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Review Article

Perspective on secondary metabolites and their exploration using biotechnological tools

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Abstract

Secondary metabolites are important bioactive compounds. They are important constituents of crude drugs. Several plants are rich in secondary metabolites, which are potential source of drugs, fragrances, pigments, food, flavours and essential oils. Biotechnology assisted production of drugs from plants is roots in physiological plant anatomy, *in vitro* culture of plant cells, the design of bioreactors, plant biotransformation and hairy root culture. Bioactive compounds are produce in very low quantity in plants. It is obvious that these products are useful to mankind and needs to be conserved and manipulated. Many plant species yet to be investigated properly for phytochemical constituent and many to be evaluated at the level of gene regulation of metabolites with the advent of newer sophisticated tools and techniques biotechnology and information technology many new information may be obtained in the near future.

Keywords: Bioactive compounds, secondary metabolites, biotransformation

Medicinal compounds are abundant in nature. They are present in plants, micro-organisms, marine-organisms, arthropods and other animals. These bioactive compounds are secondary metabolities i.e., derivatives of primary metabolities. Apparently they do not take part in any of the fundamental metabolic processes in plants and as such, are referred to as secondary metabolities. However, these are not less important than primary metabolites and play a vital role in medicine as analgesics (morphine), anti-tussives (codeine) anthypertensives (reserpine), cardiotonics (digoxin), anticancer agents (vinblastine, taxol) and anti malarial agent (artimisin, quinine), antiulcer compounds (phenylpropanoids) and anti- HIV (castanospermine) etc. In this review article we have dealt only with bioactive compounds that are present in higher plant taxa. Generally these bioactive compounds are produce in very low quantity in plants, however, as the demands of bioactive compounds are very high, such chemicals are extremely high priced. A list of important medicinal plants and their active principles are depicted in Table 1 and Table 2 indicates annual demand of 32 prioritised medicinal plants.

Limitation of field production of plant secondary metabolites

An improvement in the product formation was felt necessary to accomplish the demand of mankind *In vivo* grown plants has certain intrinsic limitations, such as production of the bioactive compounds are seasonal in many cases and dependant on organ development, thus limiting the production throughout the year. Plants are fixed in the environment and as such, they are often subject to natural calamities and pathogen attack, which restrict the stability of product formation. The yield often varies in different ecological conditions. Some of the most widely used drugs are still extracted from whole Plant source. For example, the tropane alkaloid; atropine and scopolanine, which are used in a wide range of medicinal applications are extended and used in a wide range of medicinal applications are extracted are purified from field grass *Duboisa* and *Datura* plants. The production of these drugs is therefore affected by the same variable that acts upon food plant production.

The idea of *in vitro* secondary metabolite production

Higher plants are valuable and the most important producers of natural products including food, fibre, timber and oils. However, several plants are rich in secondary metabolites, which are potential source of drugs, fragrances, pigments, food, flavours and essential oils. Biosynthesis of metabolites although, controlled genetically, are also affected strongly by environmental factors and soil conditions. UNESCO (1960) has published a survey report, which details of old world medicinal plants growing in different zones belonging to different families and production of secondary metabolites.

It was in 1950s that the potentialities of isolated cells of higher plants in producing the useful metabolities were recognized. At that time the tissue culture technique was not very elaborate and thus not be taken up for general use. Since then, technique of tissue culture has been developed for large-scale cultivation of plant cells and production of secondary metabolites has created keen interest among the researchers. The metabolites includings terpenoids, flavonoids, antimicrobials etc. have been detected from the tissues developed *in vitro*.

The path breaking route on secondary metabolite research commenced from the analysis and production of plant drugs is marketed by medieval herbals, by the

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Table 1 : Examples of Crude Drugs that have received clinical /pharmacological support for their therapeutic claims (Sukh Dev, 1997; Schulz *et al.*, 1998 and Huang, 1999)

