CHAPTER 1

GENERAL INTRODUCTION

1.1. The Plant - *Mucuna pruriens*

*Mucuna pruriens* belongs to the family *Fabaceae* (*Leguminosae*) is grown predominantly in Asia, Africa and in parts of Americas. The genus of *Mucuna* includes about 150 species of annual and perennial legumes of pantropical distribution. Many species of the genus offer an excellent source as cover crop and green manure, in addition to their traditional use as feed and food [1]. India is one of the natural centres of origin of the *Mucuna* sp. in the world [2].

*Mucuna* has long been known and valued in Indian system of medicine and it is integral part of popular herbal remedy in India. It has been recognized as a powerful aphrodisiac in Ayurvedic medicine and shown to increase testosterone levels leading to the deposition of protein in the muscles and increased mass and strength [3]. *Mucuna used* as emetic and the bean if applied as a paste on scorpion stings is thought to absorb poison and the aqueous extracts of leaves shows anti-venom activity. Furthermore, *Mucuna* is widely used to treat nervous disorders, arthritis and sexual disease in Ayurvedic system of medicine. The seeds and other parts of this bean found to contain bio-active alkaloids like mucunine, *Mucuna dine*, mucuadine, pruriendine and nicotine, besides β-sitosterol, glutathione, lecithin, oils, venolic and gallic acids along with L-Dopa. The presence of higher amount of non-protein amino acid, L-Dopa, (L-3, 4, dihydroxy phenylalanine) i.e., 40 mg g⁻¹ is noticed, only in the species of velvet bean [4]. L-Dopa has been shown to be an effective drug for the treatment of Parkinson’s disease [5].

Traditionally, velvet bean has been used as a nerve tonic for nervous system disorders. Due to the high concentration of L-Dopa in the seeds, it has been studied for its possible use in Parkinson's disease. Dopamine does not cross the blood-brain barrier and therefore cannot be used directly for a treatment. *Mucuna* exhibited a neuroprotective effect by significantly restoring the levels of Dopamine in the
substantia nigra and norepinephrine in the nigrostriatal tract of a Parkinsonian animal model [6].

1.2. Parkinson’s Disease

Parkinson’s disease (PD) is a progressive neurodegenerative disorder with clinical features of bradykinesia (slowness), postural instability (balance), rigidity and resting tremor. The principal neuropathologic feature of PD is selective degeneration of midbrain Dopamine-producing neurons of the substantia nigra pars compacta (SNpc) that make up the nigrostriatal pathway, which results in depletion of Dopamine in the caudate nucleus and putamen (collectively termed the striatum).

The number of individuals with PD over the age of 50 in ten most populated countries was between 4.1 and 4.6 million in 2005 and will double to 8.7 - 9.3 million by 2030 due to increasing number of elderly people [7]. In the majority of populations, men are more likely to develop PD [8-9]. Inherited changes in the genetic makeup (DNA) can cause PD. However, only a small proportion of cases are due to genetic changes alone.

The drugs widely used in the treatment of PD motor system include Amantadine, MAO-B inhibitors (selegiline, rasagiline), carbiDopa/levodopa, pramipexole, ropinirole and apomorphine. In many parts of the world, wealth of knowledge in non-western medical systems, such as traditional Chinese or Ayurvedic medicine, may yield important new approaches to treat PD. One of the earliest descriptions of PD originates from the ancient Indian medical system, Ayurveda. According to the Ayurvedic system, the disorder was treated with seeds of *Mucuna*; *Mucuna* are most preferred for their enhancement of low efficacy rate of treatment of PD. L-Dopa has been considered the golden standard for the symptomatic treatment [10]. *Mucuna* sp. are one of nature’s best plant sources of L-Dopa and clinical studies have shown that seed sprouts have anti-Parkinson’s effects without any normal side-effects of the pure synthetic form [11].

Although the causes for degeneration of Dopaminergic neurons in PD are not well understood, emerging data from recent studies show that any sustained adverse interaction between neurotoxins arising from environmental, dietary and life style
factors, or from normal metabolism influenced by genetic factors also could initiate
degeneration in Dopaminergic neurons. One of the common mechanisms of action
of these neurotoxins is mediated by oxidative stress [12].

1.3. Free Radical Biology and Antioxidant Mechanism in Disease Prevention
Recent interest in dietary phenolics has increased due to their role as antioxidants,
antimutagens and scavengers of free radicals. Polyphenolic compounds are
ubiquitous in all vascular plant organs and are integral part of the human diet. Plant
phenolics have potential to function as antioxidants by trapping free radicals
generated in the oxidative chemistry [13]. The synthesis of free phenolics is
hypothesized to be regulated via the proline-linked pentose phosphate pathway,
shikimate pathway and phenylpropanoid pathway. This route is vital for the
biosynthesis of secondary metabolite including L-Dopa.

One of the major obstacles is the low yield of plant secondary metabolites in plant
cell cultures. Since the major roles of plant secondary metabolites are to protect
plants from invading insects, herbivores and pathogens, or to survive in biotic and
abiotic stress, some strategies for culture production of the metabolites based on this
principle have been developed to improve the yield of such plant secondary
metabolites. It includes treatment with various elicitors, signal compounds and
abiotic stress [14-19]. Many such treatments indeed effectively promote the

1.4. Elicitor Mediated Enhancement of Plant Secondary Metabolites
Small molecules of polysaccharides and proteins produced by an invading plant
pathogen act as ‘elicitors’ triggering the synthesis of phytoalexins in plant tissues
surrounding the infection site. Elicitors could also play an important role in
biosynthetic pathways and in the enhanced production of commercially important
compounds. Fungal elicitation has been an effective tool to enhance the yield of
secondary metabolites and also helping to elucidate the mechanism(s) of plant
responses to biotic stress agents. Elicitors have been shown to induce a range of
other plant secondary metabolites. The production of these dynamic defence
response exhibited by plant cells when challenged by an elicitor. Tissue culture is an
in vitro propagation technique of a wide range of excised plant parts, through which
mass of cells (callus) is produced from an explants tissue. The callus produced, can be utilized directly to regenerate plantlets or to extract or manipulate some primary secondary metabolite.

In this context, the present research project is aimed at the enhancement of L-Dopa from callus cultures of *Mucuna* seeds to ascertain their neuroprotective effect in suitable animal model and neuronal cell line.

1.5. OBJECTIVES OF THE STUDY

Aim of the study
The aim of this research is to achieve elicitation mediated enhancement of L-Dopa and other phenolic metabolites, which provides a mechanism to improve their nutraceutical functionality to cure PD. Further, to establish the neuroprotective mechanism by suitable animal model.

Objectives

- To carry out *in vitro* propagation of *Mucuna* seeds followed by the application of biotic and abiotic elicitors.
- To analyze L-Dopa and other poly phenolic compounds in elicitor treated plantlets.
- To study signal transduction mechanism in elicited *Mucuna* plantlets (Through Calcium ion channel- Calmodulin Pathway)
- To prove neuroprotective effect of elicited Calli extract of *Mucuna* on Dopaminergic neuronal cell line (N27) and *in vivo* studies conducted on MPTP intoxicated Model of PD.
1.6. OUTLINE OF THESIS

The present research work is aimed to enhance the L-Dopa production in *Mucuna pruriens* seeds through micropropagation for the treatment of PD and also to ascertain neuroprotective by suitable *in vitro* and *in vivo* model (Fig 1.1). Thesis is organised into 4 chapters, Chapter I deals with Introduction, provides a short review of the literature related to the objectives of the research. Further, a detailed literature review concerning *M. pruriens*, development of drug PD and alteration of L-Dopa based therapy, the demand and alternative strategy pursued to improve the production of L-Dopa. Chapter II deals with elicitor and precursor mediated enhancement of L-Dopa through micropagation and antioxidant activity efficacy also was reported. Chapter III depicts signalling transduction mechanism in connection with L-Dopa production. While, Chapter IV deals with the studies on neuroprotective effect of L-Dopa content by suitable *in vitro* and *in vivo* model.
1.7 LITERATURE REVIEW

1.7.1. Traditional Medicinal System
Herbal medicines are being used by about 80% of the world’s population particularly in the developing countries for primary health care. They have stood the test of time for their safety, efficacy, consumer acceptability and lesser side effects.

