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A Review on *in-vitro* Techniques for Increase the Production of Secondary Metabolites in Plants

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Abstract

Plants generate different kinds of secondary metabolites. These metabolites are employed in modern medicine development for a variety of purposes. Thus, they are significant in the pharmaceutical industry. These secondary metabolites are only used by plants to strengthen their defenses against herbivores, a variety of biotic and abiotic stresses, and the development of interspecies defenses. However, secondary metabolites are employed in human civilization for a wide range of products, including drugs, flavoring agents, pigments, and medicines. Consequently, the pharmaceutical industry is developing quickly and relies on a wide variety of medicinal plants. To fulfil the high demand of those industries, medicinal plants become gradually endangered day by day. Plant tissue culture is a cost effective and easy micro propagation technique which can improve the secondary metabolites of plant by applying various techniques like elicitation, hairy root culture, suspension culture etcetera. Plant tissue culture is an easy opportunity to study and alter the pathways of secondary metabolites production through physiological and genetic up gradation of plant species.

It is easy to produce secondary metabolites by using plant tissue culture as needed. Simultaneously, it is feasible to alter the pathways leading to the production of secondary metabolites and extract valuable components from economically significant plants. Since medicinally significant plants are the primary source of raw materials used in the pharmaceutical industry, the process Plant tissue culture is demanding to preserve those valuable endangered plants with successful continuous formation of desired plant derived metabolites.

Keywords *- Plant tissue culture, Metabolites, Suspension culture, Elicitation, Hairy root culture, Callus.*

1. Introduction

In case of Global health tissues plant derived medicines are very popular. From ancient time traditional medicines were developed by depending on plant kingdom [1]. Generally, plant derived secondary metabolites have no functions in plant physiology but participate in to development of defensive mechanisms against environment or others like herbivores, microbes, allelopathic agents, pollinators, seed dispersing animals

etcetera [2]. In the other hand plant metabolites are used in many purposes to upgrade human civilisation as secondary metabolites are used as medicines, pigments, flavouring agents, drugs etcetera [3]. So, there are many medicinal plants dependent pharmaceutical industries are developed rapidly. To fulfils the high demand of those industries, medicinal plants become gradually endangered day by day. Generally, plants are produced very large number of primary metabolites and used them directly in their respiration, photosynthesis like important metabolic activities for growth and development of plants and afterwards those primary metabolites are produced very below number of secondary metabolites through various metabolic intermediates but they have no functions into the plant's physiological activity [4]. In case of primary metabolites production into plant's body, various pathways remain involve like Embden-Meyerhof-Parnas pathway, Hexosemonophosphate pathway and Entner–Doudoroff-pathway. In the other hand in case of secondary metabolites production into plant's body, pathways remain involve like acetate-malonate pathway & shikimic acid pathway [5]. Through plant tissue culture it is possible to develop uniform, sterile, compatible, and novel plantlets which are used in biochemical characterisation [6]. Also, biologically active constituents can easily isolate from it through suitable extraction process and purification of those bioactive constituents become easy and cost effective as there the content of impurities remains minimal. But in many cases the process becomes fail. Again, in many cases during plant tissue culture, when callus culture treated with elicitor like chitosan, methyl jasmonate etcetera, then the yield formation becomes boosted up [7]. Again, using the technique hairy root and multiple shoot culture yield achievement become higher production of secondary metabolites. As for example here the hairy root culture increases alkaloids formation [8] where as shooty teratomas can boost up mono terpenes formation [9]. Again, in case of industry, plant tissue culture of *Coptis japonica* used to increase barberine formation. Suspension culture of *Lithospermum erythrorhizon* used to increase the production of shikonin [10]. When depends on the field of agriculture, the common problems related to secondary metabolites production are below quantity and quality as well as concentration of metabolites, instability of metabolites depending on geographical, environmental and season based climatic alteration etcetera. But when depends upon the field of cell or tissue culture, those problems become solved out easily [11]. During plant tissue culture various strategies are adopted to increase the secondary metabolites production as well as biomass accumulation. Some common strategies are elicitation, strain improvement, culture media and culture condition optimization, permeabilisation, precursor and nutrition feeding, biological transformation and immobilization technique etcetera [12]. Suspension culture is commonly used to produce secondary metabolites in large scale. Say for example *Berberis Willsoniae, Coleus Blumei*, *Coptis Japonica*, *Lithospermum erythrorhizon* etcetera. But suspension culture become fail in case of some plant species like *Atropa belladonna ,Digitalis lanata, Cinchona ledgeriana, Duboisia leichhardtii* etcetera [13].

