Studies on the genetic diversity of Adhatoda vasica and Adhatoda beddomei and optimization of their callus culture conditions

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A Thesis Submitted to the Avinashilingam Institute for Home Science and Higher Education for Women (Deemed University), Coimbatore-641 043 in partial fulfilment of the requirements for the Degree of Doctor of Philosophy

April, 2000

CERTIFICATE

This is to certify that the thesis entitled **"Studies on the genetic diversity of *Adhatoda vasica* and *Adhatoda beddomei* and optimization of their callus culture conditions"**, submitted to the Avinashilingam Institute for Home Science and Higher Education for Women (Deemed University), Coimbatore, for the award of the Degree of **Doctor of Philosophy in Biochemistry** is a record of original research work done by **Indira A. Jayraaj** during the period of her study in the Department of Biochemistry and Biotechnology, Avinashilingam Institute for Home Science and Higher Education for Women (Deemed University), Coimbatore, under my supervision and guidance and the thesis has not formed the basis for the award of any Degree / Diploma / Associateship / Fellowship or similar title to any candidate of any University.

3/5/2008

Signature of the Guide

DECLARATION

I hereby declare that the matter embodied in this thesis is the result of investigation carried out by me in the Department of Biochemistry, Avinashilingam Institute for Home Science and Higher Education for Women (Deemed University), Coimbatore, under the supervision and guidance of **Dr. S. Saroja**, Professor and Head, Department of Biochemistry and Biotechnology, Avinashilingam Institute for Home Science and Higher Education for Women (Deemed University), Coimbatore, and it has not been submitted for the award of any Degree / Diploma / Associateship / Fellowship etc. of any other university or institute.

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INTRODUCTION

1.0 INTRODUCTION

Traditional medicine based on herbal remedies has always played a key role in the health system of many countries. The aborigines are using the wild plants in a variety of ways since time immemorial (Sudhersan, 1998). Among various forest products, aromatic and medicinal plants occupy a place of considerable importance, as these are used by Ayurvedic practitioners, not only to cure common diseases like cough, bronchitis and asthma, but also serious diseases like tumor, cancer and AIDS (Dai *et al.*, 1998; Das and Yadav, 1998). Western medicine was introduced into India by the British in a big way in the early nineteenth century. With the shift in the educational system, social practices and way of life over the years, Western medicine or allopathy became increasingly popular and sent traditional medicine to the back seats. But in the last few decades, however, there has been probably more interest in drugs obtained from vegetative source than at any time in history, because of the success with the antibiotics and other plant drugs such as Rauwolfia (for treatment of mental diseases), Podophyllum, a cathartic (for the treatment of atomic radiation burn) and Sapogenins (for the treatment of rheumatoid arthritis) (Khan and Khanum, 1998).

The world population is nearing 5 billion and with this rate of growth, it is likely to touch 7.5 billion by the year 2000. Global estimates indicate that over $3/4^{th}$ of the world population cannot afford the products of the Western pharmaceutical industry and thus have to rely upon the use of traditional, plant-derived medicines (Mukunthan, 1998). Developing countries can ill afford the products of the Western pharmaceutical industry due to their high and rising cost. The import of such medicines or payment of royalties for local manufacture of the medicines causes a heavy financial burden to the developing countries. The World Health Organization, therefore, recommends the inclusion of herbal medicines which are time tested, safe and available at affordable prices in the national health programmes (Souza *et al.*, 1998). More over, traditional medicines show better cultural acceptability, better compatibility and lesser side effects than synthetic ones. This recognition has made the herbal system once again the center stage of health care programme, not only in

India, but the world over (Banerjee *et al.*, 1999). It is estimated that the market potential for herbal drugs in the Western world alone could range from US $ 4.9 billion to 47 billion (Kamboj, 2000).

India, having a very old and rich tradition of folk medicine for centuries, has provided very simple but effective remedies to various ailments using plants which were available in village backyards or in nearby forests. Medicinal plants and trees have a prominent place in our mythology and religion and have been cultivated by villagers with great reverence.

Today, however, many of these traditional medical practices have faded out long with much of the forests themselves. Modern medical services, especially in villages, have failed to provide adequate and effective health care for the community due to high cost of medicines, shortage of doctors and overcrowding of hospitals. The side effects from drugs to treat even minor disorders have also become an increasingly serious problem.

The only way to make rural primary health care service effective is to revive and promote traditional health practices based on the use of medicinal plants. Many programmes have been initiated for *in situ* propagation of medicinal plants in recent times by Non Government Organizations (NGO) as well as government and semi government agencies (Swamy, 1997). Some organizations like the Center for the Application of Science and Technology to Rural Areas (ASTRA) and the Foundation for Revitalisation of Local Health Traditions (FRLHT), have worked out simple low cost propagation techniques for several medicinal plants (Thirunavoukkarasu, 1996).

In Coimbatore and in Kanjikode (located very close to Coimbatore) Center for Indian Medical Heritage is embarked upon a low cost and culturally familiar health care system for India's millions. If it succeeds, India could conceivably become one of the healthiest nations in the world by the year 2050.

India is recognized as one of the twelve mega diversity centers of the world and includes two of the eighteen biodiversity hot spots viz., Eastern Himalayas and Western Ghats (Varghese and Menon, 1999). The richness of the floristic diversity of this region is due to varied climate, topography, soil structure and landscape. The great diversity of Indian flora is well reflected in the vast variety of medicinal plants which are scattered throughout the hills and plains. About 15,000 species of higher plants identified and enumerated in Indian flora contribute to its wealth. Over 63% of the tree species of the low and medium elevation evergreen forests of Western Ghats are endemic and unique (Nayar, 1996). More than 1000 species are being used in various formulations in our traditional system of medicine. Annual herbal drug production of our country has been estimated to be around Rs.2,000 crores and is expected to reach upto Rs.4,000 crores very soon (Shete, 1999).

Asthma is characterized by airway hyperresponsiveness which is an exaggerated airway narrowing in response to many stimuli, such as histamine, exercise, cold air and allergens. More recently, it has become evident that the airway narrowing in asthma is a consequence of a special type of infection and chronic inflammation in the lungs (Barnes, 1991). Asthma has become the most common chronic disease in the world and epidemiological studies suggest that its prevalence, severity and mortality are rising at a time when mortality from other common treatable conditions is falling (Chowgule *et al.*, 1998). The reasons for the above statistics are environmental factors such as increased exposure to allergens and atmospheric pollutants. The commonly used antiasthmatic drugs like bronchio-dialators and β-agonists may rapidly relieve asthma symptoms, but they may not control the underlying inflammation. Since asthma is a chronic inflammatory condition, it may be more logical to adopt anti-inflammatory treatments to control the inflammation. Anti-inflammatory treatment includes corticosteroids which are very effective in the treatment of asthma. But corticosteriods are costly and, if given systematically, have many severe adverse effects.

Adhatoda vasica Nees and *Adhatoda beddomei* Clarke which were selected for the present study belong to the family *Acanthaceae*. *Adhatoda vasica*, a plant native to India, is popularly known by its name *vasaka*. It is a well known drug in the Ayurvedic and Unani systems of medicine. The leaves of vasaka and their extract have been used for the medication of cough, bronchitis and asthma for more than 2000 years. *Adhatoda beddomei*, is more powerful and active than *A. vasica* and its crude extract is used for respiratory ailments. The main advantage of these herbal preparations is that they are virtually free from side effects unlike allopathic medicines.

Reactive Oxygen Species (ROS) such as $O_2^{\cdot-}$, H_2O_2 and $^{\bullet}OH$ are highly toxic to cells. Cellular antioxidant enzymes and the free radical scavengers normally protect a cell from the toxic effects of ROS. However, when generation of the ROS overtakes the antioxidant defense of the cells, it creates the situation of oxidative stress (Thomas and Kalyanaraman, 1997). Severe oxidative stress can produce major derangements of cell metabolism, including DNA and RNA damage, rises in intracellular "free" Ca^{2+} and "free" iron ions, damage to membrane ion transporters and other proteins and lipid peroxidation. Many disorders, like rheumatoid arthritis, hemorrhagic shock, cardiovascular diseases, gastro intestinal ulcerogenesis, AIDS, cancer, Parkinson's disease, Down's syndrome and ischemic reperfusion injury to different tissues including heart, brain, kidney, liver and gastro intestinal tract have been reported as the ROS – mediated disorders (Li and Trush, 1995; Das *et al.*, 1997). The major role played by ROS in stress induced gastric ulcer, inflammatory bowl diseases and in aging has been documented well (Starke – Reed, 1997). One of the significant benefits of the studies on ROS will perhaps be in the designing of a suitable antioxidant therapy to control the ROS-mediated oxidative damage and the disease processes.

Medicinal plants are one of the most important sources of antioxidants (Tripathi *et al.*, 1999). So it is essential to investigate the antioxidants in *Adhatoda*

species with a view to throwing the light on the possible relationship between these free radical quenchers and its medicinal property.

A. vasica and *A. beddomei* contain quinazoline group of alkaloids namely vasicine and vasicinone (an autooxidized product of vasicine) which are claimed to have therapeutic value (Thapa, 1985). However, the production of alkaloids in both plants is too low to be exploited commercially. The safety and complex procedures involved in the chemical synthesis of these products indicate a greater demand for natural products from plants and hence seek for biotechnological approach. Plant tissue culture offers a potential alternative for phytochemicals which are of importance to food and pharmaceutical industries (Fowler and Stafford, 1992). Routier and Nickell (1956) first discussed the use of plant tissue culture technique for commercial production of secondary metabolites. Since then, a wide range of compounds viz., alkaloids, steroids, terpenes, flavanoids have been obtained by this technique (Phillipson, 1990). Even though plant cell culture technique offers an attractive alternative for production of secondary metabolites, their commercial exploitation has been restricted to only a few compounds. The low yield and the scaling up of these products have been the bottlenecks for commercial enterprise. During the last decade, a distinct progress has been made to stimulate the formation and accumulation of secondary metabolites by cell culture techniques. It begins with a search for healthy, disease free and highly productive plants for culture initiation followed by the development of special production medium to obtain the highest yield of desired products. Apart from selection, nutrients in the culture media were found to regulate secondary metabolism (Dicosmo and Towers, 1984). Nitrogen in most nutrient media is present in the form of a mixture of nitrate and ammonium. The type and amount of nitrogen source in the substrate affects the pattern and yield of secondary products. The concentration of micronutrients in the culture medium also influences the production of secondary metabolites (Roja and Rao, 1998). In addition, a group of triggering factors known as elicitors, isolated from microorganisms have been reported to stimulate particular facets of secondary metabolism (Narayanaswamy, 1994).

A. vasica and *A. beddomei* are endowed with a wide spectrum of pharmacological uses. However very little of their genetic architecture is known. It is therefore necessary to detect the amount of variation between and within the population for selection. The objective of selection is to choose the most desirable individuals for use in drug preparations and as parents in tissue culture programme for better yield of the secondary metabolites. When the variation existing between and within the population is not detectable through visual / morphological means, there is a need to go in for a highly reliable and precise method to detect the variation in which the environmental effect is over looked. The advent of molecular marker technique, an analysis based on the polymorphism found in proteins or DNA has greatly facilitated research activities in the fields of phylogeny, taxonomy, genetics and plant breeding.

For quite a long time, isozymes have been the molecular markers of choice for determining the genetic relationship within and between populations (Huen and Helentjaris, 1993). In the present situation, there are a number of molecular markers available for the detection of variability, as they are not influenced by developmental or environmental effects. Randomly Amplified Polymorphic DNA (RAPD), the polymerase chain reaction (PCR) based DNA marker system, which is simple, cost effective and gives highly reliable and reproducible results are being used by several authors in various crops (Chalmers *et al.*, 1992). Polymorphism results from changes that affect the amplification of DNA located between the two flanking primers. These changes include probably single base substitutions as well as deletions or insertions that either change the primer sequence or the size of the amplified DNA (Williams *et al.*, 1990). The polymorphic DNA fragments revealed by RAPD would be highly useful in assessing the extent of genetic diversity present between and within the populations.

Against this backdrop, the present investigation has been designed with the following objectives:

a) Collection of plant material from various places
b) Isolation and purification of alkaloids from the above plants
c) Determination of the antioxidants
d) Induction and maintenance of callus cultures
e) Optimization of a growth medium for increasing the production of alkaloids in callus cultures
f) Effects of elicitors on the production of total alkaloids
g) Determination of genetic variability within and between *A. vasica* and *A. beddomei* using isozyme and RAPD analyses

The study was formulated with the aim of exploiting the potential lying in the strains of the medicinal plants like *A. beddomei* that do not grow in all geographical locations and increasing the yield of active principles in them. Keeping the above objectives and aims in view, the experiments carried out and the results obtained are presented in the chapters that follow.

REVIEW OF LITERATUR

2.0 REVIEW OF LITERATURE

The review of literature pertaining to the present study "***Studies on the genetic diversity of Adhatoda vasica and Adhatoda beddomei and optimization of their callus culture conditions***" has been discussed under the following headings:

2.1 Introduction
2.2 Herbal medicines
2.3 Herbal medicine market in the world
2.4 Herbal medicine scenario in India
2.5 Herbal medicine standardization
2.6 Primary vs. secondary metabolites
2.7 Secondary metabolites as pharmaceutical products
2.8 Enzymes in *Adhatoda* species
2.9 Medicinal plant Biotechnology
2.10 *In vitro* production of secondary metabolites
2.11 Production of alkaloids by plant tissue culture
2.12 Strategies to increase secondary metabolites
2.13 Optimization of culture media
2.14 Manipulation of nutrients to improve yield
2.15 Elicitation
2.16 Genetic diversity studies

2.1 INTRODUCTION

The use of plants as medicine is as old as human civilization. Plants have been an integral part of traditional medicine across the continents since time immemorial. The earliest record of use of medicinal plants for the prevention of disease and cure of ailments can be traced far back to "Rigveda", perhaps the oldest repository of human knowledge having been written between 4,500 and 1600 BC (Singh, 1995). India, being one of the top twelve mega diversity centers, has immense biotic wealth marked

by remarkable ecosystem, species and genetic diversity. This rich biological diversity is matched equally by rich cultural diversity and health tradition (Padmesh *et al.*, 1999). Because of this, in our country, the use of medicinal plants is well entrenched. But during the mid century, synthetic drugs were used in large measure replacing herbal medicine to a great extent. Drugs obtained through chemical synthesis are either less effective as compared to the same drug of natural origin or have several side effects (Ramawat *et al.*, 1991). The phenomenon of drug resistance also poses another problem in the use of synthetic drugs (Jain *et al.*, 1992).

Most of the secondary metabolites found in the plant kingdom have a complex structure involving many chiral centers which determine their pharmaceutical and biological activity. Therefore, many of these complex biomolecules cannot be synthesized economically on a commercial basis (Thengane and Krishnamurthy, 1998). Examples of secondary metobolites having a high degree of structural complexity include the catharanthus alkaloids (vinblastine and vincristine) and the naturally occurring plant derived insecticide azadirachtin. This is the reason for medicinal plants being important resources of material for pharmaceutical industry and being considered as "best organic chemists" in the world.

Herbal medicine is still the mainstay of about 75-80% of the world population, mainly in the developing countries, for primary health care because of better cultural acceptability, better compatibility with the human body and lesser side effects. The last few years have seen a major increase in their use in the developed world also. In the USA, herbal drugs have been sold in health food stores with a turnover of about $4 billion in 1996 which is anticipated to double very soon. According to the recent report of Eximbank, India produced herbal bulk drugs worth Rupees 240 crore in 1984-1985, whereas the figure rose to Rupees 1500 crores in 1994-1995 (Souza *et al.*, 1998). Herbal medicines also find market as nutraceuticals (health foods) whose value is estimated at about $80-250 billion in the USA and also in Europe (Brower, 1998).

2.2 HERBAL MEDICINES

The World Health Organization (WHO, 1991) has recently defined traditional medicine (including herbal drugs) as comprising therapeutic practices that have been in existence, often for over hundreds of years, before the development and spread of modern medicine and are still in use today. In other words, traditional medicine is the synthesis of therapeutic experience of generations of practicing physicians of indigenous systems of medicine. Herbal drugs constitute only those traditional medicines which primarily use medicinal plant preparations for therapy. The earliest recorded evidence of their use in Indian, Chinese, Egyptian, Greek, Roman and Syrian texts dates back to about 5000 years. The classical Indian texts include *Rigveda, Atherveda, Charak Samhita* and *Sushruta Samhita*. The herbal medicines/traditional medicaments have, therefore, been derived from rich traditions of ancient civilizations and scientific heritage (Brower, 1998).

2.3 HERBAL MEDICINE MARKET IN THE WORLD

The herbal medicine market in 1991 in the countries of the European Union was about $6 billion (which may be over $20 billion now), with Germany accounting for $3 billion, France $1.6 billion and Italy $0.6 billion. Incidentally, in Germany and France, herbal extracts are sold as prescription drugs and are covered by national health insurance. In 1996, the US herbal medicine market was about $4 billion and with the current growth rate may be more than double by the turn of the century. A reasonable guesstimate for current herbal medicine market worldwide may be around $30-60 billion. The Indian herbal drug market is about one billion dollars and the export of herbal crude extracts is about $80 million (Rawls, 1996).

The ten best selling herbal medicines in developed countries (Kamboj, 2000) are as follows:

DRUG	*SOURCE*	*MARKET RANK AS PER SALE*
Echinacea	*Echinacea species*	1
Garlic	*Allium sativum*	2
Goldenseal	*Hydrastis canadensis*	3
Ginseng	*Panax species*	4
Ginko	*Ginko biloba*	5
Saw palmeto	*Serenoa repens*	6
Aloe gel	*Aloe barbadensis*	7
Ephedra	*Ephedra species*	8
Eleuthero	*Eleutherococcus senticosus*	9
Cranberry	*Vaccinium macrocarpon*	10

The sales of these drugs account for almost 50% of the herbal medicine market. These drugs have been well standardized and some of them namely *Echinacea*, garlic, ginko, ginseng and saw palmeto are supported with mode of action and clinical studies. Amongst the developed countries ,Germany holds the lead and has published individual monographs on the therapeutic benefits of more than 300 herbs. In developing countries, China has compiled / generated data on over 800 medicinal plants and exports large amounts of herbal drugs. India has prepared only a few monographs and its exports are dismal.

2.4. HERBAL MEDICINE SCENARIO IN INDIA

Three of the 10 most widely selling herbal medicines in developed countries, namely preparation of *Allium sativum*, *Aloe bardensis* and P*anax* species are available in India. India is the largest grower of Psyllium (*Plantago ovata*) and Senna (*Cassia senna*) plants and one of the largest growers of Castor (*Ricinus communis*) plant. These are also exported in large amounts and yet our market share is dismal because of export of crude extracts/drugs (Kamboj, 2000). Twenty other plants are commonly exported as crude drugs worth $ 8 million. Five of these, namely *Glycyrrhiza glabra, Commiphora mukul, Plantago ovata, Aloe barbadensis* and *Azadirachta indica* are even used in modern medicine. The plants *Glycyrrhiza glabra, Piper longum, Adhatoda vasica, Withania somnifera, Cyperus rotundus, Tinospora cordifolia,*

Berberis aristata, Tribulus terristris, Holarrhena antidysenterica and *Boerhavia diffusa* have been used in 52 to 141 herbal formulations and triphala (*Terminalia chebula, Terminalia belerica and Embelica officinalis*) alone has been used in 219 formulations (BCIL, 1996). The frequency of occurrence of medicinal plants in herbal formulations in India is shown below :

COMMON NAME	*BOTANICAL NAME*	*NO. OF HERBAL FORMULATIONS*
Triphala	*Terminalia chebula* *Terminalia belerica* *Emblica officinalis*	219
Yashtimadhu	*Glycyrrhiza glabra*	141
Pipali	*Piper longum*	135
Vasaka	*Adhatoda vasica*	110
Ashwagandha	*Withania somnifera*	109
Mastak (Motha)	*Cyperus rotundus*	102
Gulacha	*Tinospora cordifolia*	88
Daruharidra	*Berberis aristata*	65
Gokshura	*Tribulus terrestris*	65
Kutaja	*Holarrhena entidysenterica*	59
Punarnava	*Boerhavia diffusa*	52

A large number of medicinal plants are being exported to other countries from India. In spite of this, efforts have not been made to preserve their germplasm from different localities, identification of active plants vis-à-vis climatic zone and development of agrotechnologies for their organized farming and use as authentic materials in herbal medicines for better economic gains.

The turnover of herbal medicines in India as over-the-counter products, ethical and classical formulations and home remedies of Ayurveda, Unani and Siddha systems of medicine is about $ 1 billion with a meager export of about $ 80 million. Psyllium seeds and husk, castor oil and opium extract alone account for 60% of the exports (Cordell *et al.*, 1991). Eighty percent of the exports to developed countries are of crude drugs and not finished formulations leading to low revenue for India. Thus the export of herbal medicines from India is negligible despite the fact that the country

has a rich traditional knowledge and heritage of herbal medicine. Considering the huge herbal medicine and nutraceutical market in developed countries, India should stop exporting crude herbal drugs, but develop specific drug molecules and large scale products.

2.5. HERBAL MEDICINE STANDARDIZATION

In indigenous / traditional systems of medicine, the drugs are primarily dispensed as water decoction or ethanolic extract. Fresh plant parts, juice or crude powder are a rarity rather than a rule. Thus medicinal plant parts should be authentic and free from harmful materials like pesticides, heavy metals, microbial or radioactive contamination. The medicinal plant is subjected to a single solvent extraction once or repeatedly, or water decoction or as described in ancient texts. The extract should then be checked for indicated biological activity in an experimental animal model(s). The bioactive extract should be standardized on the basis of active principle or major compound(s) along with fingerprints. The next important step is the stabilization of the bioactive extract with a minimum shelf life of over a year. The stabilized bioactive extract should undergo regulatory or limited safety studies in animals. The trend in the export market of medicinal product is encouraging, however, lack of quality control measures in value added products and problems like admixtures, adulteration and spoilage and poor storage conditions have prevented exports from growing faster. In order to catch the export market, we have to concentrate more on standardization of herbal extraction procedures and isolation of the active ingredients. The growth of value-added medicinal product as estimated will fetch us a revenue of US $ 5 million annually (Shete, 1999).

2.6. PRIMARY vs. SECONDARY METABOLITES

Plant derived organic compounds may be classified as either primary or secondary metabolites (macromolecules such as structural and catalytically/ functionally active i.e. enzymes, proteins and informational biopolymers like DNA & RNA are excluded from this classification). Primary metabolites are directly involved

in the growth and development of cell and are widely distributed in the nature, occurring in one form or another in virtually all organisms.

When plants are subjected to any stress, to encounter the imbalance created by the stress, plants produce new or more proteins which are believed to have protective functions (Frendo *et al.*, 1992). Most of the proteins are constitutively present, markedly induced under stress conditions and/or expressed in certain developmental stages (Forreiter and Nover, 1998).

Secondary metabolites are biosynthetically derived from primary metabolites, but are more limited in distribution in plant kingdom, usually being restricted to a particular taxonomy namely, species, genus, family or closely related group of families (Flores, 1992).

Although they have no obvious role in a plant's primary or main stream metabolism (since they are non-nutritive and are thus not directly essential for growth), secondary metabolites often play ecologically significant roles in how plants deal with their environment and therefore important in their ultimate survival. Plant's secondary metabolites may serve as pollinator attractant (e.g. for insects, humming birds, small rodents and other mammals), may represent chemical adaptations to environmental stress, or may serve as defensive, protective or offensive chemicals against microorganisms, insects and higher herbivorous predators and even against higher plants. Plant secondary metabolites also serve basically to combat infections (Edwards and Gatehouse, 1999).

In terms of cellular economy, secondary metabolites are generally metabolically expensive to produce and accumulate and are therefore frequently present in plants in much smaller quantities than the primary metabolites. In addition, secondary metabolites, in contrast to primary metabolites, tend to be biosynthesized in specialised cell types and at distinct developmental stages, making their extraction, isolation and purification difficult (Bousquet and Kollmann, 1998). As a result, secondary metabolites that are used commercially, as biologically active compounds

(e.g. as pharmaceuticals, flavors, fragrances and pesticides) are generally higher value – lower volume products than primary metabolites. Thus, compared to primary metabolites, many bioactive secondary metabolites can be considered as special materials or fine chemicals (Khan and Khanum, 1998).

Medicinal plants have their values in the secondary metabolites present in various plant tissues. These produce specific physiological action in human body. The more important of these substances are alkaloids. Alkaloids are derived mostly from plants and are basic substances which contain one or more nitrogen atoms, usually in combination as part of a cycle system. The alkaloids, of which, some 10,000 are known, comprise the largest single class of secondary metabolite plant substances (Facchini and Bird, 1998). In plant, many alkaloids serve the purpose of chemical self defense to deter herbivores or to protect against pathogen challenge. The biological function of specific alkaloids, and the evolutionary pressures that selected for their biosynthesis, often provide clues about the qualities of these natural products that make them so useful as pharmaceuticals (Hamburger *et al.*, 1990). Some pharmaceutically important alkaloids, their sources and uses are listed below:

PLANT SOURCE	***ALKALOID***	***USES***
Catharanthus roseus	Vinblastine	Anticancer
Catharanthus roseus	Vincristine	Antileukemia
Catharanthus roseus	Ajmalicine	Antihypertensive
Ochrosia elliptica	Ellipticine	Anticancer
Papaver somniferum	Codeine	Analgesic
Atropa belladonna L.	Atropine	Anticholinergic
Cinchona ledgeriana	Quinidine	Antimalarial
Nicotiana tabaccum	Nicotine	Analgesic

In contrast to the relatively low cost of primary or bulk metabolites, secondary plant metabolites are often highly costly and go up to several thousand dollars per Kilogram. For example, purified opium alkaloids (codeine and morphine) are valued in the range of $400 - $600 per pound. The anticancer *Catharanthus* alkaloids have a wholesale value of about $5000 per gram and their retail value may be more than $20000 per gram. These high prices are due both to the costly methods currently used

to produce these compounds and to the extremely low yields obtained from the source plants. For example, the yield of vincristine from *Catharanthus roseus* is 0.0003–0.0005% (i.e.) some 500 kg of leaves are needed to produce just 1 g of purified vincristine. In addition, upto 16 weeks of processing may be required to produce the purified compound (Sasson, 1991). In India, valuable plant secondary metabolites are likely to account for an export earning of Rs.3,500 million. Some examples of plant products with their values are listed below:

PRODUCT	*PLANT*	*PRICE (DM/g)*
Ajmalicine	*Catharanthus roseus*	56.00
Vinblastine	*C. roseus*	15,800.00
Vincristine	*C. roseus*	37,800.00
Ellipticine	*Ochrosia elliptica*	3940.00
Camptothecin	*Camptotheca acuminata*	720.00
Emetine	*Cephaelis ipecaccuanha*	39.50
Berberine	*Captis japonica*	16.50
Sanguinarine	*Papaver*	72.00
Colchicine	*Colchicum antumnale*	75.00

(Khan and Khanum, 1998).

2.7 SECONDARY METABOLITES AS PHARMACEUTICAL PRODUCTS

Certain biologically active secondary metabolites have found medicinal application as drug entities or as model compounds (templates) for drug synthesis and semisynthesis (Ali and Mikage, 1991). The commercial value of drug products still derived directly from higher plants is considerable and should not be underestimated. Plant derived drugs represent stable markets upon which both physicians and patients rely. Etoposide is a semisynthetic antineoplastic agent derived from Mayapple reported to be useful in the chemotherapeutic treatment of refractory testicular carcinomas, small cell wing carcinomas and leukemias. Forskolin, a naturally occurring labdenone diterpene is useful in preventing the clotting of platelets, in reducing intraocular pressure in cases of glaucoma, as a hypotensive, and as an aid to serve regeneration following truama.

In the future, advances in our understanding of immunology and its related areas should permit the development of new selective and sensitive bioassays to guide the isolation of bioactive natural products. These developments should provide the means for identifying new plant derived antiviral, antitumor, immuno stimulating and adaptogenic agents (George and Eapen, 1999).

2.8 ENZYMES IN ADHATODA SPECIES

The historical perspectives in the enzymology of oxygen relates to the period 1858 – 1886 and it was known that the biological oxidations involve activation of oxygen by intracellular enzymes. Even after many milestones of progress in the following century, the enzymology of oxygen continues to remain a fascinating area of work addressing to an astonishing array of over 200 enzymes (broadly divided into oxygenases and oxidases) known to have molecular oxygen as one of its substrates.

The generation of free radicals is now accepted as universal attribute of aerobic life. Aerobic organisms began the evolutionary process of evolving antioxidant defense systems to protect themselves against oxygen toxicity. Plants and animals have developed antioxidant defenses to protect against 21 % oxygen, (the current atmospheric level), but not greater than that. When there is compulsory oxidation of bivalent organic molecules to univalent molecules, free radicals are produced as intermediates. About 5% or more of inhaled O_2 is converted to reactive oxygen species (ROS) or Active Oxygen Species (AOS) such as $O_2^{\cdot}$, H_2O_2 and $^{\cdot}OH$ (Lunee, 1990). Thus cells under aerobic conditions are always threatened with the insult of ROS, which however are efficiently taken care of by the highly powerful antioxidant systems of the cell without any untoward effect. When the balance between ROS production and antioxidant defense is lost, oxidative stress results which through a series of events deregulates the cellular functions that results in the oxidation of membrane lipoproteins, glycooxidation and oxidation of DNA; subsequently cell death occurs (Cross *et al.*, 1995).

Plants have evolved a battery of antioxidative mechanisms to detoxify and eliminate the highly reactive oxygen species (del Rio *et al.*, 1992). The antioxidant defense system includes hydrophilic (ascorbic acid and glutathione), hydrophobic (α-tocopherol, carotenoids) antioxidants and enzymes like superoxide dismutase, catalase, hydrogen peroxidase and glutathione reductase (Shalata and Tal, 1998). When plants are subjected to environmental stress the balance between the production of AOS and quenching activity of antioxidants may be upset and oxidative damage may result (Spychalla and Desborough, 1990; Cakmak and Marschner, 1992).

The increase of AOS seems to occur as a response to all abiotic stresses including drought (Smirnoff, 1993; Jagtap and Bhargava, 1995; Zhang and Kirkham, 1996), salt (Hernandez *et al.*, 1994; Gossett *et al.*, 1994a,b; 1996), extreme temperatures (Walker and Mckersie, 1993; Mahan 1994; Prasad *et al.*, 1994), nutrient deficiency (Iturbe–Ormaetxe *et al.*, 1995) and air pollution (Badiani *et al.*, 1993). The results of most of these studies suggest that the resistance to environmental stresses is usually correlated with a more efficient antioxidative system.

Environmental stresses are known to result in significant metabolic changes most of which are adaptive responses that enable the plants to survive in stressed environment (Wellburn *et al.*, 1996; Fukao *et al.*, 1998). It was therefore felt imperative to assess the enzymes in *Adhatoda* selected from the different locations. Given below is a brief account of some of the enzymes that play an important role in combatting stress conditions.

2.8.1. ENZYMES INVOLVED IN THE DETOXIFICATION OF REACTIVE OXYGEN SPECIES (ROS)

2.8.1.1. SUPEROXIDE DISMUTASE (SOD)

A variety of toxic oxygen species such as superoxide, hydrogen peroxide, hydroxyl radicals and singlet oxygen are generated in chloroplasts during photosynthesis. The active oxygen species are very reactive and cause a cascade of

oxidative reactions resulting in chlorophyll bleaching and membrane destruction. Superoxide can be formed during conditions of photoinhibition whereby an electron from PSI is accepted by dioxide. Superoxide can be a potent oxidant by itself or after dismutation to hydrogen peroxide by SOD, which may initiate subsequent formation of hydroxyl radicals by Fenton's reaction. The first enzyme involved in the antioxidant defense is superoxide dismutase (Bandyopadhyay *et al.*, 1999). Superoxide dismutase, catalase and peroxidase constitute a mutually supportive team of defense against reactive oxygen species. While SOD lowers the steady state level of O_2^-, catalase and peroxidases do the same for H_2O_2. H_2O_2 generated through the action of SOD is removed by catalases and peroxidases. SOD protects cells and tissues from the damaging effects of oxidative stress by removing O_2 before it reacts with cellular H_2O_2 to form highly reactive hydroxyl radicals.

Kangasjarvi *et al.* (1996) reported an increased SOD activity in *Betula pendula* during ozone exposure. Grace and Logan (1996) observed an increased level of SOD in *Vinca major*, *Schefflera arboricola* and *Mahonia repens* when these were exposed to radiance. Streb *et al.* (1997) have investigated SOD as a sensitive marker of light.

2.8.1.2. PEROXIDASE (POD)

Many biochemical changes are associated with the exposure of plants to harmful conditions. Among them, increased peroxidase activity has been cited as indicative of physiological stress. Bohnert and Jenson (1996) observed higher peroxidase activity in metabolically engineered plants for increased salt tolerance. Brus (1996) showed more activity of peroxidase in polluted than in unpolluted Slovenian populations of beech.