BOTANICAL NAME	ENGLISH/HINDI/ SANSKRIT NAME	KEY CONSTITUENTS	TYPE OF ACTIVITY
Acorus calamus	Vacha	?	Tranquilizer
Adhatoda zeylancia	Vasa	Vasicine	Bronchodilator
Aesculus hippocastanum	Horse chestnut	Aescine	Astringnt, antiedemic
Allium sativum	Garlic	Allin allinsase	Cholesterol-lowering, antihypertensive
Andrographis paniculata	Bhuinimba	Andrographolide	Hepatoprotective
Arctostaphylos uva-ursi	Uva-ursi	Phenolic heterosides	Antidote for urinnry tract inflammations
Artemisia annua	Qinghao	Artemisinin	Antimalarial
A. absinthium	Wormwood	Sesquiterpene lactons	Gastrointestinal disorders
Asparagus racemosus	Shatavari	Shatavarin-I	Antiabortifacient
Azadirachta indica	Nimba	Gedunin	Antimalarial
Bacopa monnieri	Brahmi	Bacosides	Memory enhancer
Boerhavia diffusa	Punarnava	?	Diuretic, anti-inflammatory
Butea frondosa	Palasha	Palasonin	Anthelmintic
Cassia angustifolia	Senna	Sennosides	Bowel stimulator, antiabsorptive
Centella asiatica	Mandookaparni	Asiaticosides	Skin diseases, psychotropic
Chamomilla recutita	Chamomile	Chamazulene, bisabolol, lypophyllic flavonoids	Anti-inflammatory antispasmodic
Crataegus monogyna, C. oxycantha	Hawthorn	Glycosyl flavonoids ? procanthocyanidins ?	Positive inotropic, antiarrhythmic
Curcuma longa	Turmeric/Haridra	Curcumin	Anti-inflammatory
Datura metel	Thorn-apple/Dhastura	Tropane alkaloids	Intoxicant, narcotic, aphrodisiac, antispasmodic, anodynous
Echinacea spp.	Coneflower	Polysaccharides	Immunomodulator
Ephedra spp	Ephedra	Ephedrine	Bronchodilator vasoconstrictor
Ginkgo biloba	Ginkgo	Bilobalide, ginkgolides, flavonoids	Anti-ischemic, antihypoxidotic, PAF - antagonistic, memory chnancer
Harpagophyatum procumbens	Devil's claw	Harpagoside	Anti-inflammatory and antiexudative
Hollarrhena antidysentierica	Kutaja	Conessine	Antidysenteric
Hypericum perforatum	St. John's wort	Hypericins	Antidepressant
Hyperzia serrata	?	Huperzine A and other alkaloids	Active cognition chnancer, facilitates memory and motor activity in the aged
Panax ginseng, P. Quinquefolius	Ginseng	Ginsenosides	Adaptogen
Phyllanthus amarus	Bhoomyaamlaki	?	Hepatoprotector
Picrorrhiza kurroa	Katukaa, Kutki	Picroside, kutkoside	Hepatoprotector
Piper methysticum	Kava	Methysticine and related pyrones	Anesthetic, anticonvulsant, central muscle relaxant
Plantago ovata	Psyllium	Mucilages, hemicelluloses	Laxative
Prunus africana	Pygeum	?	Benign prostratic hyperplasia
Silybum marianum	Milk thistle	Silymarin	Antihepatotoxic, promotes ribosome formation and protein synthesis
Serenoa repens	Saw Palmetto	?	Benign prostratic hyperplasia
Swertia chirayita	Kairrata, Chirayata	?	Febrifuge
Valeriana officinalis	Valerian	Valepotriates	Hypnotic
Withania somnifera	Ashwagandha/ Ashvagandha	Withanolides	Aphrodisiac, rejuvenator

Table 2 : Annual demand of 32 prioritised medicinal plants

Sl.No.	Species	Demand (in tonnes)		Annual Growth Rate (%)
		2001-2002	2004-2005	
1	Amla	22729.5	41782.9	22.5
2	Ashok	7051.3	10724.2	15.0
3	Ashwagandha	7028.7	9127.5	9.1
4	Atis	270.1	448.4	18.4
5	Bael	5381.2	7084.5	9.6
6	Bhumi amalaki	2212.6	2985.3	10.5
7	Brahmi	3822.5	6621.8	20.1
8	Chandan	635.2	1073.1	19.1
9	Chirata	965.2	1284.7	10.0
10	Daru haridra	1187.3	1829.4	15.5
11	Giloe	2258.3	2932.6	9.1
12	Gudmar	N.A.	N.A.	N.A.
13	Guggal	1505.0	2548.9	19.2
14	Isabgol	N.A.	N.A.	N.A.
15	Jatamansi	674.9	866.8	8.7
16	Kalihari	65.4	100.5	15.4
17	Kalmegh	2005.0	2197.3	3.1
18	Kesar	N.A.	N.A.	N.A.
19	Kokum	N.A.	N.A.	N.A.
20	Kuth	1414.1	1826.3	8.9
21	Kutki	220.3	317.0	12.9
22	Makoy	2077.9	2192.2	1.8
23	Mulethi	873.4	1359.8	15.9
24	Patharchur	37.8	60.8	17.2
25	Pippali	3992.5	6280.4	16.3
26	Safed Musali	N.A.	N.A.	N.A.
27	Sarpgandha	423.6	588.7	11.6
28	Senna	6462.5	11677.3	21.8
29	Shatavari	10924.7	16658.5	15.1
30	Tulsi	3296.8	5402.9	17.9
31	Vatsnabh	322.3	3426.8	30.0
32	Vidang	N.A.	N.A.	N.A.