In recent years, there has been known interest on usage herbal drugs in the treatment against different diseases using herbal drugs as they are generally non-toxic. Plants are directly used as medicines by a majority of cultures around the world, especially in Chinese and Indian system of medicine. World Health Organization (WHO) recommended the evaluation of the effectiveness of plants in finding alternate source of modern drugs. Plant derivatives with hypoglycaemic properties have been used in folk medicine and traditional healing systems around the world since ancient time [20].

Medicinal plants are one of the most exclusive sources of life saving drugs for the majority of the world’s population. Use of plants as a source of medicine has been inherited and is an important component of the health care system. Plant based medicines have served the human race over the ages in treating various ailments and a traditional medicine system. There are about 45,000 plant species in India with concentrated hotspot in the region of Eastern Himalayas, Western Ghats and Andaman Nicobar Islands. The officially documented plants with medicinal potential are 3000 but traditional practitioners use more than 6000 plants. India is the largest producer of medicinal herbs and is appropriately called the botanical garden of the world [21].

Medicinal plants are now universally recognised as the basis for a number of factors including critical human health, social and economic support systems and benefits [22]. There has been a major resurgence in interest in traditionally used medicinal plants with a lot of international and local initiatives actively exploring the botanical resources of Southern Africa with an intention to screen indigenous plants for pharmacologically active compounds [23].
Medicinal plants are natural sources for different forms of phytochemicals like alkaloids, steroids and other chemical substances which, are being used to cure a variety of diseases. These chemicals with medicinal properties are present in one or all of their parts like root, stem, leaf, flower, fruit and seed [24]. The medicinal properties of many plants being well known, these plants have become important raw materials for the modern pharmaceutical industries. These medicinal plants are of great interest to researchers not only in the field of pharmaceuticals, foods and pesticides but also in biotechnology [25].

1.7.2. Tribal Medicinal plants
In India, information on chemical composition of seeds of trile pulses and wild progenitors of cultivated legume is relatively meager. While searching for newfood sources, nutritionally improved plants are now receiving more attention. [26] Pradhan(1995) emphasizesthe key role the legumes play in daily human diet, owing to their immense nutritional value with high protein, carbohydrates, fats vitamins, minerals, essential for tissue formation. Less known tribal pulses could make a useful contribution and, in some instances and pest resistance and processing good nutritional quantities [27].

1.7.3. Mucuna pruriens
The genus *Mucuna*, belonging to the family Fabaceae, sub family Papilionaceae, includes approximately 150 species of annual and perennial legumes. Among the various under-utilized wild legumes, the *Mucuna* (Fig. 1.2) also known as velvet bean is widespread in tropical and sub-tropical regions of the world.

Scientific Classification

<table>
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<tr>
<th>Kingdom: Plantae</th>
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<tr>
<td>Order: Fabales</td>
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<tr>
<td>Family: Fabaceae</td>
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<tr>
<td>Subfamily: Faboideae (Tribe: Phaseoleae)</td>
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<tr>
<td>Genus: Mucuna</td>
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<td>Species: Mucuna  pruriens</td>
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Fig. 1.2: Natural strands/habitat of the plant *Mucuna pruriens.*
1.7.4. Description of Plant

The genus *Mucuna* comprises a species of annual and perennial legumes with vigorously climbing habits that originated in southern China and eastern India, where the plants were widely cultivated as a green vegetable crop. *Mucuna* is self-pollinating hence natural out-crossing is rare; the life cycles range from 100 to 300 days to harvest of the pod and the genus thrives best under warm, moist conditions, below 1500 m above sea level, and in areas with plentiful rainfall [28].

The velvet bean has been traditionally used as a food source by certain ethnic groups in a number of countries. It is cultivated in Asia, America, Africa and the Pacific Islands, where its pods are used as a vegetable for human consumption, and its young leaves are used as animal feed. It is considered as a viable source of dietary proteins due to its high protein concentration (23–35%) in addition to its digestibility, which is comparable to that of other pulses such as soybean, rice bean, and lima bean [29-31].

1.7.5. Traditional Medicinal uses of *Mucuna*

*Mucuna* is widely used in Ayurveda, which is an ancient traditional medical science that has been practiced in India since the Vedic times (1500–1000 BC). It has long been, for treating diseases including Parkinsonism [32].

All parts of *Mucuna* possess valuable medicinal properties and it has been investigated in anti-diabetic, aphrodisiac, anti-neoplastic, anti-epileptic, and antimicrobial activities [32]. Its anti-venom activities have been investigated by and its anti-helminthic activity has been demonstrated by Jalalpure (2007) [33-34]. *Mucuna has* also been shown to be neuroprotective analgesic and anti-inflammatory activity [35-36].

1.7.6. Phytochemical Constituents

Seeds of velvet beans are known to produce the unusual nonprotein amino acid 3-(3,4-dihydroxyphenyl)-l-alanine (L-Dopa), a potent neurotransmitter precursor that is, at least in part, believed to be responsible for the toxicity of *Mucuna* seeds [37]. Besides it also contain some other compounds like glutathione, lecithin, gallic acid and beta-sitosterol. It has unidentified bases like mucunine, *Mucunadine*, prurienine, prurieninine. Other bases isolated from the pods, seeds, leaves and roots include
indole-3-alkylamines-N, N-dimethyltryptamine. Leaves gave 6-methoxyharman. Serotonin is present only in pods [38]. The seeds also contain oils including palmitic, stearic, oleic and linoleic acids [39].

1.7.7. Pharmacological Applications

*Mucuna* thoroughly studied for natural antioxidants, antitumor, hypoglycemic and hypolipidemic activities [40-44]. All parts of *Mucuna* possess valuable medicinal properties [45] like anti-diabetic [46] aphrodisiac, anti-neoplastic, anti-epileptic, antimicrobial activities [32] *Mucuna* has shown to enhancement learning and memory power of human beings [47]. It also demonstrated for the aphrodisiac, antivenom activities and antihelmintic activity [33-34, 48-49].

1.7.8. L-Dopa in various plants

*Mucuna* is used in case of spasms associated with Parkinsonism (or) Bell’s palsy [50]. L-Dopa is extracted from various *Mucuna* sp. seeds which have reported the yield of L-Dopa as 1.9% where as a simple hot water extraction method that gave excellent recovery of L-Dopa (3.1-6.1%) from the seeds of nine species of *Mucuna* [51]. Extract of *Mucuna* showed antiparkinson’s activity, compared with synthetic L-Dopa when demonstrated in Parkinsonian animal models. *Mucuna* extract has been reported to contain unidentified antiparkinsonian compounds in addition to L-Dopa (or) adjuvant responsible to enhance the efficacy of L-Dopa [52] on quantitative evaluation, *Mucuna* had a quick onset of action and significantly more active than L-Dopa [53].