2.8.1.3. CATALASE (CAT)

Catalase catalyses the destruction of hydrogen peroxide radicals. This is expected to limit the concentration of free radicals which cause membrane damage through lipid peroxidation (Runeckles and Vaartnov, 1997). Catalase is considered as

the most effective antioxidant enzyme in averting cellular damage (Scandalios, 1993). Rising tropospheric ozone concentrations and increasing levels of UV-radiation at the earth's surface constitute severe environmental problems because of their negative effects on growth, morphology and physiology of plants (Coldwell *et al.*, 1995). Polle (1997) showed increased level of catalase activity in plants that were exposed to UV radiation in combination with other climatic conditions such as high light irradiation, drought and elevated ozone concentrations. A declined catalase activity has been reported in water stressed corn (Zhang *et al.*, 1990) and sunflower (Quartacci and Izzo, 1992).

2.8.1.4. GLUTATHIONE REDUCTASE (GR)

Glutathione reductase plays an important role in the protection of plants from environmental stresses. Many functions of glutathione (GSH) result in its oxidation to GSSG which is converted back to GSH by GR. Jimenez *et al.* (1997) purified GR from pea leaves. GR activity has been known to increase under various stress conditions.

Doulis *et al.* (1997) showed increased GR activity under temperature stress. GR activity was increased in spruce and pine needles when they were exposed to ozone (Baumbusch *et al.*, 1998). Mahan *et al.* (1994) determined the thermal dependencies of the apparent Km of the GR from spinach, corn and cucumber.

2.8.2. ENZYMES THAT ARE KNOWN TO BE SENSITIVE TO CHANGES IN ENVIRONMENTAL STRESSES AND INVOLVED IN OXIDO REDUCTION REACTIONS

2.8.2.1. NITRATE REDUCTASE (NR)

Nitrate is an important source of nitrogen in plants and the reduction of nitrate to nitrite catalysed by nitrate reductase has been shown to be the rate-limiting step in nitrogen assimilation (Vezina *et al.*, 1992). Nitrate reductase is more sensitive to temperature than amylase, glucose 6-phosphate dehydrogenase and phosphoenol

pyruvate carboxylase. Salalkar *et al.* (1999) reported the depletion of nitrate reductase activity to varying extent by low light conditions in different crop species.

2.8.2.2. INDOLE ACETIC ACID OXIDASE (IAAO)

Indole acetic acid oxidase is the enzyme involved in the catabolic degradation of IAA to 3-methylene oxindole. Nagaraja and Thite (1995) showed decreased activity of IAA oxidase in young infected leaves of *Strychnos nux-vamica* Linn. Mittal and Dubey (1995) observed increased IAAO activity on NaCl salinity in rice seedlings differing in salt tolerance.

2.8.2.3. ASCORBIC ACID OXIDASE (AAO)

Ascorbic acid is widespread in plant tissue and it regulates the level of oxidized and reduced glutathiones and NADPH. Lopex-Huertas *et al.* (1996) investigated the effect of salt stress on the activity of ascorbic acid oxidase and the level of its mRNA in radish.

2.8.2.4. GLUCOSE 6-PHOSPHATE DEHYDROGENASE

Among the enzyme systems involved in cellular defense, glucose 6-phosphate dehydrogenase forms an important and integral part of the auxiliary enzymes which act in conjunction with other detoxifying enzymes to deactivate ROS. Biological oxidations are coupled to energy production. During substrate oxidation, electrons flow towards a suitable sink and the enzymes catalyzing such redox reactions are obligately linked to organic coenzyme molecules acting as electron acceptor (or donor in reverse reactions) and in other cases a transition metal facilitates the rate of transfer. NAD(P)H / $NAD(P)^+$ couple has relatively more negative redox potential compared to other cofactor or redox active metals paving way for the electron flow in the respiratory chains of animal, plant or prokaryotic systems.

The mitochondrial generation of H_2O_2 has been demonstrated by Chance and Oshino (1971) and subcellular distribution studies (Flohe *et al.*, 1971) have shown the

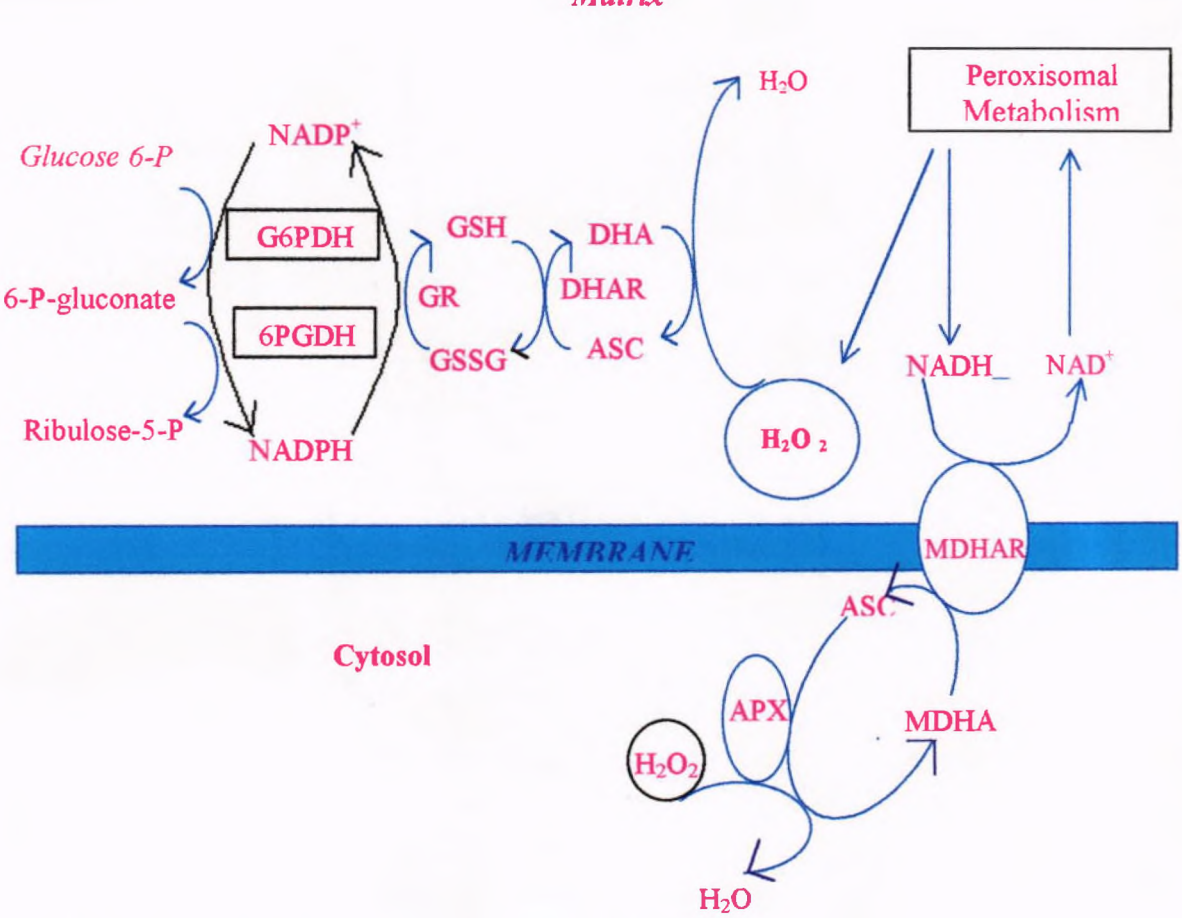
presence of GSH-Px and GR in the mitochondrial matrix space, with catalase being quite low. With GSH-Px involved in the mitochondrial matrix space with catalase, flow of reducing equivalents through the mitochondrial GSH system has to be necessarily balanced by the continuous formation of mitochondrial NADPH, maintaining the steady state. This is enabled by GR, forming an important constituent in this regulatory function (Jimenez *et al.*, 1997).

It is well established that glucose-6 phosphate dehydrogenase in the soluble fraction provides NADPH for GR. Energy linked nicotinamide nucleotide transhydrogenase of mitochondria could also be relevant in this regard which catalyzes the following reaction (Schnarrenberger *et al.*, 1995).

$$NADH + NADP^{+} \rightleftharpoons NAD^{+} + NADPH$$

It is well established that glucose 6-phosphate dehydrogenase, a regulatory enzyme, catalyzing the first reaction of hexose monophosphate shunt is the major source of NADPH in the cytosolic compartment and continuously generates NADPH for the utilization of ascorbate-glutathione cycle which in turn is used for eliminating ROS from the cell (Corpas *et al.*, 1998). The interrelationship of phosphopentose pathway, GR and glutathione peroxidase is illustrated as follows :

MODEL PROPOSED FOR THE FUNCTION OF THE ASCORBATE-GLUTATHIONE CYCLE AND THE DEHYDROGENASE-MEDIATED RECYCLING OF NADPH IN LEAF PEROXISOME



G6PDH	= Glucose 6-Phosphate Dehydrogenase
6PGDH	= 6 Phosphogluconate Dehydrogenase
APX	= Ascorbate Peroxidase
DHAR	= Dehydro Ascorbate Reductase
GR	= Glutathione Reducatase
ICDH	= Iso Citrate Dehydrogenase
MDHAR	= Mono Dehydro Ascorbate Reductase
MDHA	= Mono Dehydro Ascorbate
DHA	= Dehydro Ascorbate
ASC	= Reduced Ascorbate
GSSG	= Oxidized Glutathione
GSH	= Reduced Glutathione

2.8.3. ENZYMES INVOLVED IN THE METABOLISM OF PHOSPHATE:- ACID AND ALKALINE PHOSPHATASES

Phosphatases liberate inorganic phosphate from organic phosphate esters. Acid phosphatases hydrolyze a number of phosphomonoesters and phospho proteins. Alkaline phosphatases catalyze the hydrolysis of numerous phosphate esters such as esters of primary and secondary alcohols, sugar alcohols, phenols and amines. Acid phosphatase is generally linked with the degradative processes and senescence of the leaf.

Chowdhury *et al.* (1994) have reported that acid phosphatase activity in rice was higher in shaded plants than in control. Arcak et al. (1997) have found a positive correlation between acid and alkaline phosphatases and soil moisture, organic matter content, total nitrogen and available N and P.

2.8.4. ENZYME INVOLVED IN THE SYNTHESIS OF SECONDARY METABOLITES :- PHENYLALANINE AMMONIA LYASE (PAL)

Phenylalanine ammonia lyase is responsible for the conversion of L-phenylalanine to trans-cinnamic acid. Cinnamic acid serves as a precursor for the biosynthesis of alkaloids, coumarins, isoflavonoids and lignin. These compounds play an important role in pest and disease resistance mechanism. Changes in PAL activity accompanying fungal, viral and bacterial infection of plants have been reported.

Cui *et al.* (1996) showed increased PAL and Chalcone synthase mRNA levels following exposure of sorghum seedlings to fungal pathogens. Leyva *et al.* (1995) showed PAL and Chalcone synthase in mRNAs accumulated in leaves of *A. thaliana* upon exposure to low temperature. Arz and Grambow (1995) found that the injection of elicitor prepared from *Puccinia graminis* caused an eight fold increase in PAL activity within 24 hours in wheat.

2.9 MEDICINAL PLANT BIOTECHNOLOGY

Medicinal plant biotechnology may be described as a methodology to enhance the formation and accumulation of desired natural products and product modification under *in vitro* conditions. This new technology will help in increasing the formation of desirable plant products and provide new inputs to pharmaceutical industry. It has given added importance in preserving threatened species through fast micropropagation of elite and selected genotypes (Sahoo et al., 1997). The technique is also handy in the genetic upgradation and quality breeding of plants. Plant cell culture has in the recent years emerged as an important tool in the field of plant biotechnology. Break through made in the production of shikonin, vanillin and rosemarinic acid under *in vitro* conditions are significant leads for many more successful stories in future.

2.10 IN VITRO PRODUCTION OF SECONDARY METABOLITES

The production of secondary metabolites by plants has been known for a long time. These products are mainly produced as a defence against herbivores, microorganisms and ecological variations (Wink, 1988). In addition, the plant kingdom remains an excellent source for phytochemicals, including pharmaceuticals, agro-chemicals, flavours, fragrances and colors (Fox, 1992). It has been estimated that 25 % of prescription in the USA contained active ingredients obtained from plants. The world market for plant products are very high, some reaching $100 million and the demand is increasing at a greater percentage. These estimates as well as the safety and complex procedures involved in the chemical synthesis of these products indicate a greater demand for natural products from plants and hence seek for a biotechnological approach (Yonemitsu *et al.*, 1990).

Plant tissue culture offers a potential alternative for phytochemicals, which are of importance to food and pharmaceutical industries (Fowler and Stafford, 1992). Routier and Nickell (1956) first discussed the use of plant tissue culture technique for commercial production of natural products. Since then, a wide range of compounds

namely, alkaloids, steroids, terpenes, flavanoids etc., have been obtained by this technique (Phillipson, 1990). Moreover, many reports are available showing that cultured cells produce products whose potentials are equal or even higher than the parent plant (Zenk *et al.*, 1977).

The production of phytochemicals *in vitro* is advantageous in many ways. It is independent of the environmental factors such as climate, disease, geographical and seasonal constraints. It is a defined production system, with production as and when required giving a close control over market supply. The product quality and yield are more constant. It is independent of political interference and it is possible to produce novel compounds of commercial interest with easy manipulation.

2.11. PRODUCTION OF ALKALOIDS BY PLANT TISSUE CULTURE

Of the valuable plant secondary metabolites obtained through tissue culture, alkaloid production has been a subject of extensive study. The possibility of utilizing plant tissue and cell cultures for the formation of compounds of medicinal value was reported as early as 1942. In addition, constant supply of sources for alkaloids from conventional procedures is restricted. The production of alkaloids in cell culture being higher would add to the advantage of economic production in large scale. The earlier efforts were empirical in nature and were primarily aimed at establishing rapidly growing tissue culture systems and ascertaining their ability to synthesise the desired compounds. During the last three decades, it has become quite clear that the biosynthesis of plant products is under the influence of a number of regulatory mechanisms. A clear understanding of these mechanisms should make the exploitation of plant tissue culture system a feasible venture and it may be possible to obtain a stable supply of bioactive secondary products (Iyer *et al.*, 1998).

It is now possible to establish tissue culture from any part of a plant either as static or as liquid suspension cultures and such cultures can be maintained over several years. Plant cell, tissue and organ cultures have been established from a large number of medicinal plants, some of which synthesise secondary products in

significant amounts, for example anthroquinones from *Morinda citrifolia*, Shikonin from *Lithospermum erythrorhizon*, diosgenin from *Dioscorea deltoides* and vinblastine and ajmalicine from *Catharanthus roseus* (Chand *et al.*, 1999).

2.12. STRATEGIES TO INCREASE SECONDARY METABOLITES

Even though plant cell culture technique offers an attractive alternative for the production of secondary metabolites, unfortunately, in most of the cases, the yield of the product is too low to exploit them for commercial enterprise (Yeoman *et al.*, 1990). During the last decade, a distinct progress has been made to stimulate the formation and accumulation of secondary metabolites (Fowler and Stafford, 1992).

The importance of the selection of highly productive plants for culture initiation has been demonstrated by Kinnersley and Dougall (1986) who showed that cultures derived from high yielding plants of *C. roseus* and *N. tabacum* produced higher amounts of alkaloids. Therefore, screening at the plant level is important for the selection of high yielding plants for the initiation of tissue culture. Zenk *et al.* (1977) proposed that the manipulation of media and screening and selection procedures are effective in stimulating secondary metabolite formation in culture. In this context, various strategies have been employed. These are dealt in the following section.

2.13. OPTIMIZATION OF CULTURE MEDIA

Optimization of culture media is an important approach for improving the growth as well as the production of secondary metabolites in tissue cultures. This study involves the testing of various media compositions to increase the biomass density and product levels.

2.14. MANIPULATION OF NUTRIENTS TO IMPROVE YIELD

Apart from selection, optimization of nutrients in the culture media was found to regulate secondary metabolism (Dicosmo and Towers, 1985). They include:

1) Phytohormones
2) Carbon
3) Other nutrients

2.14.1. PHYTOHORMONES

Changes in hormone levels were found to be very effective in the development of media for secondary metabolite production (Rhodes *et al.*, 1994). The type and concentration of auxin or cytokinin or auxin to cytokinin ratio alters dramatically both the growth and product formation in cultured plant cells. The regulatory role of auxins was reported in many systems (Gamborg *et al.*, 1971; Rokem and Goldberg, 1985 and Yeoman, *et al.*, 1990).

2.14.2. CARBON

The carbon source in plant cell culture media is usually supplied as carbohydrate, the most common being sucrose. This serves various purposes like synthesis of cell constituents, providing energy for growth and maintenance and providing carbon skeleton necessary for product formation (Dougall, 1980). In the context of secondary metabolite production, higher yields were obtained when the sucrose concentration was increased (Nozue *et al.*, 1987). The osmotic stress created by sucrose alone and with other osmotic agents were found to regulate anthocyanin production in *Vitis vinifera* cultures (Do and Cormier, 1990). A dual role of sucrose as a carbon source and an osmotic agent was observed in *Solanum melongena* (Mukherjee *et al.*, 1991).

2.14.3. OTHER NUTRIENTS

The nutrients employed in plant tissue culture media influence both primary and secondary metabolism. It is envisaged that an increase in the levels of these nutrients support rapid cell growth (prolonged lag and exponential phase) in culture systems (Caldentey *et al.*, 1994). Of these, the major nutrients, nitrate and phosphate are known to influence a wide range of secondary metabolites (Ravishankar and

Grewal, 1988). Decreased levels of nitrate and phosphate, the source of nitrogen and carbon to nitrogen ratio are found to regulate secondary metabolite production (Mukherjee *et al.*, 1991). Further, changes in basal media from a high salt medium to a low salt medium or *vice versa* have proved to be effective for induction of secondary metabolites as in the case of *Catharanthus roseus* and *Lithospermum erythrorhizon* (Fujita *et al.*, 1981). In tobacco cultures, nicotine production was found to be increased by 24 folds when ammonia is totally replaced by nitrate (Roper *et al.*, 1985).

2.15. ELICITATION

Different strategies have been applied to increase the productivity of phytochemicals, of which elicitation was found to support vigorous and active secondary metabolism. Any substance that stimulates the production and accumulation of secondary metabolite is an elicitor (Das *et al.*, 1999).

The microbe-derived molecules, which cause the production of secondary metabolites, are termed biotic elicitors and the non-biological ones are called abiotic elicitors.

2.15.1. BIOTIC ELICITORS

These include the elicitors prepared from fungal, yeast or bacterial cultures. Of these, the fungal mycelial extracts, culture filtrates, fractions or compounds obtained from the cell wall were extensively used as elicitors. The stimulation of alkaloids, codeine and morphine by fungal spores in *Papaver somiferum* cultures has been reported (Heinstein, 1985). Autoclaved fungal mycelia induced the accumulation of diosgenin in *D. deltoidea* cultures (Rokem *et al.*, 1984). The production of berberine and shikonin was enhanced in cultured cells treated with fungal extracts (Kim and Chang, 1990).

Apart from fungal elicitors, preparations from yeast and bacteria were also reported to enhance secondary metabolites. A β-glucan elicitor prepared by ethanol precipitation of the yeast *Saccharomyces cerevisiae* was found to elicit alkaloid

production in cultured cells of *Eschscholtzia*. In *Tabernaemontana divaricata* cultures triterpene accumulation was increased by treating the cells with an elicitor preparation from *Candida albicans* (Wijsnma *et al.*, 1985). Influence of several bacterial preparations was studied for their elicitor activity and antimicrobial activity in *Tagetes* cultures (Buitelaar *et al.*, 1992). Purified fractions from bacteria were found to elicit diosgenin and capsaicin production (Johnson *et al.*, 1991).

2.15.2. ABIOTIC ELICITORS

In addition to biotic elicitors, compounds of non-biological origin were also found to act as elicitors. They included metal ions, chemically defined compounds and physical agents such as UV-light (Darvill and Albersheim, 1984). Of these metal ions were reported widely to induce secondary metabolite production (Threlfall and Whitehead, 1988a,b). The elicitor activity of calcium and several calcium dependent processes have been reported in many systems (Stab and Ebel, 1987). Vanadium was found to mimic the elicitor activity of fungal cell walls in cell culture systems (Smith *et al.*, 1987). Darvill and Albersheim (1984) proposed that abiotic elicitors function by activating biotic elicitors present in an inactive form.

2.16. GENETIC DIVERSITY STUDIES

The genetic diversity existing in the medicinal plant species is of immense significance to mankind. This heritage bestowed by nature provides valuable material like medicinal compounds, timber etc. for the humans. With the increasing trend of urbanization, the magnitude of concern and importance should be attributed to *ex situ* conservation of germplasm. As still many medicinal plants are in their wild status, genotypical characterization becomes important before employing them in genetic improvement programmes. This helps in better understanding of diversity, utilization and conservation of genetic resources. In order to foster a better appreciation of the diversity among plants, along with conservation, diversity analysis is substantial. Though the forest plant populations are considered as the valuable resources and kept undistributed over years, the enormous growth of human population resulted in larger

deforestation and land clearing which is becoming a matter of concern for the present and future.

The analysis of genetic diversity and relatedness between individuals or within different species or population is a central task for many disciplines of biological science. Till recently, the genetic diversity and phylogenetic studies were conducted mostly based on morphological traits using various statistical methods which are highly influenced by environmental effects and require tedious statistical procedures.

In the past, genetic variability was assessed by adopting studies such as comparative anatomy, morphology, embryology, physiology and biochemistry. The advent of molecular marker technique, an analysis based on the polymorphisms found in proteins or DNA has greatly facilitated the research activities in a variety of disciplines such as taxonomy, phylogeny, ecology, genetics and plant breeding.

2.16.1. ISOENZYME ANALYSIS

Isoenzyme analysis has been widely used over the past several decades to conduct investigations of the genetics of a large number of organisms, from fruit flies and wild herbs to humans. Isoenzyme analysis will become increasingly feasible and important in laying a sound basis for genetic management of medicinal tropical forest resources and is considered to be a powerful technique for the study of population genetics of plant kingdom (Mandal *et al.*, 1994; Kertadikera and Prat, 1995).

A genetic change causing an alteration in the amino acid sequence of an enzyme may alter the surface charge and cause damage in the rate of movement in electric field. Therefore, electrophoresis of one enzyme can indicate genetic differences originating from gene mutations. In this way, basic genetic differences among individuals or population can be investigated simply and rapidly. The study of genetics using enzymes has several advantages, as enzyme expression is unaffected by differences in environment, heterozygotes can be distinguished from homozygotes and as each individual contained thousands of enzymes, many loci can be studied

simultaneously. Isoenzymes are therefore very valuable in studying genetic variations.

However, there are a number of problems in using isoenzyme analysis for this purpose. Firstly, not all gene mutations will result in changes in the surface charge of the enzyme produced; therefore not all such mutations are detectable. In fact, only about 27% of amino acid substitutions are detectable (Lewontin, 1974). Secondly, enzymes represent only one class of genes, which may not be representative of the whole genome. Thus, estimates of genetic variation arising from studies of isozymes and other characters may differ. Despite these problems, the major advantage of studying isozyme variation and data from isozyme analysis can be used for many purposes.

2.16.2 RANDOMLY AMPLIFIED POLYMORPHIC DNA (RAPD) ANALYSIS

Unlike isozymes, DNA markers enable investigators to analyse the whole geneome, which has a wider option to observe genetic variation. DNA markers in fact have added new dimensions in understanding the genetic structure of populations in plants. A wide variety of techniques to visualise DNA sequence polymorphism have been developed in the past few years and are being employed in the genetic diversity analysis.

DNA marker studies have been successfully employed in plant to understand provenance variations (Chalmers *et al.*, 1992), genotypic identity (Keil and Griffin, 1994). molecular characterisation of germplasm (Machado *et al.*, 1996), quantitative trait loci detection (Grattapaglia and Sederoff, 1995), molecular systematics (Harris, 1995), genetic diversity (Varghese *et al.*, 1997) and hybrid identification (Rosetto *et al.*, 1997).

Of the various genetic markers currently used for the analysis of genetic diversity or for the establishment of systematic relationships, RAPDs are considered to be efficient and cost effective (Hadrys *et al.*, 1992). RAPDs have been used

effectively as genetic markers in genome mapping, population genetics, linkage mapping and taxonomic studies of numerous crop genera (Demeke *et al.*, 1992) and in detecting intraspecies variation. Analysis of RAPDs is less technically demanding and more rapid than RFLPs (Restriction Fragment Length Polymorphism) and is potentially capable of sampling more loci than isozyme analysis. With a virtually unlimited number of commercially synthesised primers available, this technique is likely to see wider use for analysing genetic variability in plant germplasm collections and in systematic studies (Hadrys *et al.*, 1992). Reproducible amplification of RAPD markers have been demonstrated in many species including rice (Fukuoka *et al.*, 1992), wheat (Devos and Gale, 1992), corn (Huen and Helentjaris, 1993) and barley (Tinker *et al.*, 1993).

Padmesh *et al.* (1999) assessed the genetic variability in *Andrographis paniculata* Nees, reputed for its antipyretic and hepato protective properties. Shah *et al.* (1994) reported an interpopulation variation in *Elaeis guinensis* using RAPD analysis. Nesbitt *et al.* (1995) observed very little genetic variation between natural populations of *Eucalyptus globulus* using 149 polymorphic bands of RAPD. But those populations showed marked variation between each other in terms of capsule and juvenile leaf morphology.

Machado *et al.* (1996) observed considerable population variation in 53 accessions of *Theobroma cocoa* using RAPD. Bucci *et al.* (1997) assessed the divergence among the two Greek populations and five Italian populations of *Pinus leucodermis* using 23 previously identified RAPD markers and 16 polymorphic allozymic markers. Divergence among Italian populations was found to be as large as that between Italian and Greek populations. Since it is likely that the split of two nuclei populations took place more than 10,000 years ago, a larger differentiation between, rather than within, the nuclei was expected.

Vicario *et al.* (1995) studied the genetic relationship between *Abis alba* and *A. nebrodensis* using allozyme, chloroplast DNA and RAPD markers. Harris (1995)

used RAPD to examine genomic diversity in taxa of the neo-tropical legume genus *Leucaena*. Distance based methods of RAPD analysis produced groups inconsistent with those identified by RFLP analysis. The major differences were grouping of the two subspecies of *L. diversifolia* in the RAPD tree, but they are separated in the RFLP tree.

Parani *et al.* (1997) used RAPD and RFLP markers to estimate intra and interspecific variations in three species of an exclusive mangrove genus, *Avicennia*. Polymorphism in *Avicennia officinalis* (Pichavaram population, 32.3%) and *Avicennia alba* (Coringa population, 37.8 %) was greater than the intra population variation observed in the populations of *A. marina* from each of the respective locations. Varghese *et al.* (1997) observed high genetic variation within the population than between the population in *Hevea brasiliensis*.

Based on the literature reviewed above, *A. vasica* and *A. beddomei*, that are used in 110 herbal formulations (Kamboj, 2000) have been chosen for the study. *Adhatoda vasica* Nees., popularly known as vasaka and *Adhatoda beddomei* Clarke., belong to the family *Acanthaceae* and are well known and highly reputed drugs in Ayurvedic and Unani system of medicine for treating respiratory tract disorders especially for asthma complaints for more than 2000 years. These plants are also used in the treatment of jaundice, fever, vomiting, skin diseases, constipation, tuberculosis, urinary disorders, hypertension, rheumatism, opthalmia and leprosy. *A.beddomei* is more active and powerful than *A.vasica* and has a very restricted natural range. Because of endemism and over exploitation *A.beddomei* is identified as one among the listed rare, threatened and endemic medicinal plants.

EXPERIMENTAL PROCEDURE

3.0 EXPERIMENTAL PROCEDURE

Asthma is a common but complex disease, that becomes the most common chronic disease in industrial areas and epidemiological studies suggest that its prevalence, severity and mortality are rising now. Asthma is a chronic condition of the airways that involves many types of inflammatory cells and the release of many mediators and neurotransmitters that have multiple effects on various target cells in the airway. Anti-inflammatory treatments include sodium chromoglycate, nedocromil sodium and corticosteroids. But corticosteroids given systematically, have many adverse side effects (Barnes, 1991). In recent years, many traditional drugs and techniques are gaining popularity in the treatment of asthma (Pairet *et al.*, 1997).

Adhatoda vasica and *Adhatoda beddomei* are highly reputed Ayurvedic medicinal plants and are used for the treatment of asthma, cough and other respiratory diseases since many years without any evidence on the toxic side effects.

In the present study, *A. vasica* and *A. beddomei* were collected from a few selected places. Alkaloids from the two species were extracted and quantified. The protein content, tannin and selected enzymes were assessed. Optimization of *in vitro* propagation media was done to elicit maximum quantity of alkaloids in the selected species. Besides these, genetic diversity was found out through isoenzyme pattern and RAPD analysis. The experimental procedure adopted is described under the following headings:

3.1 Collection of plant materials
3.2 Extraction of alkaloids
3.3 Estimation of proteins
3.4 Estimation of ascorbic acid and β-carotene
3.5 Assay of selected enzymes
3.6 Tissue culture studies
3.7 Genetic diversity studies
3.8 Statistical analysis

3.1 COLLECTION OF PLANT MATERIAL

Adhatoda vasica and *Adhatoda beddomei* were collected from the following places (Plate 1) and used for the subsequent studies.

Adhatoda vasica was collected from

1) Suruli falls
2) Kanjikode, Kerala
3) Thaimudi, Mudis
4) Kilkothagiri, Nilgiris
5) Coimbatore civil aerodrome region
6) Institute of Forest Genetics and Tree breeding, Coimbatore

Adhatoda beddomei is an endemic and endangerd species. It was collected from Kanjikode, Kerala (Fig. 1).

3.2 EXTRACTION OF ALKALOIDS

Secondary metabolites are biosynthesized from primary metabolites. Although their exact role is still not clear, many of them have an ecological role. The synthesis and accumulation of secondary metabolites might vary in *Adhatoda* species selected from different environmental conditions. Pharmacological studies of *Adhatoda* species revealed that anti-asthmatic property resides in the alkaloid fraction. The alkaloid content in the selected species was quantified after extraction, isolation and purification.

Dragendroff reagent

(a) 0.6 g Bismuth subnitrate in 2 ml concentrated HCl and 10 ml water.
(b) 6 g Potassium iodide in 10 ml water.

These solutions were mixed together with 7 ml concentrated HCl and 15 ml water and the whole diluted with 400 ml water.

Fig. 1 : Collection spots of Adhatoda species

- Collection spots

Plate -1 : *Adhatoda* species

Adhatoda vasica

Adhatoda beddomei

Vasaka or *A. beddomei* leaves were dried in the shade and powdered. The coarse powder was refluxed with 90% alcohol for 1 hour. The extract was filtered and the alcohol was removed under reduced pressure. The dark green mass obtained was extracted thrice with hot distilled water and the aqueous extract was filtered. The filtrate was extracted five times with chloroform to remove the pigments, then made alkaline with 5 % NaOH and repeatedly extracted with chloroform till no reddish orange colour was obtained with Dragendroff reagent. The combined chloroform extracts were extracted with 5% hydrochloric acid repeatedly, when the alkaloid fraction was extracted into the acid phase. The acid solution was made alkaline with ammonia and the alkaloids once again extracted with chloroform. After repeating this operation twice, the final chloroform extract was filtered and the chloroform was removed under reduced pressure. Crude fraction of total alkaloids was obtained as a brown powder.

3.2.1 PURIFICATION OF ALKALOIDS BY PARTITION CHROMATOGRAPHY

The crude total alkaloids were dissolved in moist chloroform (6 ml). A column of silica gel (10 g) suspended in 10% H_3PO_4 was prepared and the crude alkaloid fraction was loaded on the column. Elution was carried out with moist chloroform till the eluate did not extract any more alkaloid. The elution was then continued with ammonia chloroform mixture (3 : 1) till the eluate did not test positive for alkaloids. Chloroform was recovered and the precipitate thus obtained was dried over calcium chloride and weighed.

3.2.2 IDENTIFICATION OF ALKALOIDS

The crystallized alkaloids were dissolved in chloroform and were then used for identification by paper chromatography.

3.3 ESTIMATION OF PROTEINS

When plants are subjected to any stress, to encounter the imbalance created by the stress, they produce proteins which are believed to have protective functions. In the present study, total protein content was estimated in *A. vasica* collected from various places and *A. beddomei* using Bradford (1976) assay.

Sample Extraction

500 mg of leaf sample was crushed and homogenized with 5 – 10 ml of 0.5 M Tris buffer pH 7.6 and then centrifuged for 15 minutes at 10,000 rpm. The supernatant was used for protein estimation.

Bradford's reagent

Dissolved 100 mg of Coomassie brilliant Blue G250 in 50 ml of 95% ethanol. Added 100 ml of concentrated ortho phosphoric acid. Made up to 200ml with distilled water.

One hundred µl of the supernatant was pipetted out into different test tubes. The volume was made upto 1.0 ml with the buffer. Bradford reagent (5.0 ml) was added to each test tube and incubated for 15 minutes in the dark. The absorbance was read at 595nm. Protein standard graph was drawn using 10- 100 µg of BSA . The protein content in the extract was calculated from the standard graph.