(Source : National Medicinal Plants Board, Ministry of Health and & Family Welfare, Department of AYUSH, Govt. of India.)

discovery of morphine in *Opium* and synthesis of acetylsalicyclic acid. Biotechnology assisted production of drugs from plants is roots in physiological plant anatomy, *in vitro* culture of plant cells, the design of bioreactors, plant biotransformation and hairy root culture (White 1939; Gautheret, 1939). Today, recognition of ecologic implications mandates that research and Development (R & D) personnel better understand the synthesis and function of plant secondary metabolites and guarantee supply, while guarding natural resources.

Primary metabolites in plant (i.e., proteins, oils and starches) maintain essential physiological processes. Secondary metabolites, such as alkaloids, terpenoids, flavonoids, and a host of glycosides mediate the relation between plants and their environment. Manipulation of secondary metabolites, therefore, not only may change the quality and quantity of products, but also may significantly change plant interaction with the environment.

The 20,000 plus organic compounds listed as secondary metabolites show an enormous diversity and thus allowed chemical description to complement conventional classification of plants by morphological features.

Research and development for the production of secondary metabolites have been directed primarily at cell technologies. Arguments in support of plant cell cultures stress the advantages of year round availability of plant material, process isolation and magnification – accentuation of chemical reactions under growth control.

The secondary metabolites have high economical and pharmacological importance and the industries are deeply interested in large variety of chemical substances being produced by plants due to their lesser toxicity. Though these substances are generally extracted from plant parts, the plant tissue culture technique has widened the scope and opened new vistas for the production of secondary metabolites.

In India, the beginning of this work goes back to 1964 when Dr. G. C. Mitra at NBRI (National Botanical Research Institute, Lucknow, 1979) showed the production of reserpine from *Rauwolfia serpentina* tissue culture. Later on, work on various metabolites was carried out in other laboratories of European countries as well as in India.

Different approaches for the production of secondary metabolites

Development of seed material for domestication and micropropagation.

Continuous improvement of plants for secondary metabolites–through somaclonal and genetic engineering.

Table 3 : High yielding cell lines which accumulate bio-active compounds at level higher the parent plant

Plant Species	Product	Yield (% dry weight)
Ammi visnaga	Visnagin	0.31
Berberis parvifolia cell line 177	Jatrorrhizine	5.83
	Palmatine	
	Berberine	
Cassia tora	Anthraquinones	6.0
Catharanthus roseus	Vindoline	
	Catharanthine	0.13
	Serpentine	0.8
	Ajmalicin	1.0
Cephaelis ipecacuanha	Emetine	
	Cephaelin	2.2
Coffea arabica	Caffeine	1.6
Coleus blumei	Rosmarinic acid	15.0
Coptis japoncia	Berberine	11.4
Dioscorea deltoidea	Diosgenin	1.5
Ephedra sp.	Pseudoephedrin e	2.25
Fedia comucopiae	Valtrate	1.38
Lithospermum erythrorhizon	Shikonin	14.0
Macleaya microcarpa	Protopine	0.4
Morinda citrifolia	Anthraquinones	18.0
Nicotiana rustica	Nicotine	0.29
Nicotiana tabacum	Nicotine	3.4
	Glutathione	
Panax ginseng	Ginsengoside	27.0
Papaver somniferum	Papuvarine	5.6

Understanding metabolic pathways.

Alternative methods using Bioreactors (biomass); immobilization, organ culture (Short/hairy root).

New compounds in tissue culture.

1. Development of seed material for domestication and micropropagation

Plants are collected from their wild habitats for medicinal uses. This supply is unable to meet the demand if a plant finds multipurpose uses or applications. Under such conditions, uniform and large supplies can be assumed only by systematic cultivation on a large scale. This requires planting materials such as seeds, tubers or other vegetative parts. There are several examples of insufficient availability of planting material necessitating, raising it through biotechnological method as the only way to get a sufficient amount. This approach has be used for the domestication of such plants such as *Digitalis* species, *Dioscorea* species, *Chlorophytum* sp. species and many others desirable compounds, non-conventional method of *in vitro* techniques are being employed.

Under uniform cultural condition, metabolities could be produced uniformly throughout the year with consistent quality, free from pests and diseases and without interference of environmental hazards. Using the established protocol, the product synthesis can be reproduced even in geographically distant places.

Tissue culture technique as it stands today is a mere emperical science, because, most of the experimental results can not yet be exploited at the cellular or molecular level. Moreover, biological materials are widely diverse in their genetic make up as such, they respond differently under the given situation. Therefore, for every plant species, standardisation of medium composition, explant types, temperature, photoperiod etc. are essential.

Various factors viz. biological, chemical and physical, play a vital role in improving product synthesis has been illustrated in the following sections, along with differentiation at the cellular and morphological levels. Use of elicitors, precursors etc. to enhance the desirable product has also shown to hold promise. Biotransformation technique has also been employed in conversion of intermediary compound produce abundantly in an organism to a desirable compound using an enzyme from microbial source (Chaturvedi 1979).

