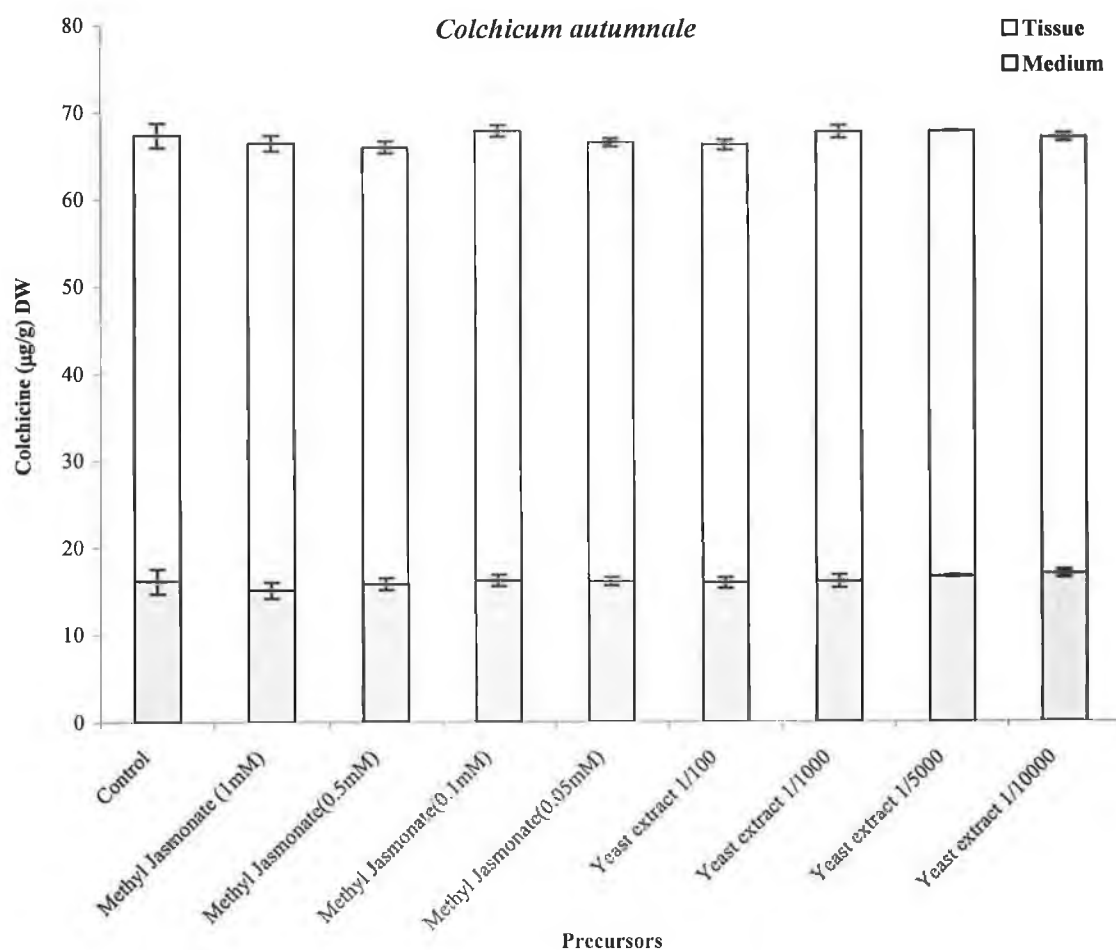


Figure 4.29: The level of colchicine accumulation from *Colchicum autumnale* suspension culture treated with different concentrations of methyl jasmonate or yeast extract, the grey and white area in the bar are the amount of colchicine ($\mu\text{g/g}$) dry weight in the medium and tissue respectively. Results are the average of three replicates and are shown with the standard error of the mean.

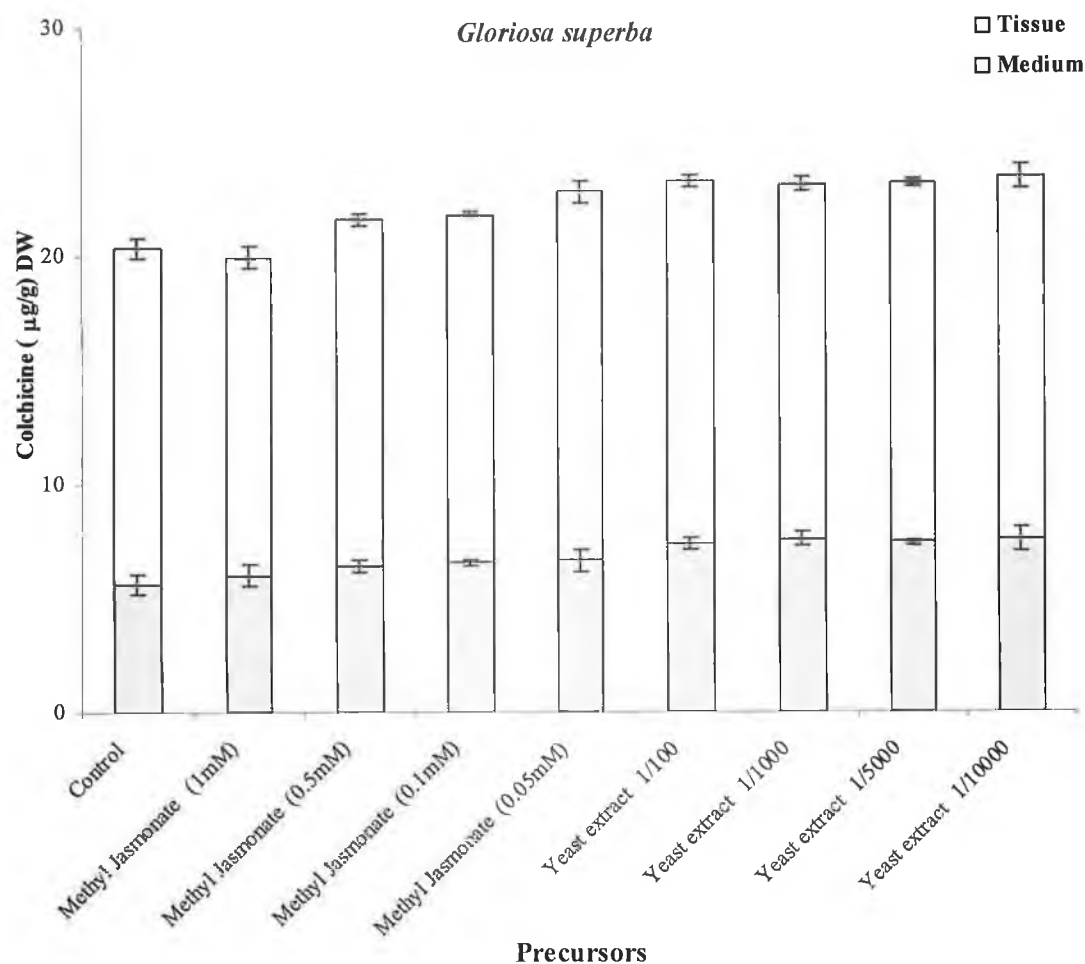


Figure 4.30: The level of colchicine accumulation from *Gloriosa superba* suspension culture treated with different concentrations of methyl jasmonate or yeast extract, the grey and white area in the bar are the amount of colchicine ($\mu\text{g/g}$) dry weight in the medium and tissue respectively. Results are the average of three replicates and are shown with the standard error of the mean.

The addition of methyl jasmonate or yeast extract into the liquid medium did not enhance the accumulation of colchicine in either culture.

4.4.7 The effect of light on colchicine accumulation

Plant tissue cultures are usually grown in diffuse light. However light is known to affect the production of several secondary metabolites (Towers and Yamamoto, 1985). Contrasting effects of light on the production of secondary metabolites have been reported; for example, the inhibitory effect of light on nicotine accumulation in tobacco cell cultures (Hobbs, and Yeoman, 1991), and stimulation of anthocyanin production by *Haplopappus gracilis* culture (Stickland and Sunderland, 1972) and of betalains by cell cultures of *Chenopodium rubrum* (Berlin, *et al.*, 1986), and caffeine and thiobromine in *Coffea arabica* (Kurata, *et al.* 1997). Light can significantly stimulate the biosynthesis of numerous secondary metabolites including alkaloids (Whitaker, *et al.* 1986) and this factor should be investigated to elucidate possible regulatory factors for colchicine biosynthesis. Two sets of 50ml flasks containing 10ml suspension culture of callus or root tissue were incubated under identical conditions except, the first set was incubated under 16h fluorescent irradiation light and 8h dark cycle and the second set was covered in aluminum foil to exclude light. Both sets were placed on an orbital shaker at 25 °C and incubated for four weeks, the medium removed and fresh medium added and the cultures incubated again for four weeks under the same conditions. At the end of second incubation, the level of colchicine accumulated in the incubation medium and in the tissue was assayed. The effect of light on colchicine accumulation are shown in Figure 4.31 and 4.32

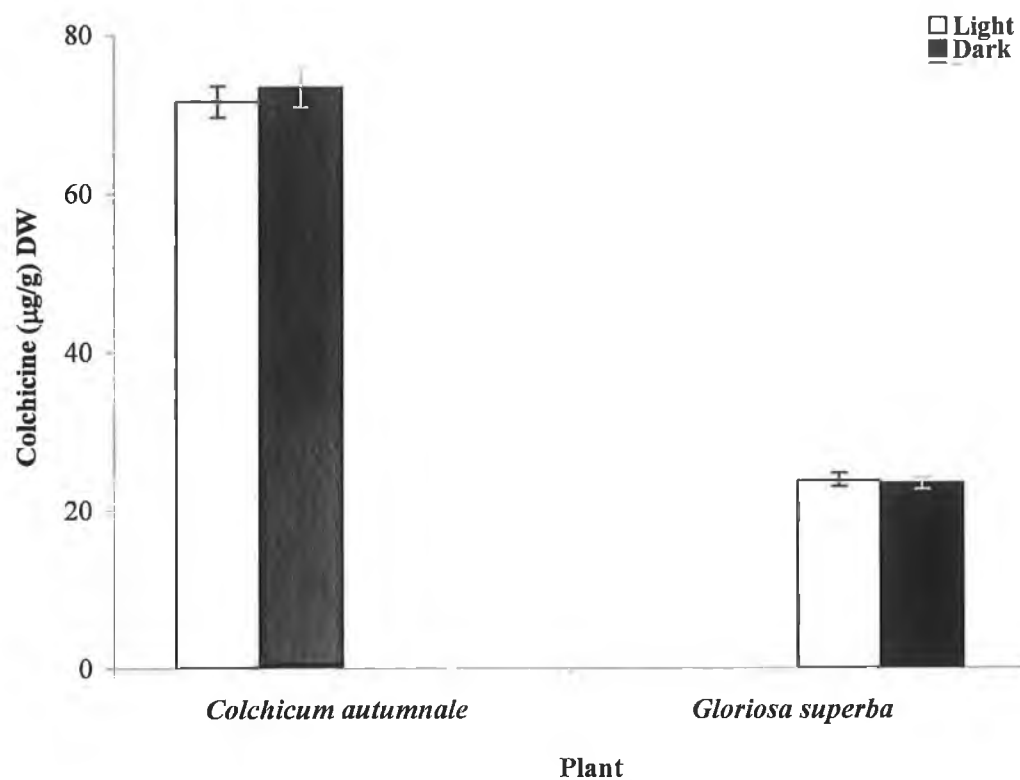


Figure 4.31: Total colchicine accumulation in *Colchicum autumnale* and *Gloriosa superba* cell suspension culture incubated under light or dark condition. The bars show the total colchicine accumulated in the medium and tissue (µg/g) dry weight. Results are the average of three replicates and are shown with the standard error of the mean.

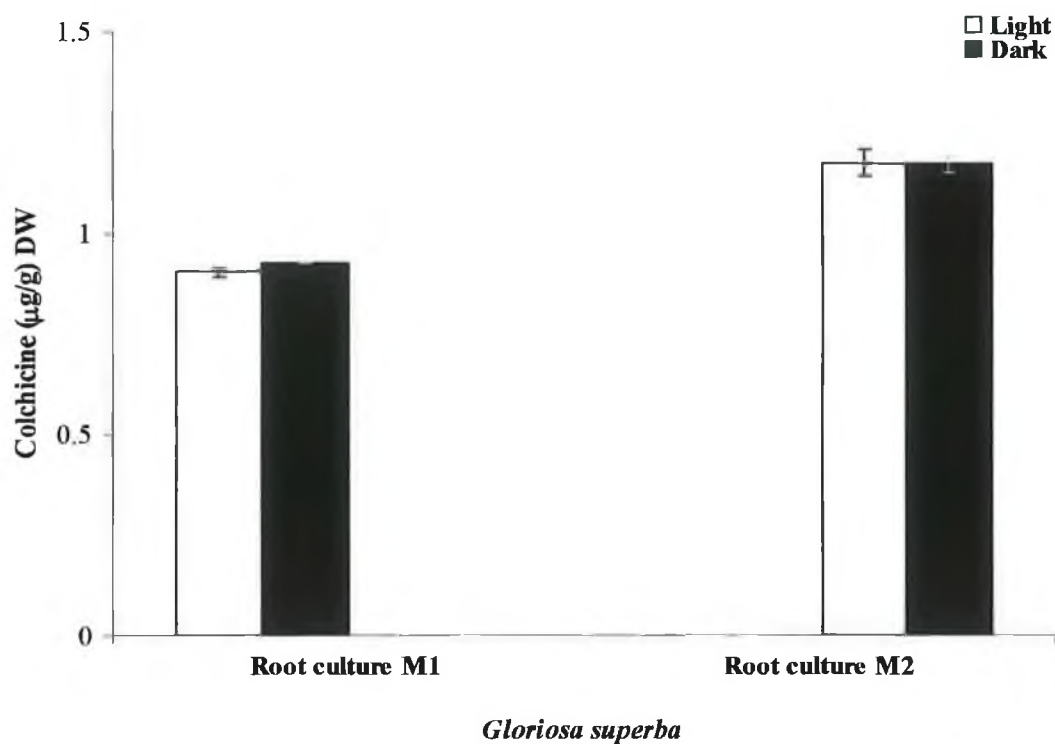


Figure 4.32: Total colchicine accumulation in *Gloriosa superba* root cultures (M1 and M2) incubated under light or dark condition. The bars show the total colchicine accumulated in the medium and tissue ($\mu\text{g/g}$) dry weight. Results are the average of three replicates and are shown with the standard error of the mean.

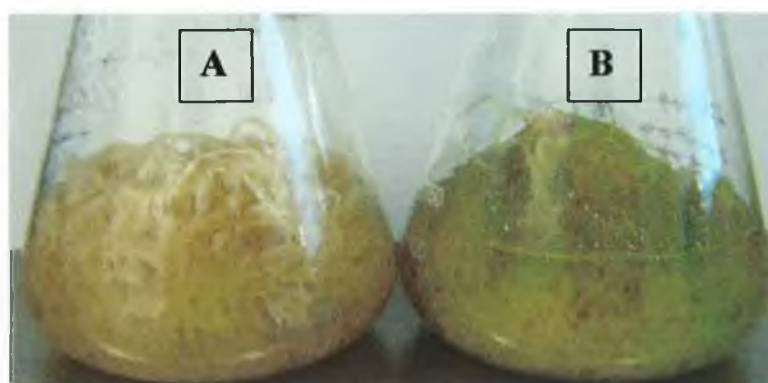


Figure 4.33: *Gloriosa superba* root culture grown in A=Dark, and B= 16h light and 8h dark cycle.

4.5 Discussion

The ability of cultured plant cells to produce secondary metabolites is of considerable interest because it offers a possible source of the production of expensive or rare compounds from cheap and plentiful available precursors or alternatively, of obtaining compounds which are difficult to synthesise.

To understand some key steps in the biosynthesis of the target secondary metabolites, the effect of exogenous precursors in cell cultures may be of use. The ability of potential precursors phenylalanine, tyrosine and their analogues to either stimulate colchicine accumulation in tissue or release into the medium was examined.

Sivakumar *et al.* (2004), indicated that the major reason for the failure of higher colchicine accumulation in callus tissue is due to absence of the precursors for colchicine biosynthesis. The choice of a 1mM concentration was based on a previous precursor feeding study by Yoshida *et al.* (1988A and 1988B). Macroscopic examinations have shown that some precursors changed the colour of the tissue, which may indicate that some precursors are toxic to the tissue. It was therefore decided to investigate lower concentration (0.5, or 0.1mM) of precursors. The total colchicine accumulated in *Colchicum autumnale* cell suspension cultures fed with 0.5mM cinnamic acid or coumaric acid was reduced to some extent compared to the culture fed with 1mM while 0.5mM 3-phenyl propionic acid resulted in accumulation of nearly the same amount of colchicine as with 1mM (Figures 4.4 and 4.5).

Colchicine accumulation was correlated with the concentration of precursor applied. Feeding cinnamic acid, coumaric acid or 3-Phenyl propionic acid at 0.1mM to cell suspension culture dramatically reduced the total colchicine accumulated in comparison to the culture fed with 1mM concentration (Figures 4.4 and 4.6). The rest of the potential precursors did not stimulate colchicine accumulation with 0.5 or 0.1mM concentration.

The proportion of the total colchicine in the medium was increased by precursor feeding. The levels of colchicine released from the tissue into the

liquid medium were significantly increased by all the potential precursors used in the feeding experiment, the amount of colchicine released into the liquid medium ranging from 43 - 73% by the culture fed by precursors relative to the control (19%) with *Colchicum autumnale* cell suspension cultures. The amount of colchicine released from *Gloriosa superba* cell suspension culture into the liquid medium ranged from 63- 80% in the culture fed by precursors compared with 42% for the control.

The levels of colchicine released from the root tissue into the liquid medium were significantly increased by all the precursors used in the feeding experiment. *Gloriosa superba* M1 root culture released from 56 - 61% and 48 – 55% colchicine into the liquid medium in the culture fed by 1mM or 0.5mM precursors respectively, compared to the control 41%. *Gloriosa superba* M2 root culture released from 53% - 75% and 57.5% – 63% colchicine into the liquid medium by the culture fed with 1mM or 0.5mM precursors respectively, compared to control 52%. However the largest proportion of colchicine was accumulated within the surrounding incubation liquid medium which was supplemented with the potential precursors.

A wide variety of compounds have been found to be released by various plant-cell cultures, these include certain alkaloids (Delfel, and Rothfus, 1977; Nakagawa, *et al.*, 1984; Pareilleux, and Vinas, 1984; Robins and Rhodes, 1986).

Nasreen *et al.*, (1997) have reported that between 40-70% of the labelled colchicine formed was found in the incubation medium of the seeds. This indicated that synthesized colchicine within the seeds was excreted into the medium.

There was a direct relationship between the proportion of colchicine released into the liquid medium and the concentration of precursors used. Intact plants usually contain specific tissues for accumulation of secondary metabolites whereas tissue cultures are generally composed of undifferentiated cells and therefore have no specificised, differentiated storage tissue for these toxic

compounds. In most cases, secondary metabolites are not stored at the site of synthesis, which may reflect toxicity of the compound to cellular components or need for accumulation at a site of action (Zhang, et al. 2002)

It appears, therefore, that precursors have significantly increased colchicine release into the liquid medium and that may suggest that the precursors may have an important effect on the permeability or structure of the plant cell membrane or they may stimulate the active transport of colchicine from the cell, or displace the colchicine from its binding site.

In previous studies on *Colchicum autumnale* cell suspensions and *Gloriosa superba* root tissue, coumaric acid and tyramine were concluded to trigger formation of colchicine, while phenylalanine and tyrosine had no effect (Yoshida *et al.* 1988A and 1988B; Ghosh *et al.*, 2002). It has been demonstrated that coumaric acid and 3-phenyl propionic acid significantly stimulated colchicine accumulation in all tissue types in which have been used. Application of the combination of two precursors was investigated, to see whether they could increase the colchicine accumulation to levels of the same or greater than the sum of the productivities of adding them individually. Hiraoka *et al* (1973) and Facchini *et al.* (2000) suggested that tyrosine, phenylalanine and their metabolites are precursors for isoquinoline alkaloids biosynthesis. It was therefore decided to combine coumaric acid or 3-phenyl propionic acid with one of the following potential precursors: cinnamic acid, dopamine, tyramine, tyrosine or phenylalanine which were added in a final concentration of 0.5mM. It has been established that no significant change in the total colchicine accumulation by adding a combination of two precursors in contrast to addition of either coumaric acid or 3-phenyl propionic acid individually. A combination of two precursors was found to have no synergistic effect on colchicine accumulation. However, there was a marginal increase in colchicine accumulated in *Colchicum autumnale* cell suspension culture fed with a combination of coumaric acid and 3-phenyl propionic acid, but no increase with a combination of 3-phenyl propionic acid with another

precursor (Figure 4.14). Also there was a slight increase in colchicine accumulated in *Gloriosa superba* cell suspension culture fed with a combination of coumaric acid with one of the following precursors, cinnamic acid, 3-phenyl propionic acid, dopamine, or tyrosine (Figure 4.15).

Feeding the cultures with potential precursor amino acids and other analogues can have unexpected results. The cell viability estimation demonstrates that cinnamic acid, tyrosine, tyramine, dopamine, and L-dopa dramatically reduced the cell viability after three days incubation with 1mM concentration to between (43% - 56%) and (38.4% - 54%) for *Colchicum autumnale* and *Gloriosa superba* cell suspension culture respectively (Figure 4.22, 4.23). Coumaric acid at 1mM reduced the cell viability to 72.1% and 74.7% after three days incubation for *Colchicum autumnale* and *Gloriosa superba* cell suspension culture respectively, with no significant changes in cell viability with cell suspension fed with 0.5mM or 0.1mM coumaric acid (Figure 4.24). This may suggest that a high concentration of 1mM of coumaric acid was relatively toxic to the cell suspension. The 3-phenyl propionic acid did not change the colour of the culture and has a negligible effect on the cell viability (Figure 4.25). Cinnamic acid, L-dopa, dopamine, tyramine, and tyrosine were also found to be toxic at higher concentrations. Cinnamic acid has been shown to be a precursor in the pathway to provide coumaric acid following catalysis by cinnamic acid 4-hydroxylase (Ghosh *et al.*, 2002), but the toxicity that it demonstrates on the cells eliminates this compound as a possible beneficial precursor (Mizukami, 1977; Yoshida *et al.* 1988A and 1988B). Culture browning may result from the oxidation of phenolic compounds and this may explain the strong browning seen with tyrosine and Dopa and their related compounds.

The production of secondary metabolites is not always enhanced when such precursors are added. It was pointed out that biosynthetic pathways have numerous steps; the precursors being modified by several enzymes before the

secondary compound is formed. The level of production of any intermediate along the pathway and of the production itself is limited by feedback inhibition, as more products accumulate. The active site of an enzyme can only cater for a certain amount of substrate and activity reaches a saturation point. It is possible that the precursors may be either incorporated directly into the product, or precursors may enter a specific product indirectly through degradative metabolism and entry into interrelated pathways (DiCosmo, and Misawa 1995).

Colchicine, while very active on cells of most species of plants, produces no effects in *Colchicum autumnale*, the most common source of this compound (Waller and Nowacki 1978). Colchicine has no significant effect on cell viability at relatively low concentration (0.1 - 1 µg/ml) on both species. There is a slight decrease in cell viability of *Colchicum autumnale* with (5 µg/ml) and higher colchicine concentrations show a dramatic decrease in cell viability. The *Gloriosa superba* cell suspension culture seems more vulnerable to higher colchicine concentrations as the cell viability dramatically decreases with increase in colchicine concentration from 5 - 100 µg/ml (Figure 4.26). This may explain the fact that *Gloriosa superba* cultures accumulate relatively lower levels of colchicine.

Study of the colchicine accumulation in tissue culture using solid medium supplemented with some precursors, Tyrosine, tyramine, dopamine, phenylalanine, and phloretic acid incorporated into the solid medium, show a negligible effect on colchicine accumulation. Cell suspensions constitute a good biological material for studying biosynthetic pathways. This is the ideal system for precursor feeding as it provides the greater surface area for uptake of precursors. Indeed, compared to callus cultures they are surrounded by the medium and that offer a good chance for precursor to enter the cells. The amount of colchicine produced in the cell and tissue cultures by using liquid medium is significantly higher than the amount from callus and tissue culture

on a solid medium, and that may suggest that liquid medium provide another benefit over the solid medium as it facilitates release of more colchicine.