3.4 ESTIMATION OF ASCORBIC ACID AND β-CAROTENE

i) ASCORBIC ACID

Sample extraction

One gram sample was extracted with 4% oxalic acid and made up to a known volume (100 ml) and centrifuged. The supernatant served as enzyme source.

Dye solution

Weighed 42mg sodium bicarbonate into a small volume of distilled water. Dissolved 52mg 2,6-dichlorophenol indophenol in it and made up to 200ml with distilled water.

Stock standard solution

Dissolved 100mg ascorbic acid in 100ml of 4% oxalic acid solution in a standard flask (1mg/ml).

Working standard

Diluted 10ml of the stock solution to 100ml with 4% oxalic acid (100 µg/ml).

Pipetted out 5ml of the working standard solution into a 100 ml conical flask. Added 10 ml of 4% oxalic acid and titrated against the dye (V_1ml). End point is the appearance of pink colour which persists for a few minutes. The amount of the dye consumed is equivalent to the amount of ascorbic acid. Pipetted out 5ml of this supernatant, added 10 ml of 4% oxalic acid and titrated against the dye(V_2ml).

Calculation

Amount of ascorbic acid mg/100g sample

$$= \frac{0.5 \text{ mg}}{V_1 \text{ ml}} \times \frac{V_2}{5 \text{ ml}} \times \frac{100 \text{ ml}}{\text{Wt. of the sample}} \times 100$$

ii) ESTIMATION OF β-CAROTENE

Principle

The High Performance Liquid Chromatography is found to be the most reliable, efficient and reproducible method for carotenoid analysis. The carotenoids in foods are generally associated with the lipid fraction and hence beta carotene in the food is saponified with 12 per cent alcoholic potassium hydroxide and extracted with

petroleum ether (60°-80°C), filtered and the carotenoid pigments separated, identified and estimated using reverse phase isocratic solvent system. Identification and quantification are based on the retention time and peak area of the individual samples.

Reagents

1. 12 % alcoholic potassium hydroxide
2. Petroleum ether (60°-80°C)
3. HPLC grade solvents – Methanol, Acetonitrile, Dichloromethane
4. Calcium carbonate
5. Anhydrous sodium sulphate
6. Beta carotene standard (Sigma, USA)

Procedure

1. 0.5 g of the sample was taken in a mortar and pestle, added small amount of fine glass powder (1 g) and macerated with a small amount of potassium hydroxide (12 %), so that the sample was completely immersed in it.
2. Transferred the ground sample into a conical flask and added the washings of the remaining sample in the mortar with 12 % potassium hydroxide.
3. Placed in a shaking waterbath at 37°C for 30 minutes to saponify the contents.
4. Mixed the conical flask – contents with little calcium carbonate and petroleum ether (60°-80°C) using a vortex mixer.
5. Transferred the contents of the conical flask into a separating funnel.
6. The upper layer was collected, discarding the lower layer and repeatedly extracted with petroleum ether until no yellow colour was observed in the supernatant layer.
7. Filtered the extract through glass wool, over which anhydrous sodium sulphate was kept to remove any moisture.
8. Volume of the extract was noted.

Determination of beta carotene by HPLC

One ml of the sample solution was filtered through 0.4 millimicron filter and 20 microlitres was used for quantification in high performance liquid chromatography.

The HPLC Shimadzu model is equipped with a variable wavelength and integrator. The wavelength set at 450 nm. A stainless steel column 150 x 4.6 mm in diameter, prepacked with shim-pack CLS ODS packing is used for carotenoid analysis. The solvent system, acetonitrile : dichloromethane : methanol, all HPLC grade solvents (70:20:10), was pumped at a flow rate of 2 ml per minute. Beta carotene standards used are of sigma chemical company (U.S.A) Type IV synthetic from carrots.

An external standard was used for quantification. Because of variation in concentration of compounds to be analysed in the food matrix, no internal standard was used. The HPLC was calibrated daily by injecting a full loop (20 microliters) of each standard solution in duplicate or triplicate. Each sample was injected in duplicate and the concentration determined from the calibration plot in which peak area was plotted against concentration (mg/ml). The detector responses are linear over the concentration range of 25 to 100 mg per 20 microlitres. All results were expressed as microgram per gram of fresh weight of the sample. Peak identification was based on the retention times and comparison with standards and co-chromatography with the standards.

3.5 ASSAY OF SELECTED ENZYMES IN Adhatoda SPECIES

The generation of free radicals are now accepted as universal attribute of aerobic life. The inevitable generation of Reactive Oxygen Species (ROS) are counteracted by multiple lines of defense at all levels namely protection, prevention, interception and repair which are provided by non enzymatic radical scavengers and quenchers as well as enzymatic defense systems.

Environmental stresses are known to result in significant metabolic changes most of which are adaptive responses that enable the plants to survive in stressed environment (Wellburn *et al.*, 1996; Fukao *et al.*, 1998). It was therefore felt imperative to assess the enzyme activities in *Adhatoda* selected from the different locations. The enzymes quantified were,

Enzymes involved in the detoxification of reactive oxygen species (ROS)

1. Superoxide dismutase
2. Peroxidase
3. Catalase
4. Glutathione reductase

Enzymes that are known to be sensitive to changes in environmental stresses and involved in oxido reduction reactions

1. Nitrate reductase
2. Indole aceticacid oxidase
3. Ascorbic acid oxidase
4. Glucose-6-phosphate dehydrogenase

Enzymes involved in the metabolism of phosphate

1. Acid phosphatase
2. Alkaline phosphatase

Enzyme involved in the synthesis of secondary metabolites

Phenylalanine Ammonia Lyase

3.5.1. ENZYMES INVOLVED IN THE DETOXIFICATION OF REACTIVE OXYGEN SPECIES (ROS)

Superoxide dismutase, catalase and peroxidases constitute a mutually supportive team of defense against ROS. While SOD lowers the steady state level of $O_2^{\bullet}$, catalase and peroxidases do the same for H_2O_2. The catalytic removal of ROS by these antioxidant enzymes is as follows

$$2O_2^{\bullet} + 2H^{+} \xrightarrow{SOD} H_2O_2 + O_2.$$

$$H_2O_2 + AH_2 \xrightarrow{peroxidase} 2H_2O + A$$

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

$$ROOH + AH_2 \xrightarrow{\text{catalases}} H_2O + ROH + A$$

3.5.1.1. SUPEROXIDE DISMUTASE (SOD)

Superoxide dismutase is a metallo protein found in both prokaryotic and eukaryotic cells (Fridovich, 1986). In eukaryotic cells, the predominant forms are the copper- containing enzyme, located in the cytosol. The second type is the manganese- containing SOD found in the mitochondrial matrix. The biosynthesis of SOD is mainly controlled by its substrate, the $^{\bullet}O_2$. SOD, an enzyme that catalyses the dismutation of the superoxide radical, provide unequivocal proof for the generation of free radicals in biological system. Most of the $^{\bullet}O_2$ generated *in vivo* probably undergoes a non-enzymatic or SOD catalysed reaction to generate H_2O_2.

Assay of superoxide dismutase enzyme was done with slight modification of the original procedure described by Beauchamp and Fridovich (1971).

Sample extraction

500 mg of leaves were ground in 50 mM potassium phosphate buffer pH 7.8, and centrifuged at 10,000 rpm in a Beckman centrifuge for 10 minutes at 4°C. The supernatant was used for the assay.

A 3.0 ml reaction mixture consisting of 50 mM phosphate buffer (pH 7.8). 13 mM methionine, 2 µM riboflavin, 0.1 mM EDTA, 75 µM nitroblue tetrazolium (NBT) and 50 µl of crude enzyme was set up with five replications. The volume was made up with double distilled water. A blank without enzyme and NBT was used to calibrate the spectrophotometer (Beckman DU64). Another control (having NBT but no enzyme) served as a reference blank.

All the tubes were exposed to fluorescent light (two 42 cm and two 60 cm fluorescent tubes) for 15 minutes. Immediately the absorbance was read at 560 nm in

a Beckman DU 64 spectrophotometer. The protein in the sample was also measured simultaneously with the available enzyme samples. The percentage inhibition was calculated and expressed as the number of units of enzyme mg/ml/min. 50 per cent inhibition is a unit of SOD .

3.5.1.2. PEROXIDASES (POD)

Peroxidases are hemeproteins that catalyse the oxidation of several substrates at the expense of H_2O_2 and are known to be present in prokaryotes and eukaryotes (Asada, 1992). There are two major classes of peroxidases in higher plants. One group guaiacol peroxide (GPx), appears to be involved in phenolic and alkaloid metabolism (Ros-Barcelo *et al.*, 1998). The other major peroxidase group is represented by ascorbate peroxidase (APx). This enzyme catalyses the oxidation of a wide variety of electron donors with the help of H_2O_2 and thereby scavenge the endogenous H_2O_2 (Mazumdar *et al.*, 1997).

Sample extraction

2.0 g of leaf samples were homogenized in cold phosphate buffer (0.05M, pH 6.5). The homogenate was centrifuged at 10,000 rpm in a Beckman centrifuge at 4°C for 10 minutes. The supernatant was used as the enzyme source.

Peroxidase was assayed following the method of Kar and Mishra (1976) with slight modifications. Five ml of the assay mixture contained 300 µM phosphate buffer (pH 6.8), 50µM catechol, 50 µM H_2O_2 and 1.0 ml of crude enzyme extract. After incubation at 25°C for five minutes the reaction was stopped by the addition of 1.0 ml of 10 per cent H_2SO_4. The colour was read at 430 nm.

For enzyme assay, the value at zero time was taken as blank and the activity of enzyme was expressed as unit g^{-1} fresh tissue.

$$\text{Enzyme Activity} = \frac{A \times TV}{t \times v}$$

where

A - Absorbance of sample after incubation minus the absorbance at zero time

TV - Total volume of the filtrate

t - The time (minutes) of incubation with substrate and

v - The total volume of the extract taken for incubation

3.5.1.3. CATALASE (CAT)

Catalase present in almost all the eukaryotic cells is localized in the peroxisomes or microperoxisomes. It is a hemeprotein and catalyzes the decomposition of H_2O_2 to water and oxygen and thus protects the cell from oxidative damage by H_2O_2 and ^{-}OH (Bandyopadhyay *et al.*, 1999).

Sample extraction

100 mg leaf tissues were crushed in 0.1 M phosphate buffer (pH 7.0) containing 1.0 per cent Polyvinyl pyrollidone and centrifuged at 4°C. The enzyme assay was carried out according to the method described by Biswas and Choudhuri (1976), in which catalysis of H_2O_2 to H_2O in an incubation mixture adjusted to pH 7.0 was recorded at 240 nm for 1 minute. The reaction mixture contained 10 mM H_2O_2, 25 mM phosphate buffer pH 7.0 and suitable aliquot of enzyme solution (0.01 – 0.04 ml). The time required for the decrease in absorbance from 0.45 to 0.40 representing the linear portion of the curve, was used for calculation of the enzyme activity. One unit of catalase activity was defined as the amount of enzyme, causing the decomposition of µmol H_2O_2 / mg protein / min at pH 7.0 at 25°C. Enzyme activity was calculated as given for peroxidase.

3.5.1.4 GLUTATHIONE REDUCTASE (GR)

The pentose phosphate pathway provides NADPH for the reduction of oxidized glutathione to reduced glutathione catalysed by GR, a flavoprotein containing FAD.

In turn, reduced glutathione removes H_2O_2 from plant cell in a reaction catalysed by glutathione peroxidase. The activity of glutathione reductase was assayed as described by Smith *et al.* (1988).

Enzyme extraction

200 mg of tissues were ground in 500 µl ice-cold 0.2 M potassium phosphate buffer (pH 7.5) containing 1.0 mM EDTA. It was centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was used as the enzyme source.

A 2.0 ml reaction mixture consisting of the following ingredients was taken in a clear quartz cuvette.

(i)	Potassium phosphate buffer (0.2M) containing 1.0mM EDTA	: 1.0 ml
(ii)	5,5'-dithio nitrobenzoate (DTNB) 3.0 mM pH 7.5 (in 0.01 M phosphate buffer, pH 7.5	: 0.5 ml
(iii)	NADPH 10 mM	: 0.1 ml
(iv)	Distilled water	: 0.25 ml
(v)	Crude extract	: 0.05 ml
(vi)	Glutathione oxidized (GSSG) 20 mM	: 0.1 ml

All the ingredients listed above except GSSG were added and used as the blank to calibrate the spectrophotometer. A kinetics Soft Pac module was used to assay the enzyme at every 30 seconds interval for a total period of 5 minutes at 412 nm. The reaction was initiated by the addition of 0.1 ml of GSSG.

The increase in absorbance was calculated from the linear portion of the enzyme reaction curve and the enzyme activity is expressed as increase in absorbance min^{-1} mg^{-1} protein.

3.5.2 ENZYMES THAT ARE KNOWN TO BE SENSITIVE TO CHANGES IN ENVIRONMENTAL STRESSES AND ALSO INVOLVED IN OXIDO REDUCTION REACTIONS

3.5.2.1 NITRATE REDUCTASE (NR)

The enzyme NR has been much studied with regard to the ecology of NO_3^- assimilation because of its almost unique kinetics. NR is substrate inducible and there was a marked positive correlation between NR activity and atmospheric pollutants (Pearson and Soares, 1995). So NR may be used as one of the biochemical stress bioindicators to monitor atmospheric pollution. The nitrate reductase activity was estimated as described by Hageman and Reed (1980).

Enzyme extraction

500 mg of leaf material was homogenized in a medium containing 1 mM EDTA, 25 mM cysteine and 25 mM potassium phosphate adjusted to a final pH 8.8 with KOH. Centrifuged for 15 minutes at 30,000 g. The supernatant served as enzyme source.

Added 0.2 ml of enzyme extract in a test tube containing 0.5 ml of 0.1M phosphate buffer pH 7.5, 0.2 ml of 0.1 M potassium nitrate, 0.4 ml NADH and 0.7 ml H_2O. Control was set up in the same way but with water instead of enzyme extract. Incubated at 30°C for 15 minutes and terminated the reaction by the addition of 1 ml of 1% sulphanilamide followed by 1 ml of 0.02 % naphthyl ethylenediamine reagent. After 10 minutes read the absorbance at 540 nm. Prepared the standard graph using 0.01 M soidum nitrite. The NR activity is calculated using the standard graph. Activity is expressed as µmole nitrite produced $min^{-1}g^{-1}$ fresh tissue.

3.5.2.2 INDOLE ACETICACID OXIDASE ACTIVITY (IAAO)

Indole acetic acid (IAA) is the best known naturally occurring auxin. IAAO is the enzyme involved in the catabolic degradation of IAA to 3-methylene oxindole.

IAAO activity was increased proportionally when plants were grown under stress (Mukherjee and Choudhuri, 1981). The activity of IAA oxidase was estimated by the method of Mahadevan and Sridhar (1986).

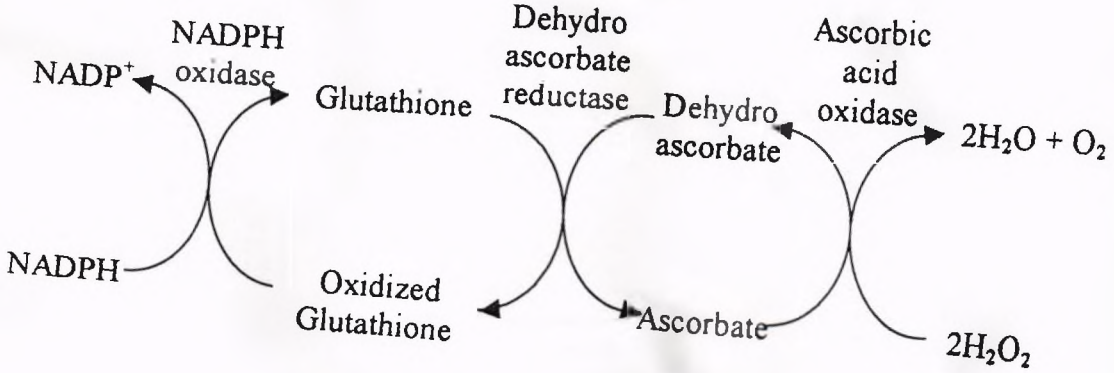
Extraction of enzyme

Exactly 1.0 g of fresh leaf was ground in 10 ml of cold distilled water, filtered through filter paper and the filtrate was centrifuged at 5,000 rpm at 4°C or 10 minutes. The supernatant was made upto 25 ml with cold distilled water.

To 1.0 ml of enzyme, 1.0 ml of phosphate buffer (0.1 M pH 6.6) and 1.0 ml of IAA (1.0 mg/ml) were added. To this, 1.0 ml of distilled water was added. The mixture was kept for incubation in a water bath at 25°C for an hour. A blank was run simultaneously without auxin. The reaction was stopped by keeping the tubes in boiling water for 2 minutes. The residual auxin in the reaction mixture was estimated by adding 8.0 ml of Gorden Weber reagent. The values were expressed as µg auxin unoxidised / hour / gram of leaf tissue.

3.5.2.3 ASCORBIC ACID OXIDASE (AAO)

It is wide spread in plant tissue. The role of this enzyme is to regulate th levels of oxidized and reduced glutathione and NADPH as shown below:



Ascorbic acid oxidase activity in plants is stimulated when plants are exposed to pollutants. Because, it is currently assumed that the negative effect of the various environmental stresses generate ROS and ascorbate plays a dominant role in the quenching of these harmful ROS (Luwe *et al.*, 1993).

Sample extraction

2.0 g of leaf samples were homogenized in cold phosphate buffer (0.05 M, pH 6.5). The homogenate was centrifuged at 10,000 rpm at 4°C for 10 minutes. The filtrate was used as the enzyme source.

For the assay of ascorbic acid oxidase, the method of Oliver (1967) was employed. The reaction mixture for this enzyme assay consisted of 1.0 ml of 100 µM ascorbic acid, 1.0 ml of 500 µM phosphate buffer (pH 6.8) and 2.0 ml of the enzyme extract. This was incubated at 37°C for 25 minutes. After incubation, 0.2 ml of 0.4 mM 2,6-dichlorophenol indophenol was added and the reading was taken at 620 nm in spectrophotometer. The enzyme activity was calculated using the formula,

$$\text{Enzyme Activity} = \frac{A \times TV}{t \times v}$$

where

A - Absorbance of sample after incubation minus the absorbance at zero time

TV - Total volume of the filtrate

t - The time (minutes) of incubation with substrate and

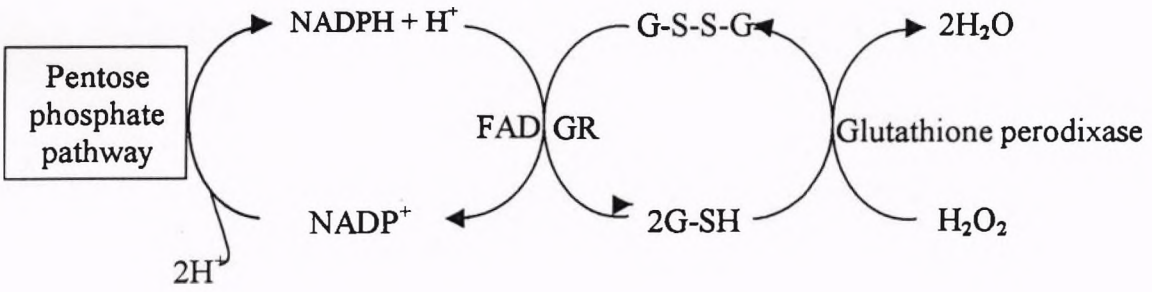
v - The total volume of the filtrate taken for incubation.

3.5.2.4 GLUCOSE 6- PHOSPHATE DEHYDROGENASE (G6PD)

Air pollutants are known to affect higher plants by damaging cell structures, disturbing physiological processes and consequently decreasing carbon fixation

(Hallgren, 1984). Moreover, increased generation of ROS is also observed when plants are exposed to adverse environmental conditions. In addition, the pentose phosphate pathway may be an important source of NADPH for the glutathione/ ascorbate H_2O_2 detoxification pathway to tackle ROS effectively (Dizengremel *et al.*, 1994).

ROLE OF THE PENTOSE PHOSPHATE PATHWAY IN THE GLUTATHIONE REDUCTASE REACTION



The glucose 6-phosphate dehydrogenase activity was measured by the initial rate of reduction of $NADP^+$ at 25°C by following the increase in absorbance at 340nm (Yoshida, 1966).

The reaction mixture contained 70μM $NADP^+$, 0.7 mM glucose 6-phosphate, 7mM $MgCl_2$, 0.05M Tris buffer pH 8.0 and suitable aliquot of enzyme solution. Enzyme activity is defined as μ moles of $NADP^+$ reduced/min at 25°C.

3.5.3 ENZYMES INVOLVED IN THE METABOLISM OF PHOSPHATE

Phosphatases liberate inorganic phosphate from organic phosphate esters. Phosphatase activities are higher in plants in polluted area than in unpolluted area. The activity of these enzymes may be used as a marker to edit the maps of air quality.

3.5.3.1 ACID PHOSPHATASE (ACP)

The activity of acid phosphatase was estimated according to the procedure described by Sadasivam and Manickam (1996).

Enzyme extraction

One gram of fresh tissue was homogenized in 10 ml of ice-cold 50 mM citrate buffer (pH 5.3) in a pre-chilled mortar and pestle and the homogenate was filtered through four layers of cheese cloth. The filtrate was centrifuged at 10,000 rpm at 4°C for 10 minutes. The supernatant was used as the enzyme source.

Substrate solution

1.49g EDTA, 0.84g citric acid and 0.039 p-nitrophenyl phosphate were dissolved in 100ml water and adjusted to pH 5.3.

Standard solution

69.75 mg p-nitrophenol in 5 ml distilled water (100 mM).

Three ml of substrate solution was incubated at 37°C for five minutes. 0.5 ml of the enzyme extract was added to it and mixed well. Immediately after addition, 0.5 ml was removed and mixed with 9.5 ml of sodium hydroxide (0.085 N). It was used as the reagent blank. The remaining solution (substrate + enzyme) was incubated for 15 minutes at 37°C. From this 0.5 ml sample was drawn and mixed with 9.5 ml sodium hydroxide solution. The absorbance was measured at 405 nm. For standard, 0.2 to 1.0 ml (4 to 20 mM) p-nitrophenol standard solution was taken, diluted to 10.0 ml with NaOH solution. Specific activity was expressed as mmoles of p-nitrophenol released per minute per mg protein.

3.5.3.2 ALKALINE PHOSPHATASE (ALP)

The activity of alkaline phosphatase was estimated following the procedure described by Sadasivam and Manickam (1996).

Sample extraction

One gram of fresh leaf was homogenized in 10 ml of ice cold 50 mM glycine - NaOH buffer (pH 10.4) in a pre-chilled mortar and pestle. The homogenate was filtered through four layers of cheese cloth and then centrifuged at 8000 rpm at 4°C for 10 minutes. The supernatant was used as the enzyme source.

Substrate solution

375mg glycine, 10mg $MgCl_2$, 165 mg p-nitrophenyl phosphate were dissolved in 42ml of 0.01N NaOH and diluted to 100ml. Adjusted to pH 10.5. The assay procedure followed was same as for the acid phosphatase.

3.5.4 ENZYME INVOLVED IN THE SYNTHESIS OF SECONDARY METABOLITES :- PHENYLALANINE AMMONIA LYASE (PAL)

Phenylalanine ammonia lyase, the first enzyme in the phenyl propanoid pathway is induced by a number of environmental stimuli including dicitation, infection, general wounding, light, temperature and other plant growth regulators (Dixon et al., 1983). PAL is responsible for the conversion of L-phenylalanine to trans cinnamic acid. Cinnamic acid serves as a precursor for the biosynthesis of a variety of secondary metabolites like alkaloids, coumarin isoflavonoids and lignin.

The activity of phenylalanine ammonia lyase was estimated according to the procedure described by Brueske (1980).

Enzyme extraction

500 mg of the plant material was homogenized in 50 ml cold 25 mM borate-HCl buffer (pH 8.8) containing 5.0 mM mercaptoethanol (0.4 ml/L). The homogenate was centrifuged at 12,000 rpm at 4°C for 20 minutes. The supernatant was used as the enzyme source.

0.5 ml of borate buffer, 0.2 ml of enzyme solution and 1.3 ml of distilled water were pipetted out into a test tube. The reaction was initiated by the addition of 1.0 ml of L-Phenylalanine solution and the incubation was done for 30-60 minutes at 32°C. The reaction was stopped by the addition of 0.5 ml of 1.0 M trichloroacetic acid. A control was also run in which phenylalanine was added after the addition of trichloroacetic acid. The absorbance was measured at 390 nm. A standard graph was prepared using trans-cinnamic acid and the activity was expressed as μ mole trans-cinnamic acid formed per mg protein per minute.

3.6 TISSUE CULTURE STUDIES

3.6.1. ESTIMATION OF TANNINS

Tannins are said to offer resistance to various stresses, diseases and pests in plants. Phenolic and tannin exudates in explants inhibit callus induction and multiplication in in vitro cultures. In order to select low tannin content sample for in vitro culture, the estimation of tannin is an essential prerequisite for choosing the best explant. The tannin content was estimated by Folins–Denis method (Schanderl, 1970).

- Folin-Denis Reagent

  Dissolved 100 g sodium tungstate and 20 g phosphomolybdic acid in 750 ml distilled water in a flask and added 50 ml phosphoric acid. Refluxed the mixture for 2 h and made up to 1 litre with distilled water.
- Sodium carbonate solution (3.5 %)
- Standard Tannic Acid Solution

  Dissolved 100 mg tannic acid in 100 ml of distilled water.
- Working Standard Solution

  Diluted 5 ml of the stock solution to 100 ml with distilled water. One ml contained 50 μg tannic acid.

Sample extraction

Weighed 500 mg of the powdered material and transferred to a 250 ml conical flask. Added 75 ml water. Heated the flask gently and boiled for 30 min. Centrifuged at 2,000 rpm for 20 minute, collected the supernatant in a 100 ml volumetric flask and made up the volume.

- Transferred 1 ml of the sample extract to a 100 ml volumetric flask containing 75ml water.
- Added 5 ml of Folin-Denis reagent, 10 ml of sodium carbonate solution and diluted to 100 ml with water.
- Shook well and read the absorbance at 700 nm after 30 minutes.
- Prepared a blank with water instead of the sample.
- Prepared a standard graph by using 0-100 µg tannic acid.
- Calculated the tannin content of the samples as tannic acid equivalents from the standard graph.

3.6.2 OPTIMIZATION OF CULTURE MEDIA

Optimization of culture media is an important approach for improving the growth as well as production of secondary metabolites in tissue culture. This study involved the testing of various media composition to increase the biomass density and product levels.

The effect of growth on secondary metabolite formation is puzzling. A number of examples have shown that accumulation occurs parallel to growth, whereas others show that maximum accumulation occurs after growth has slowed or ceased (Zenk *et al.*, 1977).

Therefore, it is necessary to have two media namely initial growth media and the production media (Stafford *et al.*, 1985) i.e., the first medium for initiation and growth and the second medium is for secondary metabolites synthesis and accumulation. Media formulation affects not only culture initiation but also growth

rate, biomass production and specific secondary product synthesis. The effect of basic compounds has been extensively studied by many workers (Delfel and Smith, 1980). Carbon, nitrogen, phosphate, vitamins and metal ions all have significant effects on secondary metabolite production (Furuya *et al.*, 1987).

3.6.2.1 PREPARATION OF CULTURE MEDIA

Erlenmeyer flasks of 150 ml capacity and test tubes (25 x 125 mm) were used as culture vessels.

MS medium (Murashige and Skoog, 1962) was used in all studies unless otherwise specified. The composition of the medium is given in Appendix-I. Stock solutions were prepared as listed and stored in the refrigerator at 4°C. They were added in the order indicated followed by the addition of supplements. pH of the medium was adjusted to 5.8 with 1 N HCl or 1 N NaOH using pH meter before the addition of 0.8 % agar (w/v).

Other basal media used included LS (Linsmaier and Skoog, 1965), SH (Schenk and Hildebrandt, 1972) and B_5 (Gamborg *et al.*, 1968). Composition of these media is given in Appendix -II.

3.6.2.2 STERLIZATION OF MEDIA AND CULTURE VESSELS

After the addition of agar, the media were heated gently with constant stirring and poured into culture vessels. The mouths of the vessels were closed with non-absorbent cotton plugs and covered with cloth. The media and glassware were sterilized by autoclaving at 1.2 kg cm^{-2} for 20 minutes. Heat labile chemicals were filter sterilized using 0.22 μ Millipore membrane filters and added to the autoclaved media under aseptic conditions.

3.6.2.3 SURFACE STERILIZATION OF PLANT MATERIAL

The leaves of plant were washed in running water to remove surface particles. Further steps were carried out in the laminar air flow chamber. The cabinet was

cleaned thoroughly with 80% ethanol and irradiated with ultraviolet light for one hour before use. The leaves were cut into small pieces with the help of a cork borer. The leaf bits were first treated with 80 % ethanol for 10 seconds and then with 0.15 % (w/v) $HgCl_2$ for 5 minutes, subsequently, they were copiously washed with sterile distilled water.

3.6.3 CALLUS FORMATION

3.6.3.1 CALLUS INITIATION

The sterilized leaves were transferred aseptically to MS solid medium supplemented with 2 mg l^{-1} 2, 4-D and 0.2 mg l^{-1} Kn and 3 % (w/v) sucrose. The flasks with the inoculated leaf bits were then incubated at 25± 2°C under dark.

3.6.3.2 CALLUS MAINTENANCE

Green callus obtained from the leaf bits were maintained on the same medium which was used for the callus initiation. Sub culturing of calli onto fresh media was done at every three week interval.

3.6.3.3 GROWTH MEASUREMENT

Growth of callus was measured in terms of fresh and dry weight. Fresh weight of the callus was determined after removing the excess moisture from the surface of the callus using blotting paper. The callus was dried at 60°C until it attained a constant weight.

3.6.4. ELICITOR EXPERIMENT

Elicitation is one of the most important strategies employed to increase the secondary metabolites in tissue culture technique. A group of triggering factors to stimulate particular facets of secondary metabolism is known as elicitors. These are of two types viz., biotic and abiotic elicitors. Biotic elicitors are derived from

microorganisms whereas abiotic elicitors include heavy metals like Cu, Zn, Hg and Cd etc.

Elicitor induction offers a novel approach to achieve rapid accumulation of certain secondary metabolites. Elicitation can improve the efficiency of product accumulation in several ways:

1. Time required for product accumulation can be reduced.
2. Use of immobilized cells during elicitation may facilitate excretion of metabolites into the medium.
3. A semi continuous production may be possible with elicitation.

Two different types of elicitors were used in this study viz. biotic elicitors and abiotic elicitors.

3.6.4.1 BIOTIC ELICITORS

In this category, the media filtrate and cell extract of various fungi, yeast and bacteria were used. The organisms included were as follows :

FUNGI

Aspergillus niger, Aspergillus flavus, Penicillium notatum, Trichoderma viridae

BACTERIA

Bacillus cereus, Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Peudomonas striata.

YEAST

Candida albicans, Saccharomyces cerevisiae.

MAINTENANCE OF MICROBIAL CULTURES

FUNGI

The fungal cultures were maintained on agar slants containing Potato-Dextrose-Agar (PDA) medium. The medium was prepared as follows : Two hundred grams of potato were cut into slices and boiled with 200 ml distilled water. The extract was filtered through four layers of muslin cloth. To this, 20 g dextrose was added and the volume was made upto 1000 ml. The pH of the medium was then adjusted to 3.5 with 10% tartaric acid before adding agar (2 % w/v).

YEAST

Yeast cultures were maintained on agar slants containing 5.0 g l^{-1} peptone, 3.0 g l^{-1} beef extract 5 g l^{-1} sodium chloride and 20 g l^{-1} agar. The pH of the medium was adjusted to 7.0 before the addition of agar.

BACTERIA

Bacterial cultures were maintained on agar slants containing 3.0 g malt extract, 3.0 g yeast extract, 5.0 g bacterial peptone and 10 g dextrose per litre of medium and solidified with 2 % (w/v) agar.

ELICITOR PREPARATION

The microbial cultures which were maintained on agar slants were transferred to 50 ml of the respective liquid medium in 250 ml Erlenmeyer flasks. These cultures were incubated at room temperature. The bacterial and yeast cultures were kept on a shaker (100 rpm), while the fungal cultures were allowed to stand without shaking. When the cells reached their stationary phase (3 weeks for fungal cultures, 72 hours for yeast cultures and 48 hours for bacterial cultures), the flasks were removed and autoclaved to kill the microorganisms. Mycelial mat/cells in the media were separated by filtration, the filtrates were pooled and made upto a volume of 200 ml. The mat/cells were washed several times with distilled water and an aqueous extract

was made by homogenizing in a mortar and pestle using acid washed neutralized sand. The extract was then filtered through muslin cloth and the volume was made equal to that of filtrate. Both the aqueous extracts and the media filtrates were autoclaved and stored at 4°C for further use.

3.6.4.2 ABIOTIC ELICITORS

Metal ions in salt form were used as abiotic elicitors. In most of the cases, they were present in normal MS medium and their levels were altered.