Depending on the objectives, a system viz. callus, cell suspension or root/shoot culture is used. Once the *in vitro* culture system is established it is used to achieve objectives through different approaches viz. micropropagation, genetic manipulation or cultivation.

Micropropagation is the most widely used application of plant tissue culture and micropropagation protocols for a large no. of medicinal plants have been reported. Micropropagation is used where ever a particular selected or unselected genotype to multiply at an enormous rate to meet the demand of cultivating to replace the existing stock of material or for introduction to a new state or country for cultivation eg. *Adhatoda* sp., *Atropa belladona, Rauvolfia serpentina, Aloe sp. Dioscorea floribunda, Mentha sp.* etc. (Chaturvedi, 1979; Zhihua *et al*, 2004; Margarita *et al*, 2005.)

2. Improvement – Somaclonal variation and genetic engineering

Decline of potential for the production of active principle or essential oil content is a common feature with cultivation of medicinal plants and a continuous required to maintain the high yield active constituents for commercially viable programmes. Therefore,

Table 4: Effect of added precursors on secondary product levels in the tissues

Species	Precursor	Metabolite	Stimulation
Ruta graveolens	4-OH-2-Quinoline	Dictamine	Trace – 0.6%DW
Cinchona ledgeriana	Tryptophan	Quinoleines	Trace – 0.9% DW
Lithospermum erythrorhizon	Phenylalanine	Shikonin	37 µg –126 µg g^{-1} FW
Ephedra gerardiana	Phenylalanine	Ephedrine	0.17% – 0.5% DW
Capsicum fruitescens	Vanillylanine + isocarpic acid	Capsaicine	Trace – 10 µg
Catharanthus roseus	Tryptamine + Secologanin	Almalicine	Trace – 0.6 mg L^{-1}

Table 5 : Production of secondary metabolites by hairy root cultures

Plant species	Agrobacterium strain/ Ri plasmid	Type of culture	Specific metabolites
Artemisia absinthium	LBA 9402	Hairy root	Volatile oils
Artemisia annua	LBA 9402	Hairy roots	Artemisinin
Atropa belladonna	15834, A4, 8196	Hairy roots	Tropane alkaloids hyoscyamine
Amsonia ellipitca	A4	Hairy roots	Indole alkaloid
Azuga reptans	*A. rhizogenes* MAFF 0301724	Hairy roots/ regenerants	20-hydroxyecdy-sone
Beta vulgaris	LBA 9402	Hairy roots	Betalin pigments
Catharanthus roseus	LBA 9402	Hairy roots	Indole alkaloids
Cinchona ledgeriana	LBA 9402	Hairy roots	Quinoline alkaloids
Coleus forskohlii	MAFF 03-01724	Hairy roots	Forskolin
Datura sp.	15834, A4, 8196 LBA 9402	Hairy roots	Tropane alkaloid
Digitalis purpurea	A4, 15834	Hairy roots	Cardenolides
Duboisia myoporoides	HRI	Hairy roots	Tropane alkaloid
Hyoscyamus sp.	A4, LBA 9402	Hairy roots	7-β-hydroxy-hyoscyamine
Lippia dulcis	A4	Hairy roots	Hernanduclin
Nicotiana tabacum	A4, LBA 9402, 15834, 8196	Hairy roots	Nicotine alkaloids
Panax ginseng	A4, 15834	Hairy roots	Ginsenoside saponins,
Rubia tinctorum	15834	Hairy roots	Anthraquinones
Solanum aviculare	15834, LBA 9402	Hairy roots	Steroidal alkaloids
Solanum tuberosum	15834	Hairy roots	Steroidal alkaloids
Vinca minor	15834, A4	Multiple shoot derived from hairy	Vincamine
Withania somnifera	A4	Hairy roots	Withanolides

(Source: Biotechnology Secondary Metabolites: Eds. K.G.Ramawat & J.D.Merillon.)

selection through conventional breeding as well as in cell culture is explored for obtaining high yielding cultivars. Once selected, this material is multiplied by the micropropagation technique to provide seedling materials to the farmers e.g. *Mentha, Citronella* and *Cymbopogon* species. Though genetic engineering offers great promise for improving the existing genotype, the work is still in infancy as compared to crop plants. Genetic manipulation has been employed to obtain hairy root cultures of a large number of Solanaceous plants and manipulation of biosynthetic pathways to obtain a desired product e.g., change in flower colour by blocking the chalcone synthase gene responsible for flower colour.