Protein contents of the callus and root tissue grown on a solid medium supplemented with 0.5mM precursors were estimated, and the results revealed that no significant changes in the content of soluble protein were observed in callus and root tissue cultures. That suggests that the potential precursors amino acids or their analogous used in the feeding experiment were not diverted or channelled into protein production.

Feeding radio-labeled 1-C¹⁴ 3-phenyl propionic acid to cell suspension cultures of *Colchicum autumnale* and *Gloriosa superba* gave a level of incorporation in to colchicine of 0.1 and 0.07% respectively. The level of radio-labeled colchicine released into the medium was significantly higher than the amount in the tissue. These results suggest that 3-phenyl propionic acid acts as precursor in the biosynthesis of colchicine, rather than as an elicitor of colchicine production and may be used to increase accumulation of colchicine by plant cell cultures and release of colchicine to the liquid culture medium. A Previous study on feeding plants with C¹⁴ 3-phenyl propionic acid have demonstrated that it is possible to produce labeled colchicine, (Herbert *et al.*, 1990) reported that addition of C¹⁴ 3-phenyl propionic acid to *Colchicum byzantinum* by wick feeding the whole plants during the flowering period lead to 0.04% incorporation to colchicine. The incorporation of radio-labelled C¹⁴ 3-phenyl propionic acid in the cell suspension cultures are relatively low, but such small incorporation of exogenously supplied precursors are typical for higher plants (Banthorp, *et al.* 1972).

Coumaric acid was found to be the most successful precursor. This is in agreement with previous research carried out by Yoshida *et al.* (1988A and 1988B); Ghosh, *et al.*, (2002).

This confirms the fact that C4H is a rate limiting enzyme, as coumaric acid was the only analogue of the phenylalanine \longrightarrow cinnamic acid \longrightarrow coumaric acid pathway to increase production. However, the high level of incorporation of 3-phenyl propionic acid demonstrates that an additional early pathway may operate consistent with precursor feeding studies on the intact plant (Herbert, *et al.*, 1990).

Compartmentalization both within the cell and within the plant is a big challenge for secondary metabolites biosynthesis. The compartmentation of alkaloid biosynthesis is even more complex, with biosynthetic enzymes localized in the cytosol, vacuole, tonoplast membrane, endoplasmic reticulum, chloroplast stroma and specific vesicles (Facchini *et al.* 2001). The results demonstrated no increase in colchicine levels following treatment with the two amino acids phenylalanine and tyrosine. Phenylalanine showed no enhancement in colchicine accumulation and that may suggest that PAL is blocked between phenylalanine and cinnamic acid. Also C4H may be blocked between cinnamic acid and coumaric acid. These bottleneck steps can sometimes be overlapped by feeding the cell cultures with a precursor metabolite that corresponds to the product of a limiting enzymatic activity although there is always the risk of activating a feedback inhibition somewhere else on the pathway (Bolwell *et al.* 1986). The failure of some supplied precursors to stimulate colchicine accumulation may be due to four reasons: First, they may be used for primary metabolites by culture. Secondly, the precursors may not have reached the synthesis site in the cell. Thirdly, the culture may lack the enzymes involved in colchicine production. Fourth, low accumulation of colchicine in cell cultures may not be due to a lack of key biosynthetic enzymes but rather due to feedback inhibition.

In spite of comparatively high concentration (1 or 0.5mM) of the potential precursors, no significant increase in colchicine accumulation was observed with phenylalanine and tyrosine, tyramine, dopamine. These compounds are important in primary metabolism and are likely to be strictly regulated to other

pathways. This suggests that they are not major rate limiting steps in the colchicine biosynthesis pathway.

In this work, the effect of elicitors in colchicine accumulation was investigated on cell cultures. Methyl jasmonate, and yeast extract were incorporated into the liquid medium with a broad range of concentration. Methyl jasmonate and yeast extract showed no significant effect on the colchicine accumulation in *Colchicum autumnale* and *Gloriosa superba* cell suspension.

It was suggested that light have induced the activity of some enzymes such as C4H in cell suspension culture of parsley (Hahlbrock *et al.*, 1971; 1976; Yoshida *et al.* (1988A and 1988B), had shown that PAL could be induced by light but C4H could not in *Colchicum autumnale* cell suspension. The effect of light on the colchicine accumulation in cell and root suspension culture was investigated. The cell and root culture in the liquid medium were subcultured and incubated under dark or light conditions. The culture incubated under dark condition turned to pale yellow or white colour and the culture which kept under light retained its yellow and green colour for the cell suspension and root culture respectively (Figure 4.32). The level of colchicine accumulated in light grown cultures was in no cases different from that in dark grown culture. That suggests that light has no significant effect on colchicine accumulation. Our results are in full agreement with Hayashi, *et al.* (1988A) showing that light did not increase the formation of colchicine. This is consistent with the finding that cinnamic acid was not an effective precursor in our studies and that C4H may be the rate limiting enzyme.

The culture conditions used in the current study gave low growth rates for the cultures of *Colchicum autumnale*, but it is not known if the production of secondary metabolites is favoured in slow growing cell lines. It is possible that under conditions of minimum increase in the mass of cell cultures secondary metabolites may be increased and vice versa, as root cultures, which are

relatively fast growing accumulate less colchicine. The different colchicine levels in *Gloriosa superba* callus and root culture are consistent with differences in growth rate.

The fact that *Colchicum autumnale* and *Gloriosa superba* callus and root tissues from which colchicine was extracted had been maintained in culture for at least two years prior to analysis, indicates that the colchicine biosynthetic pathway is operational in such tissues and that the colchicine present was not that carried over from the original explant.

4.6 Conclusion

A number of parameters were examined with a view to stimulating secondary metabolite production in cell suspension cultures. It can be concluded that cell and tissue cultures derived from *Colchicum autumnale* and *Gloriosa superba* can be stimulated by precursor treatment to increase colchicine accumulation to some degree. The hypothesis behind precursor feeding seems sound. The results suggest that the *p*-coumaric acid is a rate limiting substance in colchicine accumulation in both cell suspension and root culture of *Colchicum autumnale* and *Gloriosa superba*.

Precursor feeding in this work supported the fact that the plant tissue culture did not lose the capability of accumulating the secondary metabolite colchicine. The genetic information for its synthesis is presumably present even in the undifferentiated tissue.

The most significant increases in colchicine accumulation were found to be produced by precursor feeding. The addition of coumaric acid or 3-phenyl propionic acid may be overcoming a major rate-limiting step in colchicine production. These results strongly suggest that coumaric acid and 3-Phenyl propionic acid are significant precursors for the biosynthesis of colchicine.

Feeding radio-labeled 3-phenyl propionic acid in this work confirmed that colchicine was synthesised in culture from 3-phenyl propionic acid, and suggests that 3-phenyl propionic acid acts as precursor in the biosynthesis of colchicine, rather than as an elicitor of colchicine production, and may therefore be used to increase accumulation of colchicine by using plant cell culture. Radio-labelled C¹⁴ 3-phenyl propionic acid was efficiently taken up and incorporated into colchicine.

Precursor feeding was found to be simple and effective for stimulating colchicine accumulation in the plant cell and tissue cultures. The effects of precursor may be attributed to the availability of the rate limiting compounds in the medium.

The proportion of the total colchicine in the medium was also significantly increased by precursor feeding. That may suggest that the relatively low levels of coumaric acid and 3-phenyl propionic acid may be used to increase accumulation and release of colchicine to the liquid culture medium. That serves to promote maximum secretion of the desired alkaloid into the surrounding medium, thereby facilitating any future extraction processes. This is a significant finding as it facilitates the overall proposed scheme to enhance production and harvesting of colchicine.

Chapter Five

**Design and development of a continuous solid phase extraction
(SPE) method to harvest colchicine**

5.1 Introduction

Plant tissue culture and cell culture have been shown to have the ability to produce secondary metabolites. Often, plant cell products are retained intracellularly or only partially secreted (Payne *et al.*, 1988). In many cases *in situ* product removal enhances secondary metabolite production, and the product may be selectively released from the cells and recovered in a solvent or adsorbent (Komaraiah, *et al.*, 2003). However, the production of these plant derived compounds is often affected by product inhibition and/or degradation (Payne, *et al.* 1992). If a certain amount of secondary metabolite may be released into the culture medium, it should be possible to increase the productivity of secondary metabolites by using an extracellular extraction site (Robins *et al.*, 1988; Strobel *et al.*, 1991).

The addition of an artificial system for the extraction of secondary metabolite may therefore be an effective tool for stimulating colchicine biosynthetic pathways in plant cell cultures by continual removal and sequestration of the product from the cultures, by removal of feed back inhibition, and by decreasing any toxic effects of colchicine to the plant cells. Rhodes *et al.* (1986A) observed that *in situ* removal could also limit the degradation of plant products.

The production of a secondary metabolite such as colchicine from plant cell culture is therefore potentially facilitated by some means of harvesting it.

To increase the production yield, it is necessary to remove colchicine during the production cycle. This can be accomplished by integrating an infinite sink that selectively removes the product of choice that has been produced from the bioreactor. The infinite sink can consist of a solid or a liquid phase. Once the product of choice has been removed from the bioreactor, further steps of recovery and purification are necessary for the successful retrieval of the product.

In general, the selection strategies for the extraction phase should be aimed at maximizing separation capacity and minimizing the toxic effect on cells.

There are two extraction systems liquid-liquid and solid-liquid that have been shown to be effective and can be used to accumulate secondary metabolites. Liquid-liquid extraction and solid phase extraction are widely practiced separation techniques for pharmaceutical and fine chemicals.

5.1.1 Liquid phase extraction

Solvents are used for extraction of products from both plant cell cultures and bacterial processes when the products that are to be harvested are or can be made to be poorly soluble in water. Organic solvents may be used for *in situ* recovery of secondary metabolites from plant cell suspension cultures. Berlin *et al.* (1984) first reported the use of adsorbents to retain volatile compounds from cell culture of *Thuja occidentalis* by using Miglyol, a water-insoluble trap for lipophilic compounds. Hexadecane, a long-chain hydrocarbon, has been employed as the second liquid phase to extract shikonin from suspension culture of *Arnebia euchroma* cells by Fu and Lu, (1999). When using solvents for the extraction of pharmaceuticals, the toxicity effects and biocompatibility of the different solvents on the plant cells and medium have to be considered. The solubility of the solvent in the medium needs to be considered and also the pH may need to be controlled.

Zhao-Liang *et al.*, (2000) studied taxol production in a two liquid phase system. Due to the extremely low solubility of taxol in the medium and its inhibiting effect on cell growth the two liquid-phase culture is regarded by the authors as a key technology. *In situ* removal of taxol from the aqueous phase into the hydrophobic phase led to a shift in equilibrium towards more production and promoted taxol biosynthesis (Manfredi and Horwitz 1986).

5.1.2 Solid-phase extraction

Solid-phase extraction (SPE) has been increasingly and routinely applied in different areas like pharmaceutical, industry and environmental fields. The method provides several major advantages over liquid-liquid extraction technique including fast, simple and direct sample application, and time and cost saving (Shamsipu *et al.*, 2005).

Solid phase extraction typically involves mainly the adsorption of the product onto the surface of the solid-phase followed by a recovery stage where the adsorbed product is removed from the solid phase, usually by using organic solvents.

Polymeric adsorbent is one type of SPE available to extract pharmaceutical product from liquid medium, and different type of polymeric adsorbent are available commercially like the non-ionic Amberlite resins series. Addition of the adsorbent has been used to increase secondary metabolite production (Stroble, *et al.*, 1991). Robins and Rhodes, (1986) used XAD-7 resin for the adsorption of anthraquinones produced by *Cinchona ledgeriana* cultures, increasing the product yield 15-fold, with most of the product being secreted into medium.

Polymeric adsorbents are highly porous structures whose internal surfaces can adsorb and then desorb. For example, in polar solvents such as water, polymeric adsorbents exhibit non-polar or hydrophobic behaviour and so can adsorb organic species that are sparingly soluble. A wide variety of different species are available, depending on the environment in which they are used.

5.1.2.1 Amberlite resins

XAD polymeric adsorbents are very porous spherical beads made from non-ionic polymers based on highly crosslinked, and macroreticular agglomerates of randomly packed microspheres permeated by a network of holes and channels, giving a high mechanical stability and great surface area. It is known that Amberlite XAD resins have a greater adsorption capacity and are easier to elute than other commonly adsorbents used such as alumina or silica gel (Aiken *et al.* 1979). Amberlite XAD can be divided into two categories:

5.1.2.1.1 Non polar Amberlite

XAD-4 and XAD-16 resins are based on a cross-linked polystyrene structure, have a hydrophobic character and have a porous structure. This leads to them being good adsorbents for a wide range of organic compounds from aqueous systems and polar solvents (Min-Woo, *et. al.* 2001).

5.1.2.1.2 Moderately polar Amberlite

XAD-7 resin is based on the cross-linked polyacrylate ester structure, is somewhat hydrophilic and has been used to remove relatively polar compounds from non-aqueous solvents.

5.2 Aim

The long term goal of this work is to develop a feasible model for continuously harvesting colchicine from plant cell suspension cultures.

5.3 Materials and methods

Several experiments were performed to investigate the feasibility of the adsorbent resins for harvesting colchicine from the liquid medium under different conditions and at different concentrations.

Three types of adsorbent Amberlite resins (XAD -4, -7, -16) were obtained (Sigma-Aldrich). The properties of these resins are shown in Table 5.1

Table 5.1: Properties of Polymeric resins used

Type of Polymeric resins	Parameter	Range
Amberlite XAD-4	Surface area	725 m ² /g
	Average pore diameter	40 °A
	Wet mesh size	20-60
Amberlite XAD-7	Surface area	450 m ² /g
	Average pore diameter	90 °A
	Wet mesh size	20-60
Amberlite XAD-16	Surface area	800 m ² /g
	Average pore diameter	100 °A
	Wet mesh size	20-60

5.3.1 Apparatus

Absorbance was measured using a UNICAM UV-vis spectrophotometer A Jeio,

Tech Shaker was used to equilibrate the resins at 25 °C \pm 1 and speed 120rpm. A weller electric soldering iron was used for welding of nylon mesh, Econo-Column were used for continuous extraction of recycled medium, and Nylon mesh sheet with pore size of 400 μ m and 800 μ m.

5.3.2 Preparation of the polymeric adsorbent resins

Untreated polymeric adsorbents typically contain impurities which can leach into the cultures, but which may be removed by solvent extraction. Ten grams of each type of the resins were placed in 100ml flask and soaked in 50ml methanol, and the flasks sealed with parafilm. The flasks were shaken several times at room temperature to disperse the beads in the methanol and left to stand for 24hr. The methanol was then decanted, and the resins washed three times with 50ml of ultra pure water. The flasks were shaken several times at room temperature to make sure that the entire methanol was removed from the resins. The resins were stored in universal containers until needed. To pre-load the adsorbent with the medium components the resins were soaked in 20ml medium and left for 24hr prior to use.

5.3.3 Preparation of resin bags

Amberlite resin was enclosed within two layers of nylon mesh with different porosities. The pore size of the external layer was 800 μ m and the internal layer was (400 μ m). The two layer bag was sealed by welding the plastics using an electric soldering iron at the four sides (Figure 5.1). The resin was washed first in methanol for 1hr and then washed twice with ultra pure water. The bags were autoclaved in medium before being used for extraction.



Figure 5.1: Amberlite resin enclosed within two layers of nylon mesh. Bar= 0.25cm.

5.3.4 Calibration curve for colchicine

Standard solutions of colchicine were prepared in the range from 1 µg/ml to 30 µg/ml from a 50 µg/ml stock solution in MS medium. The absorbance at 350nm was plotted against colchicine concentration to produce the colchicine calibration curve shown in Figure 5.2.

The regression line, slope and intercept of the calibration curve were obtained by using Excel software, and used to interpolate the concentration of colchicine in the medium.

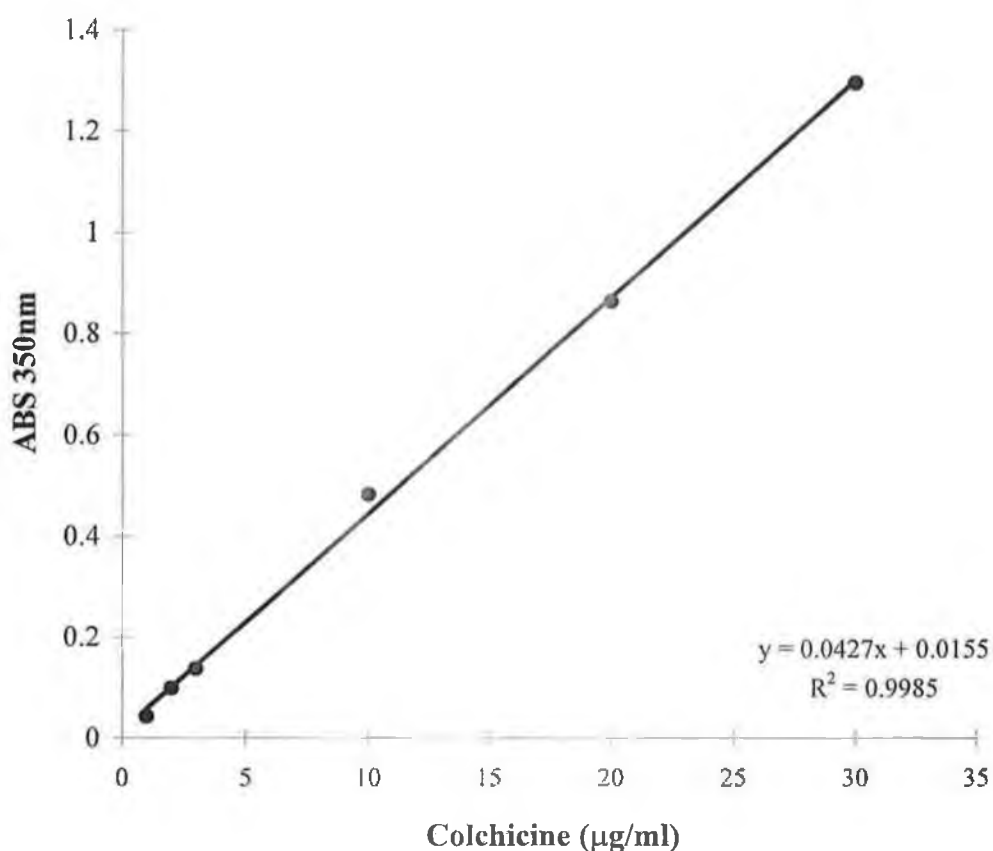


Figure 5.2: Colchicine calibration curve determined by absorbance at 350nm using a spectrophotometer.

5.4 Results

From the previous chapter, colchicine produced by tissue culture was observed in the medium. The results suggest the possibility of using an adsorbent to recover the secreted colchicine.

5.4.1 Preliminary determination of the effect of quantity of Amberlite resin on colchicine uptake

Prior to detailed studies on the affinity and saturation kinetics of a potential infinite sink, it is necessary to demonstrate that the sink shows some affinity for the substrate and to estimate the quantities necessary for uptake studies. According, the amount of resin that is required for uptake of colchicine from the medium was investigated.

Colchicine solution (1mg/ml) was made up in MS medium and dispensed into 1ml aliquots in 1.5ml microcentrifuge tubes. Amberlite resins (0.01g, 0.03g, 0.1g, 0.2g and 0.3g) of XAD-4, XAD-7, and XAD-16 were then added and equilibrated overnight on an orbital shaker (120 rpm). Each tube was then centrifuged at 13,000rpm for 10min to pellet the resins, and the supernatant transferred to a cuvette. Colchicine in the medium was assayed by spectrophotometry and the percentage of colchicine adsorbed by the resins calculated.

Table 5.2 shows the percentage of colchicine recovered from the medium with varying amounts of Amberlite resins.

Table 5.2: The effect of varying the amount of Amberlite resins on the percentage of colchicine uptake.

Resins wt. (g)	% of colchicine recovered from the medium		
	XAD-4	XAD-7	XAD-16
0.01	54	50	65
0.03	90	80	98
0.1	98	94	99.5
0.2	99.5	98	99.3
0.3	99.3	99	99.3

All the resins showed reasonably high affinity for colchicine with 98%, 94% and 99.5% of medium colchicine taken up by 100mg of adsorbent resins XAD-4, XAD-7 and XAD-16 respectively. Also apparent is that the level of adsorption was related to the weight of beads. This suggests that there is an equilibrium between the sink and the medium for colchicine which will need to be investigated.

5.4.2 Investigation of the affinity and loading capacity of the Amberlite resins

The choice of a sink depends upon the binding capacity and affinity of each resin towards colchicine. To work out the binding capacity and affinity, a broad range of colchicine concentrations were used with a fixed amount of resins (100mg). After equilibrium the amount of colchicine left in the medium was measured and the amount of colchicine adsorbed on the resins calculated. A concentration range 1 - 10 mg/ml of colchicine was prepared for each adsorbent in 1ml MS medium, 0.1g of adsorbents (XAD-4, XAD-7 or XAD-16) added to the colchicine solution and this allowed to equilibrate overnight on an orbital shaker (120 rpm). The concentration of colchicine remaining in the medium was determined by spectrophotometry, and the amount taken up

by the resins then calculated from the difference between the initial and equilibrium concentrations.

The adsorption isotherm of colchicine onto the Amberlite resins was examined to evaluate the effectiveness of resins in removing the colchicine from the liquid medium. In order to work out the binding capacity and affinity of the polymeric resins for colchicine, a modified Langmuir isotherm was used for interpretation of the equilibrium data. The Langmuir isotherm, originally derived for the adsorption of gas molecules on solid surfaces, was modified to fit the adsorption isotherm of solutes onto a solid surface (Seungman and Dongsu, 2005). The Langmuir model represents a simplified case of ligand adsorption, since this model assumes: reversible adsorption, no change in the properties of the adsorbed molecules, all adsorption sites have the same affinity for ligand, one adsorbent site for each molecule. Under such assumptions, the binding of a ligand onto an immobilised complex can be represented (Ucan and Ayar, 2002).

In any given adsorbate-adsorbent system, a dynamic equilibrium between adsorption and desorption of adsorbate, X onto an adsorbent M, can be expressed as following (Seungman, and Dongsu 2005)



At equilibrium the rate of forward interaction becomes equal to the rate of the reverse interaction (Ucan and Ayar, 2002, and a plot of the equilibrium sorption data C_e/Q_e versus C_e theoretically should produce a straight line.

Binding capacity or the maximum capacity (Q_m) = 1/Slope (g substrate bound/g adsorbent)

Affinity or dissociation constant (K_d) = $Q_m \times$ Intercept (Concentration at which 50% of the medium substrate is adsorbed by resin)

The maximum adsorption capacity of colchicine on the three adsorbents was obtained according to the modified Langmuir equation (Tunceli, and Turker 2000) (Ucan and Ayar, 2002).

$$C_e/Q_e = K_d/Q_m + C_e / Q_m$$

C_e = Amount of colchicine left in the medium g/ml

Q_e = Amount of colchicine taken up by the resins g/g resin

Figures 5.3, and 5.4, shows the linear modified Langmuir isotherm plot for adsorption of colchicine from various concentrations of colchicine in MS medium solution using XAD-4, XAD-7, XAD-16 respectively.

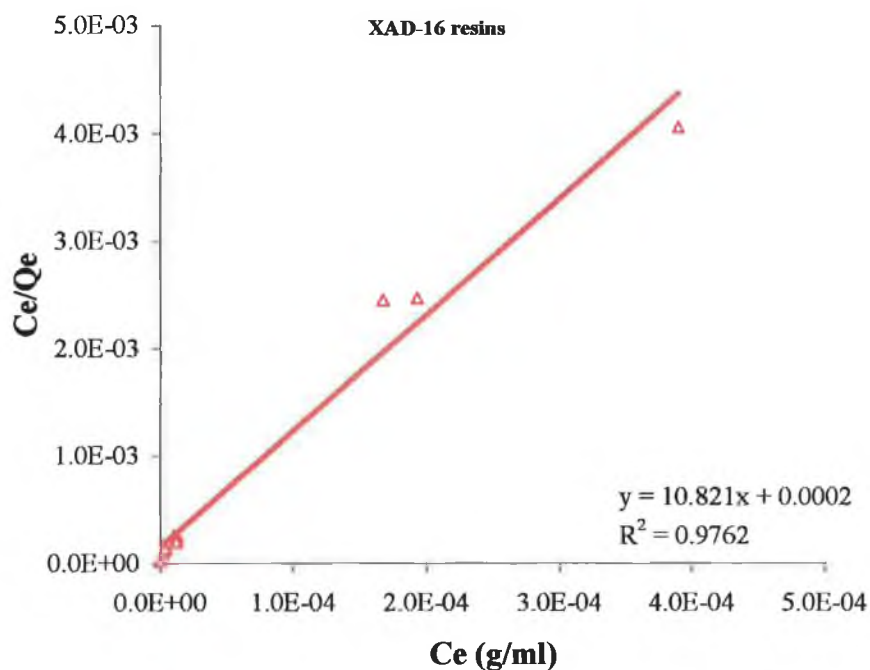


Figure 5.4: The linear modified Langmuir isotherm plot for adsorption of colchicine from MS culture medium by Amberlite resin XAD-16

The Amberlite resins binding capacity and affinity were calculated and the results are shown in table 5.3.