Calcium ($CaCl_2$ $2H_2O$), Zinc ($ZnSO_4.7H_2O$), Copper ($CuSO_4.H_2O$),

Cobalt ($CoCl_2.2H_2O$).

3.7 GENETIC DIVERSITY STUDIES

The genetic diversity existing within *A. vasica* and between *A.vasica* and A.beddomei was assessed by both isozymes and Random Amplified Polymorphic DNA (RAPD) analysis.

Over the past 30 years, isozymes have been used as markers to analyse the genetic structure of natural population. One limitation with these studies is that isozymes are products of a few functional genes within the genome. On the other hand, DNA markers enable investigators to analyse the whole genome, which has wider option to observe genetic variation. Of various genetic markers currently used for the analysis of genetic diversity, RAPD is considered to be efficient and cost effective (Hadrys *et al.*, 1992). In our present study, we determined the genetic diversity among *A. vasica* collected from various places and between *A. vasica* and *A. beddomei* using both isozyme and RAPD markers.

3.7.1 ISOENZYME ANALYSIS

PREPARATION OF SAMPLES

One gram of young leaves were taken and ground with Tris-HCl buffer in a precooled mortar and pestle. The mortar was placed on a polystyrene box containing crushed ice. The grinding of each sample took less than 10 seconds since a longer period of grinding can destroy enzymes as a result of heating from friction. Between each sample, the grinding head was thoroughly cleaned by wiping with tissue.

PREPARATION OF GELS

SOLUTION A : (Stock Acrylamide solution)

Acrylamide 30 %	30 g
Bisacrylamide 0.8%	0.8 g
Water	100 ml

SOLUTION B : (Separating gel buffer)

1.875 M Tris – HCl	22.7 g	(pH = 8.8)
Water	100 ml	

SOLUTION C : (Stocking gel buffer)

0.6 M Tris – HCl	7.26 g	(pH = 6.8)
Water	100 ml	

POLYMERISING AGENTS

a) Ammonium persulfate (5 %):- 0.5 g / 10 ml, prepared freshly before use.

b) TEMED :Fresh from the refrigerator.

ELECTRODE BUFFER

0.05 M	Tris	12g	pH 8.2 – 8.4
0.293 M	Glycine	28.8 g	
Water		2 litre	No adjustment required.

May be used 2-3 times

PROCEDURE

1. Thoroughly cleaned and dried the glass and spacers of Biorad electrophoresis unit, then assembled them properly. Held the assembly together with bulldog clips. Clamped in an upright position. White petroleum jelly or 2 % agar (melted in a boiling water bath) was then applied around the edges of the spaces to hold them in place and sealed the chamber between the glass plates.
2. Prepared a sufficient volume of separating gel mixture for 15 % gel by mixing the following.

   Solution A 20 ml

   Solution B 8 ml

   Degassed on water pump for 3-5 minutes and then added

   Ammonium persulfate solution = 0.2 ml

   TEMED = 20 µl.
3. Mixed gently and carefully poured the gel solution in the chamber between the glass plates. Layered distilled water on top of the gel and left to set for 30 – 40 min.
4. Prepared stacking gel (4 %) by mixing the following solutions.

   Solution A = 1.35 ml

   Solution B = 1.0 ml

   Water = 7.5 ml

   Degassed as above, and then added

   Ammonium persulfate (5 %) = 50 µl

   TEMED = 19 µl

Removed the water from the top of the separating gel and washed with a little stacking gel solution. Poured the stacking gel mixture, placed the comb in the stacking gel mixture, and allowed the gel to set (30 – 60 min).

5. After the stacking gel had polymerized, removed the comb without distorting the shapes of the well. Carefully installed the gel after removing the clips, agar etc. in the electrophoresis apparatus. Filled it with electrode buffer and removed any trapped air bubble at the bottom of the gel. Connected the cathode at the top and turned on the DC – power briefly to check the electrical circuit. The cathode buffer and the plates can be kept cooled using a suitable facility so that heat generated during the run is dissipated and did not affect the gel and resolution.

APPLICATION OF SAMPLES INTO THE WELLS

Prepared samples for electrophoresis, following suitable extraction procedure. Adjusted the protein concentration in each sample using the 5 strength sample buffer and water in such a way that the same amount of protein was present per unit volume. Again the concentration should be such as to give a sufficient amount of protein (50-200 μg) in a volume not greater than the size of the sample well (25–50 μl).

The required volume of the sample was taken in a microsyringe and carefully injected it into a sample well through the electrode buffer.

Turned on the current to 10 – 15 mA for the initial 10 – 15 min, until the samples traveled through the stacking gel. The stacking gel helped in the concentration of the samples. Then continued the run at 30 mA until the sample reached the bottom of the gel (about 3h). However, the gel may be run at a high current (60 – 70 mA) for short period (1 h) with proper cooling.

After the run was complete, carefully removed the gel from between the plate and immersed in respective staining solution for at least 3 h or overnight with uniform shaking.

STAINING

After termination of electrophoresis, the gel was removed from the stand and transferred to the staining trays. Care was taken to avoid tearing the gel during transfer to the staining tray. Each gel was stained for a different enzyme system (Appendix -III). Staining trays may be glass, baking dishes or plastic boxes. Each tray was labeled to identify the enzyme being stained.

Staining trays and chemicals should be prepared prior to the removal of gels from the stand. Some chemicals were light sensitive and others were hydrophilic and should not be exposed before the gel was ready. Most chemicals can, however, be pre-weighed and stored in small plastic vials in a freezer until required.

After the gels have been transferred to the staining trays, the staining chemicals and solutions were mixed and poured over the gels. The tray was gently agitated to ensure that the gel was evenly covered with the staining solution. The tray was covered with plastic wrap and incubated at 37°C. Protective gloves should always be worn when handling any chemical especially with the staining chemicals, many of which are highly toxic or carcinogenic.

Incubation time varies with the enzyme system, so the trays should be regularly inspected because over staining may obscure some variations. When adequately stained, the tray was removed from the incubating oven, the excess stain solution poured out, and the gel rinsed with tap water. Enough water was added to cover the gel and a gel fixative solution was used (100 ml acetic acid, 400 ml methanol and 1000 ml distilled water) to preserve the gel. The excess staining solutions should be collected and destroyed or disposed of in a safe fashion.

3.7.2 RAPD ANALYSIS

REAGENTS AND CHEMICALS FOR PCR REACTION

1. Liquid Nitrogen

2. IM TRIS-HCl (pH 8.0)

   Tris base 12.1 g

   Dissolved in distilled water, pH adjusted to 8.0 using HCl and volume made up to 100ml.

3. 0.5 M EDTA (pH 8.0)

   Sodium EDTA 18.6 g

4. 2X CTAB Buffer (100 ml)

CTAB	2.0 g	2%
1 M Tris HCl	10.0 ml	100 mM
0.5M EDTA	4.0 ml	20 mM
NaCl	8.2 g	1.4M
PVP	1.0 g	1%

5. TE Buffer

1 M Tris HCl	1.0 ml	10 mM
0.5 M EDTA	0.2 ml	1 mM

   Dissolved in distilled H_2O and volume made to 100 ml.

6. RNase

   100 mg of RNase was dissolved in 10 ml of TE.

   Chloroform : Isoamylalcohol (24 : 1)

7. Absolute ethanol (100%)

8. 70% Ethanol

9. Isopropanol

10. 50X TAE Buffer

Tris base	24.2 g	2M
0.5 M EDTA	10.0ml	50mM

    Dissolved in distilled H_2O, pH adjusted to 8.0 with acetic acid and volume made up to 100 ml.

11. Ethidium Bromide

    10 mg was dissolved in 1 ml of distilled H_2O

12. 6X Loading dye

Sucrose	4.0 g	40 %
Bromophenolblue	25.0 mg	0.25 %

Dissolved in water, volume made to 10 ml with distilled H_2O

13. 10X PCR Buffer

1 M Tris-HCl	1.0 ml	100 mM
$MgCl_2.H_2O$	30.0 mg	15 mM
KCl	372.8 mg	500 mM
Gelatin	1.0 mg	0.01%

Dissolved in 10 ml of distilled H_2O

14. $MgCl_2$ (10 mM)

$MgCl_2.6H_2O$	20 mg	10 mM

Dissolved in 10 ml of dH_2O

15. dNTPs Mix – 1mM Stock

16. Primer

17.

OPF 04	5'GGTGATCAGG3'	OPA 01	5'CAGGCCCTTC 3'
OPF 06	5'GGGAATTCGG3'	OPA 02	5'TGCCGAGCTG 3'
OPF 07	5'CCGATATCCC3'	OPA 05	5'AATCGGGCTG 3'
OPF 08	5'GGGATATCCC3'	OPA 11	5'CAATCGCCGT 3'
OPF 15	5'CCAGTACTCC3'	OPA 17	5'GACCGCTTGT 3'

18. Mastermix for PCR (25 µl per reaction)

10X PCR Buffer	2.5 µl	1 X
10 mM $MgCl_2$	2.5 µl	1.0 mM
dNTP mix	2.5 µl	0.1 mM
10 µM Primer	1.6 µl	0.64 µM
Taq polymerase	0.2 µl	1U
DNA template	50 – 100 ng	

Volume made to 25 µl with sterile distilled H_2O

19. Mineral Oil

20. X-ray Film Kodak

EXTRACTION OF GENOMIC DNA FROM PLANT LEAVES

One gram of leaves were taken and ground in liquid nitrogen in a mor pestle. These ground leaves were immediately transferred to 5 ml 2X CTAB preheated to 65°C in a 50 ml Greiner tube. It was incubated for one hour at 65 water bath with occasional mixing. Now, 5 ml of chloroform : isoamylalcoh added and it was incubated on a platform shaker for 15 minutes with slow sh Whole mix was then spun at 3000 rpm for 10 minutes. Aqueous layer was t carefully using a cut tip into another 50 ml sterile tube. 5 – 6 ml of isopropanol wa added and incubated for 15 minutes at room temperature for precipitation of DNA. It was then spun at 3000 rpm for 20 minutes, the supernatant discarded, the pellet washed with 70 % ethanol, vacuum dried and dissolved in 1 ml of TE buffer. RNase was added to a final concentration of 10 µg/ml and incubated at 37°C for 45 minutes. Again DNA was precipitated with 100 % ethanol, spun at 3000 rpm for 2 minutes, the pellet was vacuum dried and dissolved in minimum volume of TE.

ESTIMATION OF DNA AND THE ASSESSMENT OF ITS PURITY

10 µl of extracted DNA was dissolved in 1 ml of TE. This was used to measure the absorbance at 260 and 280 nm wavelength. Blank was set using TE. 260 / 280 ratio was taken as the criteria for purity of DNA.

Also, 10 µl of the extracted DNA was loaded in 0.7 % agrose gel and electrophorised at 100 volts. The gel was stained in ethidium bromide for 15 minutes and observed under UV light. The amount of DNA present was estimated based on the intensity of the band.

PCR REACTION CONDITIONS

25 µl of mastermix was taken for different templates and was over layered with 20 µl of mineral oil. Mineral oil was added to prevent the evaporation of the contents due to high temperature of denaturation. The samples were then spun for a short

duration and then the tubes were kept on ice. Amplification of DNA was carried out in COY thermocycler model 110s.

The parameters used for PCR reaction were

one cycle of

94°C	30 sec
36°C	30 sec
72°C	60 sec

45 cycles of

94°C	30 sec
36°C	30 sec
72°C	120 sec

final extension

72°C	7 min

The resulting amplified products were run in a high percentage agarose gel along with molecular weight marker.

HIGH PERCENTAGE AGAROSE GEL ELECTROPHORESIS

The required amount of water was taken in a flask with magnetic stirring bar. It was covered with aluminium foil and weighed. Agarose was weighed and was sprinkled into the flask with continuous stirring. It was kept on Hotplate / Stirrer till it became clear. It was then weighed again to check any evaporation and later kept at 65°C in water bath. Now, TAE buffer was added to the final concentration of 1X and the final volume was made using distilled H_2O. It was mixed properly and then poured onto the gel platform. It was kept for 30 minutes for solidification and then PCR products mixed with 6X dye were loaded. The gel was run at 100 volts in 1X TAE till the bromophenol blue dye front reached the end. Manipulations in run time were done depending upon the type and concentration of agarose.

DATA ANALYSIS

All the electromorphs observed from the electrophoretic field were scored for their presence or absence of bands using binary codes as 1 and 0 respectively. The similarity indices were calculated using the formula,

$$\text{Similarity Index} = \frac{2 \times n_{11}}{(2xn_{11}) + (n_{01}+n_{10})} \qquad \text{(Nei and Lei, 1979)}$$

where

n_{11} = Total No. of occurrences where x = 1 and y = 1

n_{01} = Total No. of occurrences where x = 0 and y = 1

n_{10} = Total No. of occurrences where x = 1 and y = 0.

Based on these coefficients UPGMA clustering was carried out and a phenetic tree was constructed using nearest neighborhood joining method. The entire analysis was performed using "RAPD istance" 1.04,a computer software developed by John Armstrong (Research School of Biological Science, Institute of Advanced Studies, A.N.U., Australia).

3.8 STATISTICAL ANALYSIS

The data were subjected to statistical scrutiny by following standard statistical procedure as given by Snedecor and Cochran (1967).

RESULTS AND DISCUSSION

4.0 RESULTS AND DISCUSSION

Two species of *Adhatoda* namely *A. vasica* and *A. beddomei* were chosen for the present study. *A. vasica* commonly occurs as an evergreen shrub and was collected from the Suruli falls, Kanjikode (Kerala), Thaimudi (Mudis) Kilkothagiri (Nilgiris), Coimbatore civil aerodrome region and Institute of Forest Genetics and Tree Breeding, Coimbatore. *A. beddomei* is considered to be more powerful and active and is used in medicinal preparation in Kerala (Asolkar *et al.*, 1992) and is listed as an endangered and endemic medicinal plant. *A. beddomei* was obtained from the Center for Indian Medical Heritage, Kanjikode, Kerala. These were chosen from the places that vary drastically in altitude, extent of atmospheric pollution, rainfall and fertility of the soil.

These samples were analysed to find the alkaloid content. Ascorbic acid, β-carotene and selected enzymes were assayed to assess the antioxidant potential of the two species and to find the interrelationship between these parameters and environmental stresses.

The alkaloids present in *Adhatoda* species are responsible for its pharmacological property. Efforts were taken therefore, to optimize the callus culture of *Adhatoda* to increase the alkaloid content. Though *A. vasica* and *A. beddomei* are different species, it is very difficult to differentiate them morphologically. Isoenzyme analysis of selected enzymes and the recently developed RAPD analysis were carried out in the leaf samples to find out the genetic variation among *A. vasica* and between *A. vasica and A. beddomei.*

The results of this study are discussed under the following heads:

4.1 Alkaloid content in the leaves

4.2 Levels of ascorbic acid and β-carotene

4.3 Enzymes

4.3.1 Enzymes involved in the detoxification of reactive oxygen species (ROS): SOD, POD, CAT and GR.

4.3.2 Enzymes that are known to be sensitive to changes in environmental stresses and also involved in oxidoreduction reactions : NR, AAO, IAAO and G6PD.

4.3.3 Enzymes involved in the metabolism of phosphate : ACP and ALP

4.3.4 Enzyme involved in the synthesis of secondary metabolites : phenylalanine ammonia lyase (PAL)

4.4 Tissue culture studies of *Adhatoda* species

4.4.1 Tannin content

4.4.2 Growth pattern of *A. vasica* and *A. beddomei*

4.4.3 Alkaloid content in the callus cultures

4.4.4 Influence of basal media on growth and alkaloid production

4.4.5 Influence of plant hormones on growth and alkaloid production

4.4.6 Effect of nutrient content

4.4.7 Screening of elicitors to optimize culture media

4.4.7.1. Effect of biotic elicitors

4.4.7.2. Effect of abiotic elicitors

4.5 Genetic diversity studies

4.5.1 Isoenzyme analysis

4.5.2 Randomly Amplified Polymorphic DNA analysis

4.1 ALKALOID CONTENT IN THE LEAVES

The alkaloids were extracted and purified by column chromatography and visualized by paper chromatography (Plate 2). The total alkaloid content of the different samples of *A. vasica* and *A. beddomei* is represented in Table –I.

Plate - 2 :
Separation of vasicine and vasicinone by paper chromotography

Adhatoda vasica samples

1. Suruli falls
2. Kanjikode,Kerala
3. Thaimudi,Mudis
4. Kilkothagiri,Nilgiris
5. Coimbatore civil aerodrome region
6. IFGTB,Coimbatore
7. *Adhatoda beddomei*

TABLE - I

TOTAL ALKALOID CONTENT IN A. vasica AND A. beddomei

Species		*Alkaloid content (g/100g dry tissue)*
A. vasica	1	0.19
	2	0.20
	3	0.16
	4	0.17
	5	0.23
	6	0.22
A. beddomei		0.26
CD (0.05)		0.03

Values are mean of four replicates in two repetitive experiments

1. Suruli falls
2. Kanjikode, Kerala
3. Thaimudi, Mudis
4. Kilkothagiri, Nilgiris
5. Coimbatore civil aerodrome region
6. IFGTB, Coimbatore

The maximum alkaloid content was observed in *A. beddomei* followed by *A. vasica* collected from Coimbatore civil aerodrome region and the least alkaloid content was found in *A. vasica* from Kilkothagiri.

Alkaloid biology is closely connected with the basic physiology of plants. The main function of alkaloids is chemical defense which works against a majority of potential enemies of the plants (Harborne, 1993). Alkaloids are certainly multi purpose components which, depending on the situation, may be active in more than one environmental interactions (Wink, 1993). To be effective, alkaloids need to be present at the right time, site and concentration. An interesting variation can be seen in some plants, where alkaloid formation is enhanced by adverse environment i.e., in the case of emergency, the production of defense compounds such as alkaloids are stimulated. So it is not surprising that *A. vasica* collected from highly polluted area (Coimbatore civil aerodrome region) showed higher concentration than those from Kilkothagiri and Thaimudi, Mudis. Alkaloid patterns usually vary between the species and in our study, *A. beddomei* contained higher alkaloid content than all the samples of *A. vasica* making it evident that this species is a better source of alkaloids.

4.2 ASCORBIC ACID AND β-CAROTENE

In cells when there is an oxidation of bivalent organic molecules to univalent, species, free radicals are produced as ROS intermediates. Plant cells are normally protected from these activated forms of oxygen and accumulation of free radicals. They are associated with damage to membranes, and build up of lipid peroxides due to various stresses, by antioxidant systems. Multiple lines of defenses have evolved from the antioxidant defense system comprising of non-enzymatic as well as enzymatic defense mechanism. Non-enzymatic scavengers include ascorbic acid and β-carotene. Most of the medicinal plants are rich in ascorbic acid and β-carotene, particularly plants which are used for curing cough, cold, asthma and other bronchial disorders. The quantification of ascorbic acid and β-carotene in *A. vasica* and *A. beddomei* would be more appropriate as they are extensively used as bronchial dilators. The levels of ascorbic acid and β-carotene in the leaves of *Adhatoda* are presented in Table II.

TABLE – II

ASCORBIC ACID AND β- CAROTENE LEVELS IN A. vasica AND A. beddomei

Species		*Ascorbic acid (mg/100g tissue)*	*β-carotene (µg/100g tissue)*
A. vasica	1	286	3920
	2	290	4032
	3	284	3863
	4	289	3891
	5	312	4300
	6	294	4120
A. beddomei		308	4298
CD(0.05)		3.19	5.00

Values are mean of four replicates in two repetitive experiments.

1. Suruli falls
2. Kanjikode, Kerala
3. Thaimudi, Mudis
4. Kilkothagiri, Nilgiris
5. Coimbatore civil aerodrome region
6. IFGTB, Coimbatore

All the samples studied, were found to be rich sources of both ascorbic acid and β-carotene. However, *A. vasica* collected from Coimbatore civil aerodrome showed the highest content of the antioxidants ascorbic acid (312 mg/100g tissue) and β-carotene in this sample (4300μg/100g tissue). The higher content of these antioxidants in this sample might be to nullify the free radicals and reactive oxygen species that might have been formed due to the adverse environmental conditions in which this sample existed.

A. beddomei was found to be on par with *A. vasica* from this region with regard to the ascorbic acid and β-carotene contents.

4.3 ENZYMES

4.3.1 ENZYMES INVOLVED IN THE DETOXIFICATION OF REACTIVE OXYGEN SPECIES (ROS)

The inevitable generation of ROS in biological systems and the obvious oxidative damage is counterpoised by an array of enzymatic defense. An increase in the oxidative enzymes upon water stress was reported by Mukherjee and Choudhuri (1981) in *Vigna* seedlings. Badiani *et al.* (1990) considered the relation between drought stress and enzymatic antioxidant systems in different species. Sehmer *et al.* (1998) showed the increase in the activities of three antioxidative enzymes (catalase, superoxide dismutase and glutathione reductase) in Spruce needles involved in the detoxification pathways when the plant was exposed to polluted air.

Considering these views and also with the assumption that medicinal plants *A. vasica* and *A. beddomei* might contain more quantity of antioxidants and antioxidases that might directly (or indirectly) be involved in the detoxification of ROS, superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and glutathione reductase (GR) were quantified and the activities of these enzymes are represented in Table III.

TABLE III

SUPEROXIDE DISMUTASE, PEROXIDASE, CATALASE AND GLUTATHIONE REDUCTASE ACTIVITIES IN A. vasica AND A.beddomei

Species	*SOD (U/mg protein)*	*POD (U/g fresh tissue)*	*CAT (U/g fresh tissue)*	*GR (U/mg protein)*
A. vasica 1	1.03	4.360	3.21	0.901
2	1.09	4.278	3.18	0.906
3	1.06	4.208	3.17	0.893
4	1.02	4.239	3.19	0.896
5	1.30	4.823	3.99	0.923
6	1.19	4.463	3.90	0.916
A. beddomei	1.06	4.396	3.02	0.908
CD(0.05)	0.01	0.045	0.02	0.004

Values are mean of five replicates in two repitive experiments.

Units :

SOD – Unit – Increase in absorbance mg^{-1} protein min^{-1}

POD – Unit – µmoles of o-dianisidine oxidised g^{-1} fresh tissue min^{-1}

CAT – Unit – Decomposition of µmol H_2O_2 g^{-1} fresh tissue min^{-1}

GR – Unit – µmoles of NADPH oxidised mg^{-1} protein min^{-1}

1. Suruli falls
2. Kanjikode, Kerala
3. Thaimudi, Mudis
4. Kilkothagiri, Nilgiris
5. Coimbatore civil aerodrome region
6. IFGTB, Coimbatore

1. SUPEROXIDE DISMUTASE (SOD)

The results revealed a significantly ($P < 0.05$) higher SOD activity (1.30 µ/mg protein) in *A. vasica* collected from the Coimbatore civil aerodrome region than in the other samples. This might probably be due to a variety of environmental stresses like elevated atmospheric CO_2 concentration, temperature and drought compared to the samples collected from other regions. The SOD activity was 1.06 µ/mg protein in *A. beddomei*, which was found to be on par with *A. vasica* samples from Kanjicode and Thaimudi, Mudis.

These observations are in accordance with those of Kangasjarvi *et al.* (1996) who found increased SOD activities in birch (*Betula pendula*) due to heat and other

environmental stresses. Karpinski (1993) has described the genes for SOD isoforms that are induced by a variety of environmental stresses.

2. PEROXIDASE (POD)

In the present study, the highest POD activity (4.823 U/g fresh weight) was noted in *A. vasica*, collected from Coimbatore civil aerodrome region, and the lowest POD value (4.208 U/g fresh weight) was noticed in *A. vasica* collected from Thaimudi, Mudis. High air pollution at Coimbatore civil aerodrome region, might have induced oxidative stress on plants which involve pronounced effects on antioxidants and related antioxidase enzymes to scavenge the free radicles like $^{\bullet}OH$ and $^{\bullet}O$ and hence the higher value of POD in *A. vasica* from this region than in other samples. Puccinelli *et al.* (1998) have also found the peroxidase activity of the trees in the urban environment to be higher than that in the trees of non-urban areas and stated that peroxidase activity may be used as a biomarker of air pollution to edit maps of air quality. Arcak *et al.* (1997) stated that variation in the enzyme activities of Pine forest vegetation in soils of Soguksu National Park, Turkey, showed a significant negative correlation between the altitude and enzyme activities. Similarly a low value of POD was found in *A. vasica* collected from high altitude and less polluted, Thaimudi, Mudis. POD of *A. beddomei* was 4.396 U/g fresh weight.

3. CATALASE (CAT)

CAT activity was found to be significantly ($P < 0.05$) more (3.21 U/g fresh tissue) in *A. vasica* collected from the region of Coimbatore civil aerodrome. The plants that grew near Coimbatore civil aerodrome region were exposed to elevated atmospheric CO_2 concentration because of heavy road traffic, high temperature, light and drought than other plant samples which were collected either from higher altitude or plains with lesser air pollution. Streb *et al.* (1997) have reported the enzyme catalase as a sensitive marker of light stress in *Retama raetam* stems and *Atriplex halimus* leaves. *A. beddomei* recorded the least catalase activity of 3.02 U/g fresh tissue.

4. *GLUTATHIONE REDUCTASE (GR)*

Glutathione reductase plays an important role in the protection of plants from stresses by preventing the oxidation of enzymes and membranes. The maximum GR activity (0.923 U/mg protein) was found in *A. vasica* collected near Coimbatore civil aerodrome. The lowest GR activity (0.893 U/mg protein) was observed in *A. vasica* collected at Thaimudi, Mudis.

When the plants are compulsorily continuously exposed to polluted conditions, they are forced to synthesise antioxidants and detoxifying enzymes in large amounts. The relevance of GR in plant species is generally linked to functional aspects of detoxification. So it is not surprising that the plant sample which was collected from a highly polluted area showed the maximum GR activity than other samples which were collected from relatively less polluted areas. Mishra *et al.* (1993) have reported that increased GR and SOD activities were adaptive responses of plants to high amount of active oxygen species in wheat at high light intensities. Creissen *et al.* (1996) also regarded GR as a key enzyme in defence against oxidative stress in peas. Mahan (1994) reported that GR played a role in the detoxification of oxidants that often resulted from exposure of plants to low/high temperature stress. The findings of this study are also very much in line with these observations. GR activity of *A. vasica* in urban environment (Coimbatore civil aerodrome and IFGTB, Coimbatore) was higher than those from the non-urban areas. These results indicated the possibility of using GR activity in *Adhatoda vasica* as a marker of air pollution. The activity of GR in *A. beddomei* was 0.908 U/mg protein. Except for the two vasica samples collected from Coimbatore civil aerodrome region and IFGTB, Coimbatore, the rest of the samples did not exhibit significant difference in their activities. This reiterates the point that the synthesis of antioxidants in this medicinal plant is enhanced in polluted regions and in plains.

4.3.2 ENZYMES THAT ARE KNOWN TO BE SENSITIVE TO CHANGES IN ENVIRONMENTAL STRESSES AND INVOLVED IN OXIDO REDUCTION REACTIONS

The activities of Nitrate Reductase (NR), Ascorbic Acid Oxidase (AAO), Indole Acetic Acid Oxidase (IAAO) and Glucose 6-Phosphate Dehydrogenase (G6PD) which are known to be sensitive to changes in environmental stresses and involved in oxido reduction reactions are presented in Table-IV.

TABLE IV

NITRATE REDUCTASE, ASCORBIC ACID OXIDASE, INDOLE ACETIC ACID OXIDASE AND GLUCOSE 6-PHOSPHATE DEHYDROGENASE ACTIVITIES IN ADHATODA SPECIES

Species		*NR (U/g fresh tissue)*	*AAO (U/g fresh tissue)*	*IAAO (U/g fresh tissue)*	*G6PD (U/mg protein)*
A. vasica	1	4.40	0.70	1600	56.80
	2	4.13	0.77	1594	56.70
	3	4.56	0.73	1585	56.19
	4	4.36	0.71	1501	55.99
	5	3.97	0.94	1630	58.00
	6	3.99	0.86	1612	56.76
A. beddomei		4.06	0.76	1602	56.72
CD(0.05)		5.36	0.02	6.57	0.15

Values are mean of five replicates in two repetitive experiments.

Units :

NR – Unit – µg of nitrate formed g^{-1} fresh tissue h^{-1}

AAO – Unit – Absorbance change of 3.58 g^{-1} fresh tissue min^{-1}

IAAO – Unit – µmol IAA unoxidised g^{-1} fresh tissue h^{-1}

G6PD – Unit – nmol NADPH utilized g^{-1} fresh tissue min^{-1}

1. Suruli falls
2. Kanjikode, Kerala
3. Thaimudi, Mudis
4. Kilkothagiri, Nilgiris
5. Coimbatore civil aerodrome region
6. IFGTB, Coimbatore

1. *NITRATE REDUCTASE (NR)*

Nitrate reductase activity (3.97 U/g fresh tissue) was low in *A. vasica*, collected from Coimbatore civil aerodrome region, on comparison with other samples. Similarly, the declined NR activity in the sample from Coimbatore civil aerodrome region might be due to the high phytotoxic potential of pollutants in that area. The highest activity of NR (4.56 U/g fresh tissue) in the Thaimudi, Mudis sample might be due to the high altitude, low temperature and lesser environmental stress in that region. Onwueme *et al.* (1971) had commented on the unique temperature sensitivity of NR. The decline in NR during drought had been attributed to a reduction in enzyme level as determined by the rate of protein synthesis and degradation as well as inactivation of enzyme activity. NR was considered to be a factor of prime importance in the cereal grain production and its activity had been correlated with grain yield, total dry matter accumulation and grain protein in maize (Deckard *et al.*, 1973). Sinha and Nicholas (1981) have opined that nitrate reductase activity is associated with protein synthesis and plant growth, both of which are affected by drought stress. Ramaniulu *et al.* (1994) stated that NR activity had declined markedly under salt stress conditions. Our findings in *Adhatoda* species are also corroborative with these statements. NR activity had declined in the samples (5, 6) from Coimbatore region due to environmental stress conditions. Klumpp *et al.* (1989) observed that spruce trees which were exposed to the pollutant mixtures of ozone, SO_2 and NO_2 resulted in a significant decrease in NR activity when compared to the control.

2. *ASCORBIC ACID OXIDASE (AAO)*

Activity of AAO which catabolises ascorbic acid, another antioxidant was reported to increase in plants upon stress condition.

From the above Table, it is evident that the maximum AAO (0.94 U/g fresh tissue) was noticed again in *A. vasica* collected from Coimbatore civil aerodrome region. The lowest value (0.71 U/g fresh tissue) was obtained in the samples collected from Kilkothagiri as well as Thaimudi, Mudis, which was found to be on par with the

A. vasica samples from Kanjikode, Suruli Falls, Kilkothagiri and also *A. beddomei*. The activity of AAO is increased in leaves of *Strychinicola nux-vomica* Linn. infected with *M. strychinicola* (Nagaraja and Thite, 1995). Dolgova and Kuchma (1977) have shown higher activity of ascorbic acid oxidase in plants grown in thiocyanate polluted area. Mukherjee and Choudhuri (1981) showed that AAO increased along with POD upon heat stress in *Vigna* seedlings. In accordance with these reports, increased AAO and POD activities were observed in *A. vasica* collected from Coimbatore civil aerodrome region which experiences high temperature, light and elevated atmospheric concentration of CO_2 and SO_2. The crucial role of AAO in regulating the levels of oxidised and reduced glutathione and NADPH might be the cause for the elevation in its activity.

3. INDOLE ACETIC ACID OXIDASE (IAAO)

IAA oxidase is the enzyme involved in the catabolic degradation of the naturally occuring plant auxin.

A careful scrutiny of Table – IV reveals that the IAA oxidase activity also was maximum in *A. vasica*, collected from Coimbatore civil aerodrome region (1630 U/g fresh tissue) and the least IAA oxidase activity (1501 U/g fresh tissue) was found in *A. vasica* , from Kilkothagiri.

The activity of IAA oxidase is altered under stress. Smith and Shortle (1990) stated that IAA oxidase activity was enhanced at the time of wounding and infection. Mukerjee and Choudhuri (1981) observed a proportional increase in the activities of IAA oxidase during water stress in *Vigna* seedlings.

Of all the samples investigated, *A. vasica* (Coimbatore civil aerodrome region) which is subjected to air pollution, light, temperature and drought stresses, exhibited marked increase in the activity of not only the antioxidant enzymes, but also of the enzymes involved in oxido reduction reactions which supply the reducing equivalent for the antioxidant system. This might be due to the acclimatization of the plant to the

environment. The plants from less polluted and low temperature places like Kilkothagiri and Thaimudi would have experienced less stress and probably produced less free radical that accounts for low levels of IAA oxidase. *A. beddomei* recorded 1602 U IAA oxidase activity per gram fresh tissue.