3. Understanding Metabolic pathways

Plant tissue culture has been extensively used to understand the nutrition of the plant cell and the secondary metabolites it can produce. All the earlier work regarding the production of the active principle concentrated on the possibility of producing a compound by the cell culture of plant species. Subsequent efforts were devoted to learn about the factors governing the production of secondary metabolites (optimization). Later, selection of cell lines for high yield of secondary metabolites and enzymes involved in the biosynthesis were investigated using radioactive precursors wherever considered necessary. Growth of the cell in a totally controlled environment of physical and chemical factors provides an excellent system for studying changes in the production of secondary metabolites, which are always present in small quantities. This basic information has provided significant clues for genes and their functioning, leading to genetic manipulation for biosynthetic pathways so as to obtain desired products by either blocking a pathway or enhancing the metabolic reaction.

4. Scaling up technology through bioreactors

Plant cell cultures grown in a Bioreactor enable biomass production of a desired medicinal plant or its active principle through transformation. Such a system has been use for a long time for production of antibiotics using fungi. The basic technology was derived from microbial systems and has been continuously improved for the growth of plant cell and organ cultures on a large scale. Bioreactors up to 20,000 litres have been designed and tetrad for the growth of plant cell cultures. Such a culture system produces a huge biomass in a short duration (10 to 20 days) but there are still several challenges to resolve before wider applications. However, several pharmaceuticals are produced or are on the verge of being produced at the commercial level using large bioreactors, e.g., Shikonin from *Lithospermum erythrorhizon*, taxol from *Taxus breisfolia* and between for *Coptis japonica*. (Paek, 2005).

5. New components/derivatives production

Plant cultivars have produced new components previously not known in the intact plant, new derivatives of known compounds, new derivatives by biotransformation of molecules incorporated in the medium. Such results provide unlimited opportunity to screen the cultivars of new compounds. It is presumed that productivity of new compound/derivatives might be due to altered gene function in cultured cells compared to the *in vivo* grown mother plants. Production of new alkaloids in cultures of *Catharanthus roseus, Ochrosia elliptica, Papaver somniferum, Ruta graveoleus* are

frequently cited examples.

Production of secondary metabolites in plants

The production of secondary metabolites in plants can be understood as the result of plant development, which differentiate in metabolism as well as in morphogenes (Constabel, 1988) or the production of secondary metabolites is the expression of plant genome under developmental controls. On the other hand, secondary metabolites accumulates as a result of stress and disease. Such metabolites may be synthesised de deno (i.e., by gene activation) and are referred to as phytoalexins. Other metabolites produced in response to stress and disease result from stimulation of enzymes active regular metabolism and are referred to as phytoanticipins. Both concepts invite genetic manipulation to better understand the biology secondary metabolites and to increase in yield.

Today, the concept of secondary metabolite products as differentiation products is supported by the following arguments :

Stages of plant cell cultures

An extensive studies have been made in varies laboratories world wide to know when plant synthesize secondary metabolites. Methodologies have been designed to know the stage for enhancement of synthesis and increase yields of secondary metabolites in cell cultures. Following stages when synthesize secondary metabolites *in vitro*. Kinetics of growth and sec metabolites synthesis and accumulation have long and repeatedly been shown to co-occur with the stationary phase of cell cultures.

A) Hormonal control

Hormonal control of metabolites formation and accumulation has been well established for many times. As a rule, deceleration of growth because of detection or reduction of auxins in culture media prompts the appearance of products viz. pigments/alkaloids. Cytokinins have been demonstrated to stimulate production, as shown in cell cultures of *Nicotiana tabacum* (Tabata *et al.* 1971) for indole alkaloids in cultures of *Catharanthus roseus* (Careu *et al.*, 1997) or for anthocyanins (Decendit, 1996).

Growth regulators affect growth and synthesis of secondary metabolites of cultivated cells. It was reported that the dose and nature of hormone supplimentation and the culture age have very marked effect on steroidal levels while studying the cultivated tissues of *Trigonella foenumgracum*. According to them centrain combinations of auxins and cytokinins had synergistic effect while other had antagonastic influence on steriodal synthesis. The nicotine synthesis in the tobacco cell cultures is strongly inhibited by 2, 4-D whereas it is promoted by kinetin. The effect of growth regulators on secondary metabolism very greatly depends upon the kinds of metabolites.

Tabata and his associates observed that the synthesis of shikonin is inhibited both by 2, 4-D and NAA but not affected at all by IAA using the *Lithospermum* sp. cell cultures. In *Morinda* cultures, Zenk *et al* (1975) observed that promotion of anthraquinones taken place in presence of NAA but not in presence of 2, 4-D. However, the synthesis of anthraquinones in *Cassia tora* remained ineffective by 2,4-D. Brain (1976) observed stimulatory effect of 2, 4-D in l-DOPA synthesis. Although some promising information have already been obtained, more researches are needed for improving biosynthetic rate of secondary metabolites in *in vitro* using biotechnological advances.