Table 5.3: Binding parameters for the adsorption of colchicine by Amberlite resins.

Adsorbent	Binding capacity (mg/g) Q_m	Affinity K_d
XAD-4	97.0	1.47E-05
XAD-7	78.0	3.85E-03
XAD-16	92.0	1.43E-05

The relationship between the isotherm and surface area of the resins shown in Figure 5.5.

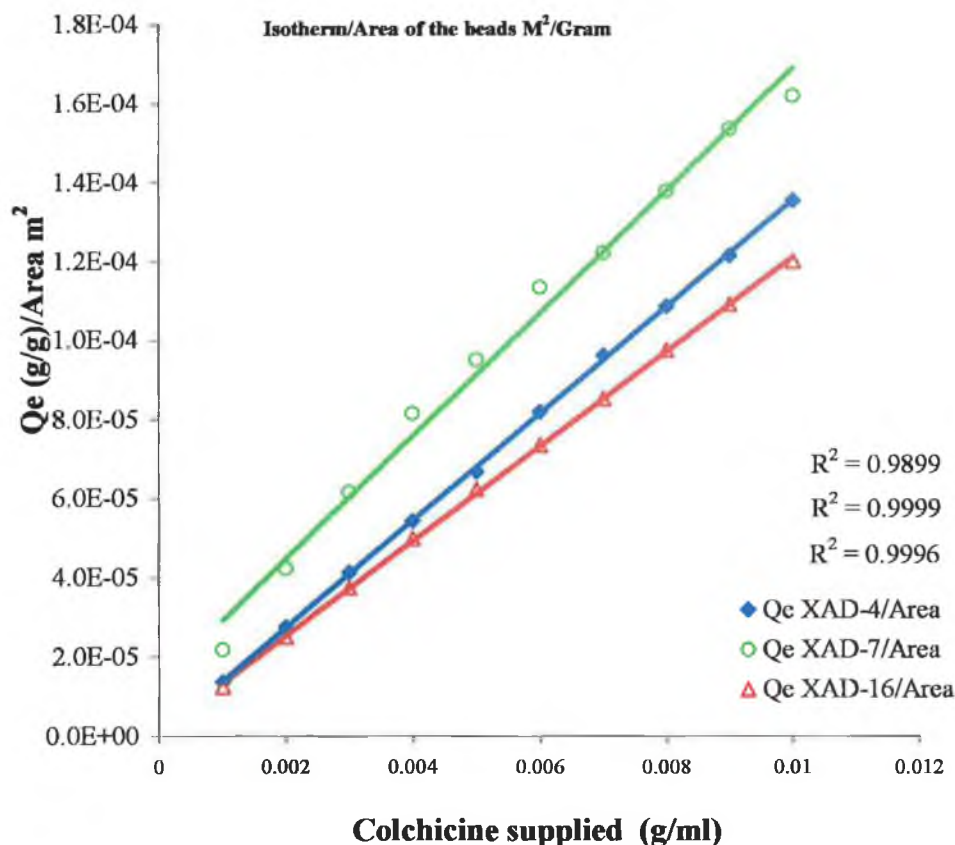


Figure 5.5: Correlation between the isotherm and surface area of the resins

The result shows that the adsorption tendency among the adsorbents used in this study is affected by their surface area. A strong relationship can be seen in (Figure 5.5) between the amount of colchicine taken up by the resins and the surface area of the resins. This may therefore account for some of observed differences between XAD-4 and XAD-16.

5.4.3 Investigation of the recovery of colchicine from resins using methanol

The adsorbents XAD4 and XAD16 have indicated their usefulness for uptake of colchicine from liquid medium. This will be greatly facilitated by an efficient method for recovering colchicine from the resin.

XAD4 and XAD16 resins (100mg) were added to microcentrifuge tubes and 1ml of colchicine solution (1-5mg/ml) in MS medium added and the tubes shaken overnight to equilibrate. The microcentrifuge tubes were then centrifuged, the supernatant assayed spectrophotometrically and the amount of colchicine removed by the resins calculated. Methanol (1ml) was then added to each microcentrifuge tube and allowed to shake (120rpm) overnight. The tubes were then centrifuged and the amount of colchicine in the methanol extract assayed spectrophotometrically. This methanol extraction step was repeated to ensure a high recovery of colchicine. The results in Figure 5.6 show the percentage of colchicine recovered from the XAD-4 resins and XAD-16 resins respectively.

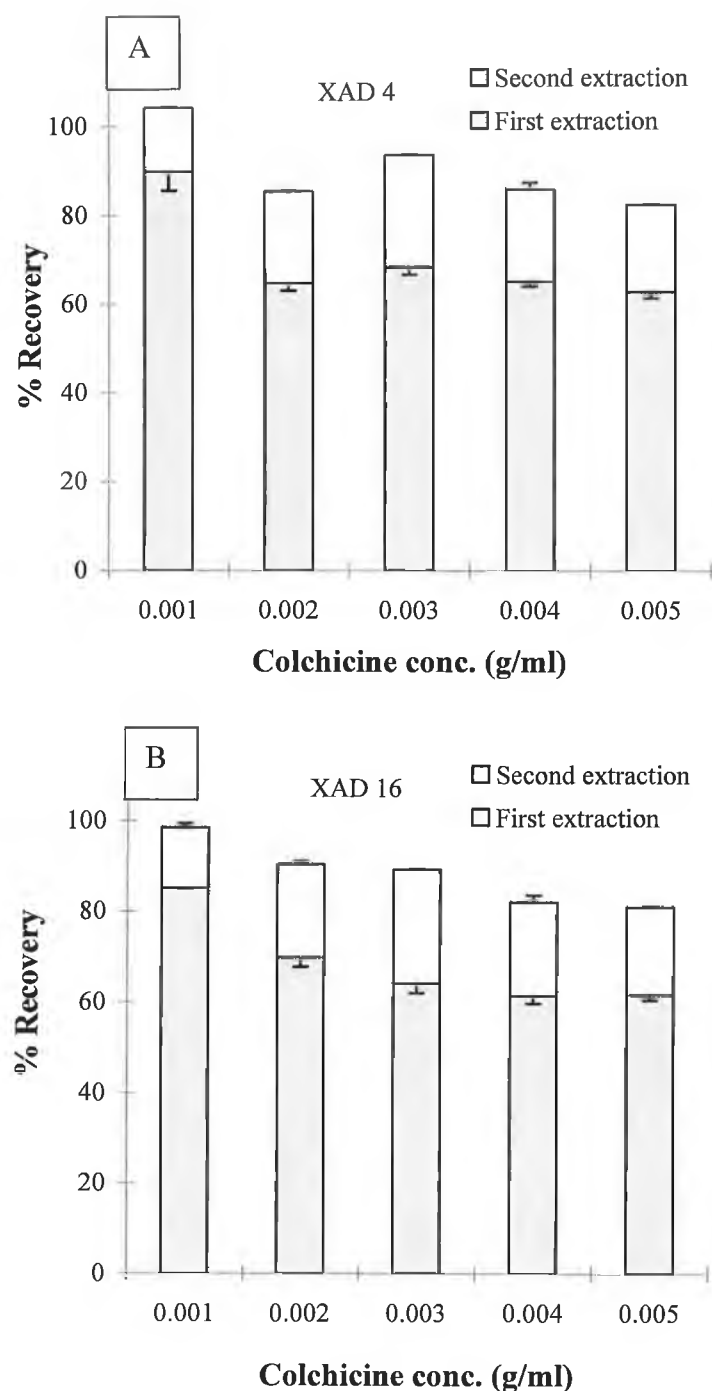


Figure 5.6: The percentage of total colchicine eluted from the A = XAD-4 and B =XAD-16 resins. The total amount of colchicine eluted is shown by the height of the bar graph; the grey colour bar corresponds to the amount of first time extraction while the white part indicates the amount of second time extraction. All values are the average of three replicates and are shown with the standard error of the mean.

5.4.4 Effects of pH on extraction efficiency

It is important to examine the performance of the resins at different plant cell and tissue culture physiological pH which ranges from 5-7.

To examine the effect of pH of the medium on the binding capability of the resins, resins were incubated at three different pH values. Three sets of colchicine solutions were made up in MS medium in a concentration ranging from (1 - 5mg/ml). The pH of each solution was adjusted to values of (5, 6 and 7) by addition of 1M KOH solution. The solutions were then added individually to microcentrifuge tubes containing 100mg resin. After overnight equilibrium, the amount of colchicine taken up by the resins (Q_e) was calculated. Figures 5.7 and 5.8 shows the relationship between (Q_e) and colchicine concentration at different pH with XAD-4, and XAD-16 resins respectively.

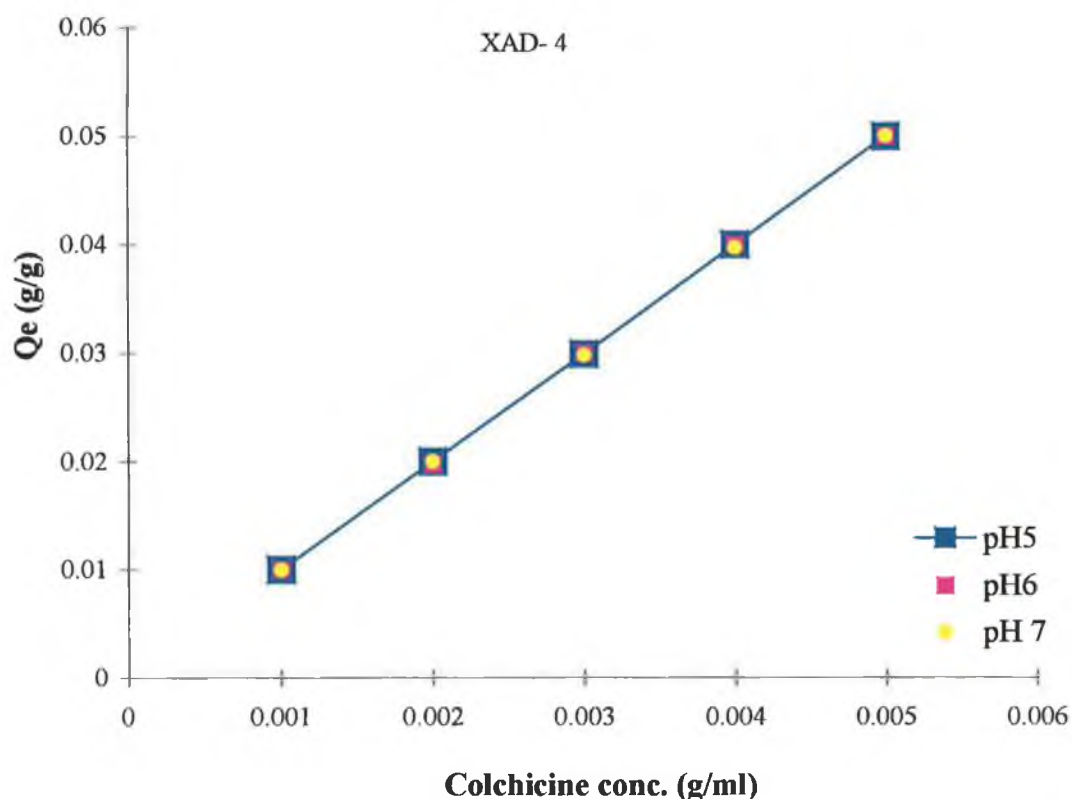


Figure 5.7: The correlation between Q_e and colchicine concentration in the MS medium with different pH values for the adsorbent resin XAD-4

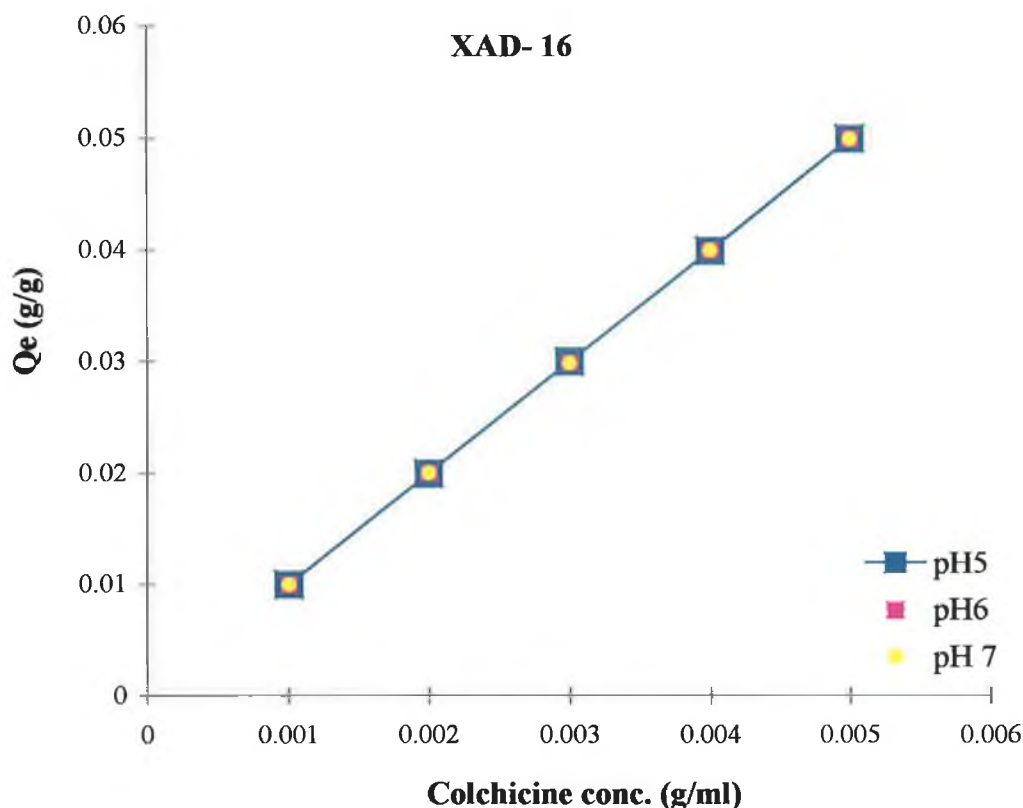


Figure 5.8: The correlation between Q_e and colchicine concentration in the MS medium with different pH values for the adsorbent resin XAD-16

There was no visible difference in colchicine uptake for either resin between pH 5, 6, 7.

5.4.5 Stability and Reusability of the resins

To use Amberlite resins economically and efficiently as a way of harvesting colchicine from the tissue culture media they must be cost-effective, and this implies that the resins should be easily and continuously regenerated.

The efficiency of the resin in repeated use was therefore studied. Five concentrations of colchicine solution were adsorbed and desorbed through ten cycle batches as described in the methanol extraction experiment (section 5.4.3), and the adsorption isotherms of the resins towards colchicine determined.

Figure 5.9 shows the colchicine equilibrium adsorption isotherm relation (Q_e) against (C_e) on the first time use of the resins and after ten adsorption/desorption cycle batches, for XAD-4 resins and XAD-16 resins respectively.

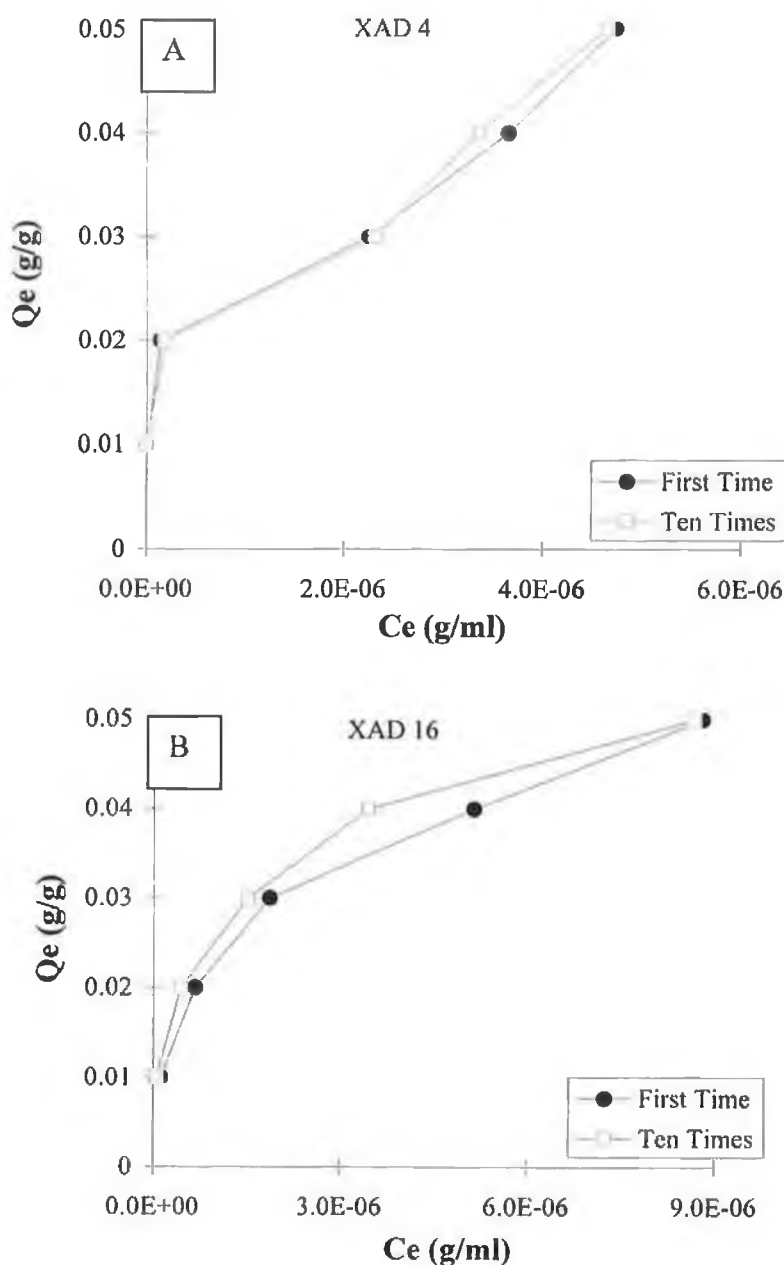


Figure 5.9: The equilibrium adsorption isotherm relation on the first time and after ten times reused resins A= XAD-4 and B = XAD-16

The above two graphs demonstrate that both XAD-4 and XAD-16 resins did not show any degeneration in binding characteristics with repeated cycles of adsorption/desorption.

5.4.6 Harvesting colchicine by using Amberlite resins enclosed in a mesh bag

The previous experiments showed that the resins have the ability to remove colchicine from the medium when added directly free into the medium with continuous shaking. However, this poses serious problems in physical separation of the beads from cell aggregates prior to the extraction process. It was therefore proposed to physically separate the two phases by placing the resins within a mesh bag. Since this may affect the rate of binding, it was necessary to investigate the ability of the resins to remove the colchicine. Mesh bags were designed for enclosing the resin which allows the medium to pass through the mesh.

300mg resin were placed between two layers of nylon mesh and sealed (as described in section 5.3.3)

Three bags were prepared for each respective resin and placed in a 250ml flask containing 50ml of colchicine solution (50 μ g/ml) in MS medium. After overnight equilibrium, the amount of colchicine taken up by the resin (Q_e) was established. The mesh bags were then transferred to 50ml flasks containing 20ml methanol (100%), the flasks sealed with cotton wool bungs, capped with a double layer of aluminium foil, and placed on a shaker at 25 °C to equilibrate overnight. The amount of colchicine released to the methanol was then determined. The results are shown in (Figure 5.10) for XAD-4 and XAD-16 respectively.

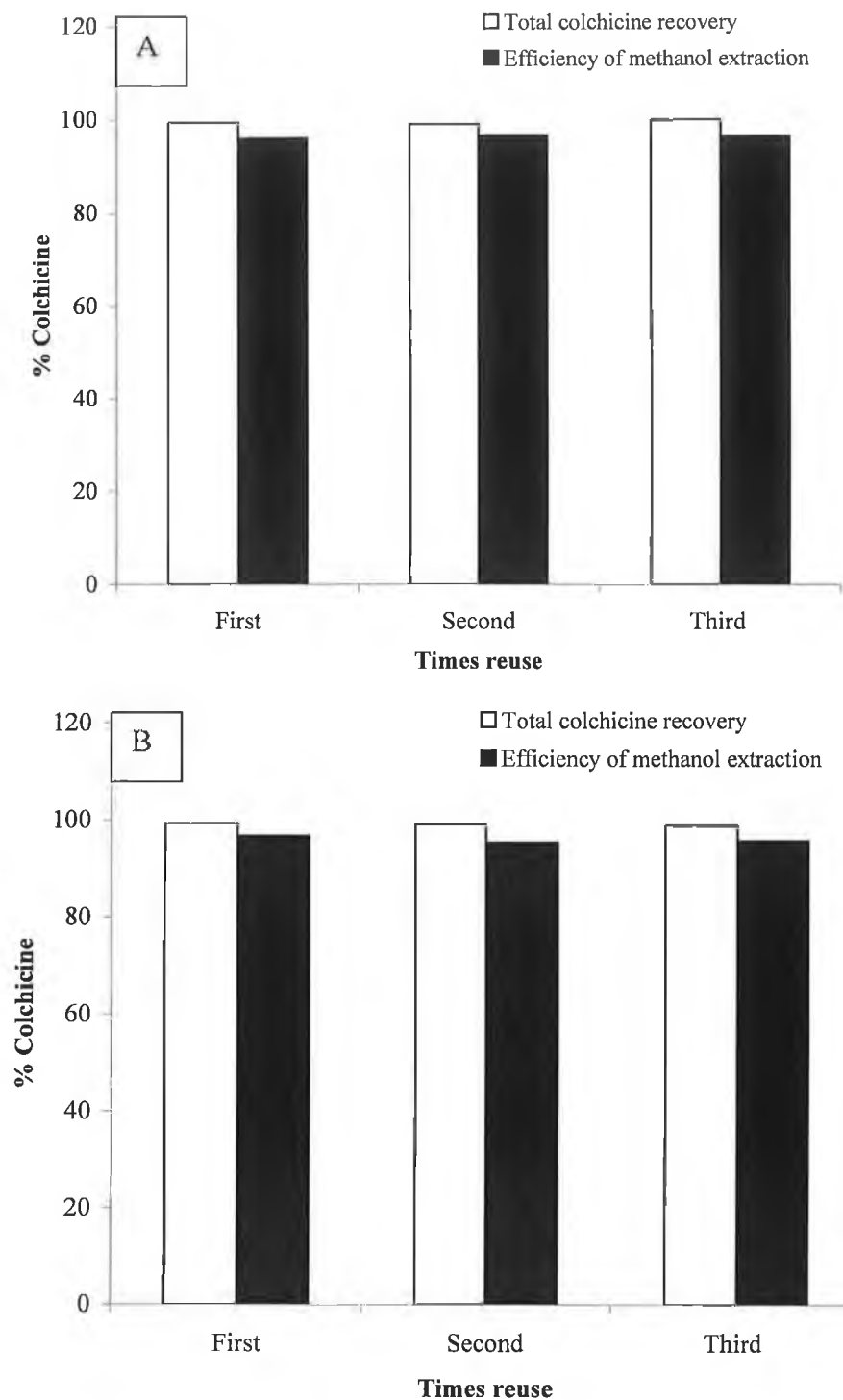


Figure 5.10: The percentage of colchicine uptake and recovered by using methanol extraction of Amberlite resins enclosed in a mesh bags reused for three times. A= XAD-4 and B= XAD-16. The results are average of three replicates.