4. GLUCOSE 6-PHOSPHATE DEHYDROGENASE (G6PD)

G6PD, is a major regulatory enzyme of pentose phosphate pathway which catalyses the conversion of G6P to 6-phospho gluconate. Similar to GR activity, *A. vasica* collected from Coimbatore civil aerodrome area showed significantly higher ($P < 0.05$) activity of G6PD (58.00 U/mg protein) and the least activity was noticed in *A. vasica* from Kilkothagiri (55.99 U/mg protein). The enhanced activity of GR could imply increased level of oxidised glutathione formation arising out of enhanced mitochondrial oxidative processes. Similar oxidative events in the cytosol would therefore call for an enhanced activity of G6PD, controlling the generation of cytosolic NADPH for the cell to provide the reducing power necessary for detoxification as well as energy and carbon skeleton involved in the repair processes. Sehmer *et al.* (1998) detected an increase in the activities of enzymes involved in detoxification pathway like SOD, GR, ascorbate reducatase and G6PD when Spruce trees were exposed to ozone and elevated atmospheric carbondioxide. Dizengremel *et al.* (1994) showed that there was a marked increase in the activity of G6PD when pine saplings were exposed to ozone fumigations. Therefore, the increased activity of G6PD along with other oxidative enzymes in *A. vasica* collected from Coimbatore civil aerodrome might be an antioxidative protection against various environmental stresses. G6PD activity in *A. beddomei* was 56.72 U/mg protein. It was on par with the values observed in *Adhatoda vasica* collected from Kanjikode, Suruli Falls and IFGTB, Coimbatore.

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4.3.3 ENZYMES INVOLVED IN THE METABOLISM OF PHOSPHATE

Acid phosphatase (ACP) and alkaline phosphatase (ALP) activites in *Adhatoda* species are given in Table V.

TABLE V

ACID PHOSPHATASE AND ALKALINE PHOSPHATASE ACTIVITIES IN ADHATODA SPECIES

Species		*ACP U/mg protein*	*ALP U/mg protein*
A. vasica	1	4.57	3.13
	2	4.58	3.21
	3	4.07	3.01
	4	4.40	3.19
	5	4.89	3.66
	6	4.62	3.39
A. beddomei		4.55	3.31
CD (0.05)		0.08	0.07

Values are mean of five replicates in two repetitive experiments.
Unit = mmoles of p-nitrophenol released mg^{-1} of protein min^{-1}

1. Suruli falls
2. Kanjikode, Kerala
3. Thaimudi, Mudis
4. Kilkothagiri, Nilgiris
5. Coimbatore civil aerodrome region
6. IFGTB, Coimbatore

Higher activities of acid and alkaline phosphatases were noticed in *A. vasica*, from Coimbatore civil aerodrome and the least activities of both the phosphatases were observed in *A. vasica* collected from Thaimudi, Mudis. These findings are in line with those of Chowdhury *et al.* (1994) who found increased activity of acid and alkaline phosphatases in plants under stress conditions. Godbold *et al.* (1993), has suggested that these two enzymes could be considered as bioindicators of stress. Arack *et al.* (1997) made a negative correlation between activities of enzymes like acid phosphatase, alkaline phosphatase and catalase of Pine forest vegetation and altitude. In accordance with this, in the present study, the

samples from high altitude exhibited lower ACP and ALP activities than those from low altitudes.

The activities of acid phosphatase and alkaline phosphatase in A.beddomi were found to be 4.55 and 3.31 U/mg protein respectively.

4.3.4 ENZYME INVOLVED IN THE SYNTHESIS OF SECONDARY METABOLITES : PHENYLALANINE AMMONIA LYASE (PAL)

The activity of phenylalanine ammonia lyase is shown in Table VI.

TABLE VI

PHENYLALANINE AMMONIA LYASE ACTIVITY IN ADHATODA SPECIES

Species		*PAL activity (U/g fresh tissue)*
A. vasica	1	2.07
	2	2.06
	3	1.99
	4	1.85
	5	2.40
	6	2.25
A. beddomei		2.26
CD (0.05)		0.01

Values are mean of five replicates in two repetitive experiments.
Unit : µmole trans-cinnamic acid formed g^{-1} fresh tissue min^{-1}

1. Suruli falls
2. Kanjikode, Kerala
3. Thaimudi, Mudis
4. Kilkothagiri, Nilgiris
5. Coimbatore civil aerodrome region
6. IFGTB, Coimbatore

It is evident from the above table that the PAL activity is significantly ($P < 0.05$) higher in the *A. vasica* sample from Coimbatore civil aerodrome region than in *A. vasica* samples 1 – 4. But it is found to be on par with those of *A. vasica* samples from IFGTP, Coimbatore and *A. beddomei*. The increase in PAL activity might be due to the enhanced alkaloid synthesis in these two samples since these contain higher alkaloid content than the rest is indicated in Table I. PAL is the key

enzyme in the branch pathway that leads to the synthesis of phenyl propanoid trans-cinnamic acid and different isoenzymes expressed in plant cells in response to stress (Edwards and Gatehouse, 1999). The higher content of PAL in *A. vasica* sample of Coimbatore civil aerodrome region might be due to the environmental stress.

4.4 TISSUE CULTURE STUDIES

4.4.1 TANNIN CONTENT

Phenolic and tannin exudates in explants inhibit callus induction and multiplication in *in vitro* cultures. So prior to selecting the plant for *in vitro* culture, it is necessary to identify the low tannin content sample.

Total tannin content of the samples selected for the study was determined to select the best *A. vasica* sample for tissue culture studies. The total tannin content of the two species of *Adhatoda* is given in Table – VII.

TABLE – VII

TANNIN CONTENT IN A. vasica AND A. beddomei

Species		***Tannin content mg equivalent***
A. vasica	1	17.00
	2	18.37
	3	16.11
	4	16.97
	5	20.26
	6	19.32
A. beddomei		18.67
CD (0.05)		0.51

Values are mean ± SD for 4 replicates in two repetitive experiments

1. Suruli falls
2. Kanjikode, Kerala
3. Thaimudi, Mudis
4. Kilkothagiri, Nilgiris
5. Coimbatore civil aerodrome region
6. IFGTB, Coimbatore

From the above table, it is evident that a high tannin content of 20.26 mg equivalents was noticed in *A. vasica* collected from Coimbatore civil aerodrome

region and a low amount (16.11 mg equivalents) was seen in *A. vasica* collected from Thaimudi, Mudis. Although *A. vasica* from Kilkothagiri, Nilgiris showed a low tannin content, it also had very low alkaloid content (Table I). In contrast, *A. vasica* from Coimbatore civil aerodrome and IFGTB, Coimbatore had not only a high alkaloid content but also higher amounts of tannin and antioxidants than the rest, that might hinder with the callus initiation and browning of callus respectively. Whereas *A. vasica* collected from Kanjikode showed intermediate value for both tannin and alkaloid (i.e.,) 18.37 mg equivalent of tannin and 0.20 g of alkaloid per 100 g dry tissue. For this reason *A. vasica*, from Kanjikode, Kerala was chosen for all further tissue culture studies which was also ideal for comparison with *A. beddomei*, collected from the same region.

4.4.2 GROWTH PATTERN OF A. vasica AND A. beddomei

The growth profile of the callus of *A. vasica* and *A. beddomei* is presented in Table VIII.

TABLE – VIII

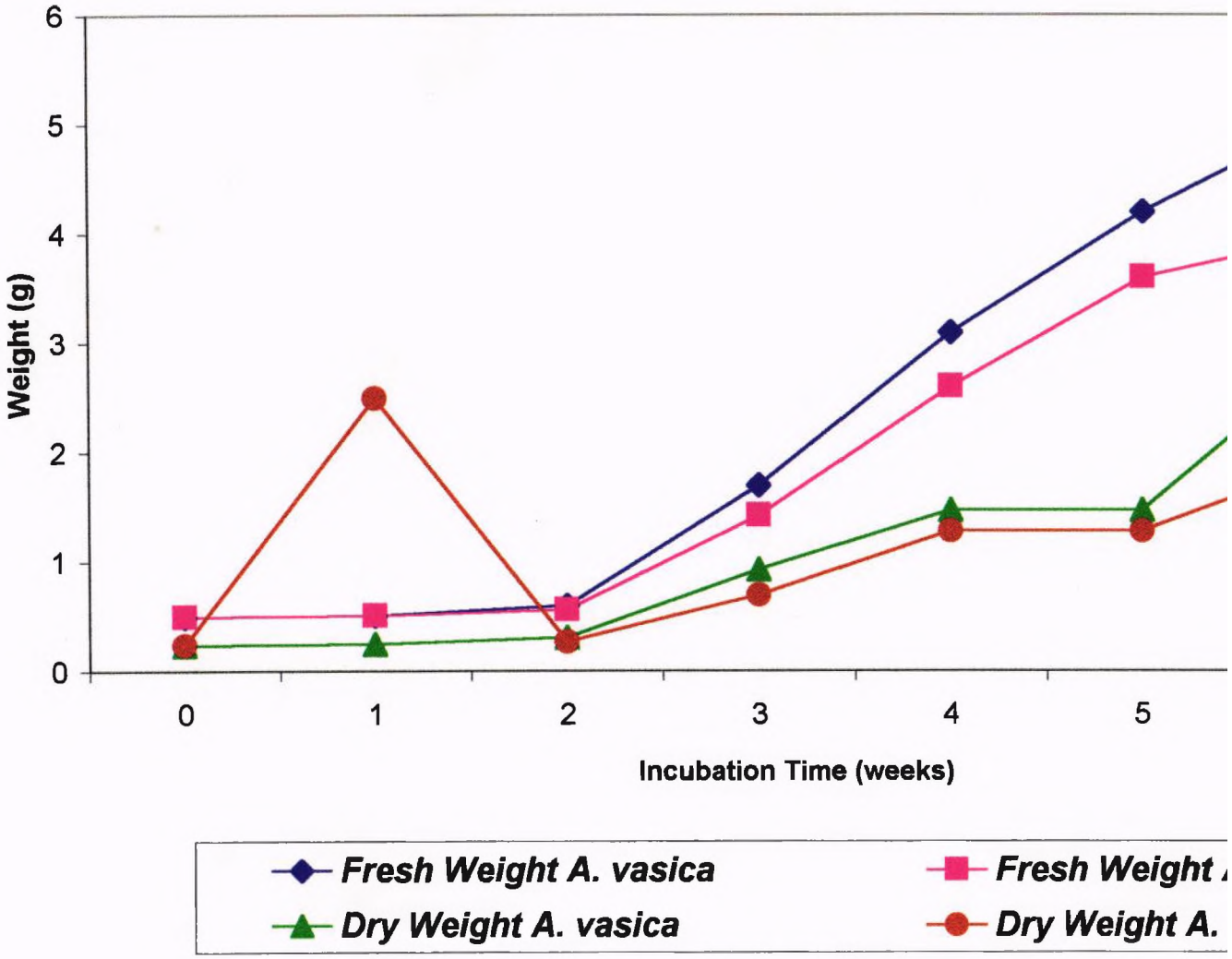
GROWTH PATTERN OF A.vasica AND A. beddomei

Weight (g)	***Species***	***Incubation time (weeks)***							
		0	***1***	***2***	***3***	***4***	***5***	***6***	***7***
Fresh	*A. vasica*	0.50	0.51	0.60	1.70	3.10	4.20	5.10	5.09
	A. beddomei	0.50	0.51	0.56	1.42	2.60	3.60	3.99	3.99
Dry	*A. vasica*	0.24	0.25	0.31	0.93	1.47	2.20	2.95	2.95
	A. beddomei	0.24	0.25	0.27	0.70	1.28	1.49	1.93	1.93

Data represent an average of 5 replicates in two repetitive experiments.
Medium : MS + 2mg l^{-1}2, 4 – D + 0.2 mg l^{-1}Kn + 3% sucrose.
Inoculum : 500 ± 50 mg fresh tissue in 25 ml medium
Incubation : At 25 ± 2°C under continuous light (2000 lux)

The growth of cultures maintained on MS medium with 2 mg l^{-1} 2, 4–D, 0.2 mg l^{-1} Kn and 3% sucrose as supplements were monitored over a period of seven weeks. The growth pattern of both *A. vasica* and *A. beddomei* showed a typical

Fig. 2 THE GROWTH PATTERN OF *A. vasica* AND *A*



sigmoidal curve (Fig. 2) with maximum growth at 6th week of culture. In *A. vasica*, the growth cycle of the culture was characterized by a lag phase of one week followed by a phase of slow increase in growth (preexponential phase) for another week, followed by an exponential growth phase in the next four weeks, before the callus cells entered the stationary phase. The fresh weight of the callus increased 10-fold over the initial inoculum during the growth cycle of *A. vasica* whereas an 8-fold increase in fresh weight was observed in *A. beddomei*. Plate 3 and 4 illustrate the callus growth of *A. vasica* and *A. beddomei* respectively.

4.4.3 ALKALOID CONTENT IN THE CALLUS CULTURE

Alkaloid content was monitored throughout the culture period and a similar pattern was observed both in *A. vasica* and *A. beddomei* which is depicted in Table IX.

TABLE – IX

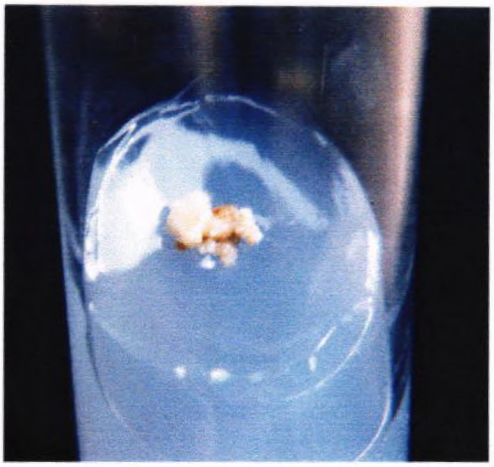
ALKALOID CONTENT IN THE CALLUS CULTURES OF A. vasica AND A. beddomei

Species	*Alkaloid content (g/100 g dry callus)*							
	Incubation time (weeks)							
	0	*1*	*2*	*3*	*4*	*5*	*6*	*7*
A. vasica	0.080	0.082	0.090	0.136	0.144	0.160	0.169	0.18
A. *beddomei*	0.084	0.088	0.094	0.148	0.160	0.172	0.180	0.19

Data represent an average of 5 replicates in two repetitive experiments.
Medium : MS + 2mg l^{-1}2, 4 – D + 0.2 mg l^{-1}Kn + 3% sucrose.
Inoculum : 500 ± 50 mg fresh tissue in 30 ml medium
Incubation : At 25 ± 2°C under continuous light (2000 lux)

Alkaloid levels were maintained fairly constant till the end of two weeks and increased rapidly for three weeks coinciding with exponential phase of growth. The alkaloid content enhanced steeply during the post exponential phase of growth. In the case of *A. vasica*, the maximum production of alkaloid was observed during the 7th week of culture which was about 0.18g% on a dry weight basis and in *A. beddomei*,

Plate -3 : Callus growth of *A.vasica* at different time points

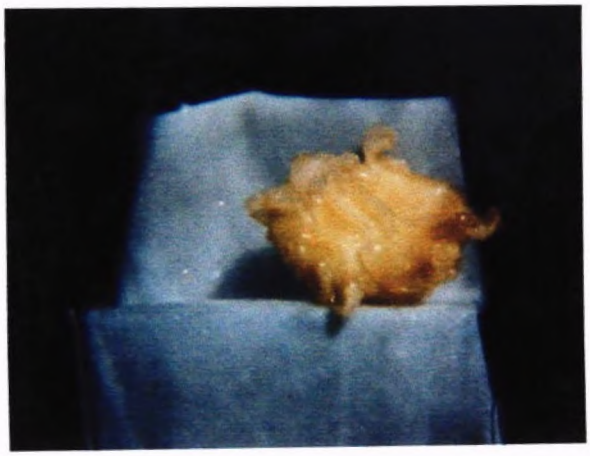


2nd week

3rd week

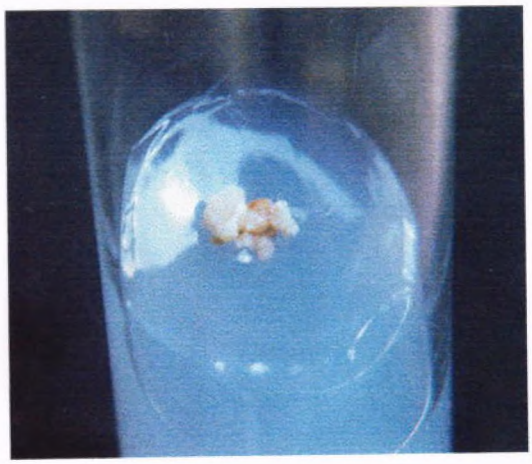
4th week

5th week

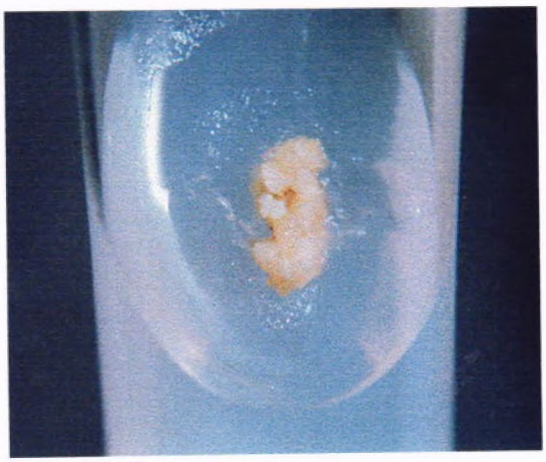


6th week

Plate -4: Callus growth of *A.beddomei* at different time points



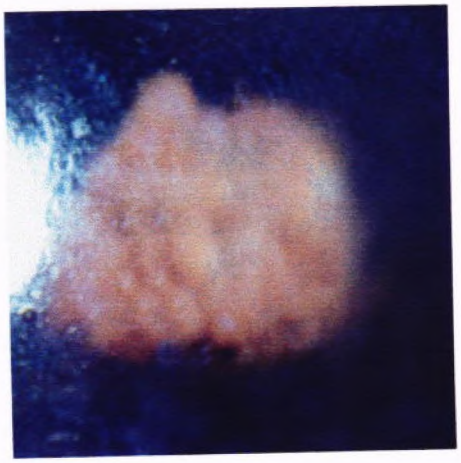
2nd week



3rd week

4th week

5th week



6th week

the highest yield of alkaloid was noted on the 7th week of culture and was about 0.19g% on a dry weight basis.

It is a well known fact that, the cells in culture, start dividing at the end of lag phase, followed by a more vigorous exponential and linear phases and the cell division ceases when the cells enter the stationary phase (Street, 1977).

In the present investigation, the callus cultures of both *A. vasica* and *A. beddomei* reached stationary phase after 6th week of culture. In the context of secondary metabolite production, investigations on the time course study of metabolite accumulation in relation to growth of the cell culture system revealed two basically different patterns. They were growth linked and non-growth linked accumulation of metabolites. Even though there were two types, it is envisaged that in most of the cultures, maximum accumulation of several secondary metabolites occurred when growth rate ceased (i.e. late exponential or stationary phase), indicating an inverse relation between growth and accumulation of secondary metabolites.

This kind of response has also been reported by Do and Cormier (1990), in the production of anthocyanin in cultures of *Vitis vinifera*. Our findings of non-growth related response is in accordance with that of Anderson *et al.* (1983) who have worked with the alkaloid producing culture system of *Ailanthus altissima*. Thus, the time course study of growth and production profile reflects the biosynthetic capacity of the culture during stationary phase.

Even though plant cell culture technology offers a great potential for the production of secondary metabolites, a major problem with these cultures is that they invariably produce low levels of the desired metabolites. This severely hampers with the commercial exploitation of these products. Hence, there is a need to find out ways to increase the yield of these metabolites.

4.4.4 INFLUENCE OF BASAL MEDIA ON GROWTH AND ALKALOID PRODUCTION IN CALLUS CULTURES OF Adhatoda SPECIES

Various strategies have been employed to enhance the production of alkaloids in the present study, since the level obtained initially was very low. They have been classified into two groups. In the first group, the constituents of the media were changed or their levels were altered to obtain an optimal production. Under this, the influence of different basal media, hormones, nitrate, phosphate and carbon source were studied. In the other group, the effect of elicitors both biotic and abiotic in origin was examined to induce or enhance alkaloid production.

In this study, four commonly used basal media were tested for their influence on both growth and alkaloid production. The growth and alkaloid content in those basal media were recorded on the 35th day of culture which is represented in Table-X.

TABLE – X

GROWTH AND ALKALOID CONTENT IN THE CALLUS CULTURES OF A. vasica AND A. beddomei IN DIFFERENT BASAL MEDIA

Basal media	*Dry weight (g)*		*Alkaloid content (g/100g dry callus tissue)*	
	A. vasica	*A. beddomei*	*A. vasica*	*A. beddomei*
MS	2.94	2.86	0.14	0.18
LS	1.75	1.73	0.12	0.14
SH	2.01	1.98	0.11	0.13
B_5	2.09	2.00	0.12	0.16

Data represent an average of 4 replicates in two repetitive experiments.
Medium : Basal media (MS, LS, SH or B_5) + 2mg l^{-1} 2,4–D + 0.2 mg l^{-1} Kn + 3% sucrose.
Basal media composition : Appendices I and II
Inoculum : 300 ± 50 mg fresh tissue in 40 ml medium
Incubation : 35 days at 25 ± 2°C under continuous light (2000 lux)

Of the four media tested, MS gave the best results in terms of both growth and alkaloid production. In cultures grown on LS, SH and B_5 media, the growth was

considerably reduced by 40%, 30%and 28% respectively when compared to MS medium.

The production of alkaloid also varied significantly with different media. Alkaloid yield was higher in MS medium which was 0.14 g/100g of dry tissue in *A. vasica* and 0.18 g/100g of dry tissue in *A. beddomei* on the 35th day of culture, whereas cultures grown in LS, SH and B_5 medium showed reduced levels of alkaloid both in *A. vasica* and *A. beddomei* compared to MS medium.

B_5 medium (Gamborg, 1968) was found to be suitable for rosmarinic acid production in *A. officinalis* cultures (De-Eknamkul and Ellis, 1985). In *Lithospermum erythrorhizon* cultures, of the different basal media tested, shikonin was found to be synthesized only in White's medium. Yamamoto and Yamada (1987) showed that in reserpine producing cultures of *Rauwolfia serpentina*, LS medium was used for maximum growth, whereas, B_5 medium was used as the production medium. LS medium was found to increase alkaloid production to over 30% in *A. altissima* cultures (Anderson *et al.*, 1987). This revealed that proper selection of basal medium is an essential influencing step in the final yield of desired product in higher quantity.

In the present study, of the four different basal media (MS, LS, SH and B_5) tested, MS medium was found suitable for both growth and alkaloid production. Therefore, MS medium was used for all further experiments.

4.4.5. INFLUENCE OF PLANT HORMONES

1. AUXINS

The influence of selected auxins on growth and alkaloid production was studied. The cultures of *A. vasica* and *A. beddomei*, maintained on MS medium with, 2, 4-D and Kn as the hormonal supplements were transferred to MS basal medium prior to transfer of tissues to the experimental media.

Auxins – IAA, NAA, IBA and 2, 4-D were incorporated into MS basal medium containing 3% sucrose. Each auxin was used at a concentration of 0.5, 1.0, 1.5 and 2.0 mg l^{-1} to study their effect on the growth and alkaloid production of *A. vasica* and *A. beddomei*. The growth and the alkaloidal content were recorded on the 35th day of culture. Tissues grown in MS basal medium served as control. The results obtained are presented in Table – XI.

TABLE - XI

FRESH WEIGHT AND ALKALOID CONTENT IN THE CALLUS CULTURES OF A. vasica AND A. beddomei ON TREATMENT WITH AUXINS

Medium + Auxins	*Auxin concentration (mg l^{-1})*	*Fresh weight (g)*		*Alkaloid content (g/100g dry tissue)*	
		A. vasica	*A.beddomei*	*A. vasica*	*A.beddomei*
Basal C	-	4.20	3.08	0.16	0.22
C + IAA	0.5	3.94	3.76	0.18	0.23
	1.0	3.99	3.90	0.19	0.24
	1.5	4.57	4.37	0.22	0.26
	2.0	5.82	5.49	0.26	0.28
C + NAA	0.5	4.12	4.00	0.14	0.18
	1.0	4.32	4.21	0.15	0.20
	1.5	5.00	4.96	0.16	0.21
	2.0	5.26	5.00	0.20	0.23
C + IBA	0.5	3.75	3.79	0.14	0.16
	1.0	3.89	4.02	0.16	0.17
	1.5	4.21	4.28	0.18	0.19
	2.0	4. 26	4.29	0.19	0.20
C+2,4-D	0.5	4.26	4.15	0.14	0.18
	1.0	5.00	4.56	0.14	0.17
	1.5	5.90	4.93	0.11	0.16
	2.0	6.14	5.86	0.10	0.14

Data represent an average of 4 replicates in two repetitive experiments.
Basal (C) : MS basal salts + 3% sucrose.
Inoculum : 500 ± 50 mg fresh tissue in 25 ml medium.
Incubation : 35 days at 25 ± 2°C under continuous light (2000 lux)

After 35 days of culture on MS basal medium (control) the growth of the callus had increased by 8 and 6 fold in fresh weight of *A. vasica* and *A. beddomei*

respectively compared to the weight of the inoculum. Alkaloid content was 0.16 g/100g dry tissue of *A. vasica* and 0.22g/100g dry tissue of *A. beddomei*.

With the increase in IAA concentration, the fresh weight of the callus and alkaloid content had increased steadily for both *A. vasica* and *A. beddomei* reaching the maximum growth at 2.0 mg l^{-1} which was 12 and 11 fold respectively. The maximum alkaloid production was also obtained with the concentration of 2.0 mg l^{-1}. For *A. vasica* it was 0.26 g/100g dry tissue and *A. beddomei* the alkaloid content was 0.28 g/100g dry tissue.

Of the different levels of NAA tested, 2.0 mg l^{-1} NAA gave good result in terms of growth and alkaloid production both in *A. vasica* and *A. beddomei*. The maximum growth of the callus was observed at 2.0 mgl^{-1}. The yield of alkaloid in *A. vasica* and *A. beddomei* at 2.0 mg l^{-1} NAA was found to be 0.20 and 0.23g/100g dry tissue respectively which was 25% more on comparison with the control in the case of *A. vasica* and 5% more in the case of *A. beddomei*.

IBA treated cultures showed increased growth and alkaloid production with increasing concentration of IBA, reaching a maximum at 2.0 mg l^{-1} IBA. The maximum yield of alkaloid was only 0.19 and 0.20 g/100g dry tissue in *A. vasica* and *A. beddomei* respectively. As the concentration of IBA increased, the fresh weight of the callus increased to a maximum at 2.0 mg l^{-1} IBA which was 8.5 fold in both *A. vasica* and *A. beddomei* when compared to the weight of the inoculum. But IBA seemed to inhibit alkaloid production.

On the contrary, 2, 4-D treated cultures showed the best result in growth but it did not have beneficial effect on alkaloid production. With increase in 2,4-D concentration, fresh weight of the callus increased parallely reaching a maximum at 2.0 mg l^{-1} which was 12 fold for *A. vasica* and *A. beddomei*. Although maximal callus growth was observed in 2,4-D containing cultures, 2,4-D was found to be inhibitory to alkaloid production. Tabata *et al.* (1971) found 2,4-D to strongly inhibit alkaloid

production in *N. tobacum*. Fujita *et al.* (1981), also found that shikonin formation was completely inhibited by 2,4-D in cell cultures of *L. erythrorhizon*.

Among the auxins studied, IAA (2 mg l^{-1}) and NAA (2 mg l^{-1}) were found to be suitable for growth as well as alkaloid production in both the species of *Adhatoda*.

2. *INFLUENCE OF CYTOKININS ON GROWTH AND ALKALOID PRODUCTION*

Cytokinins stimulate cell division and their influence on callus formation and alkaloid production was studied. This is represented in Table XII.

TABLE – XII

FRESH WEIGHT AND ALKALOID CONTENT IN THE CALLUS CULTURES OF A. vasica AND A. beddomei TREATED WITH CYTOKININS

Medium + Cytokinin	*Cytokinin concentration (mg l^{-1})*	*Fresh weight (g/culture)*		*Alkaloid content (g/100g dry weight)*	
		A. vasica	*A. beddomei*	*A. vasica*	*A.beddomei*
Basal C	-	4.35	3.98	0.17	0.20
C + Kn	0.5	4.29	4.05	0.15	0.16
	1.0	4.86	4.52	0.17	0.17
	1.5	4.82	4.69	0.19	0.21
	2.0	3.95	4.00	0.17	0.20
C + BAP	0.5	4.00	3.96	0.16	0.18
	1.0	3.86	3.26	0.17	0.19
	1.5	3.20	3.14	0.14	0.15
	2.0	3.14	2.99	0.13	0.14

Data represent an average of 5 replicates in two repetitive experiments.
Basal (C) : MS basal salts + 3% sucrose.
Inoculum : 500 ± 50mg fresh tissue in 25 ml medium.
Incubation : 35 days at 25 ± 2°C under continuous light (2000 lux)

The same concentrations (i.e.) 0.5, 1.0, 1.5 and 2.0 mg l^{-1} of cytokinins (kinetin (Kn) and benzyl amino purine (BAP) were used. Of the different concentrations of kinetin tested Kn at 1.5 mg l^{-1} level resulted in a fairly good yield of alkaloid and growth. Cultures tested with 2.0 mg l^{-1} did not support any significant increase in terms of both growth and alkaloid production. BAP at all concentrations did not have any beneficial effect on growth and alkaloid content.

Consequent to the results obtained from the above experiments, cultures were conducted to determine the optimum concentrations of auxins and cytokinins. Two mgl^{-1} concentration of the auxins, IAA and NAA was selected and combined with cytokinins (Kn) at three concentrations (0.5, 1.0 and 1.5 mgl^{-1}) to study their combined effect on growth and alkaloid production. The data obtained are shown in Table XIII.

TABLE – XIII

DRY WEIGHT AND ALKALOID CONTENT IN THE CALLUS CULTURES OF A. vasica AND A. beddomei ON TREATMENT WITH AUXIN AND KINETIN COMBINATIONS

Medium + Auxin	*Concentration of Kn (mg l^{-1})*	*Dry weight (g)*		*Alkaloid content (g/100g dry tissue)*	
		A. vasica	*A. beddomei*	*A. vasica*	*A. beddomei*
Basal (C)	-	2.82	2.80	0.14	0.18
C + IAA ($2mgl^{-1}$)	0.5	2.32	2.06	0.18	0.24
	1.0	2.82	2.75	0.24	0.28
	1.5	2.96	2.86	0.28	0.29
C + NAA ($2mgl^{-1}$)	0.5	2.00	1.98	0.13	0.17
	1.0	2.07	2.04	0.12	0.16
	1.5	2.43	2.26	0.13	0.16

Data represent an average of 5 replicates in two repetitive experiments.
Medium : MS + basal salts + 3% sucrose.
Inoculum : 500 ± 20mg fresh tissue in 25 ml medium.
Incubation : 35days at 25 ± 2°C under continuous light (2000 lux)

The growth and production of alkaloid were reduced considerably when NAA (2.0 mgl^{-1}) was supplemented with Kn. From the table, it is evident that the highest yield of alkaloid was in the presence of Kn at 1.5 mg l^{-1} i.e., 0.28 g and 0.29 g/100g dry tissue weight in *A. vasica* and *A. beddomei* respectively.

It was evident that for the maximum production of alkaloid, the optimum concentration of IAA was 2 mg l^{-1} together with 1.5 mg l^{-1} Kn. This concentration was used in all further experiments.

4.4.6. EFFECT OF NUTRIENT STRESS ON GROWTH AND ALKALOID PRODUCTION

1. NITRATE AND PHOSPHATE

The media for plant cell culture usually provide nitrogen as a mixture of NO_3^- and NH_4^+ salts. When these sources are altered, either alone or in combination or when their ratio is altered or when the levels are changed, they influence growth and product formation. In most of the cases, a decrease/depletion of nitrogen leads to an enhanced secondary metabolite production.

Growth and alkaloid production in callus cultures of *Adhatoda* was studied using low concentrations of nitrate and phosphate. These were added to the MS medium containing IAA (2.0 mg l^{-1}) and Kn (1.5 mg l^{-1}). The results obtained are shown in Table – XIV.

TABLE – XIV

GROWTH AND ALKALOID CONTENT IN THE CALLUS CULTURES OF A. vasica AND A. beddomei DUE TO NITRATE AND PHOSPHATE STRESS

Concentration	*Dry weight (g)*		*Alkaloid content (g/100g dry weight)*	
	A. vasica	*A. beddomei*	*A. vasica*	*A. beddomei*
Control	2.90	2.86	0.28	0.29
N_1 420 mg l^{-1}	2.08	1.70	0.29	0.30
N_2 210 mg l^{-1}	1.96	1.56	0.38	0.43
P_1 85 mg l^{-1}	2.10	1.76	0.28	0.30
P_2 42.5 mg l^{-1}	1.78	1.64	0.39	0.42

Data represent an average of 5 replicates in two repetitive experiments.
Control : MS + IAA (2.0 mg/l) + Kn (1.5 mg/l) + 3% sucrose.
Inoculum : 500±10mg fresh tissue in 30ml medium
Incubation : 35 days at 25±2°C under continuous light (2000 lux)
Treatment : N_1 = Nitrogen in MS medium reduced to half.
N_2 = Nitrogen in MS medium reduced to quarter.
P_1 = Phosphate in MS medium reduced to half.
P_2 = Phosphate in MS medium reduced to quarter.