2. Plant cell culture-based increment of secondary metabolites formation:

In industry, this method is very popular for secondary metabolites production because here the metabolites production does not affect by geographical or environmental alteration [14]. Also, here as per requirement the modification of secondary metabolites production is possible by applying various growth parameters. Idea of this technique was developed after 1960. In this technique according to mother plant the quality and quantity of desired secondary metabolites production become alter as per requirements [15]. According to the procedure here the plant cells are separate out from mother plant and cultured into suitable culture media with suitable environment. After successful incubation period the plant cell culture provide the pure and high-quality desired product with sufficient quantity. The significance of this technique is secondary metabolites formation in commercial level to develop the area of research in biology, biochemistry and genetics [16].

3. Suspension culture

In industry this technique is very popular& reliable because here high quantity secondary metabolites are produced [17]. During initial stage of suspension culture, the rate of production of desired secondary metabolites remains high but later the rate of production become decrease gradually due to lack of nutrition and genetic differences. So, to overcome this problem during suspension culture select and maintain high yielding cell line throughout the process is very much important factor [16]. Step by step the general procedure of suspension

culture started from selective appropriate mother plant and calli formation is occurs in suitable culture media. Here the culture media is help in differentiation technique. As this step of suspension culture is experimental and crucial, & it can be replaced by surface response method or incomplete factorial experiments [18]. In next step, sub culturing of calli become involved in organogenesis, embryogenesis, propagation and suspension culture. In developmental stage of suspension culture friable callus need to inoculate into suitable broth medium under desired environmental condition like light, heat, aeration, agitation and others as per requirement. It is mandatory to maintain the homogeneity of suspension culture throughout the process. During suspension culture optimum observation is required about cells which are depends on composition of culture media, quality of induced callus, genetic differences of cells etcetera [19].

TABLE A.1 Suspension culture derived secondary metabolites production

Serial number	Name of plant	Major Secondary metabolite	Observation	Reference
1	*Taxus Chinesis*	Paclitaxel	Increase the production of paclitaxel when temperature alteration from 24 degree centigrade to 29 degree centigrade	[20]
2	*Podophyllum hexandrum*	Podophyllotoxin	Biomass and yield product of podophyllo toxin increases rapidly when culture media is supplemented with polyvinyl pyrrolidone (PVP) and pectinase	[21]
3	*Bacopa monnieri*	Bacoside & saponin	By using modified murashige and skoog media in *Bacopa monnieri* (L.) cell suspension culture, then achieve important saponine & bacoside A, up to 1 g/100 g dry wt. as yield, from selected cell line	[22]
4	*Tinospora Cordifolia*	Protoberberine	During both callus culture and cell suspension culture, berberine and jatrorrhizine were accumulated as yield. Jatrorrhizine is comes under protoberberine alkaloids. The quantity of jatrorrhizine were higher than berberine in the root extract of *Tinosporacordifola*	[23]
5	*Ipomoea batatas*	Various caffeoylquinic acids like caffeic acid, chlorogenic acid,3,4-dicaffeoylquinic acid,	By using modified murashige and skoog high anthocyanin production medium (APM)inanthocyaninaccumulating sweet potato's cell	[24]

		3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid&3,4,5-tri-caffeoylquinic acid	suspension culture, it is possible to get phenolic compounds as yield, after 24 days of successful incubation, from selected cell line	
6	*Linum album*	Podophyllotoxin& 6-methoxypodo-phyllotoxin	During suspension culture of *Linum album*, when methyl jasmonate (MeJas) was added into specified media.Then the production of podophyllotoxin (PTOX) and 6-methoxypodophyllotoxin (6MPTOX) as yield were double	
7	*Taxus baccata*	Taxol	By using modified Gambourg B5 media in Taxus *baccata* stage-2cell suspension culture, it is observed that the growth rate was increased as well as the yield production of secondary metabolite that is taxol, was also increased	[25]
8	*Azadirachta indica*	Azadirachtin	In case of secondary metabolites production by bioreactor cultivation, the major growth limiting nutrients were glucose and phosphate	[26]
9	*Lycopersicon esculentum*	Lycopene extract	During standardised tomato cell cultures with appropriate conditions as per RSM, it is confirmed that, as end product lycopene production were increased threefold	[27]
10	*Cocos nucifera*	Phenylpropanoid	In *Cocos nucifera* (coconut) endosperm cell suspension cultures, when chitosan is used as elicitor during dark incubation, then the rate of phenylpropanoidderivatives formation become increased as yield product. This phenyl propanoid derivatives are desirable mimics in in-vivo biochemical changes of coconut palm, which induced by defence	[28]