5.4.7 Determination of the half-life of colchicine in the medium with resin adsorption

In order to compare the performance of the resins enclosed in a mesh bag to that of free resins in the liquid medium, a time course experiment was designed to examine the adsorption kinetics of the resins and determine the colchicine half-life in the medium contain either free resins or resin enclosed in a mesh bag. (A time course study was carried out to find out the rate of uptake of the colchicine by the resins). 300mg resins were enclosed in nylon mesh; three bags were prepared for each respective resin and placed in 250ml flasks containing 50ml of colchicine solution (50 μ g/ml). This was duplicated with free resins. The flasks were covered with aluminium foil and shaken at 100rpm at 25 °C. Samples (0.5ml) were then taken from each flask after an incubation period at 0.5h, 1h, and subsequently every hour to 12h and a final set of samples collected after 24h. The samples were assayed and the percentage of colchicine taken up by the resins was established. Figure 5.11 shows the uptake curves for colchicine for XAD-4 and XAD-16 resins respectively.

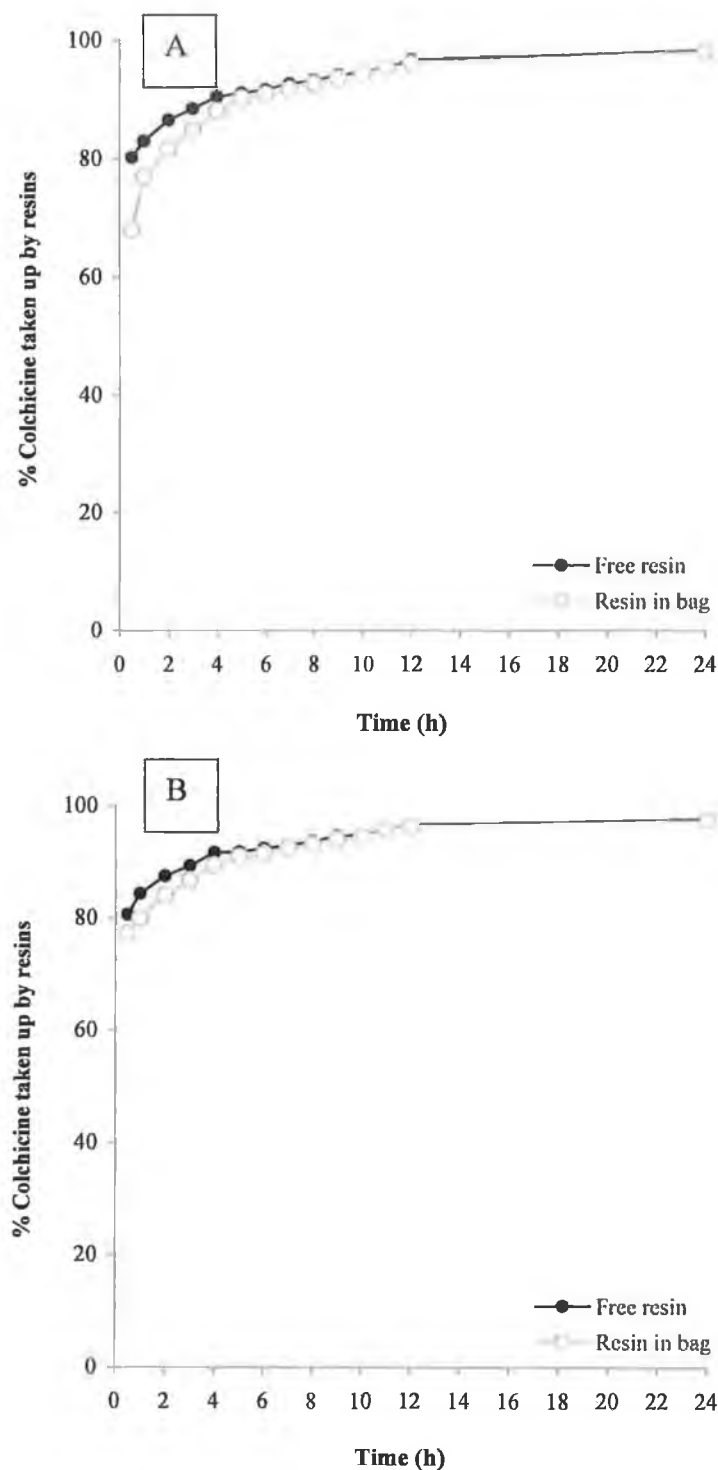


Figure 5.11: The uptake curves for colchicine taken up by Amberlite resin against incubation time A= XAD-4 and B = XAD-16. Results are average of three replicates.

Results seem consistent with a constant proportion of the available colchicine being taken up per unit time. A semi-log plot of colchicine in the medium over

time should theoretically yield a linear relationship, allowing to test the above hypothesis and calculate a half life for colchicine. Figures 5.12 and 5.13 shows the Log linearisation of the amount of colchicine remaining in the medium at a certain incubation time, XAD-4, and XAD-16 resins respectively.

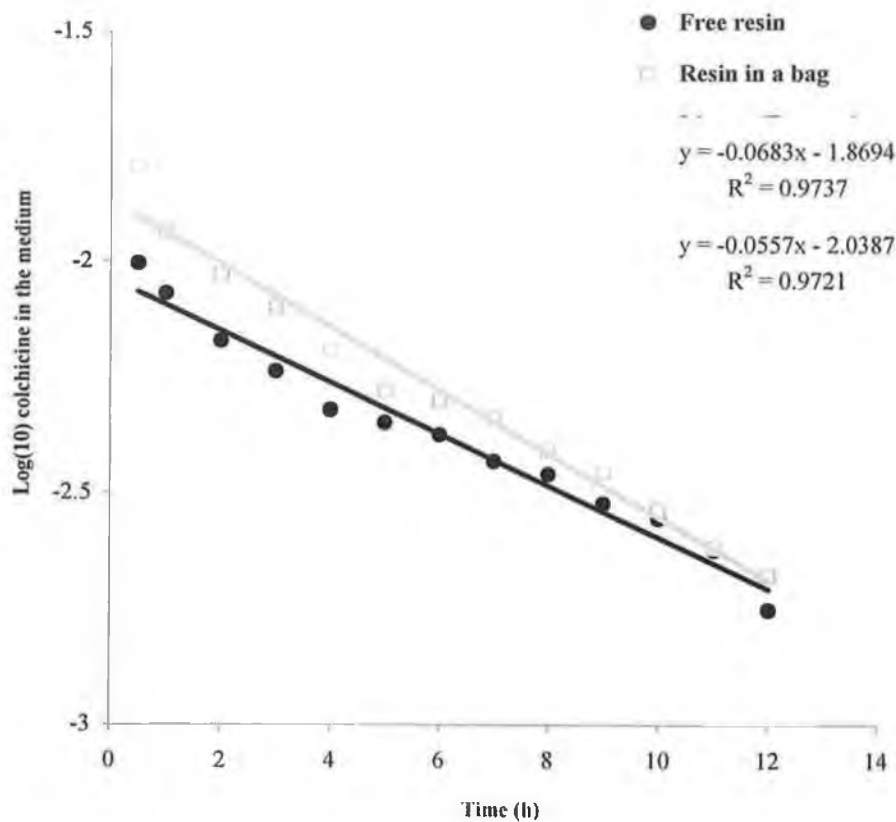


Figure 5.12: Linear relationships between Log_{10} colchicine remaining in the medium against time with (XAD-4) resin in the medium.

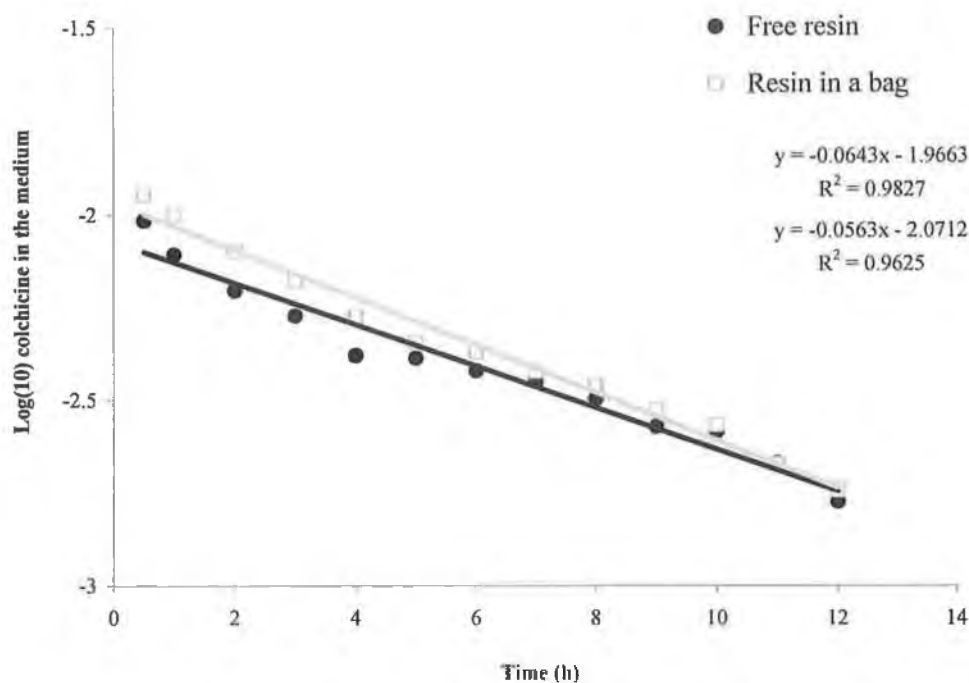


Figure 5.13: Linear relationships between Log_{10} colchicine remaining in the medium against time with (XAD16) resin in the medium.

The half life of colchicine take up from the medium is the time necessary for the resins to reduce the concentration of the solution to half and can be calculated using the following equation.

$$\text{Half-life (t}_{1/2}\text{)} = \log_{10}(0.5) / \text{Slope}$$

Table 5.4: Colchicine half-life with different resin treatments.

Treatment	Half-life (h)	
	Free	In a bag
XAD-4	5.3	4.7
XAD-16	5.4	4.4

Results show insignificant differences in uptake kinetics between free resins and resins contained in a mesh bag.

5.4.8 Application of the Amberlite resins in a column packed with the resins

To estimate the effectiveness of using a column packed with the resins for removing colchicine from a solution, colchicine solution was run through a packed column at a steady flow rate and the amount of colchicine removed over time was recorded.

Two Econo-Columns (ID =1cm and length =20cm) (Figure 5.14) were packed with 2g of resin, one containing XAD-4 and the second containing XAD-16. 50ml of 50 μ g/ml colchicine solution was prepared in MS medium. Initial column loading disturbed the beads at the top of the column and the column was therefore allowed to re-settle for five minutes before running the solution through the column. The solution was then allowed to pass through at a flow rate of 1ml/min for each column. 1ml Samples were taken every 3 min directly from the valve at the bottom of the column, and a 1ml sample was taken from the collecting flask at the end of the experiment to estimate the total quantity of colchicine taken up by resins. All the samples were assayed as before by using spectrophotometry. The results are shown in Figure 5.15.



Figure 5.14: The Econo-Column (ID =1cm and length =20cm)

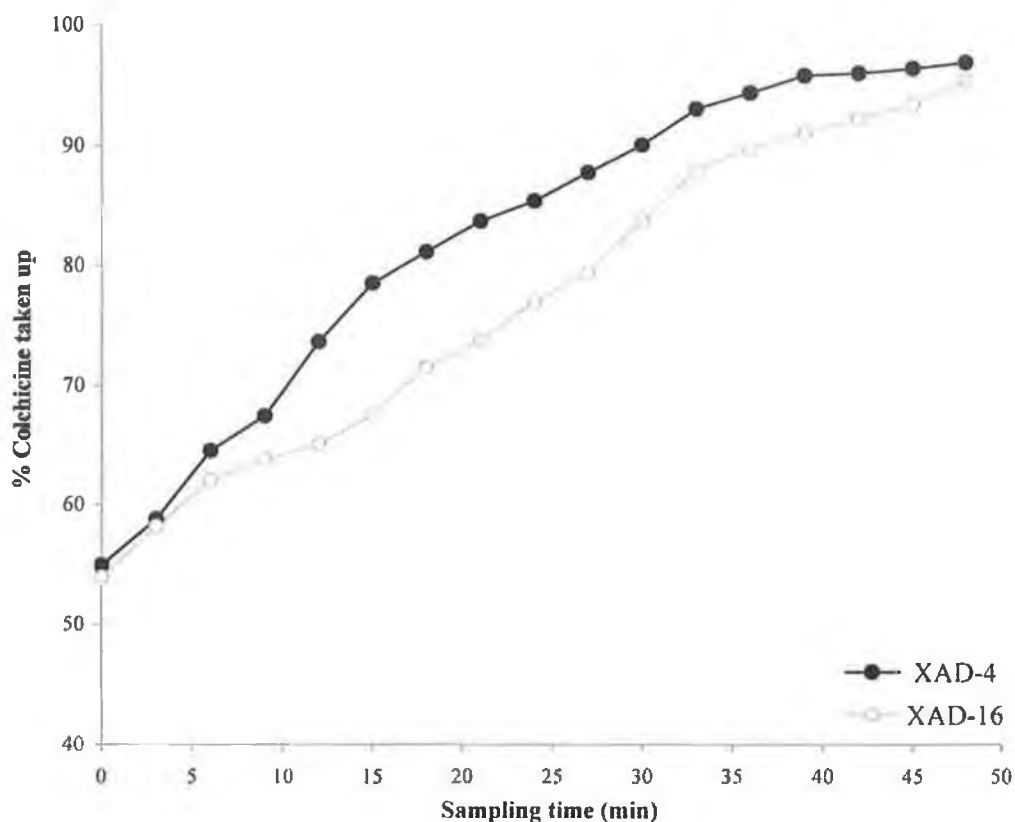


Figure 5.15: The percentage of colchicine taken up from colchicine solution (50 μ g/ml) passed through a column packed with adsorbent resins.

The total colchicine taken up by the resins from the solution was estimated by taking samples from the collection flasks at the end of the experiment, and it was found that 75.9% and 78.7% of the colchicine was removed by XAD-4 and XAD-16 resins respectively.

5.4.8 Investigation of the effect of Amberlite resin on precursors added to the medium

It is important to examine the effect of Amberlite resins on precursor feeding since the *in situ* colchicine removal by Amberlite resin may remove the precursors from the culture medium. This may lead to changes in culture metabolism and consequently may reduce the colchicine production profile. Three precursors were investigated namely cinnamic acid, coumaric acid and 3-phenyl propionic acid.

XAD-4 and XAD-16 resins (100mg) were added to microcentrifuge tubes and 1ml of precursor solution (0.1-1.5mg/ml) in MS medium added individually and allowed to shake (120rpm) overnight. The microcentrifuge tubes were then centrifuged, the supernatant assayed by spectrophotometry at 220nm for cinnamic acid, and coumaric acid and 280nm for 3-phenyl propionic acid, and the amount of the precursors taken up by the resins (Q_e) calculated. The results presented in Figure 5.16 shows the relationship between (Q_e) and the concentration of precursors supplied with XAD-4, and XAD-16 resins respectively.

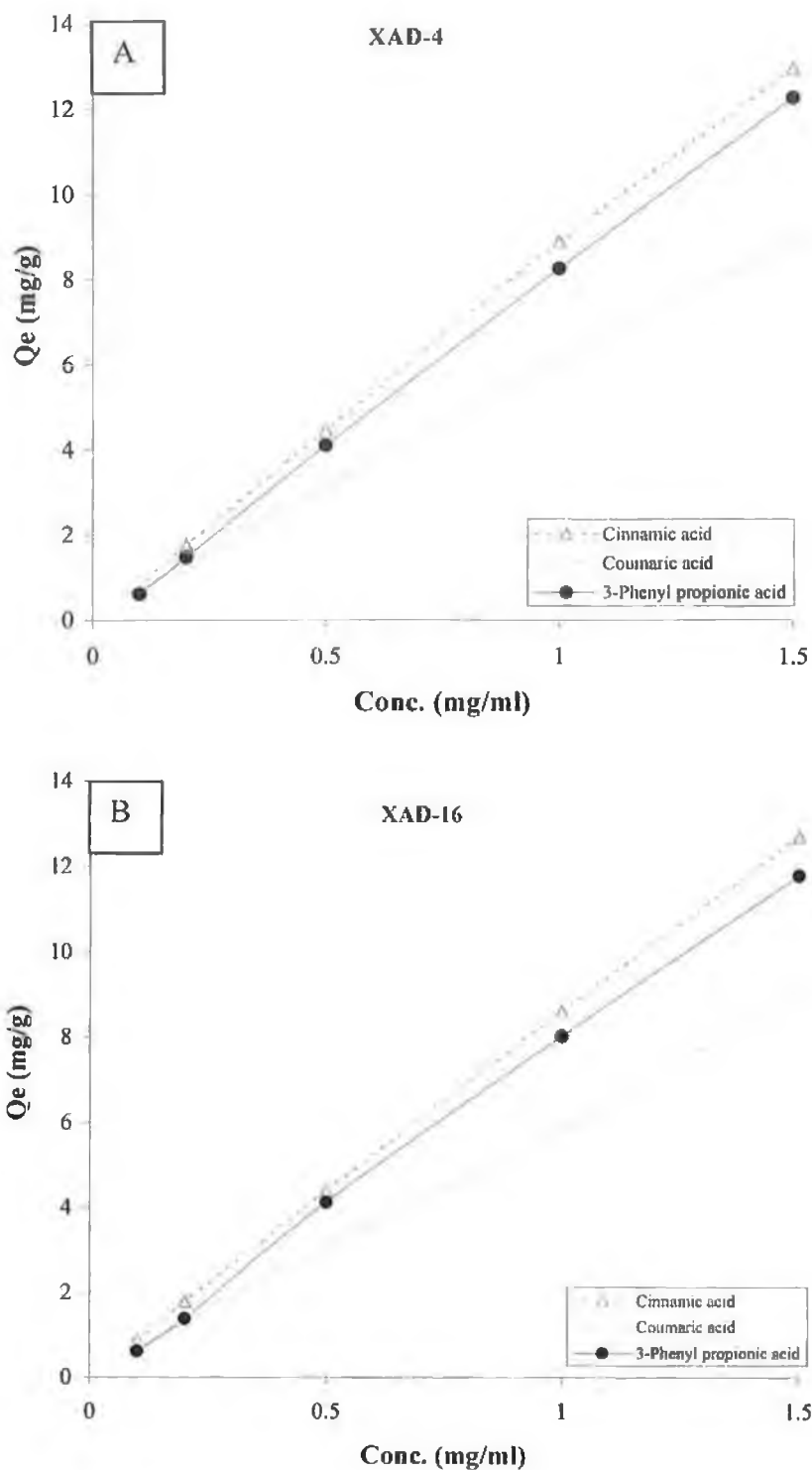


Figure 5.16: The relationship between Q_e and the concentration of precursors supplied with resins A= XAD-4 and B = XAD-16

Results show that both resins adsorb the three precursors. Coumaric acid demonstrated a lesser binding capability Q_e in comparison with the cinnamic acid, and 3-phenylpropionic acid.

5.4.8 Application of solid phase extraction (SPE) system for harvesting colchicine from cell suspensions

The SPE system developed in this chapter was then applied for *in vivo* harvesting of colchicine from *Colchicum autumnale* and *Gloriosa superba* cell suspension culture. Resins enclosed in nylon mesh bags were incubated with the cell suspension cultures. Callus tissue (approximately 200mg fresh weight) was placed in a 50ml Erlenmeyer flask containing 20ml medium, the flasks sealed with cotton wool bungs and capped with a double layer of aluminium foil, and incubated on a shaker at 100rpm at 25 °C. Liquid medium was collected after four weeks and assayed for colchicine. Bags containing resins were removed aseptically from the culturing flask and placed individually in a 50ml flask containing 20ml methanol. The flasks were then sealed with cotton wool bungs and capped with a double layer of aluminium foil and placed on a shaker at 25 °C for overnight extraction. Methanol extraction was repeated. The two methanol extracts were combined together and dried by lyophilisation and the residue was resuspended and assayed. The results are shown in Figure 5.17.

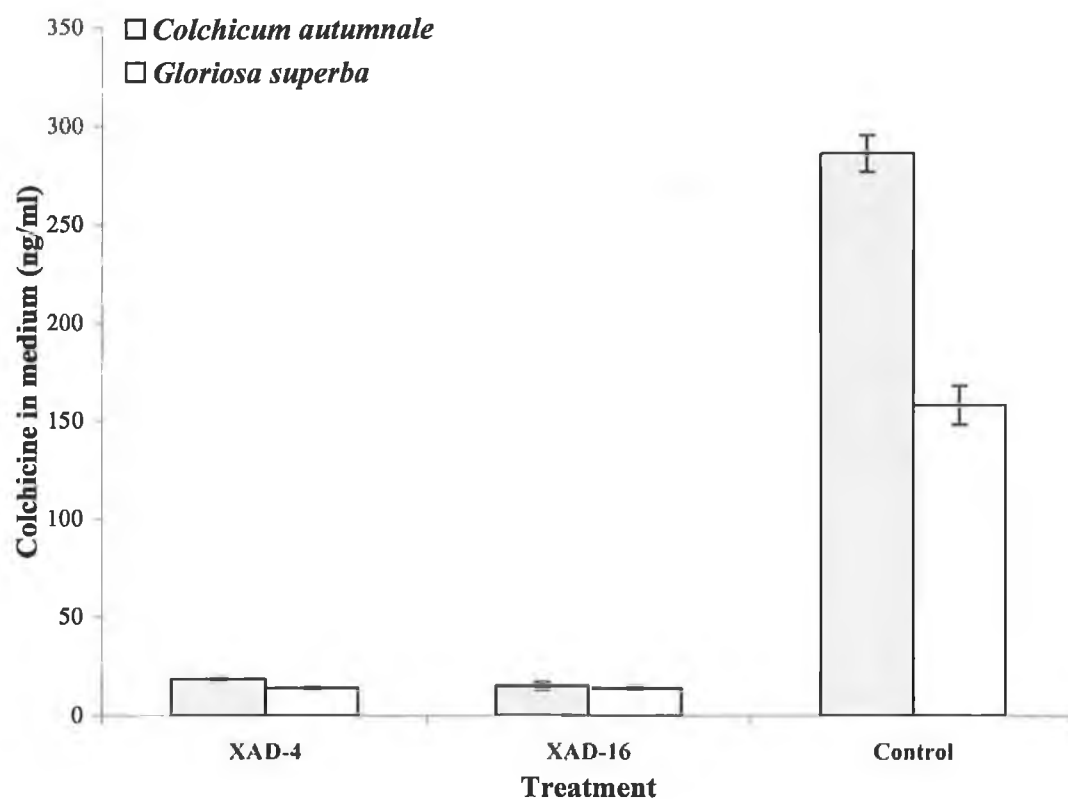


Figure 5.17: Colchicine concentration in the medium of cell suspension culture after four weeks incubation with and without resin treatments. Results are the average of three replicates and the standard error bars are included.

The total colchicine accumulated by resins and in the medium of cell suspension culture after four weeks incubation with and without resins treatments, from *Colchicum autumnale* and *Gloriosa superba* cell suspension culture are shown in Figure 5.18. Total extracellular colchicine was increased by resin adsorption and preferentially retained in the resins.