The concentration of the major inorganic components in MS medium, namely nitrate and phosphate, was varied to study their effect on growth and alkaloid

production in cultures. Two levels of ammonium and potassium nitrate (corresponding to 420 and 210 mg $l^{-1}$of nitrogen) and two levels of phosphate, in the form of potassium dihydrogen orthophosphate (85 and 42.5 mg l^{-1} phosphate) were tested. Control received normal MS salts (840 mg l^{-1} nitrogen and 170 mg l^{-1} phosphate).

A reduction in nitrate level to ¼th the normal level (210 mg l^{-1}) led to increased alkaloid production. The alkaloid content in *A. vasica* increased in nitrate stress medium with a yield of 0.38 g% in comparison to 0.28 g% in the control on the 35th day of the culture whereas in *A. beddomei* the increase was from 0.29 g% to 0.43 g% on dry weight basis.

Increased production was also observed in phosphate depleted medium (42.5 mg l^{-1}) which was 0.28 to 0.39 g% in *A. vasica* and in *A. beddomei* it was from 0.29 to 0.42 g% on a dry weight basis. A decrease in growth was observed when the cultures were under nitrate and phosphate stress.

There are some exceptions, wherein, higher levels of nitrogen resulted in increased product formation. Increase in shikonin production was reported when the nitrate level was increased from 3.3 to 6.7 mM (Fujita *et al.*, 1981) and a similar increase in conessine was noted with increase in nitrate level from 20mM to 60mM in the cell cultures of *Holarrhena antidysenterica* (Panda *et al.*, 1992).

Apart from the changes in the levels of these nutrients, the source and ratio of NO_3^- to NH_4^+ were found to influence product formation. For example, in *N. tabacum* cultures, ubiquinone formation was enhanced when NH_4^+ was the sole nitrogen source (Ikeda *et al.*, 1976). On the contrary, in *L. erythrorhizon*, the cells were not able to tolerate NH_4^+ in the medium (Fujita *et al.*, 1981). In most of the cases, an inverse relation with growth was noticed during nitrate stress.

Two important observations emerged from this study on the influence of nitrogen on growth and alkaloid production in *Adhatoda* cultures. Firstly, the cultures required both NO_3^- and NH_4^+ salts as a nitrate source for maximum growth and

alkaloid yield. Secondly, decreased levels of nitrogen had a positive effect on alkaloid production. When the levels of NO_3^- and NH_4^+ in the MS medium was reduced to half the strength (420 mg l^{-1} nitrogen) and quarter the strength (210 mgl^{-1} nitrogen) of original concentration (840 mg l^{-1} nitrogen) in normal MS medium, reduced growth was observed. Such a finding has been reported in other systems also (Ravishankar and Grewal, 1988).

It was reported that under nutrient stress, the synthesis of only those secondary metabolites which share close precursors with primary pathways would be enhanced as the stress limits the processes associated with cell division. The enzyme involved in phenylpropanoid metabolism such as phenylalanine ammonia lysase (PAL) showed increased activity under these conditions (Hahlbrock and Grisebach, 1979). It was envisaged that the rate of protein synthesis might slow down under nitrogen depletion and the unutilized carbohydrates could be diverted to phenolic synthesis. This may be the reason for the increase in alkaloid production in *Adhatoda* cultures.

The next major nutrient which has been shown to regulate secondary metabolite production was phosphate. It is well known that phosphate regimes trigger production of various metabolites including antibiotics and alkaloids in microbial cultures. When the cultures of *Peganum harmala* were treated with reduced phosphate, production of carboline alkaloid and seratinin increased considerably (Sasse *et al.*, 1982).

In the present investigation, a significant increase in alkaloid content of the *Adhatoda* culture was observed under phosphate stress. As in the case with nitrate stress an inverse relation with growth was observed.

The regulatory role of phosphate in controlling secondary metabolism has not been studied thoroughly in plant cell culture systems. In tobacco cultures, high phosphate concentration resulted in a decrease in PAL enzyme activity and phosphate depletion of culture media led to marked increase in the activity of PAL followed by increase in the biosynthesis of cinnamoyl putrescines. It is quite probable that, cells

might take up phosphate within two days of inoculation from fresh medium and store it in vacuoles from where it was depleted during growth. Mantell and Smith (1984) reported that increased levels of phosphates inhibit phosphatases, thus inhibiting the dephosphorylation step. This might in turn affect the activity of enzymes involved in secondary metabolite production.

2. *SUCROSE*

Sucrose and mannitol are used as osmotic agents to study their effects on growth and alkaloid production. Sucrose is also used as a carbon source in the tissue culture media, whereas mannitol is exclusively an osmotic agent. The levels of sucrose alone and in combination with mannitol were increased to create an osmotic stress. Four different levels of sucrose (4, 6, 8 and 10% w/v) and mannitol (1 to 3% w/v) were used in this study. The effect of sucrose alone on the growth and alkaloid content is given in Table XV.

TABLE – XV

GROWTH AND ALKALOID CONTENT IN THE CALLUS CULTURES OF A. vasica AND A. beddomei ON VARYING SUCROSE CONCENTRATION

Concentration of sucrose (g%)	*Dry weight (g)*		*Alkaloid content (g/100g dry weight)*	
	A. vasica	*A. beddomei*	*A. vasica*	*A. beddomei*
Control	2.10	2.16	0.27	0.28
S_4	1.78	1.64	0.30	0.34
S_6	1.30	1.08	0.33	0.36
S_8	1.28	1.04	0.34	0.37
S_{10}	1.26	1.00	0.28	0.29

Data represent an average of 5 replicates in two repetitive experiments.
Control : MS + IAA (2.0 mg/l) + Kn (1.5 mg/l) + 3% sucrose.
Inoculum : 400 ± 20 mg fresh tissue in 25 ml media.
Incubation : 35 days at 25 ± 2°C under continuous light (2000 lux)
Treatment : MS + IAA (2.0 mg/l) + Kn (1.5 mg/l) + Sucrose (4 to 10% w/v)
S_4, S_6, S_8 and S_{10} : Sucrose supplemented at 4.0, 6.0, 8.0 and 10.0% (w/v) levels respectively.

In experiments with increasing concentration of sucrose, growth of the callus was found to be decreased. It was reduced with increase in sucrose concentration when compared to control. Maximum reduction in the callus growth occurred in the

medium containing 10% sucrose. On the contrary, alkaloid production was found to be enhanced with increased levels of sucrose attaining a maximum at 8% level. In 8% sucrose treated cultures, the yield of alkaloid was 0.34g % in *A. vasica* and 0.37 g% in *A. beddomei* on a dry weight basis. However, the alkaloid content decreased with further increase in sucrose concentration to 10% level. Although higher alkaloid production resulted in the medium containing 8 % sucrose, this concentration was not tried as it would not be economical.

3. SUCROSE AND MANNITOL

Sucrose was also thought to play a physical role as an osmotic agent (Ilker, 1987). To check this concept, in the present investigation sucrose alone and in combination with mannitol (a well known osmotic agent) was used to study the effect on alkaloid production and the data are depicted in Table-XVI.

TABLE – XVI

GROWTH AND ALKALOID CONTENT IN THE CALLUS CULTURES OF A. vasica AND A. beddomei ON TREATMENT WITH DIFFERENT CONCENTRATIONS OF MANNITOL

Concentration of mannitol(g%)	*Dry weight (g)*		*Alkaloid content (g/100g dry weight)*	
	A. vasica	*A. beddomei*	*A. vasica*	*A. beddomei*
Control	2.93	2.80	0.28	0.29
C+1	2.00	1.96	0.30	0.33
C+1.5	1.68	1.54	0.32	0.36
C+2	1.48	1.34	0.35	0.39
C+2.5	1.40	1.30	0.37	0.41
C+3	1.38	1.26	0.39	0.45

Data represent an average of 5 replicates in two repetitive experiments.
Control : MS + IAA (2.0 mg/l) + Kn (1.5 mg/l) + 3% sucrose.
Treatment : Mannitol supplemented to control medium at 1% (C+1), 1.5% (C+1.5), 2% (C+2), 2.5% (C+2.5) and 3% (C+3) levels
Inoculum : 500 ± 20 mg fresh tissue in 25 ml media
Incubation : 35 days at 25 ± 2°C under continuous light (2000 lux)

An inverse relation of growth with increasing mannitol concentration was observed. The extent of reduction in the growth of callus was higher in comparison to

the cultures treated with higher concentration of sucrose alone. A combination of 3% sucrose and 3% mannitol reduced the growth to a maximum of about 50% when compared to control (3% sucrose). Interestingly, this combination enhanced alkaloid production which was 0.39% in *A. vasica* and 0.45% in *A. beddomei* on a dry weight basis.

4.4.7. SCREENING OF ELICITORS TO OPTIMISE CULTURE MEDIA

Even though many reports in recent years showed the possibility of using biotic and abiotic elicitors for the enhancement of secondary metabolites in culture systems, no elicitor has been found to have a general effect on many systems or no system has been found to respond to all the elicitors. Hence, the screening of elicitors has been done based on the available reports and on the availability of the material. Moreover, since the concentration of elicitors and the incubation time required for maximum elicitation differs with the kind of elicitor and the system, an empirical screening program has been adopted to find out the suitable elicitors for maximum alkaloid production.

4.4.7.1. EFFECT OF BIOTIC ELICITORS

The elicitors screened in this study included the media filtrate and mat / cell extracts of various fungi, yeast and bacteria. During the preliminary studies on *Adhatoda* species cultures, it was observed that the time of elicitation response of media filtrates (MF) and mat/cell extracts (ME/CE) was different. In the case of MF, the response being slower, the elicitation was monitored at 5 days interval, whereas, for ME/CE, it was monitored at 3 days interval. Moreover, for maximum elicitation, the concentration of elicitors required in the case of MF was higher than that of ME/CE. Thus the MFs were used at three different levels (5, 10 and 20 % v/v) and elicitation was monitored at every 5 day interval. On the other hand, the ME/CE were used at a concentration of 1, 3 and 5% (v/v) and monitored at 3 day interval for all the biotic elicitors tested.

4.4.7.1.1. INFLUENCE OF FUNGAL ELICITORS

(i) *Aspergillus niger* :

The effect of the media filtrate and mat extract of this fungus is shown in Table-XVII.

TABLE – XVII

ALKALOID CONTENT IN THE CALLUS CULTURES OF A. vasica AND A. beddomei TREATED WITH Aspergillus niger ELICITORS

Species	Incubation time (days)	Alkaloid content (g/100 dry tissue)				Incubation time (days)	Alkaloid content (g/100 dry tissue)			
		Control	Media filtrate concentration				Control	Mat Extract concentration		
			5%	10%	20%			1 %	3 %	5 %
A. vasica	7	0.17	0.17	0.18	0.19	5	0.15	0.16	0.18	0.20
	12	0.18	0.20	0.27	0.29	8	0.16	0.17	0.20	0.28
	17	0.19	0.23	0.30	0.38	11	0.18	0.20	0.24	0.39
A.beddomei	7	0.18	0.19	0.20	0.33	5	0.14	0.15	0.18	0.22
	12	0.19	0.22	0.30	0.33	8	0.16	0.17	0.21	0.34
	17	0.21	0.27	0.40	0.42	11	0.17	0.18	0.23	0.42

Data represent an average of 5 replicates in two repetitive experiments.
Medium : MS + IAA (2.0 mg/l) + Kn (1.5 mg/l) + 3 % Sucrose with or without (control) elicitor
Inoculum : 500 ± 50 mg fresh tissue in 15 ml medium
Incubation : at 25 ± 2°C under continuous light (2000 lux)

The alkaloid production increased with the incubation time and also with the concentration of the media filtrate and mat extract. Maximum alkaloid elicitation was observed on the 17th day of incubation with media filtrate and on the 11th day with the mat extract. Quantity of alkaloid elicited by both the media filtrate and mat extract was same, proving that both are effective. There was a 100% increase in the alkaloid content due to *Aspergillus niger* over the control.

ii) Aspergillus flavus

Influence of *Aspergillus flavus* elicitors on alkaloid production in callus cultures of both the species was studied which is given in Table - XVIII.

TABLE – XVIII

ALKALOID CONTENT IN THE CALLUS CULTURES OF A. vasica AND A. beddomei TREATED WITH Aspergillus flavus ELICITORS

Species	Incubation time (days)	Alkaloid content (g/100g dry tissue)				Incubation time (days)	Alkaloid content (g/100g dry tissue)			
		Control	Media filtrate concentration				Control	Mat extract concentration		
			5%	10%	20%			1%	3%	5%
A.vasica	7	0.18	0.20	0.25	0.24	5	0.18	0.21	0.21	0.24
	12	0.19	0.22	0.37	0.32	8	0.19	0.22	0.26	0.32
	17	0.21	0.24	0.42	0.30	11	0.21	0.26	0.29	0.39
A.beddomei	7	0.18	0.20	0.29	0.31	5	0.18	0.21	0.24	0.24
	12	0.19	0.27	0.38	0.44	8	0.19	0.26	0.39	0.44
	17	0.21	0.29	0.43	0.46	11	0.21	0.32	0.45	0.48

Data represent an average of 5 replicates in two repetitive experiments.
Medium : MS + IAA (2.0 mg/l) + Kn (1.5 mg/l) + 3 % Sucrose with or without (control) elicitor
Inoculum : 500 ± 50 mg fresh tissue in 15 ml medium
Incubation : at 25 ± 2°C under continuous light (2000 lux)

The elicitation of alkaloid on percentage basis at 10% MF was maximum on the 17th day of culture in *A. vasica*. MF of *A. flavus* at 20% level inhibited the total yield of alkaloid in contrast to *A. niger* where it was found that the total production of alkaloid was maximum at 20% level, whereas in *A. beddomei*, increase in elicitation was observed as the concentration of MF and inoculation time increased and the elicitation on alkaloid at 20% MF on the 17th day of culture was maximum and 2.2 times that observed in control.

The ME at 5% level was effective in eliciting alkaloid production both in *A. vasica* and *A. beddomei* on the 11th day of culture. It was 0.39 g and 0.48g/100g dry tissue respectively. The ME was found to be more effective than the MF.

iii) Penicillium notatum

Effect of *Penicillium notatum* elicitors in stimulating alkaloid production was investigated. The data obtained are depicted in Table - XIX.

TABLE – XIX

ALKALOID CONENT IN THE CALLUS CULTURES OF A. vasica AND A. beddomei TREATED WITH Penicillium notatum ELICITORS

Species	Incubation time (days)	Alkaloid content (g/100g dry tissue)				Incubation time (days)	Alkaloid content (g/100g dry tissue)			
		Control	Media filtrate concentration				Control	Mat extract Concentration		
			5%	10%	20%			1%	3%	5%
A.vasica	7	0.17	0.20	0.28	0.26	5	0.17	0.21	0.24	0.26
	12	0.18	0.26	0.34	0.32	8	0.18	0.29	0.24	0.36
	17	0.19	0.35	0.41	0.34	11	0.19	0.36	0.42	0.43
A.beddomei	7	0.17	0.19	0.22	0.22	5	0.17	0.19	0.23	0.25
	12	0.18	0.23	0.30	0.26	8	0.18	0.22	0.32	0.33
	17	0.19	0.25	0.34	0.31	11	0.19	0.26	0.35	0.37

Data represent an average of 5 replicates in two repetitive experiments.
Medium : MS + IAA (2.0 mg/l) + Kn (1.5 mg/l) + 3 % Sucrose with or without (control) elicitor
Inoculum : 500 ± 50 mg fresh tissue in 15 ml medium
Incubation : at 25 ± 2°C under continuous light (2000 lux)

The MF and ME were effective in elicitation of alkaloid in both *A. vasica* and *A. beddomei*. The MF of *P. notatum* at 10% level showed the highest yield of alkaloid both in *A. vasica* and *A. beddomei* (0.41 and 0.34 g/100g dry tissue) respectively on the 17th day of incubation. However, at higher concentration (20% level), the yield was reduced.

The total yield of alkaloid had increased with increasing concentration of ME and incubation time in both species of *Adhatoda*. The total yield of alkaloid was higher at 5% level of ME on the 11th day of incubation which was 2.5 times and 2.0 times higher than control in *A. vasica* and *A. beddomei* (0.43 and 0.37g/100g dry tissue) respectively.

iv) Trichoderma viridae

The elicitation of alkaloids by the MF and ME of *Trichoderma viridae* was investigated in both the species of *Adhatoda* which is presented in Table-XX.

TABLE – XX

ALKALOID CONTENT IN THE CALLUS CULTURES OF A.vasica AND A.beddomei TREATED WITH Trichoderma viridae ELICITORS

Species	Incubation time (days)	Alkaloid content (g/100g dry tissue)				Incubation time (days)	Alkaloid content (g/100g dry tissue)			
		Control	Media filtrate concentration				Control	Mat extract Concentration		
			5%	10%	20%			1%	3%	5%
A.vasica	7	0.18	0.21	0.26	0.32	5	0.18	0.22	0.29	0.22
	12	0.19	0.28	0.34	0.41	8	0.19	0.29	0.38	0.30
	17	0.21	0.34	0.38	0.50	11	0.21	0.35	0.49	0.31
A.beddomei	7	0.18	0.20	0.27	0.34	5	0.18	0.21	0.28	0.23
	12	0.19	0.26	0.32	0.43	8	0.19	0.28	0.37	0.30
	17	0.21	0.37	0.39	0.52	11	0.21	0.34	0.49	0.28

Data represent an average of 5 replicates in two repetitive experiments.
Medium : MS + IAA (2.0 mg/l) + Kn (1.5 mg/l) + 3 % Sucrose with or without (control) elicitor
Inoculum : 500 ± 50 mg fresh tissue in 15 ml medium
Incubation : at 25 ± 2°C under continuous light (2000 lux)

MF of *T. viridae* at all concentrations elicited a high amount of alkaloids. At 20% concentration, the elicitation was maximum in both *A. vasica* and *A. beddomei* and was found to be 2.5 times higher than that obtained with the control on 17th day of incubation, whereas ME at 3% level showed the maximum elicitation on 11th day both in *A. vasica* and *A. beddomei*. Increasing the ME concentration beyond this level resulted in browning of the culture and alkaloid yield was reduced considerably.

4.4.7.1.2. INFLUENCE OF YEAST ELICITORS

i) Candida albicans

Table -XXI represents the data obtained with the elicitors of *Candida albicans*

TABLE – XXI

ALKALOID CONTENT IN THE CALLUS CULTURES OF A.vasica AND A.beddomei TREATED WITH Candida albicans ELICITORS

Species	*Incubation time (days)*	*Alkaloid content (g/100g dry tissue)*				*Incubation time (days)*	*Alkaloid content (g/100g dry tissue)*			
		Control	*Media filtrate concentration*				*Control*	*Cell extract Concentration*		
			5%	*10%*	*20%*			*1%*	*3%*	*5%*
A.vasica	7	0.17	0.19	0.18	0.15	5	0.17	0.21	0.21	0.22
	12	0.19	0.23	0.21	0.17	8	0.19	0.29	0.22	0.21
	17	0.20	0.29	0.28	0.16	11	0.20	0.35	0.24	0.23
A.beddomei	7	0.17	0.18	0.18	0.15	5	0.17	0.21	0.17	0.17
	12	0.19	0.21	0.20	0.18	8	0.19	0.28	0.20	0.18
	17	0.20	0.28	0.21	0.17	11	0.20	0.34	0.23	0.21

Data represent an average of 5 replicates in two repetitive experiments.
Medium : MS + IAA (2.0 mg/l) + Kn (1.5 mg/l) + 3 % Sucrose with or without (control) elicitor
Inoculum : 500 ± 50 mg fresh tissue in 15 ml medium
Incubation : at 25 ± 2°C under continuous light (2000 lux)

MF of *C. albicans* at 10 and 20% concentrations was ineffective as there was no significant increase in elicitation over the control. At 20% concentration the cultures became brown and there was a partial loss of viability. However, MF at 5% level showed maximum elicitation both in *A. vasica* and *A. beddomei* (0.29 g and 0.28 g/100 dry tissue respectively) on the 17th day.

Maximum yield of alkaloid content was obtained at 1% level of CE on the 11th day of incubation by the two species of Adhatoda (0.29g and 0.28 g/100g dry tissue). The other concentrations of CE were found to be ineffective.

ii) Saccharomyces cerevisiae

The influence of *Saccharomyces cerevisiae* on the elicitation of alkaloid production is presented in the Table – XXII.

TABLE – XXII

ALKALOID COTENT IN THE CALLUS CULTURES OF A. vasica AND A. beddomei TREATED WITH Saccharomyces cerevisiae ELICITORS

Species	*Incubation time (days)*	*Alkaloid content (g/100g dry tissue)*				*Incubation time (days)*	*Alkaloid content (g/100g dry tissue)*			
		Control	*Media filtrate concentration*				*Control*	*Cell extract Concentration*		
			5%	*10%*	*20%*			*1%*	*3%*	*5%*
A.vasica	7	0.17	0.18	0.17	0.16	5	0.17	0.17	0.16	0.16
	12	0.18	0.18	0.16	0.15	8	0.18	0.18	0.16	0.15
	17	0.19	0.20	0.19	0.18	11	0.19	0.19	0.18	0.18
A.beddomei	7	0.17	0.17	0.16	0.15	5	0.17	0.17	0.16	0.16
	12	0.18	0.18	0.17	0.17	8	0.18	0.19	0.18	0.16
	17	0.19	0.19	0.18	0.18	11	0.19	0.20	0.18	0.15

Data represent an average of 5 replicates in two repetitive experiments.
Medium : MS + IAA (2.0 mg/l) + Kn (1.5 mg/l) + 3 % Sucrose with or without (control) elicitor
Inoculum : 500 ± 50 mg fresh tissue in 15 ml medium
Incubation : at 25 ± 2°C under continuous light (2000 lux)

It is evident from the above Table, that the elicitation response using *S. cerevisiae* was very poor.

4.4.7.1.3. INFLUENCE OF BACTERIAL ELICITORS

Our preliminary studies showed that the level of MF and CE of bacteria and the harvesting periods in the elicitation of alkaloid were very much similar when compared to those of fungi and yeast. The same schedule was therefore followed for bacteria also.

i) Bacillus subtilis

The influence of *Bacillus subtilis* elicitors on alkaloid production in A*dhatoda* cultures is presented in Table XXIII.

TABLE - XXIII

ALKALOID CONTENT IN THE CALLUS CULTURES OF A. vasica AND A. beddomei TREATED WITH Bacillus subtilis ELICITORS

Species	*Incubation time (days)*	*Alkaloid content (g/100g dry tissue)*				*Incubation time (days)*	*Alkaloid content (g/100g dry tissue)*			
		Control	*Media filtrate concentration*				*Control*	*Cell extract Concentration*		
			5%	*10%*	*20%*			*1%*	*3%*	*5%*
A.vasica	7	0.17	0.19	0.23	0.28	5	0.17	0.19	0.24	0.26
	12	0.18	0.24	0.29	0.36	8	0.18	0.29	0.36	0.42
	17	0.18	0.26	0.34	0.42	11	0.18	0.24	0.33	0.40
A.beddomei	7	0.17	0.19	0.23	0.29	5	0.17	0.18	0.23	0.23
	12	0.18	0.23	0.30	0.37	8	0.18	0.22	0.29	0.33
	17	0.18	0.27	0.33	0.40	11	0.18	0.26	0.34	0.39

Data represent an average of 5 replicates in two repetitive experiments.
Medium : MS + IAA (2.0 mg/l) + Kn (1.5 mg/l) + 3 % Sucrose with (or) without (control) elicitor
Inoculum : 500 ± 50 mg fresh tissue in 15 ml medium
Incubation : at 25 ± 2°C under continuous light (2000 lux)

Enhancement of alkaloid production was observed both in *A. vasica* and *A. beddomei* with increasing MF concentration and incubation period. The increase was about 2.5 times and 2.25 times that obtained in control for *A. vasica* and *A. beddomei* respectively. With CE the maximum yield was achieved at 5% level on 8th day in *A. vasica.* In contrast to this, the maximum alkaloid production in *A. beddomei* was obtained at 5 % level on the 11th day. MF was found to be better than CE in enhancing alkaloid production in both the species of *Adhatoda* considered.

ii) Bacillus cereus

Elicitation of alkaloids in *A. vasica* and *A. beddomei* callus cultures by the varying concentration of the media filtrate (MF) and cell extract (CE) of *Bacillus cereus* is given in Table - XXIV.

TABLE - XXIV

ALKALOID CONTENT IN THE CALLUS CULTURES OF A. vasica AND A. beddomei TREATED WITH Bacillus cereus ELICITORS

Species	Incubation time (days)	Alkaloid content (g/100g dry tissue)				Incubation time (days)	Alkaloid content (g/100g dry tissue)			
		Control	Media filtrate concentration				Control	Cell extract Concentration		
			5%	10%	20%			1%	3%	5%
A.vasica	7	0.17	0.19	0.22	0.23	5	0.17	0.18	0.21	0.23
	12	0.18	0.21	0.28	0.28	8	0.18	0.19	0.27	0.28
	17	0.18	0.26	0.31	0.33	11	0.18	0.25	0.30	0.31
A.beddomei	7	0.17	0.18	0.21	0.23	5	0.17	0.18	0.20	0.23
	12	0.18	0.21	0.27	0.29	8	0.18	0.19	0.27	0.29
	17	0.18	0.27	0.30	0.34	11	0.18	0.26	0.29	0.32

Data represent an average of 5 replicates in two repetitive experiments.
Medium : MS + IAA (2.0 mg/l) + Kn (1.5 mg/l) + 3 % Sucrose with or without (control) elicitor
Inoculum : 500 ± 50 mg fresh tissue in 15 ml medium
Incubation : at 25 ± 2°C under continuous light (2000 lux)

Enhancement of alkaloid production was observed on the 17th day at 20% level of MF. The increase was about 100% over the control on a dry weight basis observed both in *A. vasica* and *A. beddomei*. With CE, maximum yield was achieved at 5% level on 11th day of culture which was 0.31 g and 0.32g/100g dry tissue in *A. vasica* and *A. beddomei* respectively.

iii) Pseudomonas striata

Table – XXV depicts the influence of alkaloid production in callus culture of Adhatoda species by the application of MF and CE of *P. striata*.

TABLE - XXV

ALKALOID CONTENT IN THE CALLUS CULTURES OF A. vasica AND A. beddomei TREATED WITH Pseudomonas striata ELICITORS

Species	*Incubation time (days)*	*Alkaloid content (g/100g dry tissue)*				*Incubation time (days)*	*Alkaloid content (g/100g dry tissue)*			
		Control	*Media filtrate concentration*				*Control*	*Cell extract Concentration*		
			5%	*10%*	*20%*			*1%*	*3%*	*5%*
A.vasica	7	0.16	0.17	0.18	0.20	5	0.16	0.19	0.23	0.24
	12	0.16	0.17	0.19	0.21	8	0.16	0.21	0.27	0.28
	17	0.17	0.18	0.20	0.24	11	0.17	0.22	0.29	0.31
A.beddomei	7	0.16	0.17	0.19	0.20	5	0.16	0.18	0.22	0.24
	12	0.16	0.17	0.20	0.23	8	0.16	0.21	0.28	0.29
	17	0.17	0.19	0.22	0.25	11	0.17	0.23	0.28	0.32

Data represent an average of 5 replicates in two repetitive experiments.
Medium : MS + IAA (2.0 mg/l) + Kn (1.5 mg/l) + 3 % Sucrose with or without (control) elicitor
Inoculum : 500 ± 50 mg fresh tissue in 15 ml medium
Incubation : at 25 ± 2°C under continuous light (2000 lux)

It is clear from the table that the MF at 20 % level increased alkaloid content on the 7th day of incubation itself and the increment doubled on the 17th day. CE of *P. striata* enhanced alkaloid content at all the concentrations tested whereas the yield and incubation time varied with the level of elicitor, with maximum at 5% CE treated cultures. The maximum enhancement in alkaloid productivity was achieved at 5% level on the 11th day which was 0.31 g/100 g dry tissue in *A. vasica* and 0.32 g/100 g dry tissue in *A. beddomei* as compared to only 0.17 g/100 g dry tissue in the control.

iv) Escherichia coli

E. coli was another bacterium studied to elicit alkaloid production. The data obtained with *E. coli* elicitors on alkaloid production by the calli of the two *Adhatoda* species is shown in Table - XXVI.

TABLE - XXVI

ALKALOID CONTENT IN THE CALLUS CULTURES OF A. vasica AND A. beddomei TREATED WITH Escherichia coli ELICITORS

Species	*Incubation time (days)*	*Alkaloid content (g/100g dry tissue)*				*Incubation time (days)*	*Alkaloid content (g/100g dry tissue)*			
		Control	*Media filtrate concentration*				*Control*	*Cell extract Concentration*		
			5%	*10%*	*20%*			*1%*	*3%*	*5%*
A. vasica	7	0.17	0.18	0.19	0.21	5	0.17	0.19	0.20	0.22
	12	0.17	0.19	0.21	0.22	8	0.17	0.20	0.22	0.25
	17	0.18	0.19	0.22	0.24	11	0.18	0.21	0.20	0.27
A. beddomei	7	0.17	0.18	0.19	0.22	5	0.17	0.18	0.20	0.21
	12	0.17	0.19	0.21	0.23	8	0.17	0.19	0.23	0.24
	17	0.18	0.21	0.22	0.24	11	0.18	0.20	0.23	0.26

Data represent an average of 5 replicates in two repetitive experiments.
Medium : MS + IAA (2.0 mg/l) + Kn (1.5 mg/l) + 3 % Sucrose with or without (control) elicitor
Inoculum : 500 ± 50 mg fresh tissue in 15 ml medium
Incubation : at 25 ± 2°C under continuous light (2000 lux)

E. coli MF also exhibited maximum elicitation of alkaloid at 20% level on the 17th day of incubation both in *A. vasica* and *A. beddomei*. This was 33% higher than in control. Increased production of alkaloid content was noticed with increase in CE concentration and harvesting periods. The maximum yield of alkaloid productivity was noticed at 5% level on the 11th day of incubation. In *A. vasica* the value was 50% higher than that in control and in *A. beddomei* it was 44% higher (0.27g and 0.26g/ 100g dry tissue respectively). Thus CE of *E. coli* rather than MF was found to be more effective in eliciting alkaloid production.

v) Staphylococcus aureus

The influence of *Staphylococcus aureus* elicitors on alkaloid production in *Adhatoda* species is shown in Table - XXVII.

TABLE - XXVII

ALKALOID CONTENT IN THE CALLUS CULTURES OF A. vasica AND A. beddomei TREATED WITH Staphylococcus aureus ELICITORS

Species	*Incubation time (days)*	*Control*	*Media filtrate concentration*			*Incubation time (days)*	*Control*	*Cell extract Concentration*		
			5%	*10%*	*20%*			*1%*	*3%*	*5%*
A.vasica	7	0.17	0.17	0.18	0.18	5	0.17	0.17	0.19	0.21
	12	0.17	0.17	0.19	0.20	8	0.17	0.17	0.19	0.22
	17	0.18	0.18	0.20	0.21	11	0.18	0.18	0.20	0.24
A.beddomei	7	0.17	0.17	0.18	0.19	5	0.17	0.17	0.19	0.20
	12	0.17	0.18	0.19	0.20	8	0.17	0.17	0.20	0.22
	17	0.18	0.18	0.19	0.22	11	0.18	0.18	0.21	0.25

Data represent an average of 5 replicates in two repetitive experiments.
Medium : MS + IAA (2.0 mg/l) + Kn (1.5 mg/l) + 3 % Sucrose with or without (control) elicitor
Inoculum : 500 ± 50 mg fresh tissue in 15 ml medium
Incubation : at 25 ± 2°C under continuous light (2000 lux)

MF of *S. aureus* at 10% and 20% levels only increased alkaloid production. A treatment with 20% MF resulted in increase of alkaloid content by 17% and 22% in *A. vasica* and *A. beddomei* respectively over the control. CE of *S. aureus* enhanced alkaloid at 3% and 5% concentration during the selected points of incubation time. The increment in the alkaloid content was 33% more in the case of *A. vasica* and 39% more in *A. beddomei* than in control, at 5% CE concentration on the 11th day of incubation.

Of all biotic elicitors screened, fungal elicitor (both MF and ME) showed maximum elicitor activity in both the species of *Adhatoda*. Both the yeast elicitors *Candida albicans* and *Saccharomyces cerevsiae* had the least elicitor activity. Although bacterial elicitors were found to be better than the yeast elicitors, these too were not as effective as the fungal elicitors.

In conclusion it can be said that, even though *A. flavus*, *A. niger* and *T. viridae*

were found to elicit a good amount of alkaloid, the elicitor preparation from *T. viridae* scored better over others. Three percent ME of *Trichoderma viridae* could be used in the callus cultures of *Adhatoda* species to elicit maximum alkaloid production, with a short incubation period of 11 days.

4.4.7.2. EFFECT OF ABIOTIC ELICITORS

Metal ions, which are present in the normal MS medium were used as abiotic elicitors at a concentration which was 5, 15 and 30 times that in the normal MS medium. Elicitation was monitored over a period of 12 days.

i) CALCIUM

The effect of calcium on alkaloid production in callus cultures of *Adhatoda* species is depicted in Table - XXVIII.