The neuroprotective effects of a standardized extract of *Ginkgo biloba* were investigated on 6- hydroxy Dopamine (6-OHDA) induced neurotoxicity in the nigrostriatal Dopaminergic system of the rat brain. A significant improvement was observed in rats that were treated with higher doses of *Ginkgo biloba* (100 mg kg⁻¹ daily) than in those treated with lower doses (50mg kg⁻¹) (or) with vehicle. It indicates a possible role for the extract in the treatment of PD [54].

In 1913, Guggenheim first isolated dihydroxy phenylalanine in its levorotatory form from the extracts of *Vicia faba* beans. Recent studies have established the dose response L-Dopa absorption characteristics of *Vicia faba*. In single-dose studies
researchers have evaluated patients with pronounced “on – off” motor oscillations for the beneficial effect [54].

Petroleum ether, n-butanol extracts of roots of (Vitex negundo) produced moderate central nervous system (CNS) depression in experimental albino mice. Laboratory studies showed that butanolic and ethanolic extracts produced marked antiparkinsonian effect [55].

A single dose administration of Acantho panax senticosus plant extract elevated the noradrenaline and Dopamine level in the whole brain of rats in a dose dependent manner. A single (or) 2 weeks administration of Acantho panax senticosus (500mg kg⁻¹) showed a marked increase in Dopamine level in the striatum and antiparkinsonian activity [56].

Neuroprotective action of the ginseng extract was examined in two rodent animal models in PD. Ginseng recently demonstrated to possess neuroprotective properties which may be useful in preventing various forms of neuronal cell loss including the nigrostriatal degeneration seen in PD [50].

All the parts of Mucuna possess valuable medicinal properties. L-Dopa, a neurotransmitter precursor, has found wide application for symptomatic relief of PD and mental disorder. The need for L-Dopa is largely met by the pharmaceuticals industry through extraction of the compound from wild populations. Due to large-scale, unrestricted exploitation of the whole plant to meet its ever-increasing demand by the pharmaceutical industry, coupled with limited cultivation and insufficient attempts for its replenishment, the wild stock of this valuable medicinal plant has markedly depleted. In nature, this species propagates only through seed. Thus, conventional propagation through seed is not an adequate solution to meet the demand for this plant. Therefore, it is useful to devise a method for large-scale multiplication for commercial production. Tissue culture technology is successfully utilized in propagation of plants with poor and uncertain response to conventional propagation.
1.8. Parkinsonism – The neurodegenerative disease

In 1817, James Parkinson described in ‘An Essay on the Shaking Palsy’ a disease called ‘Paralysis angitians’. Parkinson’s disease (PD) is a progressive neurodegenerative disorder with clinical features that include bradykinesia (slowness), postural instability (balance), rigidity and resting tremor. The principal neuropathologic feature of PD is selective degeneration of midbrain Dopamine-producing neurons of the substantia nigra pars compacta (SNpc) that make up the nigrostriatal pathway, which results in depletion of Dopamine in the caudate nucleus and putamen (collectively termed the striatum).

The most common drugs used in the reatment of PD motor system, Amantadine, MAO-B inhibitors (Selegiline, Rasagiline), CarbiDopa/levoDopa, Pramipexole, Ropinirole and Apomorphine. In many parts of the world, wealth of knowledge in non-western medical systems, such as traditional Chinese or Ayurvedic medicine, may yield important new approaches to treat PD. *Mucuna* are most preferred for their enhancement of low efficacy rate of treatment of PD.

![Biosynthesis of Dopamine](image-url)

**Fig.1.3: Biosynthesis of Dopamine**
1.9 Epidemiology of Parkinson’s disease

1.9.1 Burden of Global Population in the World for Parkinson’s disease

PD ranks among the most common late life neurodegenerative diseases, affecting approximately 1.5% to 2.0% of the population older than age 60. The causes of PD, the second most common neurodegenerative disorder, are still largely unknown. Current thinking is that major gene mutations cause only a small proportion of all cases and that in most cases; non-genetic factors play a part, probably in interaction with susceptibility genes. Numerous epidemiological studies have been done to identify such non-genetic risk factors, but most of them are methodologically limited [57].

Between 1998 and 2004, WHO invested in improving the conceptual, methodological and empirical basis of assessments of burden of disease and injury attributable to major risk factors [58-62]. The WHO defines the burden of disease as a measurement of the gap between current health status and an ideal situation where everyone lives into old age free of disease and disability. For PD, the burden of disease in 2001 was estimated as 2,325,000 cases [63]. These estimates are based on incomplete knowledge of the numbers of people with PD world wide; the true impact is likely higher. The burden of PD is expected to rise as the number of persons with PD increases in the next decades.
1.9.2 Burden of Global Population in India

PD also has a heavy social and economic burden. Several epidemiological studies have been done about the Parkinson's disease [64]. Population of elderly Indians has increased from 5.6% (51 million) in 1961 to 7.1% (71 million) in 2001.

Along with other neurodegenerative diseases like Alzheimer’s disease and motor neurone disease, PD is expected to surpass cancer as the second most common cause of death by the year 2040 [65]. Assuming that 1% of the population over 60 years has PD, it was expect about 930,000 PD patients in 2010 and more than 1 million (1,130,000) PD cases by the year 2016 in India.

Of the world’s 580 million elderly (>60 yrs), 355 million (61%) living in developing countries and of these, 77 million (22% of total) live in India. To caring for this increasing elderly population can be challenging as 80% of elderly Indians live in rural areas, 73% are illiterates, 60% are women and 60% live below the poverty line.

As these demographic age groups are growing rapidly due to general aging of the population and increasing life spans, neurodegenerative diseases will represent an ever-growing economic and social burden for society [66-67]. If the total populations of India1162 million by 2010 that in 2010 the total Indian population would be 1162 million, of whom 93 million would be over 60 years of age. Projecting further, it is estimated that in 2016, of a total population of 1263 million, 113 million would be over 60 years [68].

PD affects people of all races, geographic areas and socioeconomic levels. Rates of getting PD are higher in men than women. The average age of diagnosis of PD is 60. Eighty percent of people with PD are diagnosed between the ages of 40 and 70, but five percent are diagnosed between 30 and 40 years old [69].
Projected Increase in Prevalence of PD by 2030
1.10 Dopaminergic Pathway in Brain

PD is a common progressively debilitating neurodegenerative disease that affects the Dopaminergic neurons and extra-nigral projection bundles that control sensory, cognitive, promoter and motor Pathways [70]. It is characterized by neuronal cell loss in the SN resulting in eventual depletion of Dopamine in the nigral striatal pathways culminating in pathologies of some key body systems and functions especially the motor function [71].

The initial loss of Dopaminergic neurons and subsequent decreased production of Dopamine in the substantia nigra in PD distorts the normal Dopaminergic and cholinergic pathway that manifests as the characteristic abnormal motor systems often seen in PD [72]. Other neurotransmitters are also affected such as norepinephrine, gamma-amino butyric acid (GABA) and serotonin.

Fig. 1.4 Dopaminergic Pathway in Brain
Most central Dopaminergic neurons originate in discrete areas of the brain and have divergent projections (Fig 1.4). Three major pathways can be distinguished such as,

1. Motor control [Nigrostriatal pathways]
2. Behavioral effects [Mesolimbic pathways]
3. Endocrine control [Tubero infundibular pathways]

Dopaminergic Neurons that originate in the hypothalamus and project to the pituitary gland. The largest Dopamine tract in the brain is the nigrostriatal system, which contains 80% of the brain’s Dopamine.