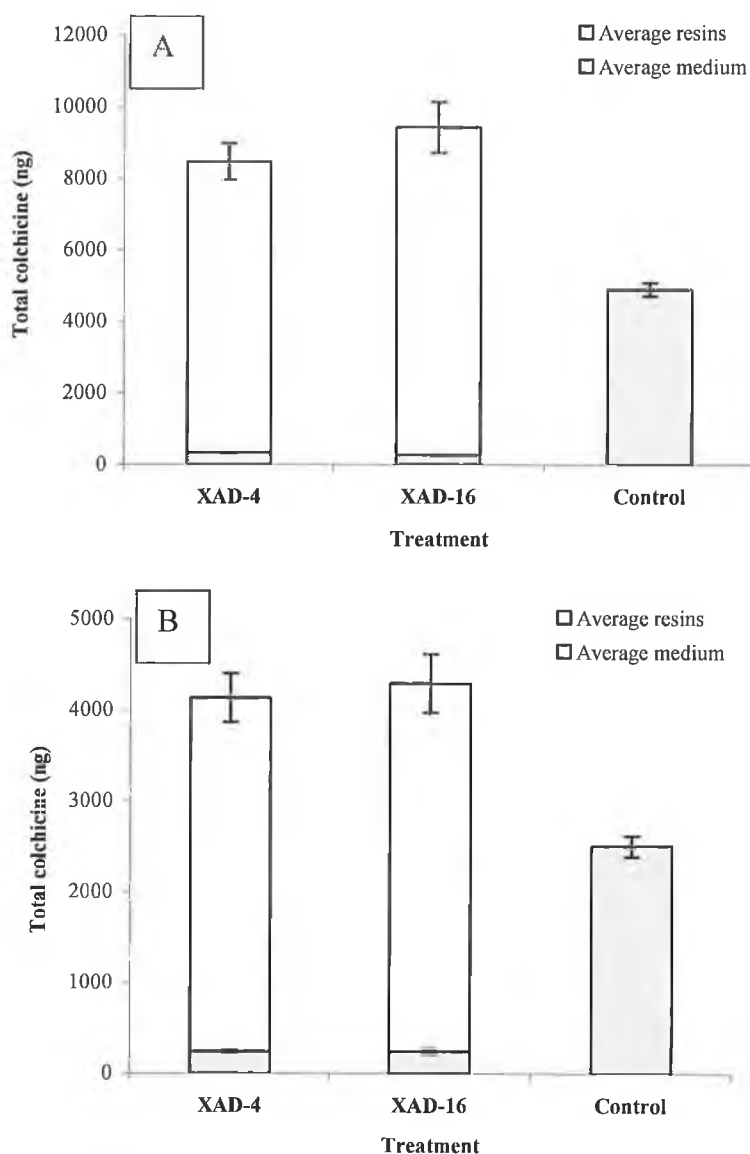


Figure 5.18: Total colchicine accumulated by resins and in the medium of cell suspension culture after four weeks incubation with and without resins treatments A = *Colchicum autumnale* and B = *Gloriosa superba*. Results are average of three replicates and the mean standard error bars are included.

5.5 Discussion:

Three commercially available adsorbents, Amberlite resins XAD-4, XAD-7 and XAD-16 were investigated in this chapter for the harvesting of colchicine from liquid medium. The effect of the resins on colchicine uptake was determined by measuring the amount of colchicine remaining in the standard solution after equilibration with the resins was performed by spectrophotometry. The initial experimental results indicated that the three Amberlite resins were highly efficient in removing colchicine from the liquid medium with up to >99% of colchicine in the medium taken up by the resins after overnight equilibration (Table 5.2). Although such removal efficiency varied slightly with the type of resin used, in general, XAD-4 and XAD-16 showed more efficiency than XAD-7.

The adsorption kinetics were investigated (Table 5.3) and the adsorption equilibria interpreted using a modified Langmuir isotherm plot $(Q_e/C_e)/C_e$. The modified Langmuir model provides the most satisfactory representation of the data. All the adsorbent resins show a relatively good binding capacity of 97, 78, and 92 (mg) colchicine per (g) of resins with XAD-4, XAD-7, and XAD-16 respectively. Thus XAD-4 and XAD-16 showed a higher binding capacity for colchicine than XAD-7.

It was found that the surface area of the three Amberlite resins varies considerably (XAD-4 =725 m²/g, XAD-7 =450 m²/g, and XAD-16 =800 m²/g). A strong relationship between the amount of colchicine taken up by the resins and the surface area of the resins was shown (Figure 5.5).

The difference in adsorption capacity and affinity may be connected with the difference in the surface area of the resins. That of XAD-16 is slightly greater than XAD-4 and is nearly twice as large as that of XAD-7. However, the difference in affinity and adsorption capacity may be explained more adequately by the physico-chemical properties of the adsorbent resins. Since XAD-4 and XAD-16 are based on a cross-linked polystyrene structure and have a hydrophobic nature, they have a high affinity for colchicine which is also hydrophobic (Dean, *et al.* 2001). XAD-7 may adsorb less colchicine

because it is based on a cross-linked polyacrylate structure that is somewhat hydrophilic. It was therefore decided to concentrate on XAD-4 and XAD-16 resins which showed a higher efficiency and binding capacity for colchicine.

Methanol was found to be effective in desorbing and recovering colchicine from the adsorption resins. Approximately 70% of colchicine was removed on the first extraction and approximately 20% on the second extraction. Harvesting total colchicine from the adsorbent resins by using 100% methanol gave a high efficiency of recovery with $90\% \pm 8.9$ and $88\% \pm 7.1$ (Mean \pm SD, $n=3$) for XAD-4 and XAD-16 respectively.

The XAD-4 and XAD-16 resins show no change in uptake characteristics with different medium pH ranged from pH5 to pH7.

It has been reported that XAD resins may be regenerated and recycled many times without affecting the efficiency of adsorption (Jain, *et al.*, 2001; Abburi, 2003). It was found that the adsorption capacity of the XAD-4 and XAD-16 resins was unchanged after ten cycle batches of adsorption-desorption indicating the possible reuse of the resins several times with no loss in binding capacity, affinity, or extraction efficiency.

Physical separation of the resins from the medium and cells by enclosure in a mesh bag showed that both resins XAD-4 and XAD-16 removed on average 99% of colchicine from the medium. Methanol extraction was able to recover 96% of this colchicine (Figure 5.10). The resins enclosed in mesh bags maintained their ability to adsorb colchicine. The mesh bags were re-used for three cycles without losing the ability for removing colchicine from the medium. Thus the resins in the mesh bags maintained their capacity to adsorb colchicine from the liquid medium, and the resins can also be regenerated when inside the mesh bags. Mesh bags may therefore be used in tissue culture with minimum contact between cells and the resins.

The performance and kinetics of the resins enclosed in a mesh bag was comparable to that of free (loose) resins in the liquid medium over short time periods (Figure 5.11) colchicine removal rate was slightly altered by enclosing the resins in mesh bags, with 68.9% and 77.8% taken up from the colchicine solution after 0.5h incubation by XAD-4 resins and XAD-16 resins respectively while the free resins take up 80.2% and 80.6% of colchicine solution. This indicates that over short time periods the free resins shaken in the liquid medium were significantly faster in removing colchicine compared to resins enclosed in mesh bags; however, this difference had narrowed dramatically with increased incubation (24h) with 97.6% and 97.4% taken up by resins enclosed in a mesh bags for XAD-4 resins and XAD-16 resins respectively, and 97.8% and 97.6% taken up by free resins.

Over a span of times of incubation there was no significant difference in the half-life of colchicine in the solution between free resins and resins enclosed in a mesh bags (Table 5.4).

Extraction of colchicine by passing medium through a column packed with adsorbant showed that the removal of colchicine from the media by both resins was relatively very effective, with 55% and 54% taken up at the beginning of the flow and (96.6% and 95%) taken up at the end of the flow with XAD-4 and XAD-16 respectively.

Passing the solution through the column packed with resins gives a simple and efficient removal of the colchicine from the solution and may possibly be applied in a large scale bioreactor.

It is clear that the resins adsorb the three precursors: cinnamic acid, coumaric acid, and 3-phenylpropionic acid. Figure 5.16 shows that the amount taken up (Q_e) by the resins are increased with the increase in concentration of the three precursors. Coumaric acid showed a lower binding capability (Q_e) in comparison with the cinnamic acid, and 3-phenylpropionic acid. That would have a negative effect on the biosynthesis of colchicine and may lead to less

colchicine produced in the culture. It is important to continue to supply the medium by precursors and to avoid their depletion caused by resins.

The developed SPE system was applied *in vivo* for harvesting colchicine from *Colchicum autumnale* and *Gloriosa superba* cell suspension culture. Resins were enclosed in a nylon mesh bags and incubated with the cell suspension cultures for four weeks. The amounts of colchicine in the liquid medium and bound to the resins were assayed. The concentration of colchicine in the medium was considerably reduced to less than 10% in the culture medium containing Amberlite resins enclosed in a mesh bags compared to the culture without Amberlite resins bags (Figure 5.17). The average total colchicine released from *Colchicum autumnale* cell suspension culture into the liquid medium (Medium + Resins) was significantly increased by 73.4% and 92.8% with XAD-4 and XAD-16 respectively, over unextracted culture medium. The average total colchicine released from *Gloriosa superba* cell suspension culture into the liquid medium (Medium+Resins) was significantly increased by 65% and 71.3% with (XAD-4) and (XAD-16) respectively, over unextracted culture medium (Figure 5.18). The effect of *in vivo* application of the SPE system not only effectively removed the colchicine from the medium, but in addition stimulated the total amount of colchicine released to the liquid medium. Since the colchicine concentration in the cells was not measured, it was not clear whether the production enhancement resulted from the enhanced secretion from the cells or from the stimulation of the biosynthesis. However, it was clear that the addition of the adsorbent increased the amount of colchicine released into the medium. One may speculate that a feedback regulatory mechanism is limiting colchicine biosynthesis.

5.6 Conclusion

This chapter represents the results from different approaches using Amberlite resins for harvesting colchicine from liquid medium. An Integrated colchicine recovery method has been developed. A simple and reliable method has been optimised to separate and concentrate colchicine from MS medium. The high binding capacity ensures that the colchicine concentration in the medium is kept very low while the majority of the product is concentrated onto the adsorbent, from which it was readily recovered by using methanol.

The resins show excellent reusability for colchicine adsorption. The adsorption capacity of the resins remains unchanged after regeneration and the XAD-4 and XAD-16 resins could be used for the continuous *in situ* extraction and recovery of colchicine from suspension cultures of *Colchicum autumnale* and *Gloriosa superba*.

Higher colchicine accumulation and recovery from cell and tissue culture were associated with the higher product removal rates of nylon mesh-enclosed resins.

Optimizing the product removal rate (i.e. *in situ* or to an external resin column) could favourably alter the balance of biosynthesis to metabolism in the cell and yield a higher level of production and recovery of secondary metabolites from plant cell cultures.

Results suggest specific productivity was enhanced by continuous removal of the secreted product.

Chapter Six

Strategy for enhancement of colchicine production from root tissue culture

6.1 Introduction

Organized tissue cultures shoot, root, and other plant organ cultures have been developed for the production of secondary metabolites and the product profiles of these organized tissue cultures has been reported to be similar to that of the field-grown plants (Endo, and Yamada, 1985). There are some reports of differentiated tissue cultures (shoots and roots) being used to produce secondary metabolites (Subroto *et al.*, 1996; Mahagamasekera and Doran, 1998). Many valuable secondary metabolites are biosynthesized in roots *in vivo*, and often biosynthesis is linked to root differentiation (Robins *et al.*, 1991). Sugimoto *et al.* (1988) found that alkaloid levels of cultured roots of *Stephania cepharantha* were much higher than those of plants. The genetic stability in these tissue cultures is much higher than in cell suspension or callus cultures with stable growth and consistent secondary metabolite production observed in shoot and root cultures of many species (Charlwood and Moustou, 1988; Miura *et al.*, 1988). This is an important finding since maintaining production consistency in cell culture through process scale-up can be challenging.

Gloriosa superba root tissues release a proportion of their intracellular colchicine into the growth medium. This is an important finding since a low accumulation of secondary compounds in cell and tissue cultures in a number of cases may not be due to a lack of key biosynthetic enzymes but rather due to feedback inhibition. In such cases, it should be possible to increase the net production by the addition of an artificial site for product accumulation. The use of *in situ* product removal of metabolites has a number of key potential advantages beyond promoting secretion. The removal and sequestering of the product in a nonbiological compartment may increase its total production (Beiderbeck and Knoop, 1988).

The application of a solid phase extraction system may therefore be feasible for the continuous harvesting of colchicine coupled with extension of the fermentation period for viable growing tissue in the bioreactor.

Bioreactors systems are also essential research tools for obtaining kinetic data for growth and productivity under well controlled, reproducible conditions. These data are essential for process optimization and for determining the economic potential of plant cell and tissue culture production systems.

A wide variety of bioreactor configurations have been proposed for organ plant culture growth. Bioreactors can be roughly divided into two main types:

1-Liquid-phase bioreactors: in the liquid-phase bioreactors, tissues are submerged in medium, and oxygen is usually supplied by bubbling air through the culture medium (Taya, *et al.*, 1989B).

2- Gas-phase bioreactors: in the gas-phase bioreactors tissues are exposed to air and the liquid medium is usually delivered to tissues as trickles. Gas-phase bioreactors can virtually eliminate any oxygen deficiency (Weathers *et al.*, 1999) while also providing a low shear stress environment.

In this study the two bioreactor systems were examined for growing *Gloriosa superba* root tissues on a pilot scale. Colchicine accumulation in *Gloriosa superba* root tissues was examined in shake flasks and compared with cultures grown in two types of bioreactor, air-lift bioreactor and a trickle column bioreactor.

6.1.1 Synergism of enhancement strategies

The combined effects of different enhancement strategies can stimulate secondary metabolite production many fold greater than any individual approach and this can be principally valuable in large-scale systems (Roberts and Shuler, 1997). Since no single strategy individually results in a large increase in colchicine accumulation, strategies of integrated process design as indicated in Chapter four and five have to be considered.

6.2 Aim

The main intent of this investigation is to demonstrate the combined effects of precursor feeding on *Gloriosa superba* root tissue culture with an extracellular accumulation site for continuous removal of colchicine, without harvesting the tissue. Precursor feeding with continuous harvesting of released colchicine into the medium may thus provide an efficient means for colchicine production in a large scale bioreactor. It is also necessary to develop methods for estimating biomass concentration.

6.3 Materials and Methods

6.3.1 Equipment

LH Fermentation system, 2l bioreactor, Peristaltic pump (ATTO AC-2110), Brookfield viscometer (DV II), 500ml Pyrex Buchner flask, glass microfiber filter 0.3 μ m (Whatman), Maxima air pump (Hagen), conductivity meter (Mettler Toledo MC-226).

6.3.2 Determination of the medium conductivity and soluble carbohydrate

Liquid medium samples (500 μ l) were collected and each one was diluted 20 times in ultra pure water. Medium conductivity was measured using a conductivity meter. The probe was simply immersed in the culture and the reading was read from the digital display. 200 μ l aliquots of the diluted samples were transferred into microcentrifuge tubes, and were adjusted to (80% v/v) ethanol by the addition of 800 μ l absolute ethanol. This solution was mixed thoroughly by repeated inversion and left at room temperature for 30min for precipitation of carbohydrate polymers. The mixture was then centrifuged at 13,000rpm for 10min, 500 μ l of the supernatant transferred to a glass test tube and 500 μ l of phenol (5% w/v) added. Concentrated sulfuric acid (2.5ml) was then added to the mixture and shaken gently to ensure homogeneity, and this was incubated at room temperature for 30 min.

The absorbance was then read at 490nm in a spectrophotometer. A calibration curve was constructed to determine the amount of soluble carbohydrate in the medium.

6.4 Results

The relatively fast-growing root cultures of *Gloriosa superba* seem useful for cultivation in bioreactors. M2 root tissue cultures have shown that they accumulate relatively more colchicine than M1 root culture, so it was decided to exploit M2 root tissue cultures and optimize colchicine accumulation using three different culturing systems, shaken flask, trickle column bioreactor and air-lift bioreactor.

A preliminary trial using a stirred bioreactor showed that the impeller damaged the root tissues, resulting in callus formation at the lowest speed (50rpm) and at a higher speed (100rpm) the tissue turned brown and was dead after one week. It was therefore decided to employ the air-lift mixing technique. The air-lift bioreactor has a draught tube to promote medium circulation. This bioreactor employs the flow of gas through the medium to mix the medium as well as to supply the required oxygen.

A draught tube was fixed in the center of the bioreactor and the stream of incoming air was used to mix the content in the bioreactor. The medium circulation induced by the difference in the hydrostatic pressure between aerated liquid rising inside the draught tube and degassed liquid descending outside the draught tube, mixes and aerates the culture.

6.4.1 Colchicine accumulation in different pilot scale culture methods

The cultures were incubated for four weeks and at the end of incubation the medium was collected, the volume of the remaining medium was determined and a 1ml sample taken for the colchicine assay. The final fresh weight of the root tissue was determined, and samples from each batch were collected and weighed then extracted for colchicine assay. The Amberlite resins were transferred to a 50ml flask containing 20ml methanol, the flasks sealed with cotton wool bungs and capped with a double layer of aluminium foil and placed on a shaker at 25 °C for overnight extraction. The methanol extraction

was repeated, the two methanol extracts combined together and dried, and the residue resuspended and assayed for colchicine.

6.4.1.1 Root tissue culture in shake flask

As a considerable amount of colchicine was found to be released into the culture medium by the *Gloriosa superba* root tissue, perfusion cultivation was performed using Amberlite resins in a mesh bag as a solid extraction system.

Weighed root tissue (approximately 4 - 5g fresh weight) was placed in a sterile 50ml conical flask containing 30ml medium. 300mg XAD-4 and XAD-16 resins, enclosed in nylon mesh bag, was incubated with the root tissue cultures. The flasks were sealed with cotton wool bungs, capped with a double layer of aluminium foil, and incubated on a shaker at 100rpm at 25 °C. After four weeks incubation the liquid medium was collected from each flask individually. Results are shown in (Figure 6.1).

Use of Amberlite resins reduced the proportion of colchicine in the culture medium to less than 10% of control values.

Average total colchicine accumulation was increased to 0.98µg/g and 0.8µg/g fresh weight with XAD-4 and XAD-16 respectively, compared to the control of 0.22µg/g fresh weight (Figure 6.1). Amberlite resins (XAD-4 and XAD-16) sequestered 90.2% and 92.6% respectively of total colchicine accumulated in the culture.

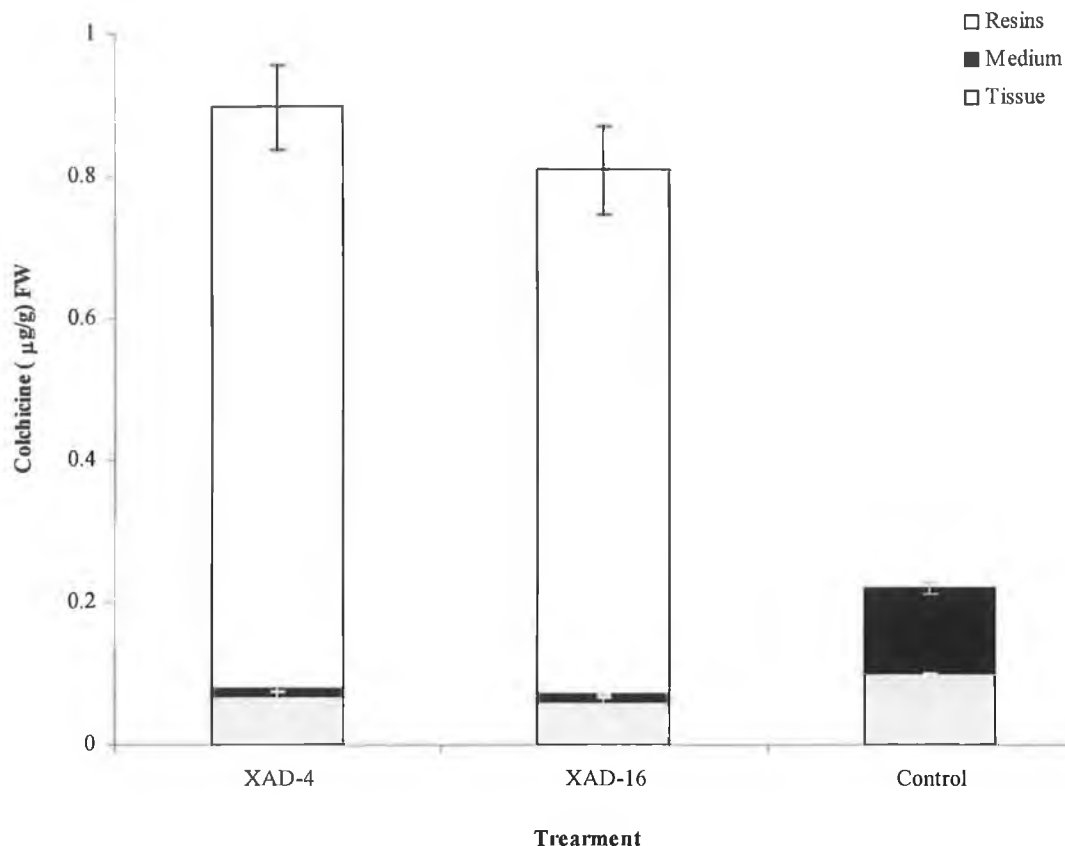


Figure 6.1: Average colchicine accumulated (µg/g fresh weight) from root culture in the shake flask experiment. The white, black, and grey areas of the bar are the amount of colchicine accumulated in the Amberlite resins, medium, and tissue respectively. Results are the average of three replicates and are shown with the standard error of the mean.

Amberlite resins greatly enhanced the total colchicine production at the end of the batch culture by 4.2 and 4.1 fold with XAD-4 and XAD-16 respectively. The amount of colchicine accumulated was significantly enhanced by using Amberlite resins. This experiment illustrated that further improvements in colchicine production and recovery were possible by using a continuous *in situ* solid phase extraction system.

6.4.1.2- Root tissue culture in a trickle column bioreactor

The trickle column bioreactor was designed and put together by myself using a one litre graduated measuring cylinder. The base and the top of the measuring cylinder were removed to yield a cylinder with the following dimensions (height 30cm, diameter 5.8cm) and a polypropylene Buchner funnel was fixed at the bottom of the bioreactor using silicon sealant. The funnel was attached to a silicone tube, and that tube was connected to the column packed with XAD-16 resins. Another tube was used to pump the medium from the Buchner flask to the top of the trickle column bioreactor using a peristaltic pump. The medium was supplied drop-wise and re-circulated via the Buchner flask (Figure 6.2).

Weighed root tissue was transferred aseptically into the autoclaved column bioreactor and 300ml of M2 liquid medium in a Buchner flask was pumped drop-wise on top of the tissue at a flow rate of 3ml/min. The extraction column containing 2g of XAD-16 was introduced on the media return line into the filter flask. The medium was aerated by water saturated, sterile air which was introduced via a tube dipped into the liquid medium. Results are shown in (Figure 6.3).

Use of Amberlite resins reduced the proportion of colchicine in the culture medium to 49% of control values.