B) Growth Factor

Biosynthetic activity of cells in a batch culture depends on cell growth and substrate utilization. Not much is known about the correlation between the rate of secondary metabolite formation and age of individual cell in culture. However, production growth pattern could be categories in 3 major types :

First type : Product - production proceeds parallel with cell growth for example antheraquinones tropane alkaloids etc.

Second type : Product – production is delayed until cell growth declines or stops e.g., shikonin etc.

Third type : Product – production declines as the cell growth increases e.g., diosgenin etc.

C) Morphogenic Differentiation

In nature, certain compounds are synthesized and stored up only in some specific plant parts via. essential oils in certain sex glands, tropane alkaloids in roots of tobacco; and latex in laticifers ducts. Such compounds reported in intact plants can not be synthesized by cells in suspension cultures but organogenesis is induced in cell cultures, there are synthesized *in vitro*. In *Scopolia parviflora* suspension culture, root initiation coupled with normal production of tropane alkaloids; alkaloid content increases many folds when organogenesis is induced in tissue culture of *D. innoxia*.

Shikonin, a devivative, found localized in cork cells only has been produced and ceptionally in suspension cultures. This finding has opened the path for *in vitro* production of certain monotrepenes, α- pinene etc. without organogenesis the futures.

D) Effect of nutrients on production of secondary metabolites

The addition of nutrients, growth hormones, vitamin etc. in culture media is primary aimed to increase cell growth in cultured condition. It has been reported that certain nutrients in culture media increase some secondary metabolites while others show an inhibitory effect.

Addition of sucrose in culture media above its ordinary level increase shikanin accumulation in cultured cells, lower concentration of sugar increases production of ubiquinone-10 in tobacco cell cultures. Carbon-nitrogen ratio (C : N) plays a vital role in the increase production of secondary metabolites in *Sycamove* sp. Unfortunately not much study has been made.

E) Environmental control of secondary metabolites

Light is considered as an important environmental factor, which controls the synthesis of most of the secondary metabolites, *in vitro*. An appriciable quantitative change has been observed in oil content in cell cultures of *Ruta graveolens* when grown in light and dark separately.

Using Parsley cell culture, Hahlbrock *et al* (1974) noted a marked increase in the accumulation of flavone and flavonol glycosides in illuminated cultured cells, specially with ultra violet light. Stimulating effect of light on the production of medicinal compound have also been reported by researchers.

Inhibitory effects of light have also been observed specially with white and blue light on shikonin derivate formation in *Lithospermum* sp.

F) Plant growth regulator and signal transduction

A balance combination of plant growth regulators (cytokinin and auxin) plays an important role in regulation of cellular and subcellular differentiation (Suri and Ramawat 1995). Inhibition of cellular and sub cellular differentiation by 2, 4-D has been clearly established. For example the auxin suppresses molecular differentiation particularly chloroplast formation (Ramawat *et al* 1989a), cellular differentiation leading to tracheid formation (Merillon *et al* 1986) and organ formation (Ramawat *et al* 1989b). Auxin are known to modulate the production of secondary metabolites, directly by modifying the growth (Tabata *et al* 1971) or indirectly by inducing cell differentiation (Suri and Ramwat 1995). The auxin 2, 4, D inhibits the production of alkaloids (Ramawat *et al* 1989, a, b) while a moderate concentration on IAA or IBA may enhance production of alkaloids (Ramawat and Arya 1979). The mechanisms behind the effects are not clearly understood and it has been suggested that involvement of membrane lipid particularly phospholipid and calcium may have vital role.

In a series of experiments it has been established that the cytokinin and zeatin derivative enhance alkaloid production where as presence of 2, 4-D in medium alone suppresses alkaloid production. Removal of 2, 4-D stimulates allocated production. With this background experiments were conducted to investigate the role of calcium in the cytokinin induced signals.

In general it has also been reported that raising the level of sucrose in the culture medium leads to an increase in the level of secondary metabolism (Yeoman and Yeoman 1996). It has recently been proved that the sugar are not only important energy sources and structural components but are capable of acting as regulatory signals that affect the expression of genes involved in several processes (Jang and Sheen 1997).

G) Role of genotypes

Like *in vivo* systems, *in vitro* study also revealed that there are qualitative and quantitative differences in secondary metabolite production with the genotype of plant under investigation. Interestingly it is not true that high yielding plants produce more metabolite under cultural condition. It has been demonstrated that serpentine concentration has been demonstrated that serpentine concentration of cell culture established from a high yielding *Catharanthus* genotype was not necessarily higher than that of cell culture raised from a plant with low serpentine content. This is not surprising because the genes controlling the quantitative feature are remarkably influenced by the cultural environment. In *Peganum*, a poor co-relationship exists between alkaloid content of callus of cell suspension culture lines and parent plants. Investigations have revealed that *Ammi visnaga* and *Ruta graveolens* produced characteristic secondary metabolites, in amounts similar to those in the plant from which they were derived. It was reported that callus cultures of two low alkaloid containing lines of *N. tabacum* L. yielded considerably lower nicotine contents than cultures from the respective high alkaloid yielding cultivars which were isogenic except for two loci for alkaloid accumulation. Thus there was a strong correlation between the nicotinic content of callus cultures and the plants from which they were derived. It was reported that high pyrethrin yielding strains were derived from high yielding parents plants of *Chrysanthemum cinerariaefolium*.