1.10.1. Nigrostriatal Pathway in Brain [Motor Control]

The *substantia nigra*, part of the extra pyramidal system, is the source of Dopaminergic neurons that terminate in the striatum. Each Dopaminergic neuron makes thousands of synaptic contacts within the striatum and therefore modulates the activity of a large number of cells. These Dopaminergic projections from the *substantia nigra* fire tonically, rather than in response to specific muscular movements or sensory input. The striatum is connected to the *substantia nigra* by neurons that secrete the inhibitory transmitter GABA at their termini in the *substantia nigra*. In turn, cells of the *substantia nigra* send neurons back to the striatum, secreting the inhibitory transmitter Dopamine at their termini. This mutual inhibitory pathway normally maintains a degree of inhibition of the two separate areas. Nerve fibers from the cerebral cortex and thalamus secrete Ach in the neostriatum, causing excitatory effects that initiate and regulate gross intentional movements of the body.

In PD, destruction of cells in the *substantia nigra* results in the degeneration of neurons responsible for secreting Dopamine in the neostriatum. Thus the normal modulating inhibitory influence of Dopamine on the neostriatum is significantly diminished, resulting in the Parkinsonism degeneration of the control of muscle movement [73-74].
1.10.2. Mesolimbic Pathways [Behavioural effect]
The cell bodies of these pathways arise from midbrain (mesencephalon) to end in the nucleus accumbens (which is situated cranial to the corpus striatum and is part of limbic system). Hyperactivity of this tract leads to schizophrenia [73-74].

1.10.3 Tubero Infundibular Pathways [Endocrine control]
The cell bodies of these pathways arise from the arcuate nucleus of the hypothalamus to end in the lactotrophs (prolactin secreting cells of the anterior pituitary). Activity of this tract causes inhibition of prolactin secretion [73-74].

1.11 Pathophysiology
These lead to profound and irreversible striatal Dopamine loss. Many factors are speculated to operate in the mechanism of cell death of the nigrostriatal Dopaminergic neurons in PD, including oxidative stress and cytotoxicity of reactive oxygen spices (ROS), disturbances of intracellular calcium homeostasis, exogenous and endogenous toxins, and mitochondrial dysfunction. Moreover, increased risk of localized oxidative damage for Dopaminergic neurons is linked to Dopamine metabolism itself. With the exception of rare familial forms, the majority of PD cases are sporadic and due, in part, to mitochondrial defects at complex I. Moreover, the presence of ubiquitinated and misfolded proteins suggests the dysregulation of protein assembly or defects in protein degradation pathway as a critical part of disease pathogenesis. Misfolding and abnormal degradation of brain proteins are linked to Dopaminergic neuronal death.

1.12 Risk Factors of Parkinsonism
The causes of PD remain essentially unknown. Although an inverse relationship has been demonstrated in a number of studies with cigarette smoking, coffee drinking and PD [75-78]. Others have insisted on the contrary or have maintained that the relationship between cigarette smoking and PD [79-80].

1.12.1. Non-Genetic Risk Factor
Environmental
Many environmental risk factors for PD have been proposed on the basis of presumed pathogenetic mechanisms of the disease. Occupational exposures to pesticides, herbicides, and heavy metals and after intravenous injection of drugs
contaminated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and the subsequent finding that MPTP selectively damages Dopaminergic cells in the substantia nigra led to the hypothesis that exposure to environmental toxins might be related to the risk of PD [81].

**Oxidative stress in Parkinson’s disease:**

\[
DA + O_2 + H_2O \\
\text{Autoxidation by MAO} \\
DOPAC +NH_3+H_2O_2 \\
Fe^2 \rightarrow \text{OH+ OH} + Fe^{3+}
\]

Autoxidation processes in the basal ganglia

Examples of reactions involving Dopamine and leading to formation of free radicals and oxidation of protective substances such as glutathione A, (Dopamine), H_2O_2, (hydrogen peroxide), 3,4-DHPA (3,4-dihydroxyphenylacetaldehyde) OH^−, (hydroxyl ion), OH, (hydroxyl radical), Ferric ion [82-84].

**Tobacco and alcohol**

**Smoking**

Smoking of cigarettes is among the most studied risk factors for PD and one of the few for which very consistent results were obtained. Many epidemiological studies have shown a reduced risk of PD among cigarette smokers.

**Alcohol consumption**

The findings on smoking and coffee consumption and the hypothesized role of Dopaminergic reward systems have led some researchers to examine the association between alcohol consumption and the risk of PD. However, results of a number of case-control studies and some prospective cohort studies have not been very straightforward, with inverse associations in some studies but no significant association in others [85-86].
Dietary factors

Various food groups and specific nutrients have been investigated as potential risk factors that are either related to a high or low risk of PD. In most epidemiological studies, dietary habits are assessed by means of a food frequency questionnaire, which unavoidably leads to a certain amount of error and thus misclassification of intake. Moreover, intakes of many nutrients are highly correlated and specific associations are therefore not always easily identified. Most epidemiological research on dietary factors comprised case-control studies. Only a few population-based prospective cohort studies have been done [87-88].

Antioxidants

The focus in nutritional epidemiology has been mainly on antioxidants, given the presumed central role of oxidative stress in the pathogenesis of PD. Antioxidants, such as vitamins E and C, might protect cells against oxidative damage by neutralizing free radicals.

Fat and Fatty Acids

The relation between dietary fat and PD is unclear. Diets with high lipid content could theoretically increase the amount of oxygen radicals by lipid peroxidation and thus increase the risk of PD.

Dietary iron

Iron may induce free radical formation and increased iron levels have been found in the substantia nigra of patients with PD. Two case-control studies found a positive association between iron intake and PD but two others reported no association. Results of prospective studies on the relation between dietary iron and the risk of PD have not been published.

Inflammation

The role of inflammation in the pathogenesis of PD is unknown. Up regulation of cytokines was found in the brains and cerebrospinal fluid of patients with PD and activated Glial cells have been observed in post-mortem material. However, whether this immune response is the cause or rather a consequence of neurodegeneration is
unclear, because no prospective studies have investigated inflammatory markers in relation to PD [89-92].

**Estrogens**
The role of estrogens in PD is disputed. The higher prevalence and incidence of PD in men in various epidemiological studies have prompted the hypothesis that female sex hormones would somehow protect against neuronal cell death. Animal studies have provided evidence for a potential beneficial effect of estrogens on PD, possibly through antioxidant properties [93].

**PD and cancer**
Some epidemiological evidence suggests a low incidence of many common types of cancers in individuals with PD did not hold, because a low incidence has been described for both smoking-related and non-smoking related cancers [94].

### 1.12.2. Genetic Risk Factors

**Causative Genes**
A mutation of gene responsible for familial Parkinsonism was located on the chromosome 4q21-q23. Shortly, the mutation was identified as an Ala53Thr substitution in the region encoding for a protein known as α-synuclein. A second mutation resulting in an Ala30Pro substitution was later identified. Interestingly, it has been shown that α-synuclein is one of the components of Lewy bodies. A mutation of the parkin gene, located on the locus 6q25.2-q27, was also reported in the autosomal case of juvenile Parkinsonism. Mitochondrial DNA mutation may also contribute to PD [91].