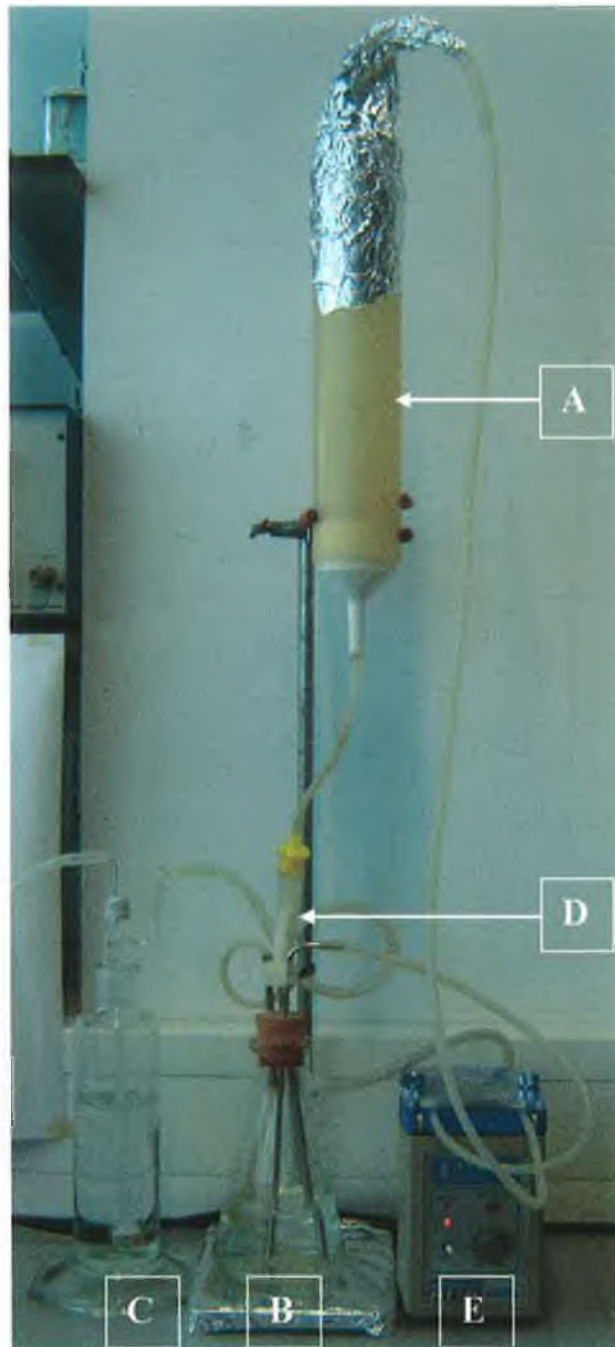


Figure 6.2: The Trickle bioreactor system; A= Bioreactor, B= Medium reservoir, C= Humidifier, D= Extraction column, E= Peristaltic pump

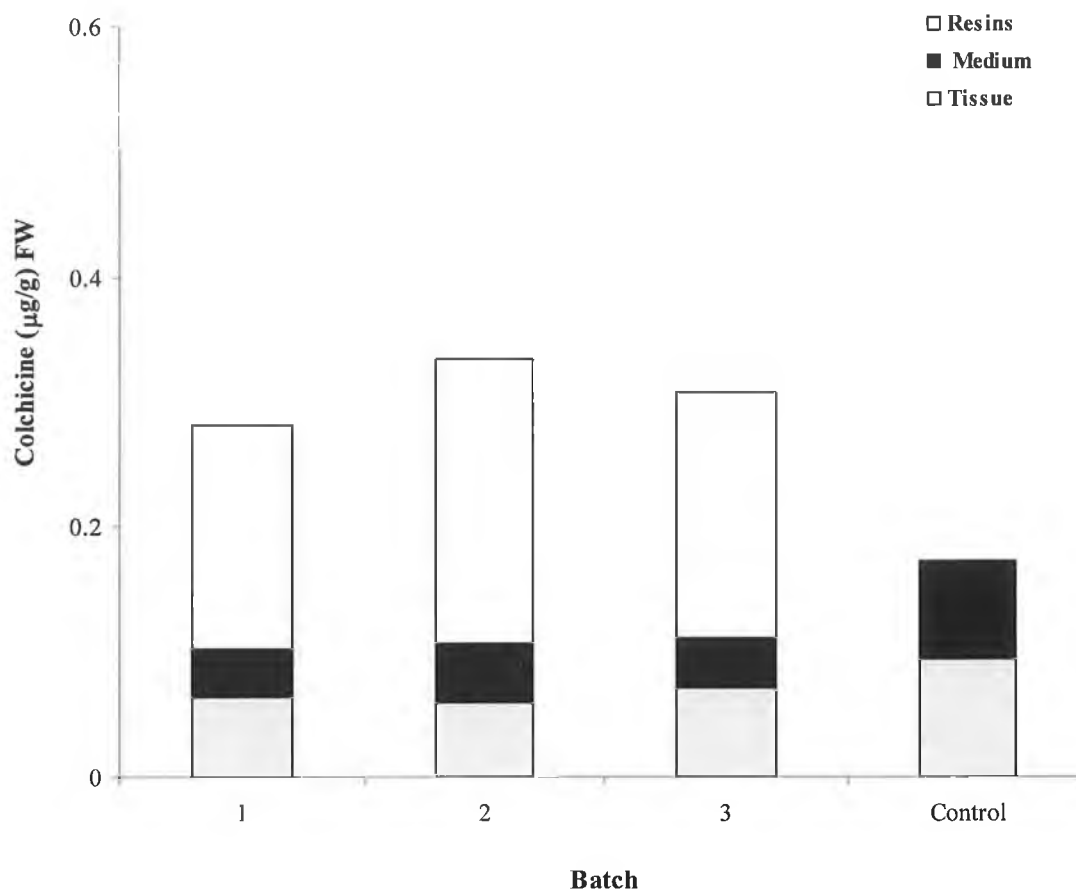


Figure 6.3: Colchicine accumulated ($\mu\text{g/g}$) fresh weight from root culture in the trickle bioreactor column. The white, black, and grey areas of the bar are the amount of colchicine accumulated in the Amberlite resins, medium, and tissue respectively.

The specific productivity of total colchicine accumulation was increased to $0.31\mu\text{g/g}$ fresh weight in the extracted cultures, compared to the non-extracted culture (control) which was $0.17\mu\text{g/g}$ fresh weight. Also Amberlite resins sequestered 65.1% of total colchicine accumulated in the culture. Amberlite resins greatly enhanced the total colchicine production at the end of the batch culture by 1.8 fold. Note the lower colchicine levels on the resins compared to shake flask (Figure 6.1).

6.4.1.3 Root tissue culture in an air-lift bioreactor

Pre-weighed root tissue was transferred into a bioreactor containing 1.7l M2 liquid medium (Figure 6.4). The medium was aerated and mixed by air-lift by water saturated, sterile air supplied at the bottom of the bioreactor. The culture was incubated for four weeks. By using a peristaltic pump the medium was passed through an adsorption column containing 2g of XAD-16 resins, and the drained medium then allowed back into the bioreactor. The flow rate of the medium through the extraction column was 3ml/min. Results are shown in (Figure 6.5).

Use of Amberlite resins reduced the proportion of colchicine in the culture medium to 61% of control values.

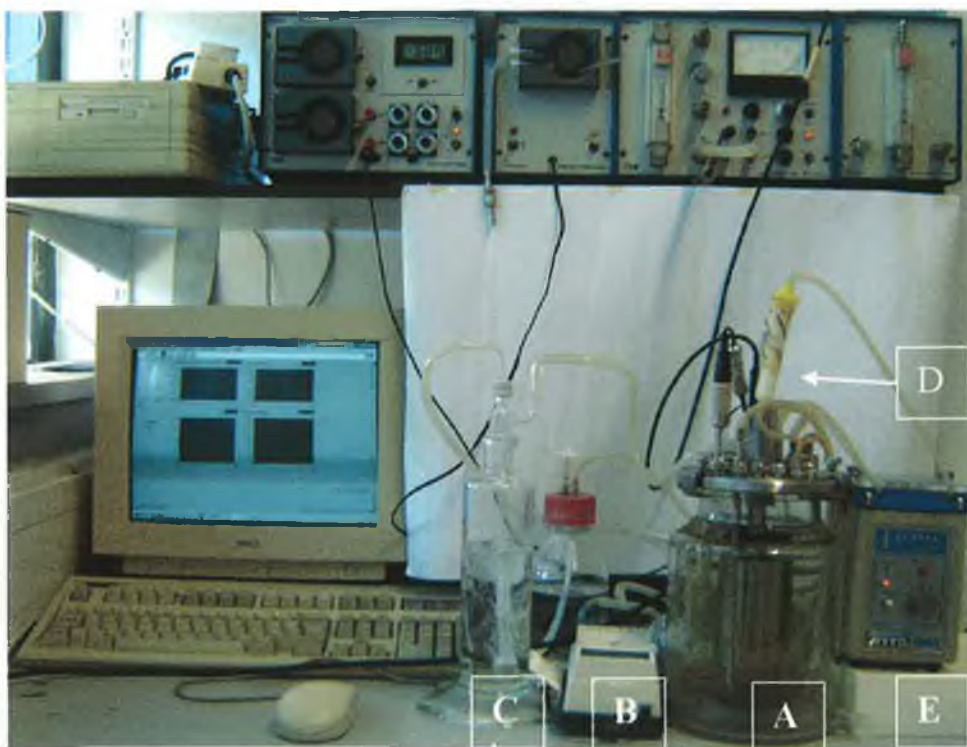


Figure 6.4: The air-lift bioreactor system; A= Bioreactor, B= Air pump, C= Humidifier, D= Extraction column, E= Peristaltic pump.

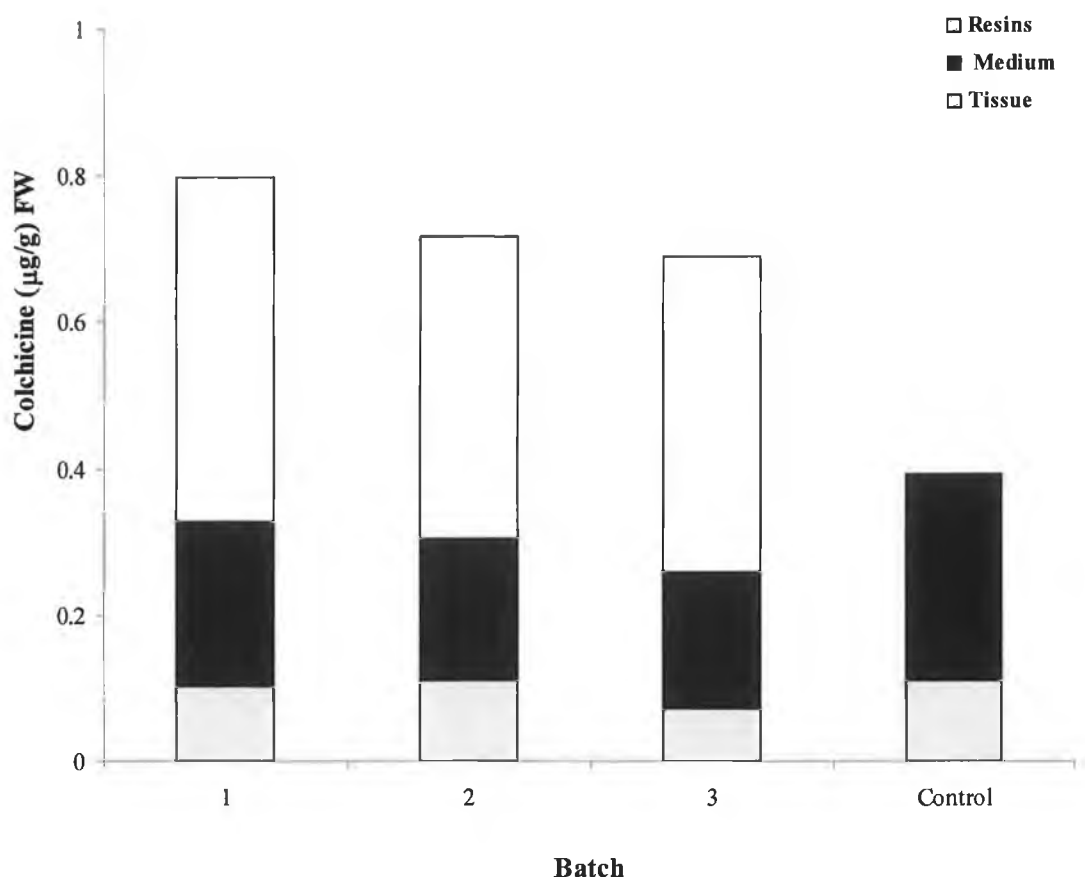


Figure 6.5: Colchicine accumulated ($\mu\text{g/g}$) fresh weight from root culture in the air-lift bioreactor. The white, black, and grey areas of the bar are the amount of colchicine accumulated in the Amberlite resins, medium, and tissue respectively.

The specific productivity of total colchicine accumulation was increased to $0.73\mu\text{g/g}$ fresh weight in the extracted cultures, compared to the non-extracted culture (control) which was $0.4\mu\text{g/g}$ fresh weight. The Amberlite resins sequestered 59.6% of total colchicine accumulated in the culture. Amberlite resins enhanced the total colchicine production at the end of the batch culture by 1.9 fold. Similar total colchicine accumulation ($\mu\text{g/g}$) fresh weight was recorded with air-lift bioreactor in comparison to shake flasks.

6.4.1.4 Combination of the adsorbent and precursors feeding using an air-lift bioreactor

Typically, no single stimulation or enhancement strategy will give a great increase in secondary metabolites accumulation, but in many cases the simultaneous application of multiple-strategies results in a synergistic interaction. The bioreactor experiment was repeated using medium supplemented with 0.5 mM coumaric acid or 3-phenyl propionic acid. Results are shown in (Figure 6.6).

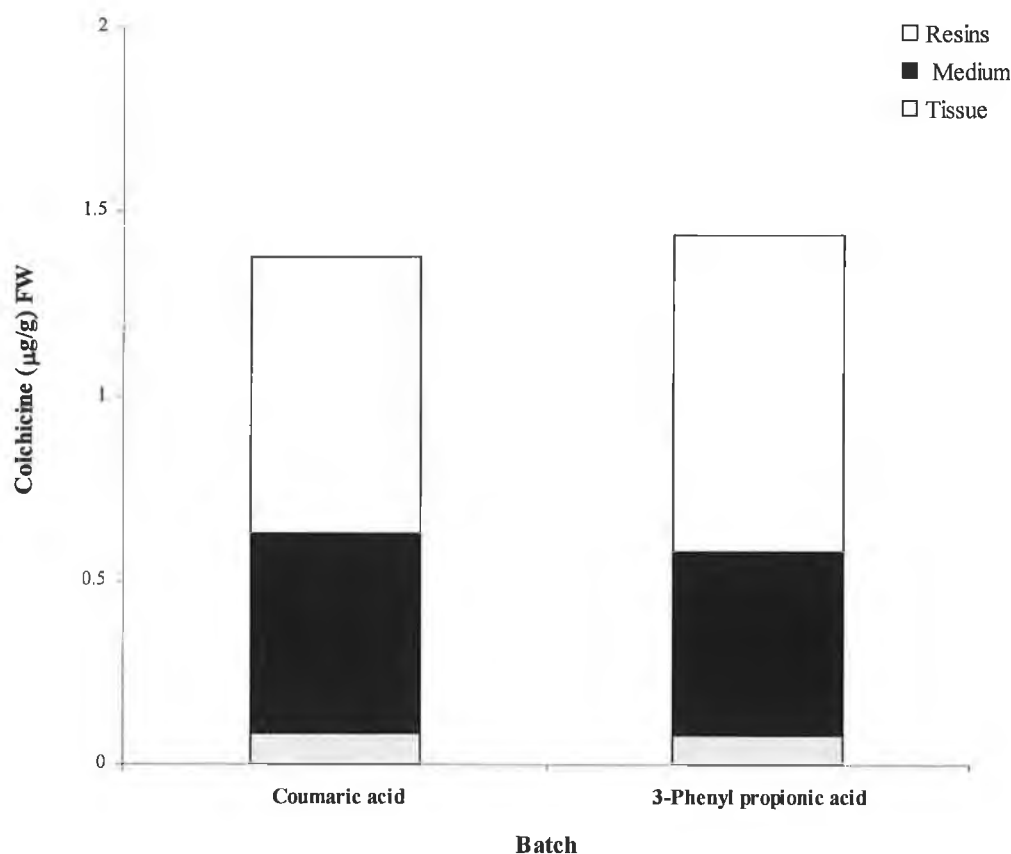


Figure 6.6: Average colchicine accumulated ($\mu\text{g/g}$) fresh weight from root culture using medium supplemented with 0.5 mM coumaric acid or 3-phenyl propionic acid in the air-lift bioreactor. The white, black, and grey areas of the bar are the amount of colchicine accumulated in the Amberlite resins, medium, and tissue respectively.

The overall colchicine accumulation was increased to 1.3 and 1.41 $\mu\text{g/g}$ fresh weight in a culture supplemented with 0.5mM coumaric acid or 3-phenyl propionic acid, respectively. Amberlite resins recovered 58% of total colchicine accumulated in the culture. Average colchicine accumulated in the extraction column (μg per day) over a period of four weeks using different culturing system is shown in (Figure 6.7).

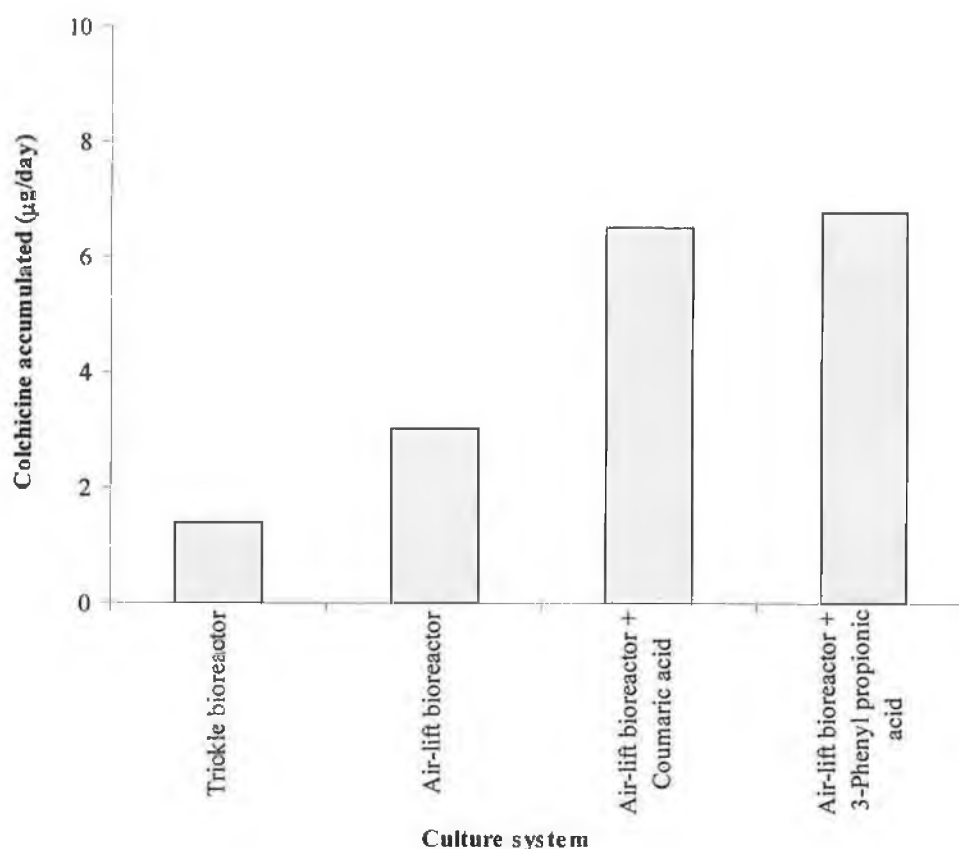


Figure 6.7: Amount of colchicine accumulated ($\mu\text{g/day}$) in the different culture systems, over a period of four weeks.

6.4.1.5 The effect of Amberlite resins system on fresh weight increment

The effect of solid phase extraction system on the growth of the root tissue fresh weight was considered. Results are shown in (Figure 6.8 and 6.9).

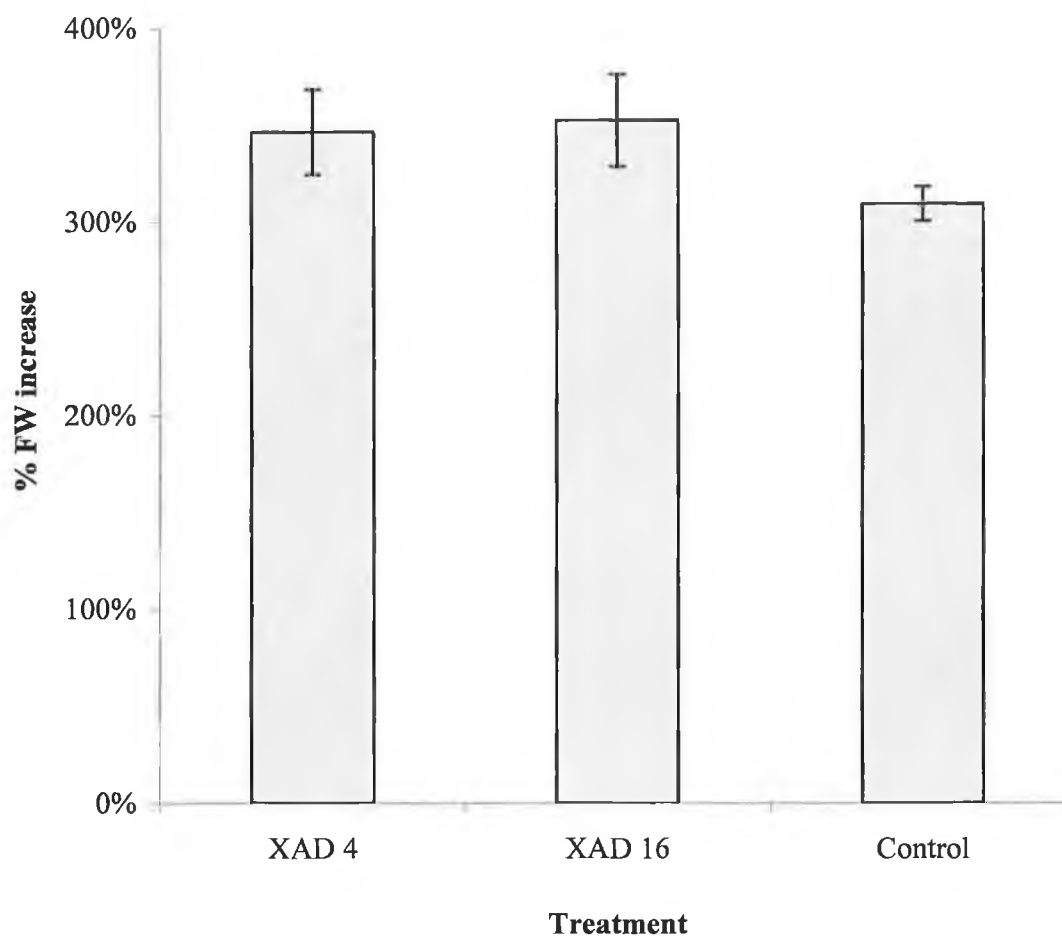


Figure 6.8: Fresh weight increment in batch culture flasks of *Gloriosa superba* M2 root over four weeks. Results are the average of three replicates and are shown with the standard error of the mean.

It can be seen from the above graph that both Amberlite resins (XAD-4 and XAD-16) slightly improved the fresh weight growth of the root tissue.

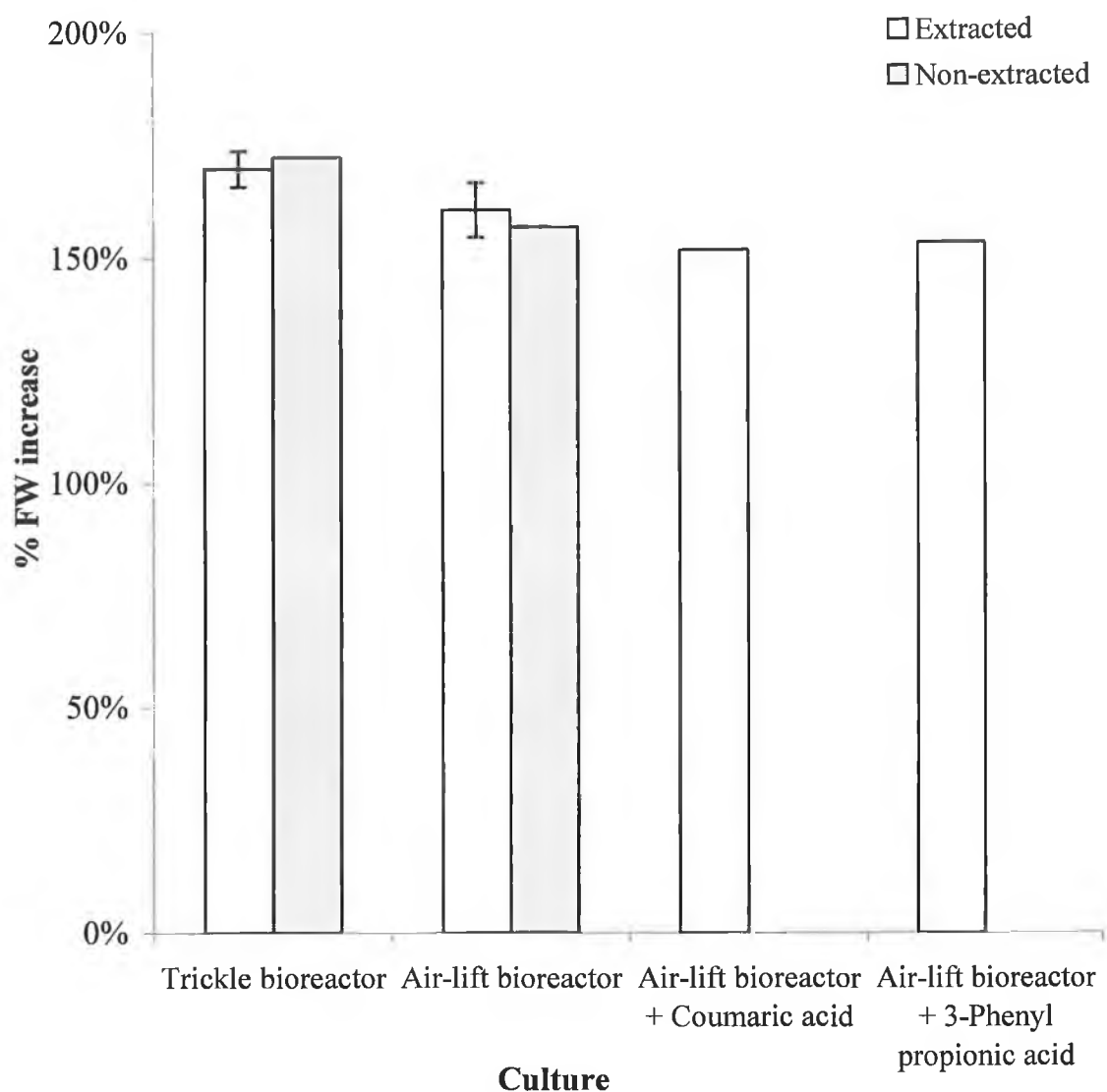


Figure 6.9: Fresh weight increment in column and air-lift bioreactor batch culture of *Gloriosa superba* M2 root culture. The white bars are the fresh weight increase with extraction culture and the grey bars are the non-extracted culture.