H) Role of Morphological and cellular differentiation in product synthesis

A major disadvantage of plant cell culture compared with the whole plant with respect to secondary metabolism is that the biosynthetic potential of the potential of the plant in question is very often not expressed in culture. Furthermore, it is desirable to use morphologically undifferential cells for the production of a large amount of desirable metabolites as a potential alternative to whole plant source. At present total synthesis of complex biochemical such as medicinal compounds from simple, primary precursors supplied in the medium is rarely achieved at a commercially significant level in suspension culture. On the other hand, there are many examples, which show high productivity of undifferentiated cells than that of the intact plants.

Therefore, studies have been initiated using differentiated cells which have roots, shoots or other organs, without knowing clearly that to what extent secondary metabolites depend on the development of specific structures/organs, or whether these two processes are genetically and/physiologically linked.

Dhar and Pal (1988) have demonstrated that pyrethrin is being synthesized more in *Chrysanthemum cinerariaefolium* cultures with shorts than the roots and its content is even lower in undifferentiated callus culture. Similar observations were also reported in cultivars of *Datura metaloides*, where tropane alkaloid production increased in the root and shoot forming cultures than undifferentiated ones.

Thus it seems that in many cases morphological differentiation may be necessary to obtain higher yield of secondary metabolites. However, this is not always

desirable in large-scale cultivation because, generally the culture period for differentiated tissue is longer than that of undifferentiated cells. It is obvious that a shorter culture tissue is required to avoid microbial contamination and to lower the production cost.

It is known that essential oils and flavour compounds in plants accumulate in swollen leaf bases in onion, secretary ducts, latex compounds found in laticifers. In *C. cinerariaefolium*, pyrethrins are localized in the oil glands and ducts present on the achens and on leaves.

Both isolated roots and root callus of *Atropa belladonna* contain measurable quantity of atropine while stem and leaf callus do not posses the alkaloid.

I) Role of elicitors

Elicitors are a special group of triggering factors, which enhance the production of bioactive compound. Mostly these compounds are isolated from microorganism and are responsible for stimulating particular facets secondary plant metabolism.

Exposer of cell culture to the action of some elicitors, such as fungal homogenates and factor are well documented. Using homogenates obtained from the pathogenic *Botrytis* sp. changes were produce in alkaloid production. The productivity of cultures significantly increased from 0.5 g/L protoberberine alkaloids to 0.8 g/L in which 3 ml of *Botrytis* hamogenate was added to 100 ml of culture medium, 8 hrs before harvesting the *B. perviflora* cells. In this treatment, release of alkaloids into the medium was mainly stimulated without loss of viability in *Berberis* cells.

Polysaccharide elicitors PmS, after obtained from *Phytopthora megasperma* var. sojae when added to soybean cell suspension cultures growing in dark induced glycolin formation after 12 hrs. and accumulation thereafter. Prior to this development a rapid increase of PAL, activity starts, reaching to maximum level 20 hrs after the addition of PmS. Comparative density – labelling technique revealed that such increases in PAL activity associated with elicited responses, as partly due to *de novo* synthesis of this enzyme.

J) Biotransformation

Biotransformation is a process through which the functional groups of organic compounds are altered by living cells. The technique of biotransformation was initially applied to the microbes due to a number of inherent advantages. Attention in recent years has been focused on the possibility of production of bioactive compounds at a commercially significant level by inducing them to perform special biotransformation reactions on organic compounds added to the medium. This way a substance of lower value is transformed to one of higher value and is also available in substantial amount.

In cases, where the final desirable product is not available from cells cultivated *in vitro*, due to non-expression of one or more enzymes in the intermediates stages of the pathway the supply of immediate and near precursor and their conversion to desired product can be possible. Alternatively the biotransformation of synthesis substances, analogues or secondary metabolites from other plant species can result in the production of noval compounds hiterto unknown in nature which have new and unique chemical and pharmacological properties.

K) Application of cell-immobilization techniques

The properties of the cell to adhere to many substances in nature is known quite for sometime. Traditional use of immobilized *Acetobactor* cells for the production of vinegar is well-known.