**Hypothesis of PD**
The experimental therapies for PD that are under investigation at present assure improvement on the limitations of existing treatments. The prospect of progress in understanding the pathogenesis of the disorder will improve the development of novel molecules and treatments that will retard, or revert, the currently inexorable progressive course of PD. PD is a chronic, progressive, unremitting neurological disorder. Nevertheless, the symptoms found at clinical onset, those that develop throughout the course of the illness, responsiveness to pharmacologic therapy and
disease progression are highly variable [95]. For years, physicians have observed that patients with tremor as the primary manifestation appear to have a better prognosis and possibly respond better to L-Dopa than those with prominent postural instability and gait difficulty [96]. Despite advances in pharmacotherapy that have improved quality of life for these patients, the mortality rate remains largely unchanged. Existing drugs are symptomatic and temporarily ameliorate the symptoms of PD. Nevertheless, symptom progression can be slowed and quality of life improved with current methods of treatment. Pharmacotherapy for PD includes L-Dopa, Dopamine agonists, monoamine oxidase (MAO) inhibitors, anti-cholinergics and most recently, Catechol-O-methyltransferase (COMT) inhibitors [97]. The most effective drug for relieving the symptoms of PD is L-Dopa. A combination of L-Dopa and carbiDopa is widely used. The carbiDopa reduces L-Dopa's peripheral conversion to Dopamine by inhibiting Dopa decarboxylase. This in turn reduces side effects and increases the amount of L-Dopa available for uptake into the CNS. Long-term L-Dopa therapy is associated with motor complications that can be as disabling as the disease itself. Dopamine agonists hold promise because of more sustained stimulation of Dopamine receptors. This class of drugs includes bromocriptine mesylate, pergolide mesylate, cabergoline, pramipexole, and lisuride and ropinirole hydrochloride. These drugs stimulate Dopamine receptors and some of them have been used for many years in the treatment of PD. Recently, they have been gaining popularity for several other reasons. For example, they are longer acting, which creates more sustained stimulation of Dopamine receptors and less pulsatility. In theory, this should reduce the progression of motor complications. Another group of drugs, which is promising and most widely used is, the selective inhibitors of MAO-B. Selegiline hydrochloride does not stop the progression of PD but has been shown in controlled trials to be neuroprotective, delaying the need for L-Dopa therapy. Many of this class of drugs have antioxidant action in Parkinson study group. Selegiline is generally used early in the illness because of its putative neuroprotection and because it has only a minimal effect on symptoms. It has few side effects, but like other MAO-B inhibitors, it cannot be used in a patient taking selective serotonin reuptake inhibitors. The other MAO-B inhibitors such as rasagiline, lazabemide, entacapone, and moclobemide are under clinical trial.
1.13 Experimental Animal Models of Parkinson’s disease

An ideal animal model of PD should reproduce the progressive, selective nigrostriatal Dopaminergic neurodegeneration and recapitulate most of the features of PD. It should cause a preferential loss of Dopaminergic neurons within the SNpc, leading to motor dysfunction. In addition to the loss of nigrostriatal Dopamine neurons, a key determinant that differentiates this disorder from other neurodegenerative diseases is the Lewy bodies’ inclusion formation.

1.13.1 6-HydroxyDopamine (6-OHDA) Model

6-HydroxyDopamine was the first chemical agent discovered that has specific neurotoxic effect on catecholaminergic pathways [98-99]. 6-OHDA is a hydroxylated analogue of Dopamine and thus uses the same catecholamine transport system. This produces specific degeneration of catecholaminergic neurons. Systemically administrated 6-OHDA is unable to cross the blood–brain barrier. Stereotaxically, 6-OHDA injected into SNpc or the ascending medial forebrain (MFB) or the striatum for the specific target to nigrostriatal Dopaminergic pathway. Following 6-OHDA injection, Dopaminergic neurons start degenerating within 24 hours and takes 2-4 weeks to induce striatal Dopamine depletion. The magnitude of the lesion is dependent on the amount of 6-OHDA injected and the site of injection. Injection of 6-OHDA directly to the striatum causes a retrograde degeneration of the nigrostriatal system over a period of weeks and has been used to mimic the slow progressive nature of PD [100]. Unilateral 6-OHDA injection leads to asymmetric rotational motor behavior after administration of Dopaminergic drugs like amphetamine or apomorphine, due to physiologic imbalance between the lesioned and the unlesioned striatum. This can be quantified and correlated with degree of neuronal lesion [98]. Drawback of the 6-OHDA model is that it differs from progressive degeneration of the Dopaminergic nigral neurons in PD. The 6-OHDA model lesion has been used to ascertain the efficacy of antiparkinsonian compounds [101].

1.13.1. 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) Model

Remarkable clinical symptoms similar to sporadic PD in humans result after injection of 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) [102]. After administration, MPTP crosses the blood brain barrier and is metabolized in astrocytes to it active metabolite 1-methyl-4-phenylpyridinium ion (MPP+), by
monoamine oxidase-B (MAO-B). MPP\(^+\) is selectively taken up by Dopaminergic neurons due to its affinity for the Dopamine transporter and these results in selective toxicity to Dopaminergic neurons [103]. Exposure to MPTP results in nigrostriatal Dopaminergic pathway with 50% to 93% cell loss in the substantia nigra pars compacta and more than 99% loss of Dopamine in the striatum [104]. Rats are resistance to MPTP toxicity and mouse strains vary widely for sensitivity to the toxin. Neurochemical changes following MPTP exposure include decreased level of Dopamine and its metabolites in the striatum, increased oxidative damage as evidenced by increased lipid peroxidation, increased 3-nitrotyrosine levels and diminished concentration of antioxidants, such as glutathione (GSH) and superoxide dismutase (SOD), etc. Limitations of MPTP model are its failure to mimic progressive nature of PD. Additionally, the MPTP model does not produce lewy bodies in rodents.

1.13.3 MPP\(^+\) Model
Infusion of MPP\(^+\) into the median forebrain bundle in rats caused significant loss of Dopaminergic neurons in the SNpc with ensuring behavioral, neurochemical and biochemical changes characteristic of the lesion [105]. Unilateral intranigral administration of MPP\(^+\) produced dose-dependent depletion of Dopamine in the ipsilateral striatum of rats following two weeks of infusion [106]. Intrastriatal infusion of MPP\(^+\) in rats caused severe biochemical lesion and behavioral symptoms and also helped in studying the neuroprotective action of non-steroidal anti-inflammatory drugs in MPP\(^+\) induced neurotoxicity [107].

1.13.4. Rotenone Model
This novel model of PD is based on chronic systemic exposure of rats to rotenone a pesticide and complex I inhibitor [108]. Rotenone is lipophilic compound that easily crosses the blood brain barrier. The rotenone model appears to be an accurate model, since chronic exposure to rotenone resulted in uniform and selective Dopaminergic neuronal damage, selective striatal oxidative damage and formation of ubiquitin and \(\alpha\)-synuclein-positive inclusions in nigral cells. These were similar to the Lewy bodies observed in human PD. The major problem associated with this animal model is its nature of variability, with only some animals.
1.13.5. Genetic Model

Genetic defects cases PD in a small percentage of populations. Mutation in three different gene, including α-synuclein, have been associated with familial PD [109]. Since α-synuclein is a major component of lewy bodies and mutations in α-synuclein may result in nigrostriatal dopaminergic degeneration in familial PD, animal model have been developed to investigate the role of α-synuclein in the etiology of PD.

This model system focused on the use of transgenic mice or drosophila, which express the wild type or mutated α-synuclein. Transgenic mice over expressing human α-synuclein demonstrated a number of features of PD, including loss of nigrostriatal Dopaminergic nerve terminal in striatum, development of α-synuclein and ubiquitin-positive cytoplasmic inclusion and motor impairments [110].

*Mucuna* exhibited a neuroprotective effect by significantly restoring the levels of dopamine in the *substantia nigra* and norepinephrine in the nigrostriatal tract of a Parkinsonian animal model. Synthetic commercially available drug, levedopa, is in practice to treat PD but it is associated with a variety of chronic side effects like fluctuations among motor performance, confusion and hallucination and limiting anti-Parkinsonian efficacy [111]. *Mucuna* is one of nature’s best plant sources of L-Dopa and clinical studies have shown that seed sprouts have anti-Parkinson’s effects without any normal side-effects of the pure synthetic form [11].