It can be seen from the above graph that there was no significant effect of the solid phase extraction system on the fresh weight growth of the root tissue.

6.4.1.6 Determination of the viscosity of the liquid medium

The medium gradually become more viscous as the culture growth progressed and may affected mixing and oxygen transfer in the bioreactor. Medium was collected at the end of incubation period and the liquid viscosity was established using Brookfield rotating cylinder viscometer.

In order to investigate the cause for the increase in the medium viscosity, it was decided to probe the amount of soluble and total carbohydrate in the medium. The collected samples were tested for both soluble and total sugar using the method described in section 6.3.2. The ethanol step was omitted for determination of the total carbohydrate. Results are illustrated in Table 6.1.

Table 6.1: The viscosity with the amount of total and soluble carbohydrate of the liquid medium from air-lift bioreactor after four weeks culture.

Batch	Viscosity (cP)	Total carbohydrate (g/l)	Soluble carbohydrate (g/l)
1	9.3	8.11	2.79
2	8.5	7.42	3.23
3	9.8	6.78	3.67
Control	8.3	6.16	3.01

cP= centiPois. Water at 20.2 °C has an absolute viscosity of one centiPoise.

6.4.2 Growth characteristics of *Gloriosa superba* root culture in liquid medium

For the development of plant tissue culture, accurate and rapid measurements of tissue growth and assessment of growth-related bioprocess kinetic are essential for the improvement of plant tissue biotechnology. Measurement of root tissue growth in cultivation devices presents a serious problem. As it is difficult to obtain representative samples from flasks or bioreactor, alternative means for estimating growth rate from the medium have to be developed. In this study two methods were considered for evaluation of root tissue culture growth: medium conductivity and soluble carbohydrate determination.

The decrease in conductivity of liquid culture media can be used to assess growth rates of plant tissue cultures. The use of electrical conductivity as a means for estimating biomass growth rate in plant cell and tissue culture systems has been investigated by a number of authors, including (Hahlbrock, 1975; Taya *et al.* (1989A); Ryu, *et al.* (1990); and Kwok *et al.* (1992). However, medium conductivity only depends on the electrolyte concentration and ignores the changes in sugars that are present in higher concentrations than the other inorganic components of the medium.

Soluble sugar (Mono and oligosaccharides) was measured spectrophotometrically using a modified phenol-sulphuric acid assay (Dubois *et al.*, 1956). An initial extraction with 80% ethanol extracts low molecular weight carbohydrates and precipitate polysaccharides which can be removed by centrifugation.

6.4.3 Development of an online method for estimating growth rate of root tissue culture in liquid medium

Erlenmeyer 100ml flasks were weighed and inoculated with approximately 7g fresh weight root tissue and the flask reweighed. Thirty ml of medium was then added and the flask incubated at 25°C on an orbital shaker (100rpm). Every 3 days the medium was transferred to sterile universal tubes, the fresh weight determined and the medium returned back to the flask immediately. A 0.5ml medium sample of each culture was placed individually in a universal tube and stored at -20°C for conductivity and soluble carbohydrate analysis. The time course of fresh weight growth as well as medium conductivity and soluble carbohydrate concentration curves of root culture are presented in Figure 6.10 and 6.11 for M1 and M2 *Gloriosa superba* root culture respectively.

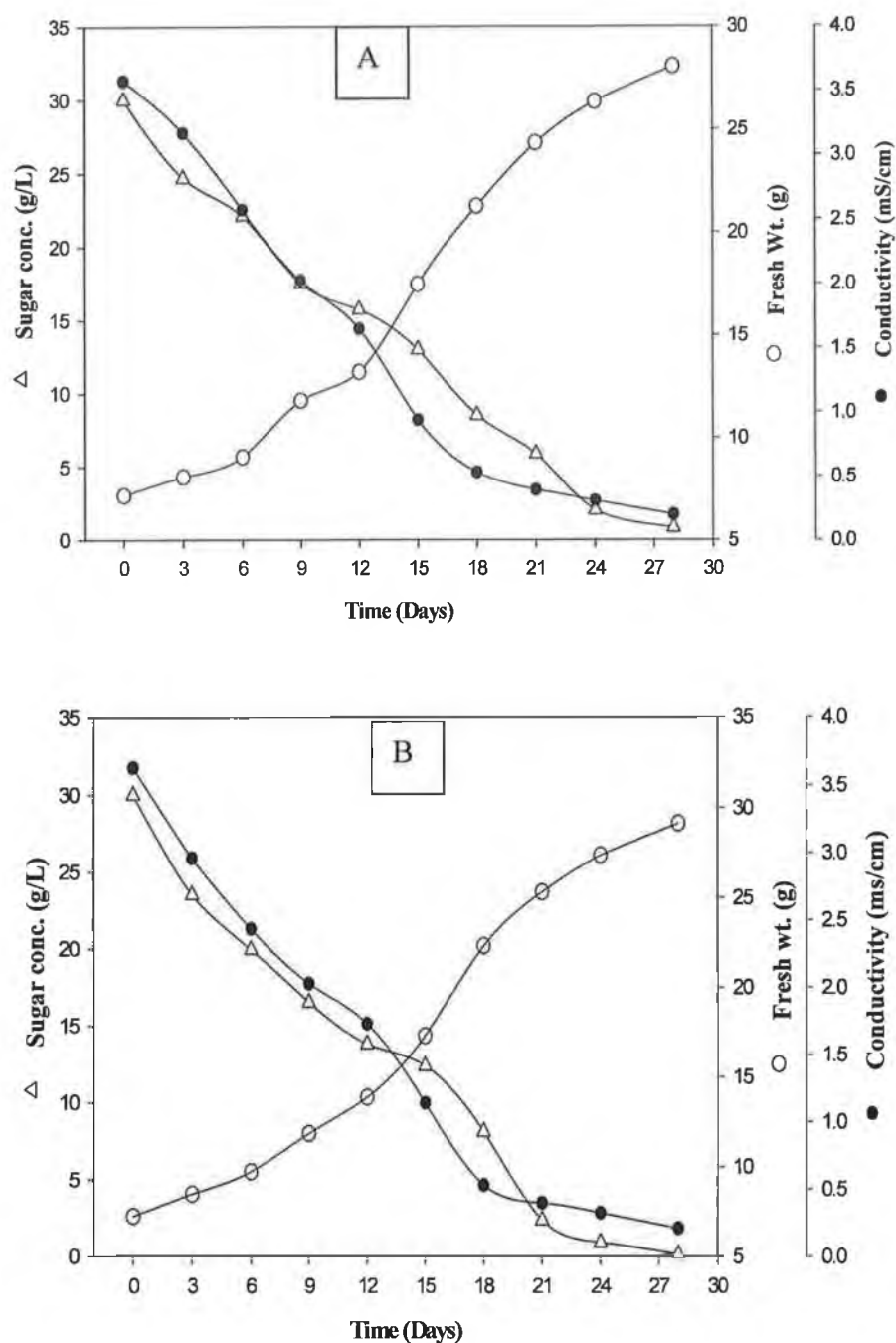


Figure 6.10: Time course profiles of fresh weight growth of *Gloriosa superba* A = M1 and B = M2 root culture with medium conductivity, and soluble carbohydrate concentration. Open circle =FW, triangle = Soluble sugar in the medium, and solid circle = medium conductivity. Results are the average of six replicates.

6.4.3.1 Fresh weight measurements

Tissues fresh weight determination is an off-line measurement made either on medium or tissue withdrawn from the culturing vessel. Figure 6.11 show the exponential fresh weight growth curves from flask batch cultures for *Gloriosa superba* M1 and M2 root culture respectively.

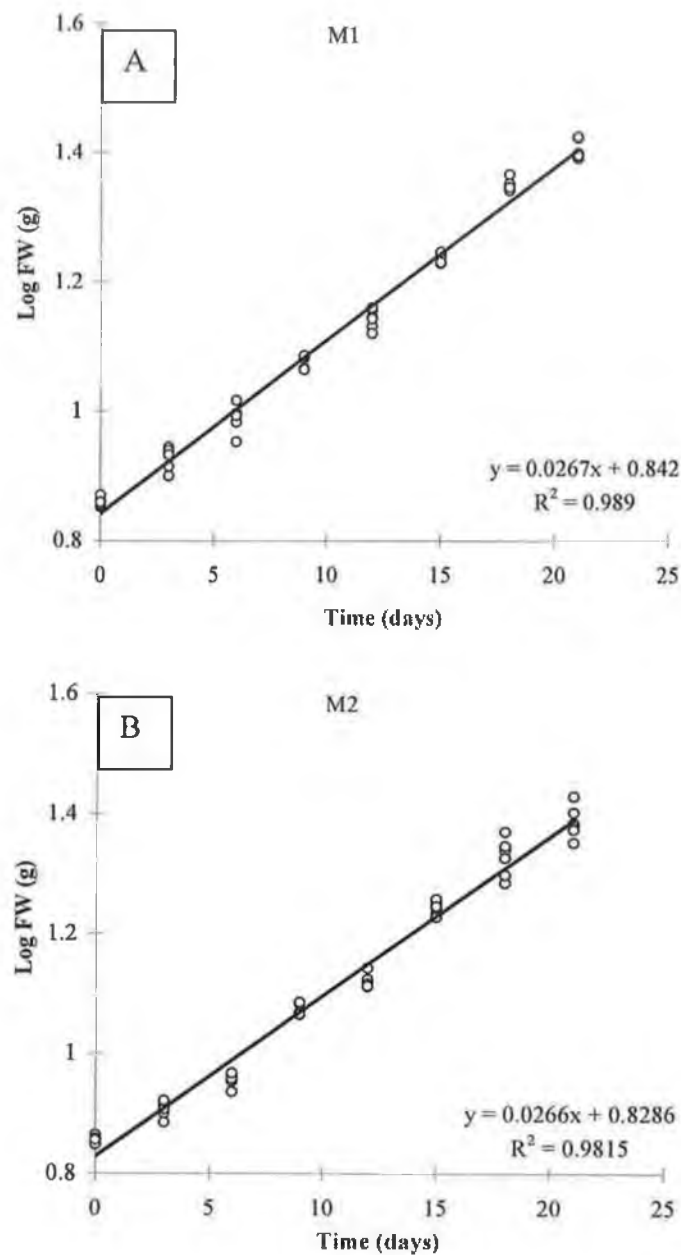


Figure 6.11: Time course profile of Log fresh weight growth curves for *Gloriosa superba* A = M1 and B = M2 root culture

6.4.3.2 Indirect measurements

6.4.3.2.1 Soluble carbohydrate

The soluble carbohydrate in the liquid medium from the flasks batch cultures are shown in (Figure 6.12) for *Gloriosa superba* M1 and M2 root culture respectively.

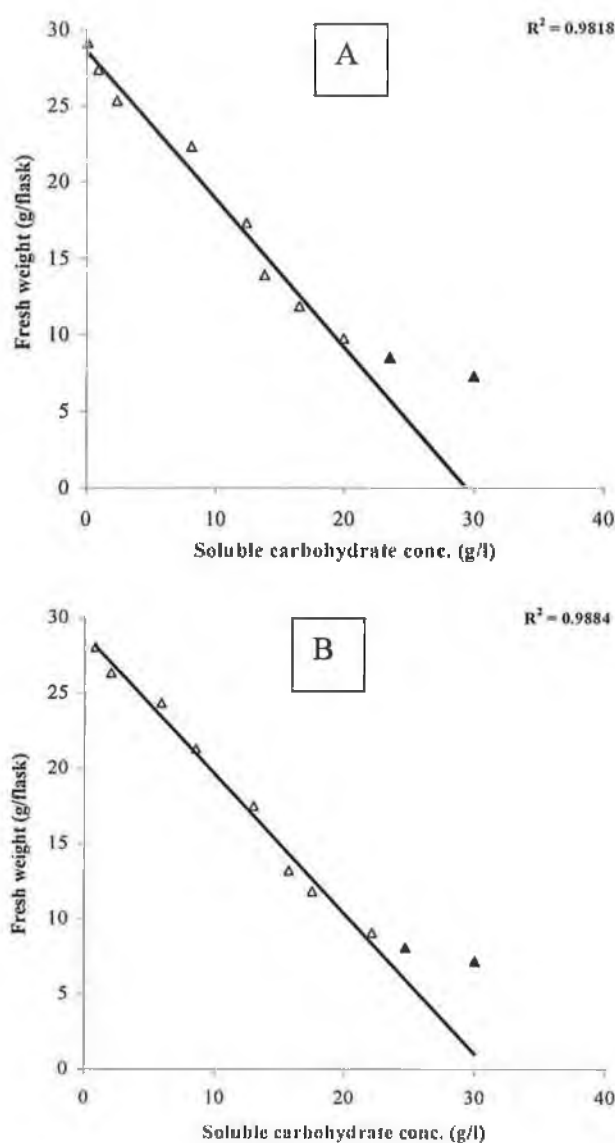


Figure 6.12: Relationship between fresh weight and soluble carbohydrate in the liquid medium of *Gloriosa superba* A = M1 and B = M2 root culture. Black triangles represent the initial six days. Results are the average of six replicates.

6.4.3.2.2 Medium conductivity

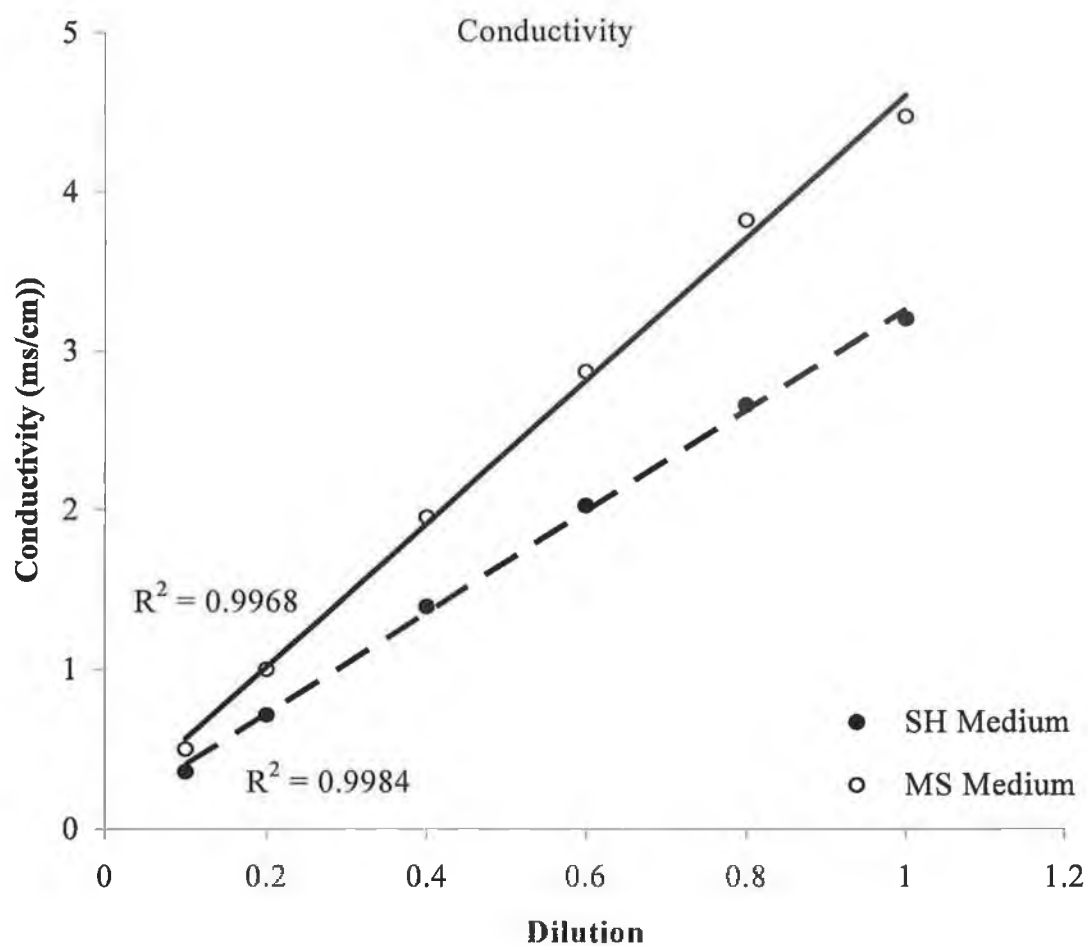


Figure 6.13: Conductivity standard curve of MS and SH medium

The medium conductivity, was measured throughout the growth cycle of the flask batch culture, to examine the possibility of using this method as a mean for assessing culture growth. (Figure 6.14A and 6.14B) shows the relationships between fresh weight biomass and medium conductivity in the flask batch cultures experiment for *Gloriosa superba* M1 and M2 root culture respectively.

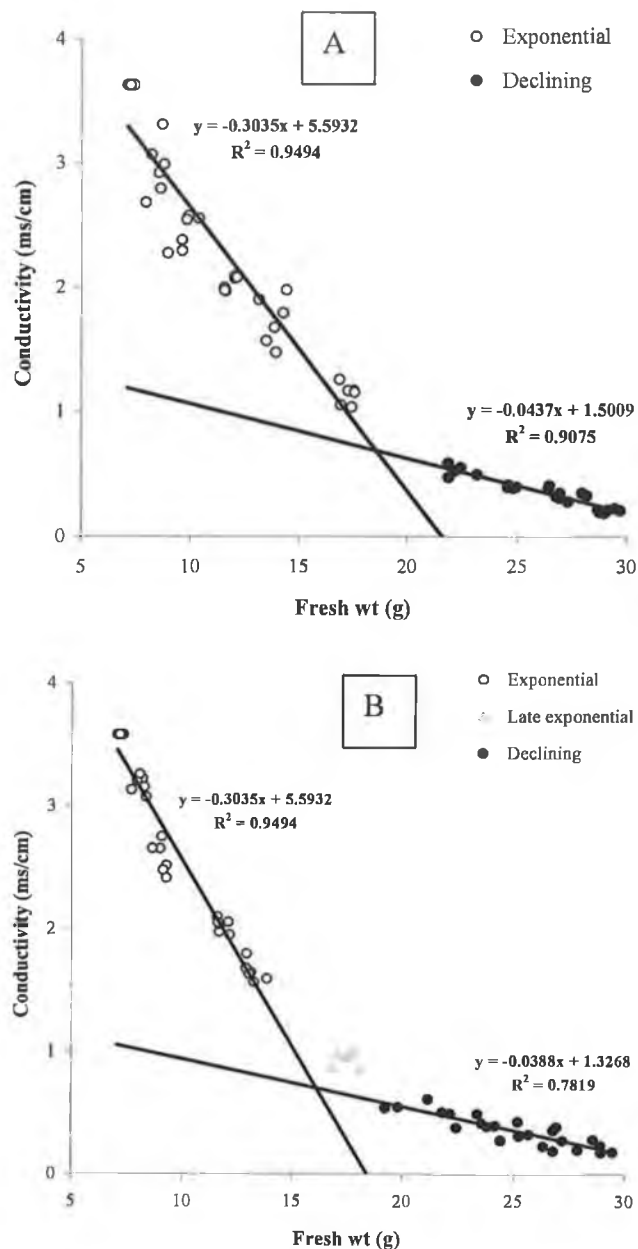


Figure 6.14: Relationship between medium conductivity and fresh weight increase of the *Gloriosa superba* A = M1 and B = M2 root culture

6.4 Discussion

The *in situ* extraction of plant natural products from tissue culture can dramatically increase the total amounts of secondary metabolites formed in a typical batch culture cycle or in a continuous bioreactor culture. If tissue and medium are left in contact for any length of time, equilibrium between release and re-uptake is established. The use of a specific adsorbent has much to recommend it; it effectively concentrates the product, so simplifying recovery procedures, and continuously removes product from the medium as it is released and as well as facilitating product release by maintaining a near-zero external concentration. Additionally, there may be biological advantages related to possible stimulation of biosynthesis, conceivably by reduction of feedback inhibition or removal of toxic compounds. A significant fraction of colchicine produced by *Gloriosa superba* root culture was observed to be released into the liquid medium.

To ensure product quality, consistency in tissue growth and secondary metabolism characteristics the culture must be maintained through process scale-up. However, lack of consistency during scale-up is a problem in many plant cell and tissue culture systems. Loss of productivity or low productivity is often observed when plant cultures are transferred from shake flasks to bioreactors. Scragg *et al.* (1987) observed a decrease in serpentine production in *Catharanthus roseus* suspension cultures on transfer from shake flasks to various air-lift bioreactors. And the maximum serpentine concentration in a larger scale bioreactor was lower than that in a smaller scale bioreactor. A shift in cell metabolism in *Catharanthus roseus* suspension cultures on transfer from shake flask to bioreactor was also observed by Schlattmann *et al.* (1993). They attributed the loss or shift in production to the different physical conditions (e.g., degree of mixing, shear stress, and gas-phase compositions) encountered between the two environments.

The differences in the physical conditions between the three culture methods may cause changes in cell metabolism and this makes maintaining the same

physical conditions in flask and small bioreactor problematical. A decrease in productivity on scale-up (Tom *et al.*, 1991) has been attributed to changes in the physical conditions of the cultures. The *Gloriosa superba* root tissues continue to accumulate relatively the same amount of colchicine on scale-up into a different culturing system used in this pilot study and this may suggest that further scale-up may be possible without loss of productivity.

Amberlite resins removed a considerable proportion of colchicine from the culture with approximately 90% of total colchicine accumulation in the shaken flask experiment compared to 51% and 39% for the trickle column bioreactor and air-lift bioreactor respectively. This may suggest that the incubation of the adsorption Amberlite resins physically within the culture system is enhancing the amount of colchicine removed. The low colchicine uptake from the air-lift bioreactor may be due to the fact that the volume of the medium in the air-lift bioreactor was larger (1.7l) than that in the trickle column (300ml). A larger volume of extracellular medium may facilitate colchicine release. The specific productivity of colchicine in air-lift bioreactor was higher than that of the trickle bioreactor and this may be due to the following reasons: first the tissues in the air-lift bioreactor is submerged in the medium, so they have a continuous contact with the medium compared to the tissues in the trickle bioreactor in which the medium was only trickling on the surface of the tissue. In both cases, the physical separation of tissues and adsorbent may make extraction economically more efficient and effective. Periodic replacement of the solid phase extractant may result in a constant production rate of colchicine. The Amberlite column can be aseptically removed *in situ* without interrupting bioreactor operation, whereas Amberlite resins incubated with the culture cannot be removed without interrupting bioreactor operation.

The reasons for the enhancement of colchicine accumulation by *Gloriosa superba* root culture may be due to the reduction of feedback regulatory mechanisms and the removal of other product synthesis inhibiting compounds.

Asad and Shuler (1989) were perhaps the first to exploit multiple strategies using production of extracellular ajmalicine from *Catharanthus roseus* as a model. A strategy using a production medium, elicitor, and *in situ* adsorption (XAD-7), and immobilisation in calcium-alginate matrix gave an extracellular concentration of nearly 90mg/l compared to levels of only 1mg/l in growth medium. An approach was set up in which production of colchicine was investigated using a combination of precursor feeding and *in situ* product removal.

Combination of precursor feeding and adsorption on an Amberlite column significantly enhanced the total colchicine accumulation at the end of the batch culture by 3.5 and 3.7 fold for coumaric acid or 3-phenyl propionic acid respectively. The amount of colchicine accumulated in the resins per day from the air-lift bioreactor culture was significantly enhanced by combining the precursor feeding with the solid phase extraction compared to using solid phase extraction alone (Figure 6.7). It seems that these two combinations (precursor's feeding and SPE system) provide a synergistic effect on colchicine productivity.