11	*Nostoc flagelliforme*	Exopolysaccharides	By including sodium nitrate & potassium hydrogen phosphate in BG-11 broth medium, then the rate of growth of *Nosto flagelliforme cells* & production of yield that is exopolysaccharides became increased. Also as elicitor phosphate show very small effect on exopolysaccharide production as yield product in case of selected cell mass. Here the cell growth of *Nosto flagelliform* was optimal at 25 degree centigrade temperature with neutral pH. But when the temperature became less or higher from 25 degree centigrade and in weak alkaline pH, more exopolysaccharides became accumulated. Again, with light intensity, the rate of growth of *Nosto flagelliform cells* & production of yield that is Exopolysaccharides are proportional	[29]
12	*Arnebia hispidissima*	Alkannin	Here the production of yield that was alkannin was depending on various culture media. By using M-9 media both callus culture and cell suspension culture showed maximum production of yield that is Alkannin	[30]
13	*Arnebia uchroma*	Various Isohexenylnaphthazarins compounds like alkannin, hydroxyisovalerylalkannin, acetylalkannin, isobutyrylalkanni, β-2’’-(S)-α-methylbutyrylalkannin, propionylalkannin, teracrylalkannin&acetylshikonin	n-hexane extract from both suspension & callus culture of Arnebia *uchroma* (Royle) Jonst. Contains numbers of isohexenylnaphthazarins as yield product which are found during phytochemical screening	[31]

14	*Stevia rebaudiana*	Stevioside	Suspension culture showed highest growth. Rate of growth for specific cell was 3.26 day $^{-1}$ & doubling time was 26.35 hour. In that case get 75% cell viability. In exponential development phase of suspension culture showed maximum stevioside production as yield product which gradually decreased during stationary phase	[32]
15	*Stevia rebaudiana*	Steviol glycoside	Steviol glycosides Accumulation become increased when salt is used as elicitor and produce abiotic stress. During callus culture increase the quantity of steviol glycosides from 0.27 (control) to 1.43 and 1.57% with 0.10% sodium chloride & 1.57% with 0.025% sodium carbonate salt as elicitor. Again, in suspension culture on 10th day of successful incubation similar quantity of sodium chloride and sodium carbonate salt as above can increase the production of steviol glycosides from1.36 (control) to 2.61 and 5.14%, correspondingly	[33]
16	*Scrophularia striata*	Acteoside	shoot of *Scrophularia striata* is an ideal explant for successful callus culture in Murashige and Skoog medium modified by adding 0.5 mg/l naphthalene acetic acid & 2.0 mg/l benzyl adenine. Here that media was Optimum. Here the Rate of induction was also maximum that was 100%. Here callus development was satisfactory & produced maximum acteoside as yield product that was 1.6 µg/g in fresh weight	[34]
17	*Satureja*	Rosmarinic acid	During culture, rosmarinic	[35]

	khuzistanica		acid production was decreased with scarcity of nitrogen. When used 1⁄4 strength of Gambourg B5 media supplemented with 8.3 mM of total nitrogen, then after three weeks of successful incubation, in the event of lowest cell growth fresh weight was 243.0 g/L, dry weight was 17.4 g/L and rosmarinic acid content was 38.0 mg/g as well as in case of highest cell growth fresh weight was 353.5 g/L, dry weight was 19.7 g/L and Rosmarinic acid content was 180.0 mg/g. Ultimately that type of culture was a standard method for produce rosmarinic acid with highest quantity & also there is a chance of further optimisation by modifying experimental design to produce higher quantity and better quality of rosmarinic acid	
18	*Saccharu officinarum*		During indirect organogenesis somaclonal variation was observed. During molecular analysis if RAPD and ISSR are used as marker Then it will be easy to detect the varieties of sugarcane in very initial stage	[36]
19	*Salvia leriifolia*	Various Phenolic acids including caffeic acid, salvianolic acid B & rosmarinic acid	Here at first maximum quantity of rosmarinic acid was produced by successful incubation where highest quantity of caffeic acid and salvianolic acid B wear produced after second week of successful incubation. Again if 40 g/L Of sucrose Was used in cell culture Then started the Rapid production of caffeic acid and salvianolic acid B and also got the Maximum dry	[37]