TABLE – XXVIII

ALKALOID CONTENT IN THE CALLUS CULTURES OF A. vasica AND A. beddomei TREATED WITH HIGH CALCIUM CONCENTRATION

Species	*Incubation time (days)*	*Alkaloid content (g / 100 g dry tissue)*			
		Control	*Concentration of calcium*		
			5 X	*15 X*	*30 X*
A. vasica	0	0.16	0.16	0.16	0.16
	3	0.16	0.18	0.19	0.19
	6	0.16	0.22	0.24	0.25
	9	0.16	0.23	0.24	0.29
	12	0.16	0.23	0.25	0.32
A. beddomei	0	0.17	0.16	0.16	0.16
	3	0.17	0.20	0.20	0.21
	6	0.17	0.22	0.24	0.28
	9	0.17	0.23	0.24	0.31
	12	0.17	0.24	0.26	0.36

Data represent an average of 5 replicates in two repetitive experiments.
Medium : MS + IAA (2.0mg/l) + Kn (1.5 mg/l) + 3% sucrose.
Inoculum : 400 ± 50mg fresh tissue in 15 ml medium
Incubation : At 25 ± 2°C under continuous light (2000 lux)

Calcium, at all concentrations tested, enhanced alkaloid production. But in the control, alkaloid content was not increased with increase in incubation time. Calcium at a concentration 30 times that in normal MS medium elicited the maximum alkaloid content on the 12th day of culture. The increase was 100% and 110% in *A. vasica* and *A. beddomei* respectively over that in control.

ii) MANGANESE

The effect of manganese on alkaloid production in callus culture of *Adhatoda* species is shown in Table - XXIX.

TABLE – XXIX

ALKALOID CONTENT IN THE CALLUS CULTURES OF A. vasica AND A. beddomei TREATED WITH HIGH MANGANESE CONCENTRATION

Species	*Incubation time (days)*	*Alkaloid content (g / 100 g dry tissue)*			
		Control	*Concentration of Manganese*		
			5 X	*15 X*	*30 X*
A. vasica	0	0.17	0.17	0.19	0.20
	3	0.17	0.17	0.19	0.20
	6	0.17	0.17	0.20	0.22
	9	0.17	0.18	0.29	0.28
	12	0.17	0.19	0.22	0.24
A. beddomei	0	0.18	0.17	0.18	0.20
	3	0.18	0.17	0.18	0.21
	6	0.18	0.18	0.19	0.22
	9	0.18	0.18	0.20	0.32
	12	0.18	0.19	0.21	0.25

Data represent an average of 5 replicates in two repetitive experiments.
Medium : MS + IAA (2.0mg/l) + Kn (1.5 mg/l) + 3% sucrose.
Inoculum : 400 ± 50mg fresh tissue in 15 ml medium
Incubation : At 25 ± 2°C under continuous light (2000 lux)

Manganese at a concentration 15 times that in normal MS medium increas the alkaloid content on the 9th day of culture which was much higher than t maximum production obtained in control during the culture period in the case *A. vasica* and was 70% increase over control.

Calcium, at all concentrations tested, enhanced alkaloid production. But in the control, alkaloid content was not increased with increase in incubation time. Calcium at a concentration 30 times that in normal MS medium elicited the maximum alkaloid content on the 12th day of culture. The increase was 100% and 110% in *A. vasica* and *A. beddomei* respectively over that in control.

ii) MANGANESE

The effect of manganese on alkaloid production in callus culture of *Adhatoda* species is shown in Table - XXIX.

TABLE – XXIX

ALKALOID CONTENT IN THE CALLUS CULTURES OF A. vasica AND A. beddomei TREATED WITH HIGH MANGANESE CONCENTRATION

Species	*Incubation time (days)*	*Alkaloid content (g / 100 g dry tissue)*			
		Control	*Concentration of Manganese*		
			5 X	*15 X*	*30 X*
A. vasica	0	0.17	0.17	0.19	0.20
	3	0.17	0.17	0.19	0.20
	6	0.17	0.17	0.20	0.22
	9	0.17	0.18	0.29	0.28
	12	0.17	0.19	0.22	0.24
A. beddomei	0	0.18	0.17	0.18	0.20
	3	0.18	0.17	0.18	0.21
	6	0.18	0.18	0.19	0.22
	9	0.18	0.18	0.20	0.32
	12	0.18	0.19	0.21	0.25

Data represent an average of 5 replicates in two repetitive experiments.
Medium : MS + IAA (2.0mg/l) + Kn (1.5 mg/l) + 3% sucrose.
Inoculum : 400 ± 50mg fresh tissue in 15 ml medium
Incubation : At 25 ± 2°C under continuous light (2000 lux)

Manganese at a concentration 15 times that in normal MS medium increased the alkaloid content on the 9th day of culture which was much higher than the maximum production obtained in control during the culture period in the case of *A. vasica* and was 70% increase over control.

In *A. beddomei* the maximum alkaloid content (0.32 g/ 100g dry tissue) was obtained on the 9th day of culture at a concentration 30 times that in normal MS medium.

iii) ZINC

The effect of zinc on alkaloid production in callus cultures of *Adhatoda* species is presented in Table - XXX.

TABLE – XXX

ALKALOID CONTENT IN THE CALLUS CULTURES OF A. vasica AND A. beddomei TREATED WITH HIGH ZINC CONCENTRATION

Species	*Incubation time (days)*	*Alkaloid content (g / 100 g dry tissue)*			
		Control	*Concentration of Zinc*		
			5 X	*15 X*	*30 X*
A. vasica	0	0.16	0.16	0.17	0.18
	3	0.16	0.16	0.17	0.19
	6	0.16	0.16	0.19	0.20
	9	0.16	0.16	0.22	0.23
	12	0.16	0.15	0.23	0.26
A. beddomei	0	0.16	0.16	0.17	0.19
	3	0.16	0.17	0.18	0.20
	6	0.16	0.17	0.20	0.20
	9	0.16	0.16	0.19	0.21
	12	0.16	0.17	0.21	0.22

Data represent an average of 5 replicates in two repetitive experiments.
Medium : MS + IAA (2.0mg/l) + Kn (1.5 mg/l) + 3% sucrose.
Inoculum : 400 ± 50mg fresh tissue in 15 ml medium
Incubation : At 25 ± 2°C under continuous light (2000 lux)

Zinc at a concentration 30 times than that in the control caused the maximum production of the alkaloid on 12th day of incubation. It was 0.26 g and 0.22g/100g dry tissue in *A. vasica* and *A. beddomei* respectively.

iv) COPPER

The effect of copper on alkaloid production in callus cultures of *Adhatoda* species is depicted in Table - XXXI.

TABLE – XXXI

ALKALOID CONTENT IN THE CALLUS CULTURES OF A. vasica AND A. beddomei TREATED WITH HIGH COPPER CONCENTRATION

Species	*Incubation time(days)*	*Alkaloid content (g / 100 g dry tissue)*			
		Control	*Concentration of Copper*		
			5 X	*15 X*	*30 X*
A. vasica	0	0.17	0.17	0.17	0.17
	3	0.17	0.17	0.17	0.16
	6	0.17	0.17	0.16	0.16
	9	0.17	0.17	0.17	0.17
	12	0.17	0.16	0.17	0.17
A. beddomei	0	0.17	0.17	0.17	0.17
	3	0.17	0.17	0.16	0.16
	6	0.17	0.18	0.18	0.16
	9	0.17	0.18	0.18	0.17
	12	0.17	0.17	0.18	0.17

Data represent an average of 5 replicates in two repetitive experiments.
Medium : MS + IAA (2.0mg/l) + Kn (1.5 mg/l) + 3% sucrose.
Inoculum : 400 ± 50mg fresh tissue in 15 ml medium
Incubation : At 25 ± 2°C under continuous light (2000 lux)

Copper did not enhance the alkaloid production at any of the concentrations tried. This indicated that copper is not an elicitor of alkaloid production in *Adhatoda* species.

v) IRON

The effect of iron on alkaloid production in callus cultures of *Adhatoda* specie is depicted in Table -XXXII.

TABLE – XXXII

ALKALOID CONTENT IN THE CALLUS CULTURES OF A. vasica AND A. beddomei TREATED WITH HIGH IRON CONCENTRATION

Species	*Incubation time days*	*Alkaloid content (g / 100 g dry tissue)*			
		Control	*Concentration of Iron*		
			5 X	*15 X*	*30 X*
A. vasica	0	0.17	0.17	0.18	0.18
	3	0.17	0.18	0.19	0.24
	6	0.17	0.18	0.26	0.22
	9	0.17	0.19	0.22	0.23
	12	0.17	0.18	0.24	0.23
A. beddomei	0	0.17	0.17	0.19	0.18
	3	0.17	0.18	0.19	0.19
	6	0.17	0.19	0.24	0.26
	9	0.17	0.20	0.25	0.26
	12	0.17	0.21	0.27	0.28

Data represent an average of 5 replicates in two repetitive experiments.
Medium : MS + IAA (2.0mg/l) + Kn (1.5 mg/l) + 3% sucrose.
Inoculum : 400 ± 50mg fresh tissue in 15 ml medium
Incubation : At 25 ± 2°C under continuous light (2000 lux)

Iron at a concentration of 15 times that in normal MS medium enhanced the maximum production of alkaloid in *A. vasica* (0.20g/100g dry tissue) on the 6th day and then it declined. In *A. beddomei*, the maximum yield was obtained on the 12th day of culture with 30X concentration of iron.

vi) COBALT

The effect of cobalt on alkaloid production in callus cultures of *Adhatoda* species is depicted in Table - XXXIII.

TABLE – XXXIII

ALKALOID CONTENT IN THE CALLUS CULTURES OF A. vasica AND A. beddomei TREATED WITH HIGH COBALT CONCENTRATION

Species	*Incubation time (days)*	*Alkaloid content (g / 100 g dry tissue)*			
		Control	*Concentration of Cobalt*		
			5 X	*15 X*	*30 X*
A. vasica	0	0.16	0.16	0.18	0.17
	3	0.16	0.16	0.18	0.18
	6	0.16	0.17	0.20	0.22
	9	0.16	0.17	0.21	0.24
	12	0.16	0.17	0.23	0.29
A. beddomei	0	0.16	0.16	0.17	0.18
	3	0.16	0.17	0.18	0.18
	6	0.16	0.18	0.17	0.22
	9	0.16	0.20	0.19	0.23
	12	0.16	0.20	0.21	0.28

Data represent an average of 5 replicates in two repetitive experiments.
Medium : MS + IAA (2.0mg/l) + Kn (1.5 mg/l) + 3% sucrose.
Inoculum : 400 ± 50mg fresh tissue in 15 ml medium
Incubation : At 25 ± 2°C under continuous light (2000 lux)

Cobalt at a concentration 30 times that in the control, elicited maximum alkaloid production on 12th day of culture in both the species. It was 81% more in *A. vasica* and 75% more in *A. beddomei* than in the control.

Among the abiotic elicitors tested, calcium was found to be most effective. The maximum increase in alkaloid content was observed on the 12th day of culture with the level of calcium 30 times that of the control (i.e., 0.32 and 0.36 g/100g dry tissue in *A. vasica* and *A. beddomei* respectively). The effect of calcium as an abiotic elicitor and a second messenger has also been reported by Buitelaar *et al.* (1992). These abiotic elicitors are also thought to regulate the enzymes involved in the secondary metabolite synthesis which are located on the plasma membrane.

A comparison of various strategies employed in alkaloid production led to the following inference. Even though decreased levels of nitrogen and phosphate in the media enhanced alkaloid content, the incubation time was longer (35 days). Sucrose

alone or in combination with mannitol at 3% concentration each increased alkaloid production significantly on the 35th day, which was 39% and 55% higher than the control in *A. vasica* and *A. beddomei* respectively. In spite of this steep increase, this strategy may not be appropriate in large scale process due to the high cost of these components and longer incubation time.

The results obtained using biotic and abiotic elicitors seem to solve some of these problems. The use of the biotic elicitor, *Trichoderma viridae* (ME 3%) or the abiotic elicitor calcium (30X) will produce a good yield of alkaloids (133 % and 100% increase over the control) in *Adhatoda* callus culture in a short incubation period. The present work carried out using biotic and abiotic elicitors is the first of its kind with respect to alkaloid production system in *Adhatoda*.

4.5. GENETIC DIVERSITY STUDIES

Protein-based polymorphism or isoenzyme analysis is still a valuable tool for determining the genetic relationship between and among plant population. But the development of DNA – based techniques is providing plentiful array of additional genomic markers like Randomly Amplified Polymorphic DNA (RAPD) and Restriction Fragment Length Polymorphism (RFLP). The practical benefits of RADP are more than RFLP technique. The advantage of RAPD technique over RFLP include faster data production, a protocol that requires less DNA, and no radioactivity.

In this study, isoenzyme analysis and RAPD analysis were successfully employed to detect the genetic variation among *A. vasica* and between *A. vasica* and *A. beddomei*.

4.5.1. ISOENZYME ANALYSIS

Isoenzymes are direct gene products and therefore, less susceptible to phenocopy responses and modification by environmental variables. Hence, the electrophoretic pattern of isozymes represent a valid tool in detecting genetic and epigenetic variations. Isozyme polymorphism could be used as a desirable marker to

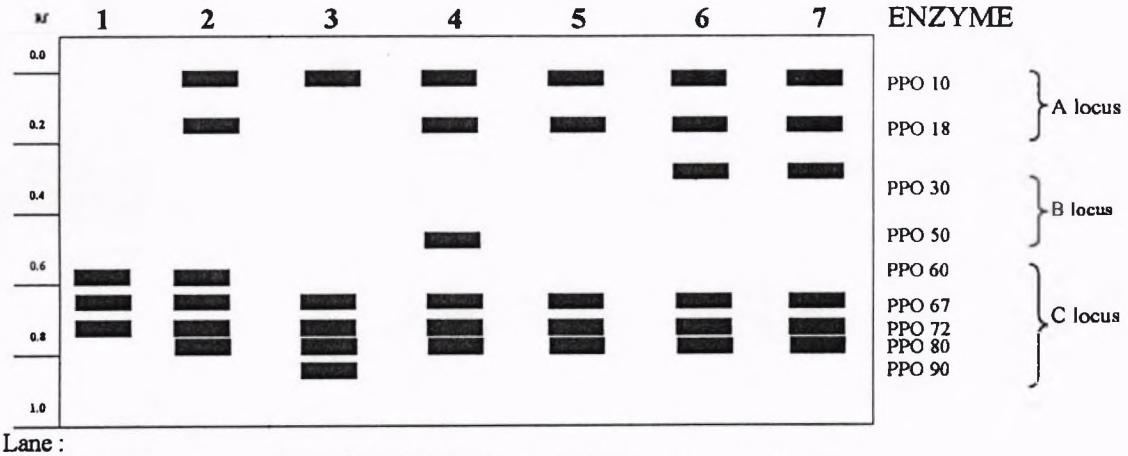
analyse the adaptability of a plant to biotic and abiotic stresses. Isozymes were the first kind of molecular markers studied (Viruel *et al.*, 1995).

Five isozyme systems namely, polyphenol oxidase, peroxidase, shikimic acid dehydrogenase, malate dehydrogenase and esterase were studied on the 6 genotypes of *A. vasica* and *A. beddomei*, using leaf samples.

POLYPHENOL OXIDASE (PPO)

All the samples of *A. vasica* and *A. beddomei* were studied for their leaf polyphenol oxidase isozyme pattern. On staining the gels, the PPO were observed as light golden brown bands on a colourless background (Fig. 3).

FIG 3 : ZYMOGRAM OF POLYPHENOL OXIDASE (PPO) OF *A.beddomei* AND *A. vasica*



Lane :

1. *A. beddomei*
2. Suruli falls
3. Kanjikode, Kerala
4. Thaimudi, Mudis
5. Kilkothagiri
6. Coimbatore civil aerodrome region
7. IFGTB, Coimbatore

2-7 indicate the places of *A. vasica* sample collection.

All the seven types chosen had totally 9 isozymes with Rf values 0.10, 0.18, 0.30, 0.50, 0.60, 0.67, 0.72, 0.80 and 0.90 and were named as PPO 10, PPO 18, PPO 30, PPO 50, PPO 60, PPO 67, PPO 72, PPO 80 and PPO 90 respectively which were resolved in to 3 loci namely, PPO A (PPO 10 AND PPO18), PPO B (PPO 30

and PPO 50) and PPO C (PPO 60, PPO 67, PPO 72, PPO 80 and PPO 99). PPO A locus had a maximum of two isozymes, B locus had two and C locus had five.

In the PPO A locus, *A. beddomei* (lane 1) was entirely different from all genotypes of *A. vasica* by the absence of PPO 10. Moreover in the PPO A locus, *A. beddomei* and *A. vasica* collected from Kanjikode (lane 3) were similar to each other and different from the other genotypes of *A. vasica* by the absence of PPO 18.

A. beddomei did not have any isozyme in PPO B locus. In locus PPO B, *A. vasica* collected from Coimbatore civil aerodrome and IFGTB, Coimbatore (lane 6 and 7) were similar by having PPO 30. PPO 30 was absent in all other genotypes studied. In PPO B locus PPO 50 was unique to *A. vasica* collected from Thaimudi, Mudis (lane 4).

The isozyme PPO 60 of the PPO C locus was found in *A. beddomei* and *A.vasica* collected from Surli falls (lane 2). *A. beddomei* and all types of *A. vasica* showed a resemblance in having two isozymes (i.e.) PPO 67 and PPO 72. The isozyme PPO 80 of PPO C locus was present in all genotypes of *A. vasica* but not found in *A. beddomei*. The fastest migrating isozyme PPO 90 was found only in *A. vasica*, collected from Kanjikode, Kerala (lane 3). The zymogram pattern indicated that *A. beddomei* behaved differently from *A. vasica* for the polyphenol oxidase system.

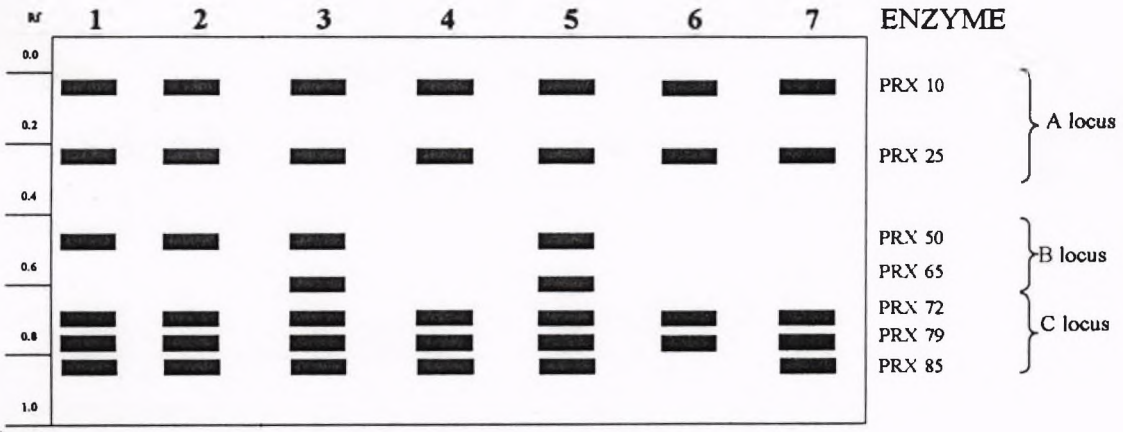
The isozyme could be visualized as low intense bands on the gel. This could be because, when the enzyme catalyses the oxidation of catechol the rate is at first linear and during its action, the enzyme becomes inactivated by o-quinone formed and hence the color of the bands did not intensify. From the zymogram, it was noticed that *A. beddomei* behaved differently from *A. vasica* as both are two different species and their genetic organization are also expected to be different. *A. vasica* collected from Coimbatore civil aerodrome and IFGTB, Coimbatore were found to be identical in their isozyme patterns as they belong to same species and subjected to more or less similar types of environmental stress. *A. vasica* collected from Thaimudi, Mudis and

Kilkothagiri showed minimum dissimilarity in zymogram indicating less genetic dissimilarity between them.

PEROXIDASE (PRX)

Leaf peroxidase isozyme system of *A. beddomei* and *A. vasica* samples was studied. PRX was visualized as blue bands (which later on turned brown) on a colorless background (Fig. 4).

FIG 4 : ZYMOGRAM OF PEROXIDASE OF *A. beddomei* AND *A. vasica*



Lane :
1. *A. beddomei* 2-7 indicate the places of *A. vasica* sample collection.
2. Suruli falls 3. Kanjikode, Kerala 4. Thaimudi, Mudis
5. Kilkothagiri 6. Coimbatore civil aerodrome region 7. IFGTB, Coimbatore

Electrophoretic pattern of PRX had 7 isozymes with Rf values 0.10, 0.25, 0.50, 0.65, 0.72, 0.79 and 0.85 and were named as PRX 10, PRX 25, PRX 50, PRX 65, PRX 72, PRX 79 and PRX 85 and were resolved into 3 loci namely PRX A (PRX 10 and 25), PRX B (PRX 50 and 65) and PRX C (PRX 72, 79 and 85).

In the first locus (PRX A) all the samples of *A. vasica* and *A. beddomei* were identical due to the presence of both PRX 10 and 25 isozymes. In PRX B locus PRX 50 was present in *A. beddomei* and *A. vasica* collected from Suruli falls, Kanjikode and Kilkothagiri and was absent in *A. vasica* collected from Kilkothagiri, Coimbatore civil aerodrome and IFGTB, Coimbatore. The isozyme PRX 65 of PRX B locus was

found only in the two samples of *A. vasica* (Kanjikode and Kilkothagiri). PRX C locus had 3 isozymes and PRX 72 and 79 were present in all the types selected for the study. The fastest moving isozyme PRX 85 was present in all samples except *A. vasica* collected from Coimbatore civil aerodrome.

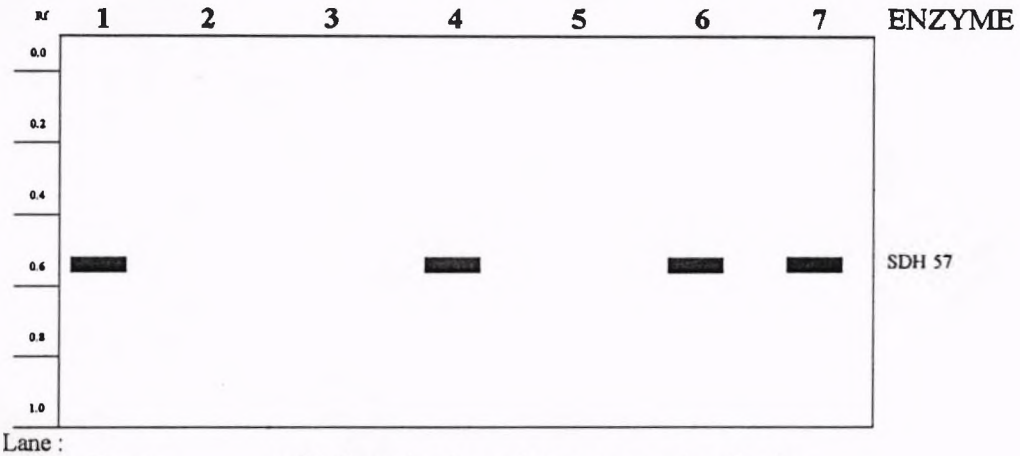
The zymogram pattern indicated that *A. beddomei* and *A. vasica* collected from Suruli falls, Kanjikode and Kilkothagiri (lanes 1,2, 3 and 5) were identical except for an extra isozyme i.e., (PRX 65) found in the latter two samples. *A. vasica* collected from Thaimudi, Mudis and IFGTB, Coimbatore (lanes 4 and 7) could be grouped together as they have isozyme profile PRX 10, 25, 72, 79 and 85. *A. vasica* collected from Coimbatore civil aerodrome region (lane 6) behaved differently from the rest.

The higher level of similarity between *A. beddomei* and *A. vasica* (Suruli falls), between *A. vasica* collected form Kanjikode and Kilkothagiri and between Thaimudi, Mudis and IFGTB implied a similarity in their genetic organization whereas *A. vasica* from Coimbatore civil aerodrome region showed a high degree of dissimilarity within the species itself. This denotes the different genetic organization of this sample probably due to adverse environmental conditions.

SHIKIMIC ACID DEHYDROGENASE

Shikimic acid dehydrogenase catalyses the conversion of 3-Dehydroshikimate to shikimate. Electrophoretic analysis of shikimic acid in *A. beddomei* and *A. vasica* showed (Fig. 5) a single form only with Rf value 0.57 (SDH 57).

FIG 5 : ZYMOGRAM OF SHIKIMIC ACID DEHYDROGENASE (SDH) OF A. beddomei AND A. vasica



Lane :

1. *A. beddomei* — 2-7 indicate the places of *A. vasica* sample collection.
2. Suruli falls — 3. Kanjikode, Kerala — 4. Thaimudi, Mudis
5. Kilkothagiri — 6. Coimbatore civil aerodrome region — 7. IFGTB, Coimbatore

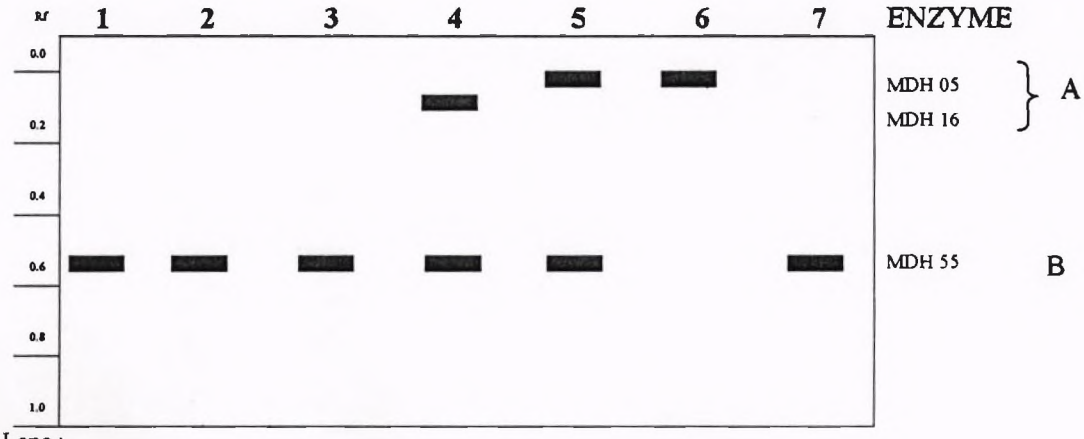
This band was present in *A. beddomei* and *A. vasica* collected from Thaimudi (Mudis), Coimbatore civil aerodrome and IFGTB, Coimbatore (lanes 1, 4, 6 and 7). The band obtained in lanes 2, 3 and 5 was faint and disappeared immediately.

SDH was a relatively simpler isozyme system with only one isozyme (SDH 57). *A. beddomei* and *A. vasica* collected from Thaimudi, Coimbatore civil aerodrome region and IFGTB, Coimbatore, showed similarity and were distinguished from *A. vasiva* collected from Suruli falls, Kanjikode and Kilkothagiri by having SDH 57. This isozyme might not be useful in comparing inter specific and intra specific types.

MALATE DEHYDROGENASE (MDH)

Malate dehydrogenase isozyme system was studied in 6 genotypes of *A. vasica* and *A. beddomei* (Fig. 6).

FIG 6 : ZYMOGRAM OF MALATE DEHYDROGENASE (MDH) OF A. beddomei AND A. vasica



Lane :

1. *A. beddomei* — 2-7 indicate the places of *A. vasica* sample collection.
2. Suruli falls — 3. Kanjikode, Kerala — 4. Thaimudi, Mudis
5. Kilkothagiri — 6. Coimbatore civil aerodrome region — 7. IFGTB, Coimbatore

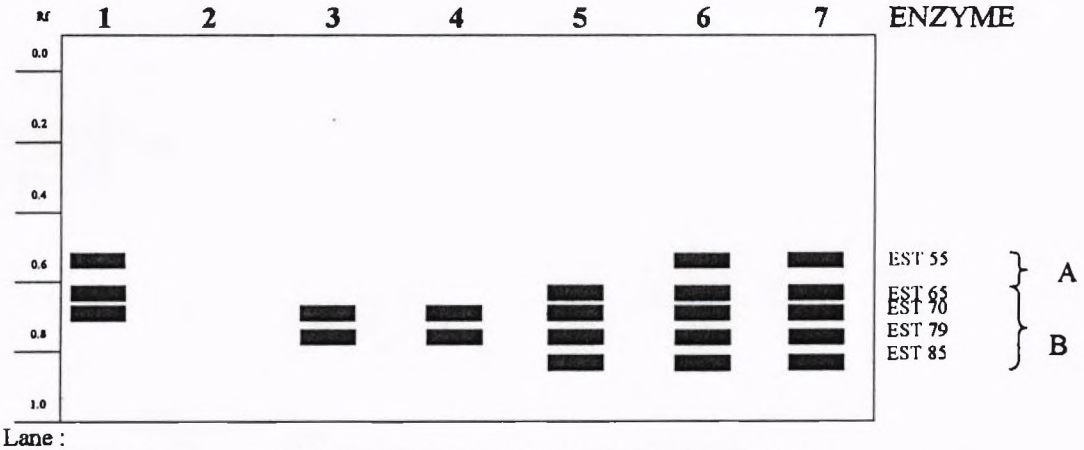
MDH isozyme was relatively simple when compared to polyphenol oxidase, peroxidase and esterase. The isozyme segregated into two loci viz., MDH A and MDH B. MDH A locus contained two isozyme profiles MDH 05 and MDH 16 with Rf value 0.05 and 0.16 respectively. MDH 0.05 was present in *A. vasica* collected from Kilkothagiri and Coimbatore civil aerodrome region (lanes 5 and 6). MDH 16 was unique to *A. vasica* collected from Thaimudi, Mudis (lane 4). MDH B showed only one isozyme MDH 55 and was present in all the samples except in *A. vasica* collected from Coimbatore civil aerodrome region (lane 6).

MDH catalyses the oxidation of malate to oxaloacetate with the simultaneous reduction of NAD^+ to NADH. *A. vasica* collected from Coimbatore civil aerodrome region had an entirely different MDH isozyme pattern from the rest. MDH 55 was not found in this sample. This variability might be due to the adaptability of the species to air pollution and environmental stresses.

ESTERASES

Esterases isozyme system was studied in the samples of *A. vasica* and *A. beddomei*. Esterases were visualized as dark brown bands on a colorless background (Fig. 7).

FIG 7 : ZYMOGRAM OF ESTERASE (EST) OF A. beddomei AND A. vasica



Lane :

1. *A. beddomei* — 2-7 indicate the places of *A. vasica* sample collection.
2. Suruli falls — 3. Kanjikode, Kerala — 4. Thaimudi, Mudis
5. Kilkothagiri — 6. Coimbatore civil aerodrome region — 7. IFGTB, Coimbatore

There were 5 isozymes in two loci EST A and EST B. EST A locus contained 2 isozymes EST 55 and 65 with Rf value 0.55 and 0.65. EST 55 was present in *A. beddomei* and *A. vasica* collected from Coimbatore civil aerodrome region and IFGTB, Coimbatore. EST 65 was noticed in *A. beddomei* and *A. vasica* collected from Kilkothagiri, Coimbatore civil aerodrome and IFGTB, Coimbatore. EST B locus contained 3 isozyme profiles EST 70, 79 and 85. EST 70 isozyme was present in all genotypes taken for the study. EST 79 was absent in *A. beddomei* and *A. vasica* from Suruli falls but all other samples of *A. vasica* exhibited this isozyme profile. The fastest migrating EST 85 was present in *A. vasica* collected from Kilkothagiri, Coimbatore civil aerodrome and IFGTB, Coimbatore.

Esterases are hydrolyzing enzymes which catalyse the addition or removal of water in biological reactions. Esterases are wide spread in nature and occur frequently

in plants. Like that of PPO, estrases in *A. beddomei* showed highest variability among other types studied and that led to the inference that esterases may be used in species identification of *A. beddomei* along with polyphenol oxidase. The observations between *A. vasica* from Coimbatore civil aerodrome region and IFGTB, Coimbatore (lanes 6 and 7), and that between *A. vasica* from Kanjikode and Thaimudi (lanes 3 and 4), showed a similar isozyme pattern. And this indicates the possibility of similar genotypic properties among them. *A. vasica* collected from Suruli falls exhibited a faint band of esterase 79 and it was transitory and disappeared immediately.

COMBINED ANALYSIS OF A. vasica AND A. beddomei FOR 5 ISOZYME SYSTEMS

The complexity in 6 genotypes of *A. vasica* and *A. beddomei* for isozyme profile was assessed and compiled from the number of isozymes recorded for individual genotypes, is given in Table XXXIV.