L-Dopa is used to increase dopamine concentrations in the treatment of PD and dopamine-responsive dystonia since it is capable of crossing the protective blood-brain barrier, which dopamine itself cannot. Once L-Dopa has entered the central nervous system, it is converted into dopamine by the enzyme aromatic L-amino acid decarboxylase, also known as dopa decarboxylase. The occurrence of this unusual amino acid (L-Dopa) in the seeds of different plant species like *Vicia faba*, *Baptisia* species and *Lupinus* species is well-known [112-113]. In 1976 Brain [114] reported that L-Dopa was accumulated in solid media on which *Mucuna* calli were grown.
1.14 *In vitro* propagation

1.14.1. Plant tissue culture

Plant tissue culture technology is being widely used for large-scale propagation.

1.14.1.1. General

Plant tissue culture, also referred to as cell, *in vitro*, axenic, or sterile culture, is an important tool in both basic and applied studies, as well as in commercial application [115]. Plant tissue culture is the aseptic culture of cells, tissues, organs and their components under defined physical and chemical conditions *in vitro*. The theoretical basis for plant tissue culture was proposed by Gottlieb Haberlandt in his address to the German Academy of Science in 1902 on his experiments on the culture of single cells [116]. He, thus, clearly established the concept of totipotency and further indicated that the technique of cultivating isolated plant cells in nutrient solution permits the investigation of important problems from a new experimental approach. This approach of using explants with meristematic cells produced the successful and indefinite culture of tomato root tips [25].

1.14.1.2 Historical Background

In the 1930s the first *in vitro* cultures were established [117] and this was followed by a period of development of culture media and of cultivation methods [118]. In these early efforts, plant cells in culture were treated in direct analogy to microbial systems, with little knowledge of plant cell physiology and biochemistry or the influence of bioreactor operation on the physiologic state of such systems. In 1982, at least 30 compounds were known to accumulate in plant culture systems in concentrations equal to or higher than that of the plant [119]. Concerning the production of natural plant metabolites, some *in vitro* systems exist that allow the large-scale production of economically important plant metabolites [120]. A survey of the historical milestones in plant cell cultures is given by Schmauder and Doebel [121]. Strategies to optimize growth and product formation began to develop separately during the period between 1975 and 1985. During the last thirty years, tissue culture-based plant propagation has emerged as one of the leading global agrotechnologies. Between 1986 and 1993, the worldwide production of tissue cultured plants increased by 50%. In 1993, the production was 663 million plants and by 1997 the production had risen to 800 million plants.
More recently, some companies from Israel, USA and UK have shifted their production requirements to countries like Costa Rica and India [122]. Application of transgenic plant culture for the production of medicines is an emerging area of biotechnology.

Plant cell cultures are able to produce secondary metabolites came quite late in the history of in vitro techniques. It had 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 [123]. Zenk and co-workers experimentally demonstrated that this theory was wrong, as they could observe dedifferentiated cell culture of *Morinda citrifolia* yielding 2.5 g of anthraquinones per liter of medium [124]. This finding opened the door to a large community of *in vitro* culturists who extensively studied the possible use of plant cultures for the production of secondary compounds of industrial interest, mainly pharmaceutics and dyes.

### 1.14.1.3 Callus Culture

Regeneration of plants by micropropagation of *in vitro* cultures can be achieved from organ primordia existing in shoot tips and axillary bud explants. Alternatively, plants can be regenerated from unorganized callus tissues derived from different explants by dedifferentiation induced by exogenous growth regulators. Plant regeneration from callus is possible by *de novo* organogenesis or somatic embryogenesis. Callus cultures also facilitate the amplification of limiting plant material. In addition, plant regeneration from calli permits the isolation of rare somaclonal variants which result either from an existing genetic variability in somatic cells or from the induction of mutations, chromosome aberrations and epigenetic changes by the *in vitro* applied environmental stimuli, including growth factors added to the cultured cells [125-127].

Datta and Srivastava (1997) [128] initiated callus cultures from different explants in *Cantharanus roseus* of different age and observed detectable quantities of vinblastine in the calli seedling origin. They also observed that as the callus differentiated into multiple shoots, the vinblastine production increased rapidly equal to that of *in vitro* seedlings of similar age. Plant regeneration and callus induction study in periwinkle
using leaf, shoot tip hypocotyls, epicotyls and root explants with and without intervening callus phase.

The effects of Murashige and Skoog (MS) medium in combination with different types of plant growth regulators i.e., 2,4-dichlorophenoxy acetic acid (2,4-D), Napthaleneacetic Acid (NAA), 4-Amino-3,5,6- trichloropicolinic acid (Picloram), 3,6-dichloro-o-aniscic acid (Dicamba), indole-3-acetic acid (IAA), 3- indolebutyric acid (IBA), 6-Benzyl Adenine (BAP), kinetin and Thidiazuron (TDZ) on callus induction were investigated. In this study, we reported the selection of elite plant material for the production of high quantities of flavonoid from callus culture’s of *Cantharanus asiatica* to ensure consistent production of active compounds.

1.14.1.4 Growth regulators

The application of IAA or IBA as the sole growth regulator was effective in inducing callus from leaf and internode explants. Cytokinins BA and KN together with auxins can be used for efficient induction of callus. Addition of 2,4,D to the culture medium had a positive effect on callus induction. Callus induction from mature leaves took place at various auxin/cytokinin concentration ratios. Callus produced from *in vitro* culture produced a vast variety of genetic changes. Such variations can result in useful agricultural, horticultural, pharmacological and biochemical products [129].

In many plant species of *Azadirachta indica* callus culture was derived from the matureseedlings grown *in vitro* from hypocotyls, epicotyl, cotyledonary node, leaves and roots of 7 day old seedlings [129-130]. Among a number of other components in the medium, phytohormones such as auxins and cytokinins have shown remarkable effect on callus growth and secondary metabolites production. There are some other reports in the literature on the need of citric acid, ascorbic acid, polyvinyl pyrrolidone for callus induction since they control the secretion of phenols.

1.15. Successful Commercial L-Dopa Production through Cell Culture

Most applications of plant cell suspension cultures in biotechnology are aimed at the production of naturally occurring secondary metabolites [Table 1.1]. This has
included production of shikonin, anthocyanins, and ajmalicine and, recently, important anti-tumor agents like taxol, vinblastine and vincristine [131].

**L-Dopa**

L-3,4-dihydroxyphenylalanine, is an important intermediate of secondary metabolism in higher plants and is known as a precursor of alkaloids, betalain, and melanine, isolated from *Vinca faba, Mucuna*, *Baptisia* and *Lupinus* [132]. The widespread application of this therapy created a demand for large quantities of L-Dopa at an economical price level, and this led to the introduction of cell cultures as an alternative means for enriched production [133]. The callus tissue of *Mucuna* accumulated 25 mg L\(^{-1}\) Dopa in the medium containing relatively high concentrations of 2, 4-D [134] induced callus tissues of *M. hassjoo*, *M. Pruriens*, and *M. deeringiana* and optimized the culture conditions. The highest concentration of Dopa was obtained when *M. hassjoo* cells were cultivated in MS medium with 0.025 mg L\(^{-1}\) 2, 4 – D and 10mg L\(^{-1}\) kinetin. The level of Dopa in the cells was about 80 m mol g\(^{-1}\)-fresh weight [135].