			biomass	

4. Elicitation:

Substance that starts and increase the biosynthesis of particular compound called elicitor of that compound. Though there are many successful traditional methods but elicitation is the best to boost up secondary metabolites formation. Very small concentration of elicitor is required for induction of desired yield by speed up specific biosynthetic pathway [38]. According to source and characteristics elicitors are many types. Depends on source externally and internally applied elicitors are very common. In the other hand depends on characteristics biotic and abiotic elicitors are popular. Various inorganic and non-biological substances, inorganic salts, ion of copper, cadmium, calcium, high pH condition, high and low heat condition, UV light exposure, salt of heavy metals etcetera are some abiotic elicitors. Some biotic elicitors are intra cellular proteins, glycoproteins, G-proteins, sterilised mycelium of pathogenic fungi, many polysaccharides like pectin, cellulose, chitin etcetera. Externally applied elicitors are those components which are produced outside of cell like polysaccharides, fatty acids etcetera. In the same manner endogenous elicitors are those components which are produced inner portion of cell like galacturonide, hepta beta glucoside etcetera [39]. When chemical or physical stresses are applied during plant tissue culture then those stressful environments also act as elicitor& increase the formation of desired yield. In the field of research application of *in-vitro* elicitation is a novel technique [40]. Chitosan & yeast extract as elicitor in the suspension culture of *Decalepis salicifolia* where the intention was to differentiate the effects of both elicitors on 2H4MB content, TPC content, TFC content and antioxidant property. As a result, it was found that effectivity of chitosan was more than yeast extract at 72 hours of incubation and 200 μM CH. There 2-hydroxy-4-methoxybenzaldehyde (2H4MB) was increased 1.4-fold than control means 10.8 μg/g. Maximum total phenolic content was observed 4.8 mg/g and total flavonoid content (TFC) was 4.0 mg/g. Gymnemic acid was the secondary metabolite of *Gymnema sylvestre* [41]. *Aspergillus niger, Saccharomy cescerevisiae, Bacillus subtilis and Escherichia coli* extract shave capability to boost up the desired yield formation of this plant. Respectively *A. niger, S. cerevisiae, A. rhizogenes, B. subtilis and E. coli* produce gymnemic acid [42]. By following the same path methyl jasmonate 50 μM, yeast extract 0.5 mg/l and chitosan 100 mg/l as elicitor with varying concentration to produce plumbag in from *Drosera burmanii.* Here the yeast extract was more efficient for plumbagin formation in root than control plant. In that case the production of root was 3.5-fold higher. Similarly, chitosan showed the highest concentration in root and methyl jasmonate showed the highest concentration in shoot [43]. So, for increase the production of secondary metabolites, particular elicitation technique for particular plant species and plant organ, is a very successful process. Actually, during elicitation, the defence mechanism of plant become activates which induce the secondary metabolite production. If in any case the triggering for secondary metabolites production was not successful then there is no confirmation that secondary metabolites productions were not satisfactory. So screening procedure is obvious [44].

Here the following table represent some specific biotic & abiotic elicitors which are suitable for specific plant species to enhance their specific secondary metabolite production. Elicitation was not successful when elicitors were not specific for particular plant species. Say for example for suspension culture of *Vanilla planifolia*, yeast extract was not suitable as elicitor because unsuccessful activation of phenyl propanoid pathway [45]. Again, the same elicitor can increase phytoalexin generation through *Glycine max culture*, & also boost up alkaloid formation through *Thalictrum rugosum* and *Eschschottzia californica* culture by triggering specified pathway. Also, chitosan as elicitor having the capability to activate phenyl propanoid pathway. So, determination of which elicitor, when show its effectiveness, that is very challenging trial and error method during plant tissue culture [44].

TABLE A.2Abiotic elicitors induced secondary metabolites production

Serial number	**Name of specified plant**	**Specific Abiotic elicitor**	**Effects on selective yield**	**Reference**

1	*Glycyrrhizae chinate*	Sodium alginate	Echinatin	[46]
2	*Datura stramonium*	Metal ions of Aluminium, chromium cobalt, nickel, copper, zinc	Sesquiterpenoids	[47]
3	*Daucus carota*	Ions of calcium salicylic acid	Chitinase	[48]
4	*Atropa belladonna*	Ions of copper and cadmium	Tropane alkaloids	[49]
5	*Arabidopsis*	Stressfor oxygen deficiency & lacking of amino acid	Camalexin	[50]
6	*Hyoscyamus albus*	Cu- sulphate	Phytoalexin	[51]
7	*Glycyrrhiza uralensis*	UV rays	Glycyrrhizin	[52]