TABLE – XXXIV

COMBINED ANALYSIS OF A. vasica AND A. beddomei FOR ISOZYME SYSTEMS

Species		*No. of isozymes in*					*Total*
		PPO	*PRX*	*SDH*	*MDH*	*EST*	
A. beddomei	1	3	6	1	1	3	14
A. vasica	2	6	6	0	1	2	15
	3	5	7	0	1	2	15
	4	6	5	1	2	2	16
	5	5	7	0	2	4	18
	6	6	4	1	1	5	17
	7	6	5	1	1	5	18
TOTAL		37	40	4	9	21	113

Lane :
1. *A. beddomei* 2-7 indicate the places of *A. vasica* sample collection.
2. Suruli falls 3. Kanjikode, Kerala 4. Thaimudi, Mudis
5. Kilkothagiri 6. Coimbatore civil aerodrome region 7. IFGTB, Coimbatore

Altogether 113 isozyme bands were observed in five enzyme systems in 7 genotypes. More isozymes were present for peroxidase system (40) and the lowest

was recorded for Shikimic acid dehydrogenase (4). The two genotypes of *A. vasica* collected form Kilkothagiri and IFGTB, Coimbatore recorded more number of isozymes (18). These genotypes had more number of peroxidase, polyphenol oxidase and esterases isozyme profile. The lowest number of isozymes was in *A. beddomei* (14).

Similarity indices between all possible pairs of 7 genotypes for five isozyme systems are presented in Table XXXV.

TABLE XXXV

SIMILARITY INDICES OF SELECTED ADHATODA GENOTYPES

	1	2	3	4	5	6	7
1	1.000						
2	0.720	1.000					
3	0.640	0.760	1.000				
4	0.600	0.720	0.720	1.000			
5	0.600	0.720	0.800	0.680	1.000		
6	0.560	0.520	0.520	0.640	0.720	1.000	
7	0.680	0.640	0.640	0.760	0.760	0.880	1.000

1. *A. beddomei* 2-7 indicate the places of *A. vasica* sample collection.
2. Suruli falls 3. Kanjikode, Kerala 4. Thaimudi, Mudis
5. Kilkothagiri 6. Coimbatore civil aerodrome region 7. IFGTB, Coimbatore

The values ranged from 0.520 between *A. vasica* collected from Suruli falls and Coimbatore civil aerodrome and between Kanjikode and Coimbatore civil aerodrome to 0.880 between Coimbatore civil aerodrome and IFGTB, Coimbatore.

A cluster tree depicting the genetic similarity of 7 genotypes based on five isozyme systems was constructed and is illustrated in Fig 7.

It could be seen that *A. vasica* collected from Coimbatore civil aerodrome region and IFGTB, Coimbatore were the most similar types. *A. vasica*, collected from Kanjikode showed similarity with *A. vasica* from Kilkothagiri. *A. vasica* collected from Suruli falls showed similarity with *A. vasica* collected from Kanjikode.

A. vasica collected from IFGTB, Coimbatore was similar to *A. vasica*, from Thaimudi, Mudis. *A. vasica* collected from IFGTB, Coimbatore showed the similarity with Kilkothagiri. *A. beddomei* was diverse from other genotypes of *A. vasica*.

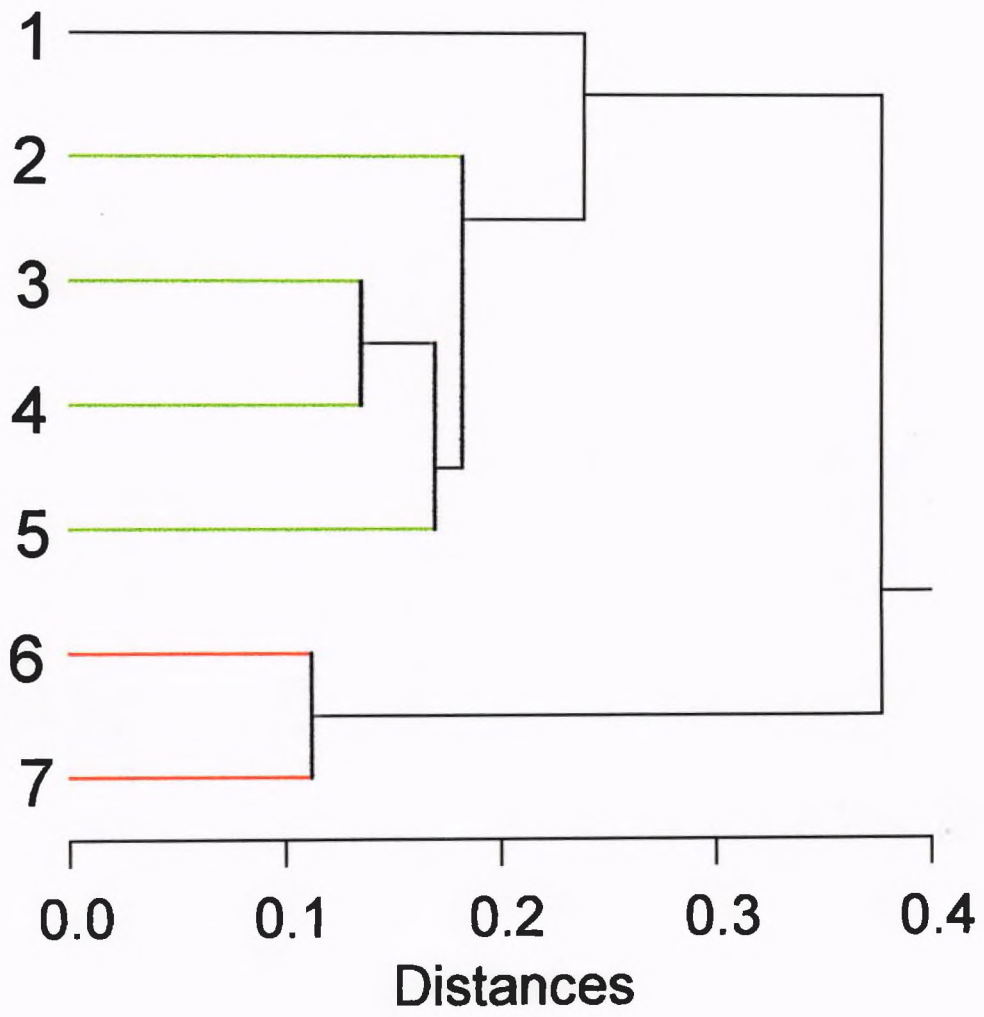
The genotypes fell into two groups while *A. beddomei* fell out separately. *A. vasica* collected from Coimbatore civil aerodrome region and IFGTB formed the first cluster. The cluster II was constituted by *A.vasica* collected from Suruli falls, Kanjikode, Thaimudi and Kilkothagiri.

The above studies involving isozymes revealed a high amount of genetic diversity in the genus. Isozyme approach is identified as a valuable means for distinguishing intra and interspecies of Adhatoda. The determination of isozyme frequencies proposed here is the absence or presence of an allelic form for each plant being visualised by electrophoresis. This study on 5 isozymes was probably optimal to bring out species difference as revealed from the cluster tree constructed from the distance matrix, between each pair of 6 genotypes of *A. vasica* and *A. beddomei*, reflecting the phylogenetic classification and overall similarity among the species. *A. beddomei* showed the highest distance among other genotypes of *A. vasica*. *A. vasica* collected from Coimbatore civil aerodrome region and IFGTB, Coimbatore have been clubbed together in view of a low extent of dissimilarity. *A. vasica* collected from Suruli falls, Kanjikode, Thaimudi, Mudis and Kilkothagiri showed resemblance to each other and formed one group.

This study is in agreement with Gottlieb (1981) whose results of analysis of isozyme variability in populations provided useful information in the organization of the gene pool in a large number of plant species.

FIG. 8

PHENETIC TREE OF SELECTED ADHATODA GENOTYPES - ISOENZYMES



1. *A. beddomi*
   2-7 indicate the places of *A. vasica* sample collection.
2. Suruli falls
3. Kanjikode
4. Thaimudi, Mudis
5. Kilkothagiri
6. Coimbatore civil aerodrome region
7. IFGTB, Coimbatore

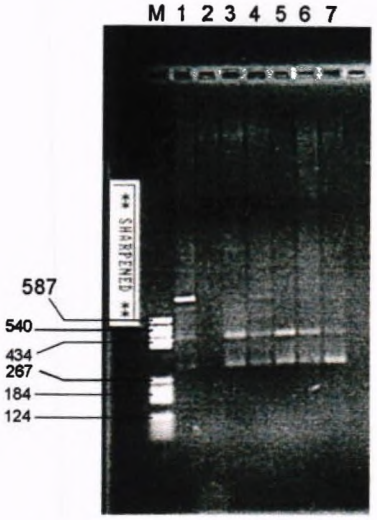
4.5.2. RANDOMLY AMPLIFIED POLYMORPHIC DNA (RAPD) ANALYSIS

The analysis of genetic diversity and relation between individuals or within a population is a central task for many disciplines of studying the diversity. Attempts were made to study the genetic diversity using RAPD analysis. The three major steps included in RAPD analysis are the isolation of genomic DNA, amplification of total DNA using arbitrary primers and scoring of amplified products followed by analysis of data. Since *A. vasica* and *A. beddomei* contain high amount of phenolics, and polysaccharides, CTAB method was followed to isolate the genomic DNA. CTAB method of DNA isolation has been followed in many tree species containing phenolic substances such as *Glyricidia spium* (Chalmers *et al.*, 1992), *Citrus deliciosa* (Machado *et al.*, 1996), *Acanthus ilicifolius* (Lakshimi *et al.*, 1997). The discussion on the results of RAPD analysis is given below.

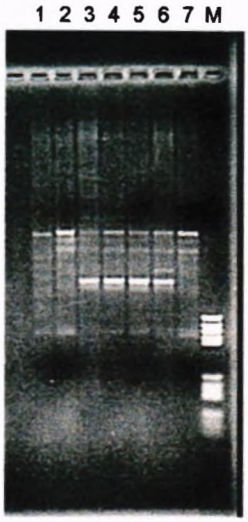
SCORING OF AMPLIFIED PRODUCTS

Out of 10 primers used, 4 primers gave polymorphic electromorphs between the selected genotypes. The amplified products varied in number and intensity among the selected genotypes. Plate 5 shows the RAPD pattern of the primers OPA 05, OPA 11, OPA 17 and OPF 15, respectively with polymorphic bands. Totally 78 bands were identified by the 4 arbitrary primers out of which 74 were polymorphic for the 7 genotypes considered. The maximum number of amplified products (36) were observed in a profile amplified by OPA 11. The electromorphs were scored for the presence or absence of bands in binaries for various primers. The binary data of electromorphs for various primers are presented in Table XXXVI.

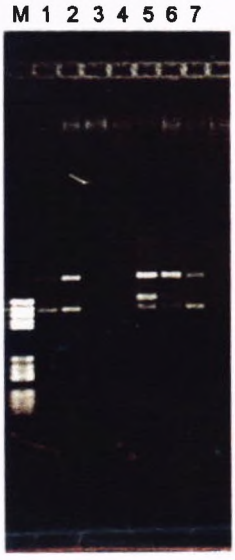
Plate - 5 : The RAPD profile of selected *Adhatoda* genotypes



Amplification with OPA 05



Amplification with OPA 11



Amplification with OPA 17

Amplification with OPF 15

Lane - 1. *A. beddomei*, 2-7. *A. vasica* collected from various places
2. Suruli falls
3. Kanjikode
4. Thaimudi
5. Kil kothagiri
6. Coimbatore civil aerodrome region
7. IFGTB, Coimbatore

TABLE - XXXVI

BINARY DATA FOR THE PRESENCE OR ABSENCE OF BANDS IN RAPD PROFILE OF A. beddomei AND A. vasica

Primer	*Band position*	*Lane number*							
		1	2	3	4	5	6	7	
OPA 05	1	1	0	0	1	0	0	0	2
	2	1	0	1	1	1	1	1	6
	3	1	0	1	1	1	1	1	6
TOTAL		3	0	2	3	2	2	2	**14**
OPA 11	1	1	1	1	1	1	1	1	7
	2	1	1	1	1	1	1	1	7
	3	1	1	1	1	1	1	1	7
	4	0	0	0	0	0	1	0	1
	5	0	0	1	1	1	1	0	4
	6	0	0	1	1	1	1	0	4
	7	1	1	0	1	1	1	1	6
TOTAL		4	4	5	6	6	7	4	**36**
OPA17	1	1	0	0	0	1	1	1	4
	2	0	0	0	0	1	1	0	2
	3	1	1	0	0	1	1	1	5
TOTAL		2	1	0	0	3	3	2	**11**
OPF 15	1	0	1	1	0	0	1	1	4
	2	0	0	0	1	0	0	0	1
	3	0	1	1	0	0	1	1	4
	4	0	0	1	0	1	1	0	**3**
	5	0	1	1	1	1	1	0	5
TOTAL		0	3	4	2	2	4	2	17

DATA ANALYSIS

The binary data were analyzed and the distance matrix (Nei and Lei, 1979) between the genotypes were derived which is depicted in Table XXXVII.

TABLE XXXVII

DISTANCE MATRIX OF SELECTED ADHATODA GENOTYPES

	1	2	3	4	5	6	7
1	0.000						
2	0.467	0.000					
3	0.750	0.529	0.000				
4	0.600	0.625	0.647	0.000			
5	0.529	0.444	0.368	0.556	0.000		
6	0.500	0.333	0.273	0.524	0.130	0.000	
7	0.333	0.250	0.412	0.750	0.444	0.333	0.000

1. *A. beddomei* 2-7 indicate the places of *A. vasica* sample collection.
2. Suruli falls 3. Kanjikode, Kerala 4. Thaimudi, Mudis
5. Kilkothagiri 6. Coimbatore civil aerodrome region 7. IFGTB, Coimbatore

The maximum genetic distance co-efficient value of 0.750 was obtained between *A. beddomei* and *A. vasica* collected from Kanjikode, Kerala and between *A. vasica* collected from Thaimudi, Mudis and IFGTB, Coimbatore, followed by 0.647 between *A. vasica* from Thaimudi and Kanjikode. The least genetic distance (0.130) was observed between *A. vasica* collected from Kilkothagiri and Coimbatore civil aerodrome region.

Based on the genetic distance matrix of 4 primers for 7 genotypes a cluster tree was constructed (Fig 9). The tree showed the grouping pattern of 7 selected genotypes.

In the present investigation, the clustering pattern of all the 7 genotypes through RAPD analysis revealed that the variation in the distance matrix ranged from 0.130 to 0.750. *A. beddomei* showed genetic variation with *A. vasica* collected from different places. This genetic variation ranged from 0.333 to 0.750. The maximum

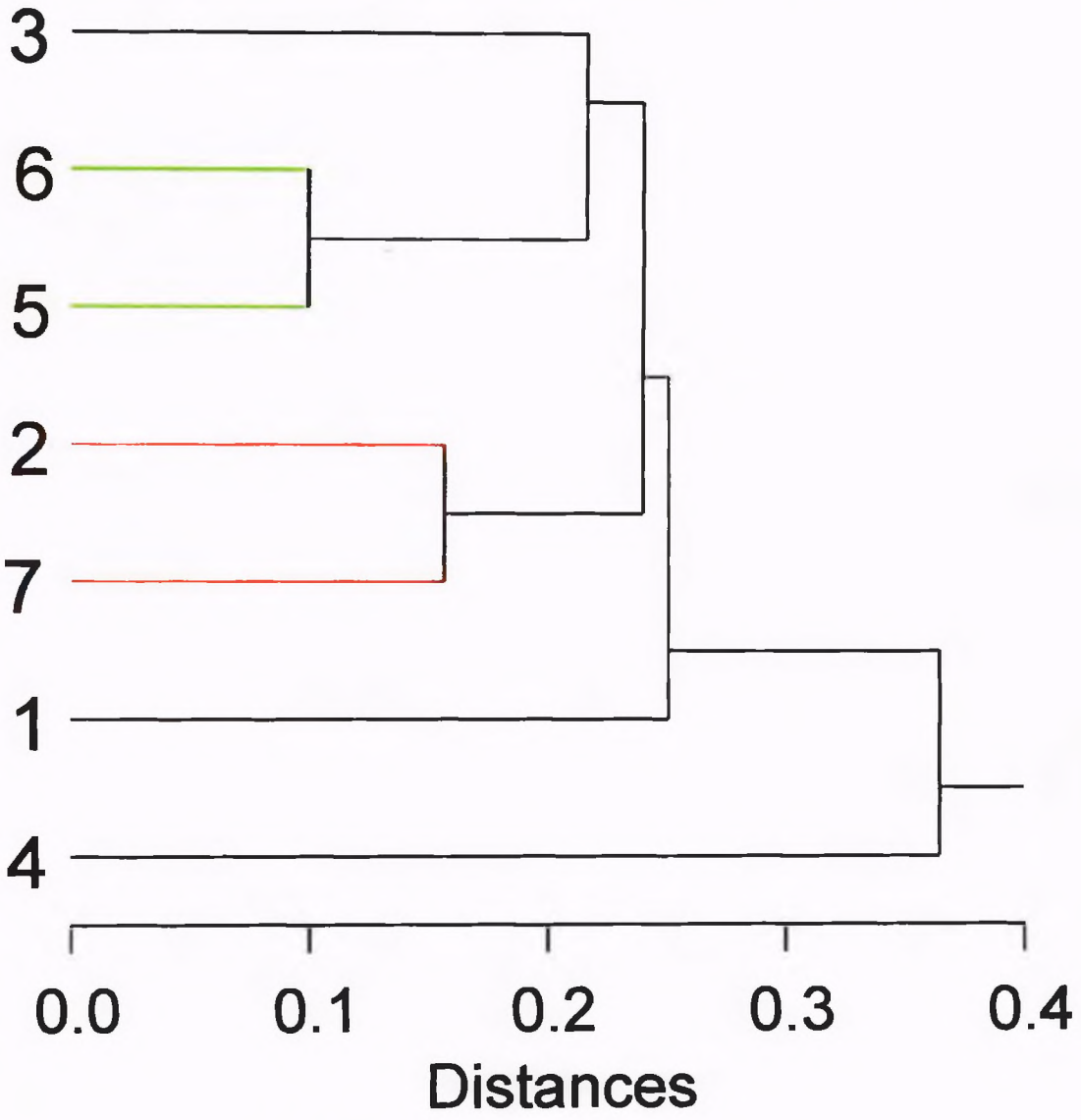
genetic variation was observed between *A. beddomei* and *A. vasica* from Kanjikode and between *A. vasica* collected from Thaimudi, Mudis and IFGTB, Coimbatore and least genetic dissimilarity was noticed between *A. vasica* from Kilkothagiri and Coimbatore civil aerodrome region. Genetic diversity studies conducted in different plants have led to a wide array of results. Chalmers *et al.* (1992) using RAPD markers, estimated high levels of genomic variation between *Glyricidia* population. Machado *et al.* (1996) reported that DNA fingerprints of phenotypically distinct *Citrus deliciosa* commercial cultivars resembled like clones in which some cultivars showed similarity indice values of 1.0. Nesbitt *et al.* (1995) showed that in *Eucalyptus globulus* distant geographic population showed marked variations in fruit and juvenile leaf characters.

The results depicted in the phenetic tree (Fig 9) indicated that the 7 genotypes were found to be grouped in to a distinct cluster revealing the identity of each population. Two closely related clusters were found comprising *A. vasica* collected from Suruli falls and IFGTB, Coimbatore and the other cluster comprising Kilkothagiri and Coimbatore civil aerodrome region. Variation was observed within *A. vasica* samples and between the species. This is in concordance with the result of Hamrick (1990), who revealed that in out breeding perennial plants, most variation is exhibited within populations.

To our knowledge this is the first attempt to study the genetic variability in *Adhatoda* species by RAPD analysis. In this study, though individual populations were found to be distinct clusters no Population Specific Markers (PSM) were identified. This may be due to the limited number of primers used in this study and it could be possible to obtain PSMs if the primer numbers are increased.

FIG. 9

PHENETIC TREE OF SELECTED ADHATODA GENOTYPES - RAPD



1. *A. beddomei*
   2-7 indicates the places of *A.vasica* sample collection
2. Surli falls
3. Kanjikode
4. Thaimudi, Mudis
5. Kilkothagiri
6. Coimbatore civil aerodrome region
7. IFGTB, Coimbatore

COMPARATIVE STUDY ON MOLECULAR MARKERS

For studies on genetic diversity, isoenzyme and RAPD techniques which are relatively simple in operation, appear more efficient since genetic similarity or diversity could be quantified in terms of the number of bands shared, or their presence vs absence. They help in quantifying genetic diversity within and between species. Therefore, these two molecular markers were employed in the basic studies for elucidating the genetic architecture and measuring the genetic diversity in the genus *Adhatoda*. The results obtained from isozymes showed slight variation with those obtained from RAPD analysis. However both isozyme and RAPD markers were proficient at distinguishing between *A. vasica* and *A. beddomei* and among *A. vasica*. Among these two techniques, isoenzyme analysis revealed that *A. beddomei* formed a separate cluster as it is a different species from the closely related *vasica* species. *A. vasica* collected from Coimbatore civil aerodrome region and IFGTB, Coimbatore showed maximum similarity index in concordance with their same environmental interactions. The maximum diversity was noticed between *A. vasica* collected from IFGTB, Coimbatore and Thaimudi and between *A. beddomei* and *A. vasica* from Kanjikode, Kerala.

RAPD dendrogram contained 3 clusters and *A. vasica* collected from Kanjikode fell on a separate cluster. RAPD markers appear more striking than those obtained from isoenzymes because of larger number of informative markers generated per primer directly at DNA level and the reproducibility of RAPD analysis was high. The two techniques contrasted most notably in pair-by-pair comparison and both matrices resulted in different dendrograms. Nevertheless, overall representation of relationships among plant samples by cluster analysis and coordination was in considerable agreement. Based up on the results of this study, it appeared that the relationships estimated from DNA markers were at variance with the broad representation described by isoenzymes. This is due to the less number of primers used.

SUMMARY AND CONCLUSION

5.0 SUMMARY AND CONCLUSION

Investigations were carried out to explore the medicinal properties of two species of *Adhatoda* namely *Adhatoda vasica* and *Adhatoda beddomei*. The study involved the collection of samples from various places and the assessment of ascorbic acid, β-carotene and selected enzymes. In this study, an efficient protocol was standardised to initiate and maintain the callus culture of *A. beddomei* and *A. vasica* collected from Kanjikode, Kerala. The pharmaceutical value of these selected medicinal plants resides in their alkaloid content. But the intrinsic alkaloid content in the two species of *Adhatoda* selected for the study is low. Therefore, an alternative biotechnological approach using the technique of plant tissue culture was developed to achieve high yield of alkaloids. Hence, the major thrust of this investigation was focused on the enhancement of yield of alkaloids by various ways, in particular, testing the potentials of several biotic and abiotic elicitors. Attempts were made to study the extent of genetic diversity among *A. vasica* and between *A. vasica* and *A. beddomei* using isoenzyme and RAPD analyses. The conclusions arising out of the salient research findings of the study are summarised below :

1) Collection of plant samples : *A. vasica* samples were collected from various places like Suruli falls, Kanjikode (Kerala), Thaimudi (Mudis), Kilkothagiri (Nilgiris), Coimbatore civil aerodrome region and Institute of Forest Genetics and Tree Breeding, Coimbatore, which vary in climate, temperature, rainfall, altitude, soil fertility and atmospheric pollutants. *A. beddomei*, which is an endangered species, was collected from Kanjikode, Kerala.

2) *A. vasica* collected from Coimbatore civil aerodrome region showed the maximum contents of ascorbic acid, β-carotene and alkaloid.

3) Investigations on the assessment of enzymes showed a possible relationship between the enzymes and pollutants in places from where the plant samples were collected.

Plants from plain and highly polluted areas like Coimbatore civil aerodrome region showed higher levels of SOD, POD, GR, NR, IAAO, AAO, G6PD, ACP, ALP and PAL than those from high altitude and less polluted areas like Kilkothagiri (Nilgiris) and Thaimudi (Mudis). This revealed that the synthesis of antioxidants and enzymes sensitive to environmental stresses in this medicinal plant is enhanced in polluted regions.

4) Tissue culture studies

1. Analysis of the samples for tannin and total alkaloid content revealed that the samples selected from Coimbatore civil aerodrome region and IFGTB, Coimbatore had higher alkaloid as well as tannin contents that might hinder with the callus initiation. Samples from Suruli falls, Thaimudi (Mudis) and Kilkothagiri (Nilgiris) had low alkaloid content. Since *A. vasica* from Kanjikode had intermediary values (0.2 g alkaloid/100 g dry tissue and 18.37 mg tannin equivalent) this was chosen for tissue culture study along with *A. beddomei* which was also ideal for comparison with *A. beddomei* collected from the same region.

2. Callus cultures of *A. vasica* and *A. beddomei* was raised from leaves on MS medium. The callus was also maintained on MS medium.

3. A time course study on the pattern of growth and alkaloid content showed maximum growth and alkaloid production at the 6^{th} week of callus culture.

4. Four different growth media (MS, SH, LS and B_5) were used for growing the callus from *A. vasica* and *A. beddomei*. Of the different media tested, MS medium was found to be ideally suited both for the growth of callus and alkaloid production.

5. The effect of modulation of the levels of phytohormones on the growth and alkaloid production in the callus cultures was evaluated. It was found that, among the auxins tested (IAA, NAA, IBA and 2,4-D), IAA at

2.0mgl^{-1} was found to elicit the maximum growth and alkaloid production. Among the cytokinins tested (Kn and BAP) Kn at 1.5 mg l^{-1} enhanced both the growth and the production of alkaloid.

6. Nitrate and phosphate stress reduced the growth of the callus but enhanced the alkaloid production.

7. The study of the effect of different concentrations of sucrose showed that sucrose at a concentration of 8% (w/v) resulted in an increase of 26% in *A. vasica* and 32% in *A. beddomei* over the control in alkaloid production. A combination of sucrose (3%) and mannitol (3%) enhanced alkaloid production by 40% and 55% in *A. vasica* and *A. beddomei* respectively. In both the cases, an inverse relationship between growth and increasing concentrations of sucrose and mannitol was observed.

8. The phenomenon of elicitation of alkaloids by biotic and abiotic elicitors was exploited for the possible enhancement of alkaloid content. Two different categories of elicitors were used namely (i) biotic elicitors–media filtrates (MF) and mat/cell extracts (ME/MC) of fungi, yeast and bacteria and (ii) abiotic elicitors – metal ions.

   Of all the biotic and abiotic elicitors tested, *Trichoderma viridae* was found to be the best elicitor of alkaloid content with a minimum contact time (11 days).

   It can be concluded that the best protocol for callus culture of the endangered *A. beddomei* and also *A. vasica* is MS medium supplemented with IAA (2.0 mg l^{-1}), Kn (1.5 mg l^{-1}) and 3% mat extract of *Trichoderma viridae* with an incubation period of 11 days.

9) The genetic diversity using isoenzyme analysis on the samples revealed that the genetic distance between *A. vasica* and *A. beddomei* was higher and that *A. beddomei* formed a separate distinct cluster from *A. vasica*

collected from various places proving that it is different from the closely related *vasica* samples. The *A. vasica* samples collected from Coimbatore civil aerodrome region and IFGTB, Coimbatore formed one cluster in concordance with their same environmental interactions.

10) The study of the genetic diversity using RAPD showed that the maximum genetic distance was observed between *A. beddomei* and *A. vasica* collected from Kanjikode, Kerala and also between Thaimudi, Mudis and IFGTB, Coimbatore.

Although genetic variability between *Adhatoda* species was observed by RAPD analysis, no Population Specific Markers (PSM) could be identified. This might be due to the lesser number of primers used for amplification. PSM can possibly be obtained by increasing the number of primers.

RECOMMENDATIONS FOR FURTHER RESEARCH

1. Investigations can be carried out to check whether the plants raised from the callus treated with the fungal elicitor, *Trichoderma viridae*, produce a higher amount of alkaloid.

2. The production of alkaloid from the callus cultures can be scaled up to commercial levels.

3. The economics of alkaloid production from the callus cultures and the plants raised from the callus can be assessed.

4. The levels of individual alkaloids (like vasicine and vasicinone) can be analysed to find out which fraction is elicited most on optimization of the culture medium.

5. The genetic diversity of *Adhatoda* samples collected from other geographical locations can be followed using isozyme and RAPD analyses.

6. The study can be extended to other, commercially important, medicinal plant species.

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APPENDICES

APPENDIX - I

COMPOSITION OF MURASHIGE AND SKOOG'S MEDIUM

Stock Solution	*Constituents*	*Conc. in stock (gm/l)*	*Stock solution in final medium (ml/l)*	*Final concentration in medium*	
				(mg/l)	*(in mM/μm)*
A	NH_4NO_3	82.5	20	1650	20.6 mM
B	KNO_3	95.0	20	1900	18.8 mM
C	H_3BO_3 KH_2PO_4 KI $NaMoO_4.2H_2O$ $CoCl_2.2H_2O$	1.24 34.00 0.166 0.05 0.005	5	6.2 170.0 0.83 0.25 0.025	100 μM 1.25 mM 5.0 μM 1.0 μM 0.1 μM
D	$CaCl_2.2H_2O$	88.0	5	440.0	3.0 mM
E	$MgSO_4.7H_2O$ $MnSO_4.4H_2O$ $ZnSO_4.H_2O$ $CuSO_4.5H_2O$	74.0 4.46 1.72 0.005	5	370.0 22.3 8.6 0.025	1.5 mM 100 μM 30 μM 0.1 μM
F	Na_2EDTA $FeSO_4.7H_2O$	7.4 5.57	5	37.35 27.85	100 μM 100 μM
G	Thiamine HCl Nicotinic acid Pyridoxin HCl Glycine	0.2 0.2 0.2 0.8	5	1.0 1.0 1.0 4.0	

Addenum : Sucrose 30 g/l

Hormones as necessary

Agar 8g/l

APPENDIX – II

COMPOSITION OF DIFFERENT BASAL MEDIA (mg/l)

Constituents	*SH*	*LS*	B_5
KNO_3	2500	1900	3000
NH_4NO_3	-	1650	-
$NH_4H_2PO_4$	300	-	-
$(NH_4)_2\ SO_4$	-	-	134
$MgSO_4.7H_2O$	400	370	500
$CaCl_2.2\ H_2O$	200	440	150
KH_2PO_4	-	170	-
$NaH_2PO_4H_2O$	-	-	150
$MnSO_4.4H_2O$	10	-	10
$MnSO_4.H_2O$	-	22.3	-
KI	1	0.83	0.75
H_3BO_3	5	6.2	3
$ZnSO_4.7H_2O$	1	8.6	2
$CuSO_4$	0.2	-	0.025
$CuSO_4.5H_2O$	-	0.025	-
$Na_2MoO_4.2H_2O$	0.1	0.25	0.25
$CoCl_2.6H_2O$	0.1	0.025	0.025
$FeSO_4.7H_2O$	15	27.86	28
Na_2EDTA	20	37.26	37.25
Myoinositol	1000	100	100
Thiamine-HCl	5	0.4	10
Nicotinic acid	5	-	1
Pyidoxine-HCl	0.5	-	1

SH = Schenk and Hildebrandt (1972)

LS = Linsmaier and Skoog (1965)

B_5 = Gamborg *et al.* (1968)

APPENDIX – III

ENZYME STAINING RECIPES

Polyphenol oxidase (Sato and Hasegawa, 1976)

The composition of staining solution was as follows :

Sodium phosphate 0.1 M	– 100 ml (pH 6.8)
Catechol	– 15 mg
Sulfanilic acid	– 50 mg

The gel was incubated at 30°C in the solution till bands of activity appeared. The gel was photographed and zymograms were drawn immediately as the bands tended to vanish.

Peroxidase (Reddy and Gasber, 1971)

The gel was incubated at room temperature in the following staining solution:

Tetramethyl benzidine	– 0.5 g
Hydrogen peroxide (3%)	– 0.675 ml
Acetic acid	– 4.5 ml
Distilled water	– 100 ml

As soon as the blue bands appeared, the reaction was stopped in 7 per cent acetic acid. The gel was photographed and zymogram recorded.

Esterase (Smith et al., 1970)

The staining solution used for esterase assay was prepared just before gel incubation as follows:

Sodium dihydrogen phosphate	- 1.4 g

Disodium hydrogen phosphate	- 0.55 g
Fast blue RR salt	- 0.10 g
Alpha-Naphthyl acetate	- 0.015 g
Distilled water	- 100 ml

The gel was incubated in the above solution at 37°C in dark for half an hour with constant shaking. The brown bands were visualized in light and photographed. Zymogram was drawn.

Malate dehydrogenase [MDH]

0.2M Tris-HCl, pH 8.0	- 12.5 ml
0.5 M DL-Malic acid, pH 7.0	- 12.5 ml
NAD (0.5%)	- 0.5 ml
NBT (0.1%)	- 0.5 ml
PMS (0.1%)	- 0.5 ml

Note : Incubated the gel in the dark about 45 min. at 37°C until dark blue bands appeared.

Shikimic acid dehydrogenase

The gel was incubated in dark at 35°C in the following staining solution.

0.2 M Tris-HCl, pH 8.0	- 10 ml
Shikimic acid (0.5%)	- 10 ml
1% $MgCl_2$ (1.0%)	- 1 ml
NADP (0.1%)	- 0.5 ml
NBT (0.1%)	- 0.5 ml
PMS (0.1%)	- 0.5 ml