**Table 1.1: Production of secondary metabolite production from callus and cell suspension culture from different plant species**

<table>
<thead>
<tr>
<th>Plant name</th>
<th>Active ingredient</th>
<th>Culture type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Capsicum annuum L.</em></td>
<td>Capsaicin</td>
<td>Suspension</td>
<td>Johnson et al., 1990</td>
</tr>
<tr>
<td><em>Coffea arabica L.</em></td>
<td>Caffeine</td>
<td>Callus</td>
<td>Waller et al., 1983</td>
</tr>
<tr>
<td><em>Podophyllum hexadrum royle</em></td>
<td>Podophyllotoxin</td>
<td>Suspension</td>
<td>Chattopadhyay et al., 2002</td>
</tr>
<tr>
<td><em>Portulaca grandiflora</em></td>
<td>Betacyanin</td>
<td>Callus</td>
<td>Schroder and Bohm, 1984</td>
</tr>
<tr>
<td><em>Salvia fruticosa</em></td>
<td>Rosmarinic acid</td>
<td>callus &amp; suspension</td>
<td>Karam et al., 2003</td>
</tr>
<tr>
<td><em>Stizolobium hassjoo</em></td>
<td>L-DOPA</td>
<td>Suspension</td>
<td>Huang et al., 2002</td>
</tr>
<tr>
<td><em>Mucuna pruriens</em></td>
<td>L-DOPA</td>
<td>Suspension</td>
<td>Wichers et al., 1993</td>
</tr>
<tr>
<td><em>Mucuna pruriens</em></td>
<td>L-DOPA</td>
<td>Callus</td>
<td>Brain et al., 1976</td>
</tr>
<tr>
<td><em>Scutellaria columnae</em></td>
<td>Phenolics</td>
<td>Callus</td>
<td>Stojakowska and Kisiel, 1999</td>
</tr>
<tr>
<td><em>Lithospermum erythrorhizon</em></td>
<td>Shikonin derivatives</td>
<td>Suspension</td>
<td>Fujita et al., 1981</td>
</tr>
<tr>
<td><em>Lithospermum erythrorhizon</em></td>
<td>Shikonin derivatives</td>
<td>Suspension</td>
<td>Fukui et al., 1990</td>
</tr>
</tbody>
</table>
1.16. Approaches to increase secondary metabolites Production

1.16.1. Optimization of Culture Conditions

Number of chemical and physical factors like media components, phytohormones, pH, temperature, aeration, agitation, light affecting production of secondary metabolites has been extensively studied [136-139]. Cultured plant cells offer various advantages over the field plants for the production of both primary and secondary metabolites. Cultures of plant cells are not limited by environmental, ecological or climatic conditions [140]. Suspended cells with high embryogenic potentials should be selected to maintain a stable production of quality somatic embryos [141]. Plant cell cultures combine the merits of whole-plant systems with those of microbial and animal cell cultures for the production of valuable therapeutic secondary metabolites [142].

Manipulation of physical aspects and nutritional elements in a culture is perhaps the most fundamental approach for optimization of culture productivity. For example, ginsenosides by *Panax ginseng* [143-146], rosmarinic acid by *Coleus bluemei* [147], shikonin by *Lithospermum erythrorhizon* [148], ubiquinone-10 by *Nicotiana tabacum* [149], berberin by *Coptis japonica* [150], were accumulated in much higher levels in cultured cells than in the intact plants.

Sucrose is the main source of carbon energy for *in vitro* cultures. Plant cell and tissues in a culture medium lack autotrophic ability and therefore, need external carbon for energy [151]. Sucrose is the main source of carbon energy for *in vitro* cultures. The growth of the shoots is also affected by differences in the concentration of sucrose [152-153]. Plant cells and tissues also require an optimum pH for growth and development in cultures. The pH affects nutrient uptake as well as enzymatic and hormonal activities in plants [154]. The changes in external pH have a small transient effect on cytoplasmic pH but the cells are readily readjusted towards their original pH [155], thus the effect of external pH on cytoplasm is not long lasting. However, this change may affect plant growth by the conversion of inorganic phosphate into organic phosphate at the extracellular region. The detrimental effects of adverse pH are generally related to an imbalance in nutrient uptake rather than to direct cell damage.
1.16.2. Addition of Precursor of L-Dopa Production

Several strategies such as manipulating the nutrient, optimizing the culture conditions, feeding of precursor and elicitation can be applied in order to substantially increase the yields of secondary metabolites in plant cell cultures [156]. As an example, feeding of 0.2 mmol L\(^{-1}\) of L-phenylalanine, one of the precursors in the phenylpropanoid pathway, to cell culture of *Cistanche deserticola* resulted in 75% higher production of phenylethanoid glycosides when compared to the cell culture without precursors [157]. The other possibility for increasing the secondary metabolite synthesis is the supplying of plants or their cultures with the precursors.

In order to enhance the synthesis of secondary metabolites, several organic compounds can be added to the culture medium [158]. The regulation of amino acids pathway under some stress condition dominated by the need for secondary metabolite derived from the pathway [159]. Isoflavones and flavonoids originated from phenylalanine, an upstream metabolic precursor through phenylpropanoid pathway. Supplementation of phenylalanine is expected to increase elevated level of target compound [160].

1.16.3. Biotic and Abiotic elicitors

Plant secondary metabolites are unique resources for pharmaceuticals, food additives, and fine chemicals. They also provide original materials used in other areas. Besides direct extraction from plants and chemical synthesis to provide those compounds or derivatives with similar uses, plant cell culture has been developed as a promising alternative for producing metabolites that are difficult to be obtained by chemical synthesis or plant extraction. Plants protect themselves against pathogens using multiple mechanisms, including synthesis of pathogenesis-related (PR) proteins, generation of reactive oxygen species and accumulation of phytoalexins [161-162].

From those studies it became apparent that elicitors could be used as enhancers of plant secondary metabolite synthesis and that the elicitors could play an important role in biosynthetic pathways and in the enhanced production of commercially important compounds. The use of abiotic elicitors in plant cell cultures has received less attention compared with the biotic elicitors [163].
Elicitors are signals triggering the formation of secondary metabolites. Use of elicitors of plant defense mechanisms, i.e. elicitation, has been one of the most effective strategies for improving the productivity of bioactive secondary metabolites [164]. Biotic and abiotic elicitors which are classified on their origin are used to stimulate secondary metabolite formation in plant cell cultures, thereby reducing the process time to attain high product concentrations [165-167]. Productions of many valuable secondary metabolites using various elicitors were reported [136, 168-171].

Elicitors are chemicals or biofactors from various sources that can induce physiological changes of the target living organism. In a broad sense, delictitors T, for a plant refer to chemicals from various sources that can trigger physiological and morphological responses and phytoalexin accumulation. It may include abiotic elicitors such as metal ions and inorganic compounds and biotic elicitors from fungi, bacteria, viruses or herbivores, plant cell wall components, as well as chemicals that are released at the attack site by plants upon pathogen or herbivore attack.

Elicitors include microbial molecules derived for example from the bacterial cell wall or the flagella, signalling compounds present in insect oral secretions, or molecules derived from the damaged plant cell walls. These elicitors activate signal transduction pathways that generate secondary signals produced by plants [172]. Three major secondary signalling molecules are jasmonates and salicylic acid [173-176]. Production of these hormones generates a signal transduction network that leads to a cascade of events responsible for the physiological adaptation of the plant cell to the external stress. The JAs, ethylene and salicylic acid signal transduction pathways act synergistically or antagonistically in a variety of responses, leading to fine-tuning of the complex defense response [177].