TABLEA.3 Biotic elicitors induced secondary metabolites production

Serial number	**Name of specified plant**	**Specific biotic elicitor**	**Effects on selective yield**	**Reference**
1	*Cicer areentium*	Ascochytarabiei	Medicarpin, Maackiain	[53]
2	*Capsicum annuum*	Cellulase	Capsidol	[54]
3	*Cells of different plants*	Erwiniacarotovora	Enzymes, End metabolites	[55]
4	*Cupressus lusitanica*	Fungus	Indole alkaloids	[56]
5	*Catharanthus roseus*	Trichodermaviride	Ajmalicine	[57]
6	*Salvia miltiorrhiza*	Yeast	Diterpenoidtanshinones	[58]

5. Organ culture-based increment of desired yield:

In those cases cell suspension culture were not appropriate for secondary metabolites production. There organ culture is very much desired technique for desired yield formation [59].

6. Hairy root culture

Hairy root culture is the culture produced after the infection of the explant or culture by *Agrobacterium rhizogene*, is a gram-negative bacterium which developed into soil, family Rhizobeaceae. These bacteria mainly target dicotyledonous plant to produce disease [60]. *Agrobacterium rhizogene* is a naturally occurring bacterium has plasmids which have capability to develop roots, known as Ri Plasmids which produce infection of roots of dicotyledonous plant as source of food that is opines, for the bacterium and as a result of that infection abnormal growth is occurs in root of plant. Here the plant genome becomes genetically transformed. Particularly those roots can produce numerous roots without any hormonal support during culture. Actually, those roots are neoplastic with indefinite growth [61]. A complex sequence is formed due to interaction between plant and *Agrobacterium rhizogene* & also the same sequence is formed during the involvement of *Agrobacterium rhizogene.* There is a background of hairy root production. When plant cell become wounded by any chance then the wounded cell produces some phenolic compounds, like acetosyringone and α-hydroxy acetosyringone etcetera. Those compounds act as signal molecule for specified bacteria that is Ri-plasmid containing bacteria *Agrobacterium rhizogene* or Ti-plasmid containing bacteria *Agrobacterium tumefaciens* & after getting the signal the specified bacteria become attracted for that wounded plant cell through chemotactic movement. Then the bacteria infect that wounded plant cell by attachment & colony formation. Then specified bacteria respectively inserted & transfer its small portion of T-DNA from plasmid to the host genome of plant, then integrated and getting expressed thereto produce hairy root tissues which are known as neoplastic crown gall tumour. In maximum cases *Agrobacterium* strains consist only one T- DNA but few Ri-plasmids who carries atropine consist two autonomous T-DNA, where one DNA is right-handed called TR-DNA is similar as Ti-plasmid of T-DNA of *Agrobacterium tumefaciens* and another one is left-handed called TL-DNA is similar as Ri-plasmid of T-DNA of *Agrobacterium rhizogene* [62]. This TL-DNA is responsible for hairy root formation where there is no significance of TR-DNA in transformed culture [63]. During the expression of cytokinin Proliferation is occurs by increasing the rate of cell division. Again, during the expression of auxin proliferation is occurs by increasing the rate of cell elongation and produce hairy roots from infection site. Here occurs *Agrobacterium*-mediated genetic transformation where vir gene show very important role. Ti-plasmid or Ri-plasmid's 40-kb region contains numerous vir genes. So, this specified region is known as virulence region [64-66]. That vir gene expression is occurs only in presence of acetosyringone due to activation of expression of genes remains encoded by T-DNA into infected plant cell which is needed for transformation as those genes encoded rapid expression of eukaryotic regulatory sequences which prohibited their expression. Rapid expression of vir gene started with different sugar molecules because those molecules are served as collegial with acetosyringone.

In the other hand during the expression food of bacteria become produced to provide carbon, nitrogen & energy source for survival of specified bacteria. In the type of that food involve some unusual amino acids like opctopine, agropine, nopaline, mannopine etcetera are called opine. Also, formation of opine facilitate the probability of hairy root induction [67].