Immobilization has been defined as a technique which confers a catalytically active enzyme or cell within a reactor system and presents its entry into the mobile phase, which carries the substrate and the product . Immobilization of plant cells was ealier reported by Hall *et al.*, 1987. They have shown that entrapment of *Catharanthes roseus* and *Daucus carota* cells in alginate matrix enhance product synthesis. The mechanism involved in the process is physically restraining the cultured cells within a solid matrix such as alginate, polyurethane etc. Secondary metabolites are generally associated with slow growing compact cells. Through immobilization of cultured cells, the cell aggregation process can be encouraged and the physical contact often induces cyto differentiation which in turn promote increased metabolite production.

L) Role of Precursors

Attempts have been made to increase the contents of secondary metabolites in plant cell cultures by feeding related precursors and intermediates. There are examples of precursors significantly enhancing the production of secondary metabolites

M) Protein and RNA

Catharanthus roseus culture have been extensively investigated (Hall *et al* 1987). It was previously established that transferring callus from a medium containing 2, 4-D to medium lacking 2, 4-D induced alkaloid biosynthesis. Most auxin effects have been investigated to find out the role of plant growth regulators in transcription, translation or post translational level. Ouelhazi *et al.*, (1993) analysized total protein of *C. roseus* cultures grown in 2, 4-D supplemented medium. Total proteins pattern by gel electrophoresis reveal that the levels of 17 polypeptides are altered during growth phases tissues. Alternation in protein synthesis is also observed in cells grown in a 2 4 -D free medium. These results indicate that the alteration in the protein pattern may affect the regulation of alkaloid pathway. Ouelhazi *et al* (1994) attempted to analyse the effect of cytokinin or 2, 4-D on gene expression in *C. roseus* cells by monitoring polypeptide patterns. Though hormone treatments did not achieve dramatic changes in polypeptide pattern, accumulation of specific RNA coding for 18 and 28 KD polypeptide

was demonstrated under conditions of alkaloid production in the cells.

N) Genetic manipulations

Biosynthesis of most secondary metabolites is a complex multistep reaction involving several enzymes and genes. With development of many new techniques in molecular biology it is now possible to engineer plant cells and plants to enable them to perform specific metabolic reaction. Therefore attempts are being made to alter secondary metabolic pathways by targeted genetic manipulation. Plant cell cultures provide an excellent system for incorporation and expression of genes regulating secondary metabolism. There is no need to regenerate a complete plant for expression of such genes. Yeoman and Yeoman 1996 described three aspects of genetic engineering for the synthesis and accumulation of plant cell cultures. These are:

Developmental regulation of genes encoding key enzymes; Manipulation of secondary metabolism by adding novel genes and Manipulation of secondary metabolism by the down regulation of specific gene using antisense RNA technology.

Use of molecular marker

Molecular marker such as restriction fragment length polymorphism) and random amplified polymorphic DNA (Williams *et al.*, 1990) appear to be a powerful tool in identification of plant species. They have been used for confirming Somatic hybrids (Takemori *et al.*, 1994) and more recently Rani *et al* (1995) reported that RAPD markers can be utilized for genetic analysis of micropropagated plantlets. This methodology also been used to determine the variation of medicinal parts among cultivars and varieties (Nakai *et al.*, 1996), to authenticate *Panax* sp. (Show and Butt 1995) and to evaluate genetic relationship between the medicinal plant species and their secondary metabolites (Yamazaki *et al.*, 1994). From the perusal of literature it has been appeared that very few studies have been made on this area. In our ongoing study of the application of plant tissue culture to the clonal propagation of medicinal plants we have adopted two approaches. One approach involves the propagation of virus free homogenous plants by meristem cultures and another aspects to confirm the homogenity of population by qualitative analysis of secondary metabolites *in vivo* and TC generated plants. However single such analysis requires a large amount of sample material as well as extended growth. We decides to apply the technique of RAPD analysis to confirm the genetic homogenity of our regenerated plants. Similar reports have been made by Shoyama *et al*, 1995, 1987, 1988, Yamata *et al* 1991, Hatano *et al.* 1989. Negi *et al* (2006) investigated the efficiency of selectively Amplified Microsatellite Polymorphic loci (SAMPL) in assessing the levels of genetic diversity among *withania somnifera* genotypes.

Concluding remarks

From the on going account it is clear that a wide range of secondary metabolites are produced by plant cells. It is obvious that these products are useful to mankind but needs to be conservation and manipulation. Still there are many plant species yet to be investigated properly for phytochemical constituent and many more yet to be evaluated at the level of gene regulation of metabolites with the advent of newer sophisticated tools and techniques biotechnology and information technology many new information may be obtained in the near future. As the plant gets importance because of it's medicinal value demand increases and resultantly the plants become an endangered species. Biotechnological approaches used to produced and such compound of interest which can be exploited in drug designing and drug development techniques in curing several

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