Beiderbeck and Knoop, (1988) had shown that using XAD-4 at a high concentration decreased *Nicotiana tabacum* cell culture growth, while Berlin and Witte, (1988) had noticed a slight stimulation in growth rate of *Thuja Occidentalis* cell culture. The solid phase extraction system (Amberlite resins) applied in this study revealed no significant effect on the fresh weight growth rate of the root culture in the column bioreactor and air-lift bioreactor batch experiment, in comparison with the control (Figure 6.9), while the batch root culture in the flasks experiment demonstrated a slight increase in fresh weight growth rate in comparison to the control (Figure 6.8).

Fresh weight growth rate was similar between culture grown in a trickle bioreactor or air-lift bioreactor and that may suggest that the availability of

nutrient uptake by the root tissue culture submerged in the medium or supplied by droplets was similar (Figure 6.9).

A strong characteristic of the *Gloriosa superba* cell and root cultures was production of viscous exudates. High fluid viscosities can affect proper mixing and oxygen mass transfer rate in the bioreactor systems and consequently, affect the culture viability and metabolism (Taticek *et al.*, 1994; Kieran *et al.* 1997). This is not unusual for plant cell culture; Kato *et al.* (1978) reported an increase in viscosity in liquid medium of *Nicotiana tabacum* cultures. The viscosities of the liquid medium from air-lift bioreactor culture were found to range between 8.3 – 9.8 cP. The medium viscosity and concentration of soluble and total sugar illustrated in Table 6.1 show that the elevated viscosity was found to be directly related to the non-soluble sugar in the medium. The increase in viscosity may therefore be attributed to the secretion of polysaccharide from the plant culture. The effect of polysaccharides on the viscosity of the liquid medium is well known and was demonstrated by Olson *et al.* (1969) with *Nicotiana tabacum* cultures.

The growth profiles of plant tissue culture normally demonstrate characteristic lag, exponential and stationary phases and the duration of each phase depends on the growth condition and on the cell line being cultivated. The growth of batch root cultures in flasks shows that a low growth rate was observed over the first 3 days of the experiment with the exponential growth phase between the 4th day and the 21st day, and a stationary phase following the exponential phase. Root tissue culture (7g fresh weight) inoculated in a liquid medium yielded 28.9 and 29.4g of roots in 28 days for M1 and M2 root culture, respectively. Under the experimental conditions, the fresh weight increased exponentially for 21 days from the time of inoculation. Analysis of growth kinetic data show a specific growth rate of 0.062 g/g/day corresponding to a doubling time 11.2 days for M1 root culture and a specific growth rate of 0.061 g/g/day corresponding and doubling time 11.3 days for M2 root culture.

After inoculation in the shake flasks the cultures enter a lag phase of approximately three days. The low growth rate in the first three days was probably due to the time the tissue biomass needed to adapt to the new medium. During this period the cultures may adjust to the fresh medium and prepare for growth. Variation in the growth hormones in the two medium resulted in relatively no observable effect on growth rate. A strong relation between log fresh weight and time with a high correlation coefficient ($R^2=0.989$) shows exponential growth (Figure 6.12).

According to literature reports, non-transformed root cultures rarely grow very fast and often have a tendency to gradually turn into callus. An average growth cycle in such cultures is 60–70 days (Wysokinska and Chmiel 1997). Based on the fresh weight results, it was observed that the roots of *Gloriosa superba* were characterised by relatively fast growth and significant production of biomass (four times the mass of inoculum) in four weeks.

The commonly used techniques for monitoring growth rate of plant tissue culture are fresh weight and dry weight. Although these conventional methods are consistent, they are time consuming and invasive to the culturing system. There is an obvious need for alternative methods, ideally suitable for on-line application during cultivation scale up in bioreactors. Fresh weight growth and simple sugar and conductivity curves of root culture are presented in Figure 6.10 and 6.11. Medium conductivity and soluble carbohydrate were measured over the course of the growth cycle, to examine the possibility of using these methods for estimating the growth rate of the root culture. Medium conductivity closely paralleled the consumption of the sugar.

A good linear correlation was observed between log fresh weight and soluble carbohydrate. The soluble carbohydrate curve, which is essentially a substrate utilization curve follows the expected pattern and correlates well, in an inverted manner, with the fresh weight (Figure 6.12). There was an initial high rate of sugar usage with low fresh weight growth rate during the first six days,

and that may suggest that the tissue was converting sugar into starch after which there is a good linear correlation between soluble carbohydrate and fresh weight. It has been reported (Sarkissian, and Gray, 1990) that in the fast growing phase, plant cells synthesize cell wall material and starch from available carbohydrate, and when they reach the stationary phase starch is metabolized.

The medium conductivity is directly proportional to the concentration of ionic components in the medium (Figure 6.13).

The use of electrical conductivity as a means for estimating biomass growth rate in plant cell and tissue culture systems has been investigated by a number of authors, including Hahlbrock (1975), Ryu and Roamani, (1990), Taya *et al.* (1989A), and Kwok *et al.* (1992). This method was investigated in this work to assess the feasibility of using conductivity measurements as a basis for monitoring *Gloriosa superba* root culture. Medium conductivity decreased rapidly during the exponential phase as the culture requirements increased. Conductivity of the both cultures is seen to fall in a manner that is inverse to that of increase in fresh weight. There is a good linear correlation between decrease in conductivity and fresh weight over the exponential phase of culture, and also in the phase of declining growth towards the end of the growth cycle the conductivity is seen to decrease more slowly (Figure 6.10). At the beginning of batch culture when the cell growth was slow or in the lag phase, the medium conductivity decreased rapidly. This is considered to be due to the active metabolic uptake of medium ions by the cultures before the start of exponential growth at the following stage (Wen and Zhong, 1996).

The linearity between fresh weight and conductivity is confined to the first 15 and 12 days of the growth cycle for the M1 and M2 root culture respectively, before the fresh weight profile enters the late exponential phase, and that suggests that the relationship is more complex. There was a good correlation

of conductivity with fresh weight in the exponential phase, ($r^2 = 0.95$) for both M1 and M2 *Gloriosa superba* root culture.

These data indicates the prospect for using conductivity as a rapid means of monitoring growth rate for *Gloriosa superba* root culture.

The potential for on-line estimation of the growth rate of *Gloriosa superba* root culture by monitoring the soluble carbohydrate and conductivity of the liquid medium is thus evident from the preliminary trial carried out in this study. However, a more comprehensive study is required to optimise this method.

6.5 Conclusion

The results of this chapter have indicated that the specific productivity of colchicine was enhanced by its continuous removal of the medium.

Combination of precursor feeding and *in situ* or on-line removal of colchicine by using Amberlite resins show synergistic effects on colchicine biosynthesis and release.

A simple model for enhanced production of colchicine has been developed.

The results show that the growth rate of *Gloriosa superba* root culture in liquid medium can be estimated by simple, reliable and non-invasive methods by measuring the conductivity and the amount of soluble carbohydrate in the liquid medium.

Chapter Seven

General Discussion

7.1 General discussion

A reason for adopting an *In vitro* culture approach was to examine the colchicine biosynthesis pathway and stimulate colchicine accumulation in *Colchicum autumnale* and *Gloriosa superba*. Colchicine is the major alkaloid in *Colchicum autumnale* (Capararo and Brossi, 1984; Fowler and Scragg, 1988) and *Gloriosa superba* (Brossi *et al.*, 1988). Also it offers a possible alternative to using whole plant material for elucidating the biosynthetic pathway in *Colchicum autumnale* and *Gloriosa superba* which grows only for a few months during the autumn. Our study using plant tissue culture did not have these time constraints and can be used as a lab tool which offers a simplified system to study the biosynthetic pathway at any time of the year, and is therefore more practical.

The initial aim of this study was to initiate callus from shoot tissue of *Colchicum autumnale* and *Gloriosa superba* and grow them to supply sufficient quantities of tissue for biochemical studies and also to establish suspension cultures. Cultures either on solid or in suspension cultures showed extremely slow growth rates. However, although the callus tissues initiated were found to be slow growing, they still accumulated colchicine. Slow growth rate was a reasonably common phenomenon in plant cell cultures (Wilson, *et al.* 1971).

Different concentration of 2,4-D were tested and compared with other auxins (NAA, IBA) which are sometimes used in plant cell culture. It was found that 2,4-D was necessary for optimum growth of callus tissue in both solid and suspension culture. Changing the concentration of the auxins in the growth medium showed that the 2,4-D had a significant affect on the growth rate of the callus culture of both species and at the same time did not affect the colchicine accumulation to any significant extent. Hence, based on biomass and the desirability of having a friable type of callus formed, MS medium supplemented with 2,4-D (3mg/l) and Kinetin (1mg/l) was found to be the most suitable culture medium for the production and maintenance of callus tissue for the

preparation of cell suspension culture and precursor feeding studies. Removing the plant growth hormones from the medium, led to a decrease in growth rate, and the culture started to differentiate in to rootlets.

The culture conditions used in this study gave relatively good growth rates for *Gloriosa superba* root culture, but it is not known if low colchicine accumulation is affected in rapidly growing root tissue. It is possible, that under condition of maximum increase in the culture tissue biomass secondary metabolism may be inhibited (Hahlbroc and Wellmann, 1970). The relationship between growth and secondary metabolite production is complex and not fully understood. Some possible explanations for the relatively low accumulation of colchicine in *Gloriosa superba* cultures could be the relatively rapid growth of cell cultures, genetic instability of production or culture conditions which were not optimal for accumulation of colchicine.

If there is competition for a common intermediate between primary and secondary metabolic pathways there will be an apparent antagonism between growth and secondary metabolite accumulation. The relatively rapid growth state in which the root tissue cultures of this study were maintained may have favoured primary metabolism for growth at the cost of colchicine accumulation. Tissue differentiation is sometimes negatively related to secondary metabolite accumulation. Sharma and Khanna (1980) found that undifferentiated cultures of *Agave wightii* yielded 1-2% dry weight sapogenins but, when differentiated to produce roots or bulbils, only trace sapogenins were accumulated.

Secondary metabolites are generally not products of single enzymes but are the results of multi-step and multi-enzyme processes. Many natural products of complex structure require 20–30 enzymes for their synthesis, and each synthetic pathway is branched into a network that leads to a multiplicity of related chemical structures (Toni and Kutchan, 2005). There are several groups of reactions through which channelling could occur (Winkel, 2004). The colchicine

biosynthetic pathway involves at least 12 steps (Rueffer and Zenk, 1997), and some of the enzymes involved in the biosynthesis are still unknown.

The difference between accumulation of colchicine in culture and in the intact plant is unlikely to result from any alterations in the genome because cultures usually retain all the genetic information. Hence, the failure of the cultures to accumulate the same level of colchicine as in intact plants must arise through a lack of gene expression in the cultured state. Yeoman *et al.*, (1982) have presented examples of different culture which appear to have all the enzymatic machinery required to form natural products although these products are themselves absent. They favoured the hypothesis that biosynthesis pathways are often present in callus and that cells are simply awaiting a trigger to switch them on Yeoman *et al.* (1982). Lindsey and Yeoman, (1984) discussed cases in which certain enzymes necessary for secondary metabolism were reported to be absent from the culture and suggest that the enzymes are, perhaps, not absent, but that their expression is being suppressed.

From this study and from the literature (Hayashi *et al.*, 1988A; Yoshida, *et al.* 1988; Ghosh *et al.*, 2002; Sivakumar *et al.*, 2004); it is known that callus cultures of *Colchicum autumnale* and *Gloriosa superba* are capable of producing colchicine.

The levels of colchicine were relatively low in comparison with the intact plant. The results obtained in this respect using tissue cultures of *Colchicum autumnale* were in agreement with what was reported previously (Hayashi *et al.*, 1988A; Yoshida *et al.* (1988A and 1988B),). While the level of colchicine in *Gloriosa superba* are contradictory, some authors detecting levels of colchicine which were similar to those observed in corms and seeds which known as the storage site for colchicine and normally accumulate the highest level of colchicine in the whole plant. Ghosh, *et al.* (2002), reported 240µg/g dry weight of colchicine in the root tissues and up to 1870 µg/g dry weight with root tissue fed with coumaric acid and tyramine, and no colchicine detected in

the liquid medium. Although Sivakumar, *et al.*, (2004) reported 40µg/g dry weight in callus tissue and 9mg/g dry weight with callus tissue treated with tyrosine. However such difference may be simply due to using plants from different origins. Large variations exist among secondary metabolites derived from plants of different cultivars growing in different regions (Fu 1999). It is evident from the literature shown that colchicine content may be affected by plant age, seasonality and locality (Seifert, 1979).

Since colchicine is formed from demecolcine in the pathway (Figure 7.1), it is unsurprising that no demecolcine was detected in all the cultures used in this study. Yoshida, *et al.* (1988) have suggested that the turnover of demecolcine to colchicine is probably very high in *Colchicum autumnale* cell culture. Also colchicoside which is only one step after colchicine in the pathway was not detected in any of the cultures used in this study and this in agreement with Yoshida *et al.* (1988A and 1988B), who did not detect any colchicoside in *Colchicum autumnale* cell culture. Yoshida, *et al.* (1988), and Poutaraud, and Girardin (2003) have suggested that the glucosylation of colchicine probably occurs in specific tissue such as corm and seeds during maturation.

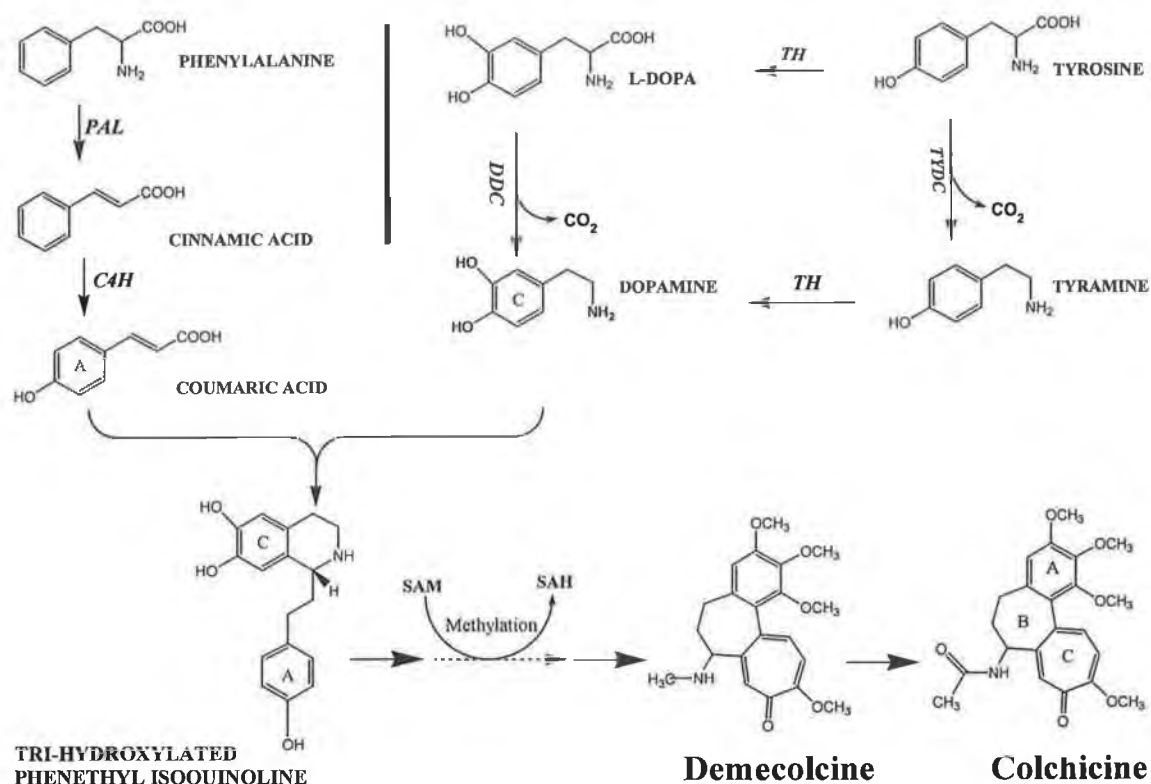


Figure 7.1: The early part of colchicine biosynthetic pathway. DDC= Dopa decarboxylase, TH= Tyrosine hydroxylase, TYDC= Tyrosine decarboxylase, PAL= Phenylalanine ammonia lyase, C4H= Cinnamic acid 4 hydroxylase. (Yoshida, *et al.* 1988B; Herbert *et al.*, 1990; Kattah, 1990; Nasreen, *et al.*, 1997).

In this study, the fact that the accumulation of colchicine did not cease with subculturing of the callus tissues of *Colchicum autumnale* and callus and root tissues of *Gloriosa superba* and was maintained at a constant level for at least two years after callus initiation, demonstrates active synthesis of colchicine.

That indicates that the callus culture did have the enzymes responsible for colchicine production, that the colchicine biosynthesis pathway was operational in such tissues, and that the colchicine present is not that carried over from the original explant. In conclusion, it is clear that callus and root culture of *Colchicum autumnale* and *Gloriosa superba* have the capacity to biosynthesis colchicine.

As has been stated, secondary metabolites are produced following long biosynthetic pathways that can involve dozens of enzymes. This synthesis is much more complex than for recombinant proteins produced with mammalian or prokaryotic biotechnologies, which usually involve one or two genes. This can partially explain the lack of success of plant cell and tissue cultures compared to other expression systems. The reasons for low accumulation of colchicine may: 1- Lack of expression in the genes which control the essential steps in the biosynthetic pathway. 2- The diversion of substrate away from secondary metabolism. 3- The non-availability of storage sites in which secondary metabolites would normally be sequestered.

Colchicum autumnale and *Gloriosa superba* callus tissue showed a similar ability for the accumulation of colchicine. This may suggest that it is likely that they possess the same biosynthetic pathway. However, they were different in the amount of colchicine accumulated as *Colchicum autumnale* accumulated relatively more colchicine than *Gloriosa superba* callus tissue. Since understanding the dynamics of metabolite production in plant cell and tissue cultures is a prerequisite to controlling productivity, it is reasonable to expect that attention focused on precursor feeding should lead to significant improvements. To enhance the formation of secondary metabolites in plant cell cultures a series of experiments were carried out in order to study the effects of precursors feeding on colchicine accumulation. The cultures confirmed the ability to produce colchicine when they were fed by precursors. Relatively low concentration of coumaric acid and 3-phenyl propionic acid significantly stimulated the colchicine accumulation while the other precursors showed no change in the amount of colchicine accumulation. Results from experiments directed to increase alkaloid production in plant cell cultures by feeding of precursors, especially of amino acids, vary considerably (Bohm *et al.*, 1979).

Precursor feeding has great potential to increase the yield of valuable pharmaceuticals. However, as outlined previously, there are many problems in realising this potential such as toxicity of the precursor.

Feeding radio labelled 3-phenyl propionic acid to callus cultures of *Colchicum autumnale* and *Gloriosa superba* showed that enzymes responsible for colchicine biosynthesis existed in the culture. The incorporation into colchicine was low, but by no means atypical of the findings from feedings of 3-phenyl propionic acid to whole plants of *Colchicum byzantium* (Herbert *et al.*, 1990).

At many steps in a secondary metabolic pathway, there is competition for the restricted pool of precursors. There are several possible approaches to increase the yield of a desired compound relative to other metabolites. One can increase the yield of a precursor primary metabolite to enlarge the pool available to secondary metabolism. This can up-regulate a large number of secondary pathways, both to the desired product, but also to other unwanted products.

Attempts to increase secondary metabolite formation have commonly involved the use of potential precursors. Sometimes this method is ineffective for various reasons; diversion of the precursor into other pathways or insufficiency of enzymes between precursor and product, the inaccessibility of the precursor to the biosynthetic site, and the inability effectively to utilize the precursor that does reach the site because of a lack of co-factors. Unless very specific precursors are used it would seem likely that diversion into primary or alternative secondary pathways is very likely.

Increasing colchicine level in the cultured plant system appears to rely on the biochemical modifications induced by precursor feeding. It was shown that precursor feeding is an important factor influencing both colchicine accumulation and release into the liquid medium of both *Colchicum autumnale* and *Gloriosa superba*. A significant fraction of colchicine produced was observed to be released to the liquid medium. The ability to increase the release of metabolites from intracellular storage compartment should decrease feedback inhibition that might otherwise shutdown metabolic pathways. From a biological point of view it makes sense because the compound should not act in the synthesizing cell but outside, where the site of action is.

One possibility is that cultures are unable to store secondary metabolites. The absence of such structures in the callus and cell suspension culture may repress biosynthesis. There may be an important change in the permeability or structure of the plasma membrane caused by the precursors. If we could characterize the mechanisms of the secondary metabolites release, it would be very helpful to us so that we can redirect cellular secondary metabolites flow from the inside to the outside of the cell.

A continuous *in situ* extraction and recovery of colchicine from the culture was developed using a solid phase extraction system, Amberlite resins XAD-4 and XAD-16 which showed a high adsorption capacity and binding affinity towards colchicine. This appears to be the first attempt in the use of a SPE in harvesting colchicine from the culture medium.

Many techniques and processes have been developed to increase secondary metabolite production in culture. The use of simultaneous application of multiple-strategies should yield a synergistic response. To increase the rate of secondary metabolite synthesis, harvesting the product is often used, together with other product-enhancing steps such as precursor feeding or elicitation (Asad and Shuler, 1989).

In an attempt to achieve better results in colchicine biosynthesis, a solid phase extraction combined with precursors feeding were employed, and these techniques were found to be positive. One advantage of precursor feeding and continuous removal of colchicine from the culture is a reduction in the toxic effect of the product on the cultures.

Bioreactors offer a great hope for the large-scale synthesis of therapeutically active compounds in medicinal plants. The production of colchicine can be enhanced using bioreactors. Continuous culture of a large quantity of root tissue offers the prospect of establishing a stable culture. The Amberlite column which facilitates the extracellular release of colchicine can be aseptically extracted *in situ* without interrupting bioreactor operation.

Colchicine accumulation in callus tissue were relatively lower than the cell suspension, and this may be due to the fact that the callus tissue only had one side in contact with the medium. As a result, nutrient and exogenously applied chemicals have to diffuse through the medium and the callus, thus making precursor feeding studies slow and uneven. Also nutrient uptake may be enhanced by submerged culture conditions which may stimulate growth rate and higher yield of secondary metabolites.

Many alkaloids are particularly difficult to produce in cell cultures and this may be due to a metabolic block of specific step in the pathway. The initiation and maintenance of culture capable of biosynthesis of a desired product from a specific plant source remains a difficult technical process, and however, tissue culture is extremely expensive, and can only be economically justified for the most expensive of products. Economic use of cell cultures will only be applicable in the production of high-value commercially required compounds, such as colchicine. Nevertheless, plant tissue and organ culture techniques have much to offer in studies of secondary metabolites biosynthesis.

7.2 Concluding remarks

It can be concluded that cultured tissues derived from the *Colchicum autumnale* and *Gloriosa superba* can be induced to elaborate colchicine to the same degree as the corresponding structure in the intact plant and the accumulation was stimulated by precursor feeding.

Neither the growth rate of the callus and root tissue nor their ability to accumulate colchicine is affected by exposure to light.

The finding of this work are in agreement with the generally held opinion that use of cultured plant systems for large-scale production of important secondary metabolites is a feasible proposition.

7.3 Recommendation for future work

1- The culture media used in the present study were SM and SH. Manipulating the culture conditions by using another known culture medium such as White or M5 it may be possible to improve the growth rate, and there is a chance that cells could be triggered to stimulate colchicine accumulation in tissue culture of *Colchicum autumnale* and *Gloriosa superba*.

2- Investigation of the enzyme activity in the colchicine pathway and identification of the rate-limiting step in the pathway. Callus and tissue culture of *Colchicum autumnale* and *Gloriosa superba* offer a good source for investigation of the enzyme activity involved in the colchicine pathway.

3- Identification of the potential bottleneck in the pathway and the genes responsible for the rate limiting enzyme which can be transferred to the cells to overexpress that gene in order to improve the rates of reaction.

4- Investigation of the effect of precursor feeding on colchicine release and the mechanism of its transport. Information on the mechanisms of transport of metabolic intermediates and products is important to this understanding.

5- Designing a continuous cultivation and extraction system where the tissues are kept in the bioreactor, the medium changed from time to time and the extraction column extracted and washed for reuse.

6- Optimization and validation of the application of on-line monitoring of the growth rate of *Gloriosa superba* root culture using medium conductivity and soluble carbohydrate in pilot and large scale.

7- Determination of the intracellular localization of colchicine and the biosynthetic site of colchicine synthesis in the cell.

Chapter Eight

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