Classification of*Agrobacterium rhizogene* according to hairy roots mediated opine synthesis [68]:

- Agropine-type strains (This particular type of strain mediated hairy roots produceagropine, mannopine and corresponding acids) as following-

  (i) A4 (ii) 15,834 (iii) HR1 (iv) LBA 9402

- Mannopine-type strains (This particular type of strain mediated hairy roots produceonlymannopine, mannopinic acid and agropinic acids) as following-

  (i)8196 (ii)TR7 (iii)TR101

According to [67] there are five classes of *Agrobacterium rhizogene* strain:

(i)Octopine (ii)agropine (iii)nopaline (iv)mannopine (v)cucumopine

Different strains of *Agrobacterium rhizogene* contains different transformation ability [69]. So comes the variation in morphology and virulence of hairy roots [70]. Hairy root culture is a very renowned biosynthesis procedure which is utilised for mother plant related secondary metabolites production enhancement without application of any growth hormone from outsides [71-72]. In the other hand by using the technique hairy root culture it is possible to achieve novel secondary metabolites which do not produce by the mother plant in general [73]. As the process hairy root culture is suitable for dicotyledonous plants but in some area monocotyledonous plants are also suitable [74]. Hairy root culture also increases that secondary metabolite formation which only produces in the uncovered portion of the plant [75]. Through the culture produced transformed roots have capacity to maintain the viability & genetic stability in each and every subculture and regenerated plant. As hairy roots are coming under transgenic root system so here remains high chance of integration between supplementary gene and Ri plasmid in host plant cells. For study about biology, Characteristics, and genetic expression into metabolic pathways, the technique hairy root culture is very much helpful. This technique is also used to detect various precursor, intermediates and involved enzymes into the biosynthetic pathway of desired yield production [76]. There are some examples of hairy root culture mediated secondary metabolites production as followings:

Brugmansia candida's hairy roots were synthesised tropane alkaloids [77]. Again, in plant tissue culture hairy roots of *Glycyrrhiza glabra* synthesised licoagrodin & glycyrrhizin with isoliquiritigenin respectively [78-79]. Again, in plant tissue culture hairy roots of *Atropa belladonna* synthesised scopolamine [80]. *Hyoscyamus reticulatus* L.'s hairy roots were also synthesised hyoscyamine and scopolamine [81]. Again, hairy roots of *Ammi majus* synthesised various furanocoumarins like psoralen, xanthotoxine, bergapten and imperatorin [82]. In plant tissue culture hairy roots of *Ophiorrhiza pumila* synthesised camp to the cin [83]. In the same manner in plant tissue culture hairy roots of *Panax ginseng* synthesised ginsenoside [84]. Again, in plant tissue culture hairy roots of *Salvia miltiorrhiza* synthesised many secondary metabolites like tanshinones, tanshinone I, tanshinone IIA, cryptotanshinone etcetera [85]. *Beta vulgaris*'s hairy roots were synthesised betalain [86]. In plant tissue culture hairy roots of *Echinacea purpurea* synthesised cichoric acid [87]. In the same manner in plant tissue culture hairy roots of *Fagopyrum cymosum* M. Synthesised rutin [88]. Again, in plant tissue culture hairy roots of *Silybum marianum* L. synthesised silymarin [89]. According to [90] in plant tissue culture hairy roots of *Rauvolfia serpentina* synthesised vomilenine & reserpine [90]. In plant tissue culture hairy roots of *Angelica gigas* Nakai synthesized pyranocoumarin [91]. In the same manner in plant tissue culture hairy roots of *Arnebia hispidissiman* synthesised shikonin. Again, the hairy roots of *Ophiorrhiza alata* Craib synthesised camptothecin. *Withania somnifera* L.'s hairy roots were synthesised anolide A, anone & aferin A [92]. *Tripterygium wilfordii* Hook F's hairy roots were synthesised triptolide, wilforine [93]. In the same manner hairy roots of *Arachis hypogaea* synthesised many secondary metabolites like resveratrol, piceatannol, arachidin-1, arachidin-3 etcetera [94]. Again, was established that in plant tissue culture hairy roots of *Linum usitatissimum* synthesised lignan [95]. According to hairy roots of *Brassica rapa* subsp. pekinensis synthesised glucosinolates (GSLs) [96]. In plant tissue culture hairy roots of *Withania somnifera* L. synthesised Withaferin-A [97]. Similarly in plant tissue culture hairy roots of *Centella asiatica* synthesised many secondary metabolites like madecassoside, asiaticoside, madecassic acid and asiatic acid [98].