Elicitation has been shown to be the most efficient strategy that direct to the enhancement in anthocyanin production in plant cell cultures [178]. The addition of methyl jasmonate (MeJA) or jasmonic acid (JA) in the culture medium as an elicitor enhanced the anthocyanin production of *Tulipa gesneriana* and *Vaccinium pahalae* [179-180]. Methyl jasmonate (MJ) has successfully used as an elicitor in other plant species for enhancing the production of secondary metabolites in the cell cultures [181-182]. Similarly, the manipulation in the components of the culture medium
(e.g. carbon source, nitrogen and phosphate) also found to be effective for the production of secondary metabolites [183]. Biotic elicitors and abiotic stresses stimulate its *in vivo* and *in vitro* synthesis. *Vitis vinifera* cell cultures have been used in several studies to explore the factors involved in the induction and regulation of stilbene biosynthesis and metabolism [184].

Furthermore, elicitors stimulated the antioxidant defense systems of plant cells [185]. Treatments of cultured cells with fungal elicitors have also been shown to induce the phenylpropanoid/flavonoid biosynthetic pathways [186-187]. The fact that fungal elicitors stimulate strongly and rapidly plant secondary metabolite accumulation has recently attracted considerable attention [172].

Among these three key regulatory signals, by far the most important molecules for induction of secondary metabolism are the JAs. JAs have been found to induce the biosynthesis of a variety of secondary metabolites in different plant species, including alkaloids, terpenoids, glucosinolates and phenylpropanoids [188].

Elicitors or avirulence determinants must be recognized by plant receptors or R proteins localized to the plasma membrane or the cytoplasm before initiating signaling pathways, which lead to defense reactions such as synthesis of PR (pathogenesis-related proteins), or defense secondary metabolites. Molecular recognition and physical interaction between elicitor signal molecules and specific plant receptors are complex processes but are required for specific elicitor signal transduction.

Because synthesis of phytoalexin-type secondary metabolites is a part of the defense reaction of plants, the question arises whether or not rol-gene signals interfere with general plant defense pathways. In order to address this question, we began evaluating the roles of some key processes of defense reactions, such as Ca\(^{2+}\) signaling, phosphorylation and dephosphorylation events, on the growth and AQ production in transgenic cultures. In this stage of the investigation, we used the pharmacological approach, which provides information for the initial assessment of signal transduction.
1.16.4. Signaling Pathway

Calcium exerts a profound influence on many biological processes [189]. Ca$^{2+}$ permeable channels exist in a variety of cell types including carrot and parsley suspension cultures and such channels can be activated by membrane depolarisation [189-190]. Thus a number of signals, including blue light, red light nodulation factors and fungal elicitors, evoke a rapid membrane depolarization resulting in an increase in the Ca$^{2+}$ channel activity [191-194]. In response to a variety of stimuli, including light, gravity, abiotic and biotic stresses and hormones, the cytosolic Ca$^{2+}$ concentration in plants is rapidly elevated via an increased Ca$^{2+}$ influx and then quickly returns to the basal level by Ca$^{2+}$ efflux – this produces a Ca$^{2+}$ spike [195-197]. In recent years, significant progress has been made in measuring \textit{in vivo} free calcium changes in plant cells using calcium-imaging techniques. Free Ca$^{2+}$ changes have been observed not only in the cytosol but also more recently in the nucleus as well [198]. Even within subcellular compartments, spatially and temporally regulated microdomains of different Ca$^{2+}$ oscillations are likely to exist.

For simplicity, signaling pathways usually have been studied in isolation, with experimenters attempting to define a single pathway through which a given stimulus evokes a response. However, cells are not simple, and for any given stimulus (input), the final response (output) is likely to be the result of complex interaction, or cross-talk, between multiple pathways [199]. Presumably, this cross-talk evolved as a mechanism to enable a relatively small number of messengers to help cells process a much larger array of potential stimuli in an appropriate fashion. In plant cells, the list of messengers used by signaling pathways includes Ca$^{2+}$, lipids, pH and cyclic GMP (cGMP). However, no single messenger has been demonstrated to respond to more stimuli than has cytosolic free Ca$^{2+}$ ([Ca$^{2+}]_c$).

Metabolism in all cells requires the presence of orthophosphate (P$_i$) and phosphorylated organic compounds, particularly for cytosolic reactions associated with transduction of free energy. The low solubility product of Ca$^{2+}$ with P$_i$ would have required the early evolution of mechanisms for maintenance of [Ca$^{2+}]_c$ at a level that would certainly be well below the millimolar concentrations that prevail in seawater. Thus, transport systems that export Ca$^{2+}$ from the cytosol are present in all cells to sustain steady state values of [Ca$^{2+}]_c$ in the submicromolar range. This
homeostatic mechanism would have been ideal for subsequent evolution of Ca$^{2+}$-based signaling pathways. Specifically, the elevation of [Ca$^{2+}$], by a factor of 10 or 20 can occur more rapidly than would be possible for ions or solutes that are maintained at millimolar levels. Ultimately, homeostasis of [Ca$^{2+}$]$_c$ must be achieved by export across the plasma membrane because both biochemical buffering and intracellular sequestration have finite capacities. Although the potential difference across the membranes of organelles, such as the vacuole and ER, is likely to be less negative than that across the plasma membrane, large driving forces nevertheless prevail and require energized removal of Ca$^{2+}$ from the cytosol. Efflux of Ca$^{2+}$ from the cytosol is mediated by pumps powered by either ATP hydrolysis or a proton motive force. By contrast, the passive entry of Ca$^{2+}$ into the cytosol is mediated by ion channels.

1.16.5. Calcium ATPase

Calcium pumps belong to the superfamily of P-type ATPases that directly use ATP to drive ion translocation. Two distinct Ca$^{2+}$ pump families have been proposed, based on protein sequence identities [200]. Members of the type IIA and IIB families, respectively, include the ER-type and plasma membrane–type Ca$^{2+}$ pumps first identified in animal cells. Previously, ER- and plasma membrane–type pumps were distinguished by three criteria: (1) localization to either the ER or plasma membrane, respectively; (2) differential sensitivity to inhibitors (e.g., ER-type inhibition by cyclopiazonic acid and thapsigargin); and (3) direct activation of plasma membrane–type pumps by calmodulin [201].

Although the effect of Ca$^{2+}$ binding is unknown, the Arabidopsis knockout mutant atnig1-1 exhibits hypersensitivity to salinity stress, suggesting that AtNIG1 plays a positive role in salt tolerance [202]. In addition, cross-talk between Ca$^{2+}$-mediated transduction pathways contribute to highly modulated plant responses. For example, a subset of CDPK and CIPK proteins may also be regulated by CaM [203] and AtCPK1 and CaM have opposite effects in regulating Ca$^{2+}$-ATPase activity of ACA2 [204]. Although some protein targets of calcium sensors have been identified, the molecular mechanisms underlying calcium signaling remain to be fully explored. As plant mitogen-activated protein kinase (MAPK) cascades are also key components in
stress signaling, the interplays between calcium and MAPK signaling pathways require future investigation.

Commercial production of L-Dopa from intact plants is hampered by the herbaccous twining habit and presence of strident trichomes on the pods, which cause a very strong itching senation creating difficulties in large scale cultivation of plants and harvesting their pods [205]. Plant cell cultures synthesize and produce several valuable compounds of industrial and pharmaceutical significance and are considered as a source of high value compounds.

The great demand of L-Dopa is largely met by the pharmaceutical industries through extract of Mucuna from wild population. But commercial exploitation for production is hampered due to its limited availability.