7. In-vitro culture of shoot

It is a very desirable technology to achieve secondary metabolites as per requirement. This technique is proceeded with similar mechanism of hairy root culture. Here the infection is induced by the bacteria *Agrobacterium tumefaciens* generally in the aerial part of plant. The method shoot culture is important for transgenic shoot that is shooty teratomas induction [99] with formation of suitable hormone for non transgenic shoot induction [100]. As the *in-vitro* culture of hairy root & shoot can boost up the desired yield formation by maintaining the stability of gene and also balance the development with formation of desired end product [101]. During shoot culture Ti plasmid of *Agrobacterium tumefaciens* become integrated with plant genome. So occurs

the genetic transformation of host plant cell which induced the growth of shooty teratomas [102]. Shooty teratomas are used in biotransformation. Say for example in *Nicotiana tabacum* shooty teratomas played the role for biotransformation of nicotine [100]. Again, in *Mentha citrate* shooty teratomas proved the presence of abundant quantity terpenes achieve from oil of mint [103]. Again, in shooty teratomas *Atropa belladonna* produced tropane alkaloids, *Nicotiana tabacum* produced nicotine & *Solarium tuberosum produced* steroidal alkaloids [104]. Again, in *Solanum eleagnifolium* shooty teratomas produced a poisonous alkaloid solasodine [105]. Others like *Catharanthus roses* G. produced vincristine as secondary metabolites [106-107], *Drosera capensis* var. alba produced naphthoquinone as secondary metabolites [108] by shooty teratomas.

8. Biotransformation involved secondary metabolites production enhancement:

Suspension culture of plants- an established technology to generates valuable metabolites. Though Results were not satisfactory for some cases [109]. During cell suspension culture of plant, exogenous components become convert into some novel component with better quality due to the process biotransformation. Biotransformation is a process where occurs the transformation of main substrate into another in presence of appropriate enzymes or microorganisms with suitable condition. Here the Properties as well as quality of the converted component will be different from initial substrate [110-116]. Enzymes remains present into plant cells are suitable for biotransformation reaction. Plant cell enzymes are powerful catalyst as enzymes present into the microorganism. So various kinds of reactions are possible in presence of the catalytic activity of plant cell enzymes like region& stereoselective reactions, various organic compounds' hydroxylation, oxido-reduction, hydrogenation, glycosylation, and hydrolysis etcetera [117]. In biotransformation labile functional groups remains without protection. This is the main difference between the chemical reaction and biotransformation [118]. In case of several plants' biotransformation is occurs successfully and novel component is produced from initial substrate as following:

TABLEA.4 Biotransformation mediated secondary metabolitesproduction

Serial number	Name of plant	Process	Initial substrate	Transformed substrate	Reference
1	*Eucalyptus perriniana*	biotransformation	thymol, carvacrol and eugenol	glycosides	[119]
2	*Nicotiana tabacum*	biotransformation	hyoscyamine	scopolamine	[120]
3	*Catharanthusroseus*	glycosylation biotransformation	capsaicin and 8-nordihydro capsaicin	1b-hydroxyl desacetylcinobufagin	[121]
4	*Platycodon grandiflorum*	biotransformation		1b-hydroxyl desacetylcinobufagin	[113]

In *Catharanthus roseus & Platycodon grandiflorum*'s suspension culture when biotransformation is occurs then some unknown novel substrate produced cytotoxic activityon the cell lines of HL-60 [113]. For desired metabolites generation, immobilised cell technology also very useful [122].

9. Genetic engineering in enhancement of secondary metabolites production:

Plant cells contains many chemical components which are produced through their metabolic pathway. Those chemical components are very important for both plant and animal kingdom. Plants are used those components for their defensive activities. In animal kingdom those components are proved as important disease curing agents directly or indirectly. From ancient time plant derived natural products and their synthetic derivatives

were used due to their magical activities to cure various chronic diseases. But here the main challenging part is the isolation of those chemical components. Because plants produced very low quantity of those metabolites. Again, the bioavailability of those metabolites is also very low and collection of those metabolites are not very easy due to environmental challenges. Here the magic of genetic engineering which can enhance the quantity and quality of plant derived useful metabolites as per requirement by altering the metabolic pathway into plant cells by utilised microbes as genetic tool in *in-vitro* condition [123]. During *in-vitro* biosynthesis of plant cell they are produced a number of intermediates and obvious the final yield where genetic engineering achieves a broad spectrum for modification or alteration the pathway according to benefits [124]. Genetic engineering's effects are established on a variety of plant cell derived secondary metabolites like isoprenoids, flavonoids, stilbenes etcetera. Produce non natural derivatives of natural components achieve from plants to boost up their biological effectivity, is one of the main objectives of that segment of engineering with gene.

10. Micropropagation based desired yield formation:

Herbal medicines are very popular from ancient time. Even now a days the acceptability of plant derived medicines are in continuous incremental phase. The medicinal values of plants are completely depending on their phytochemical constituents. Plant derived phytochemicals are actually biologically active chemical compounds with complex composition. Plant cells are produced those bioactive complex chemical components for utilising in various physiological activities during stressful condition. Various pharmaceutical industries are utilised those plants for their bioactive phytochemical constituents as medicines, cosmetics, dietary supplements, fragrances, dyes etcetera. So for industry compliance there is create a demand for those plants as well as their bioactive phytochemical constituents. To fulfill that type of demand plant tissue culture technique was developed. Through this technique large scale secondary metabolites production were successful by using bioreactors. The main advantage of this micropropagation technique is that the process do not depends on the climatic or geographical location. In spite of that the process is very economic & stable. Here required secondary metabolites are produced from viable sources [125]. So in that case the conclusion is, *in-vitro* micropropagation method is very helpful for biologically effective different phytoconstituents generation like glycosides, tannins, pterocarpans & many others [126].

11. Desired yield formation by culturing callus:

Callus suspension culture is a best method to increase desired yield formation [127]. During *in-vitro* culture of plant, their growth & different metabolites formation are depending on the presence of plant hormones. Here concentration of media is also showing the valuable role on callus development with metabolites formation. During desired yield formation by in-vitro culture of specific plant species then it is mandatory to maintain the culture conditions like different chemical environmental conditions as well as physical parameters as per requirements. Oxidative stress is one of the great parameters has to maintain during plant tissue culture to produce desire metabolites from particular plant. Plants are produced phenolic compounds as secondary metabolites which are produced through phenyl propanoid pathway and plants are utilised that component to defence from different biotic and abiotic stresses. When plants are gone through some unfavourable circumstances then also reactive oxygen spacies (ROS) are produced into plant cells.

12. Discussion

Plants produced limited quantity of secondary metabolites in its body but those metabolites are directly or indirectly very much important in the field of pharmaceutical industries. To overcome the scarcity of medicinal plant derived secondary metabolites and complies the demands of pharmaceutical factories, methods are developed from the field of biotechnology and metabolic engineering. They introduced the culture with cells, tissues & organs of plants. Those are more capable procedure to produce large scale yield from plants which have therapeutic values. Different types of in-vitro culturing related to plants and elicitation are some novel strategies in the field for expansion of the desired yield formation. Here another one of the important and successful strategy is hairy root and multiple shoot culture by using a specified bacteria that is *Agrobacterium* species to increase the production of plant derived secondary metabolites with pharmaceutical importance.

Sometimes hairy root and multiple shoot culture is proved as so convenient in the field of commercial production of secondary metabolites than others techniques. Tissue culture of plants is very economical technique and do not depend on geographical location, seasonal climate and environment. Though plant derived secondary metabolites are very useful in the field of pharmaceutical, but the grate disadvantage is the poor bioavailability of those metabolites. In the other hand by altering the chemical structure of those metabolites it is possible to improve its bioavailability as well as effectivity. In that case biotransformation of those metabolites is very much useful to produce novel components with better pharmacological functions. Cell suspension culture of medicinal plants is one of the useful areas for biotransformation to produce novel components. Again, in the field of genetic engineering having some suitable techniques to modify the biosynthetic pathways occurs into plant cells to get the better quality with higher amount of yield in commercial level from therapeutically valuable plants. During plant tissue culture to get the better quality with higher amount of yield from therapeutically valuable plants, various molecular biology techniques are showing successful role for *in-vitro* regulation as well as expression of different biosynthetic pathways occurring into plants. *In-vitro* cell culture of plants also provide some knowledge about the biosynthetic pathways & its maintenance for generation of desired yield from medicinal plants in commercial level. So these techniques in the field of tissue culture are very much successful, reliable & stable to overcome the scarcity of present era by producing different known and unknown constituents derived from various medicinally important plants.

Appendices

Table A.1 : Suspension culture derived secondary metabolites production

Table A.2 :Abiotic elicitors induced secondary metabolites production

Table A3 :Biotic elicitors induced secondary metabolites production

Table A.4 : Biotransformation mediated secondary metabolites production

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