Chapter

# Root Cultures for Secondary Products

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## Abstract

Plants are source of many high-value secondary compounds used as drugs, food additives, flavors, pigments and pesticides. The production of these compounds in nature faces to many difficulties because of the dependence on weather, soil ... Furthermore, these compounds are usually limited by species, periods of growth or stress. The utilization of plant cells *in vitro* for the secondary compounds has gained increasing attention over past decades. However, the yield is still low, probably due to the degree of cell differentiation. Therefore, root culture is focused on research as an alternative to cell cultures to produce secondary compounds because of high rate proliferation, great potential in the production with high and stable yields. Hairy roots and adventitious roots have a high ability to biosynthesize secondary compounds *in vitro* with high and fairly stable in yield in comparison with plant cell suspension cultures. Nowadays, it is feasible to expand the scale of root cultures in bioreactors, which makes it possible to produce secondary compounds on an industrial scale.

**Keywords:** adventitious roots, *Agrobacterium rhizogenes*, elicitors, hairy roots, secondary products

## 1. Introduction

Plant secondary products are natural sources of bioactive compounds which used in traditional medicine and in industrial applications. In 1976, Farnsworth and Morris said that: higher plants-the sleeping giant of drug development [1]. Indeed, many chemicals derived from plants are important drugs, which are used as antibacterial and antitumour agents. Furthermore, they are used in antioxidant foods ... Besides, natural products presented chemical structures, which are very important for scientists to pursue new chemical for drugs [2]. In plants, these valuable compounds are usually limited by species, periods of growth or stress and the yield is still low. The production faces to many difficulties because of the dependence on weather, soil .... So the utilization of plant cell, tissue and organ culture for these compounds has gained increasing attention over past decades.

## 2. Plant primary and secondary products

Plants synthetize efficiently organic compounds via photosynthesis from inorganic materials and the pathways involved are metabolic pathways. They are primary metabolism and secondary metabolism. Carbohydrates, lipids, proteins and nucleic acids are necessary for normal growth, development, and reproduction

of plants (primary products). Besides, there is a large, diverse array of organic compounds that have no direct function in growth and development of plants. These substances are known as secondary products (secondary compounds, secondary metabolites or natural products) [3].

Secondary products are restricted distribution in the plant kingdom, that is found in only one plant species or related group of species. For many years, these compounds were thought to be simply functionless end products of metabolism or metabolic wastes. But now, secondary products have been suggested to have important ecological functions in plants. They protect plants against being eaten by herbivores and against being infected by microbial pathogens (**Figure 1**). Furthermore, they serve as attractants for pollinators, seed dispersing animals and as agents in the competition of plants [4].

Secondary metabolism is connected to primary metabolism by using intermediate products and biosynthetic enzymes derived from primary metabolism. Secondary compounds are synthesized through mevalonate, non-mevalonate (MEP (methylerythritol phosphate) shikimate and malonate pathway (**Figure 2**). These metabolisms rely on environmental conditions, physiological states and stages of plant growth, and yields are often very low.

There are many ways of classification of secondary products, but in general, they are divided into three chemically distinct groups: terpenes, phenolics, and nitrogen containing compounds.

The terpenes (terpenoids, isoprenoids) seem to be the largest class of secondary products. They are biosynthesized from acetyl-CoA – intermediates of many biological reactions. Terpens are widely used in pharmaceuticals, food and cosmetics industries. They possess antitumor, anti-inflammatory, antibacterial, antiviral, antimalarial effects, promote transdermal absorption, prevent and treat cardiovascular diseases, and have hypoglycemic activities [5].

The phenolics in plants are a chemically heterogeneous group of nearly 10,000 individual compounds. Many kinds of phenolics are used as agents of anti-aging, anti-inflammatory, antioxidant and anti-proliferative activities. They are used as therapy agents for chronic diseases, diabetes, cancers, cardiovascular diseases ... through the management of oxidative stress [6].

Alkaloids are organic compounds that contain at least one nitrogen atom at any position in the molecule, which does not include nitrogen in an amide or peptide bond. Alkaloids have a wide range of biological activities such as antiviral, antibacterial, anti-inflammatory, antitumor .... [7]. Many of these compounds possess

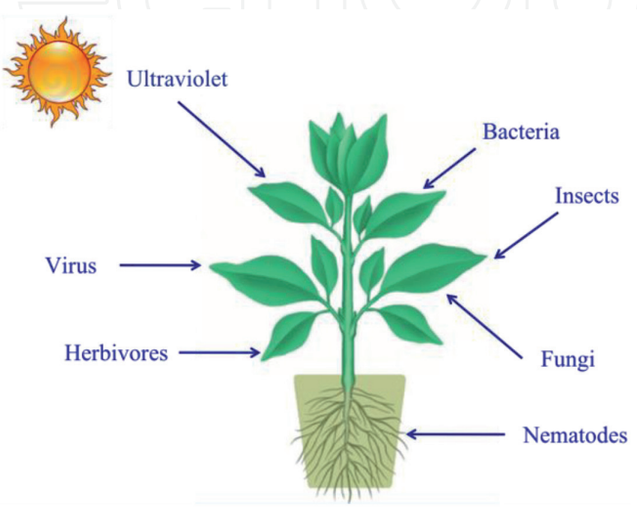


**Figure 1.**
*The effects of exogenous factors on plants.*

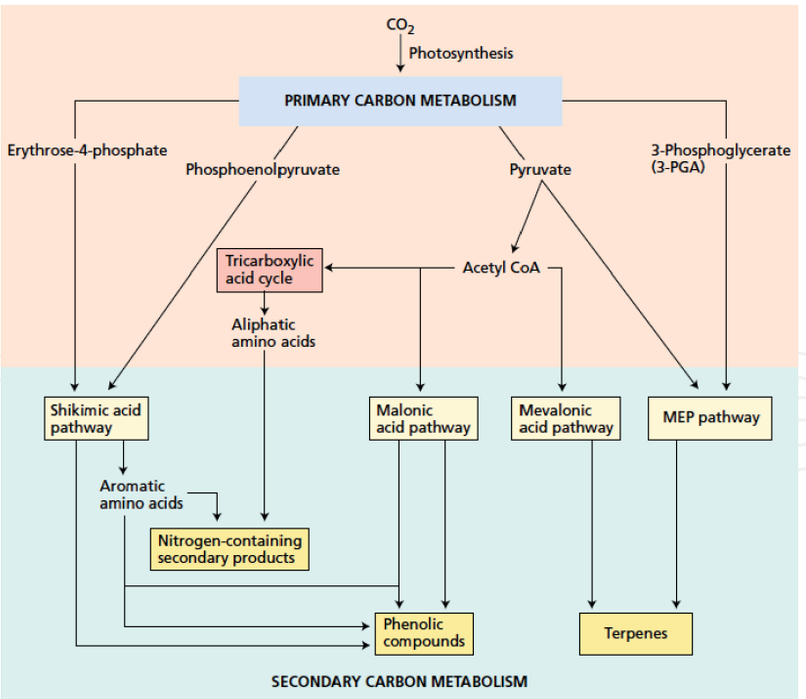


**Figure 2.**
*A simplified view of the major pathways of secondary-metabolite biosynthesis and their interrelationships with primary metabolism [4].*

potent pharmacological effects, for example, the well-known plant alkaloids include the narcotic analgesics (morphine, codeine, apomorphine (a derivative of morphine) used in Parkinson's disease, the muscle relaxant papaverine, the antimicrobial agents sanguinarine and berberine. Also several potent anti-cancer drugs have been developed from plant compounds such as vinblastine, vincristine, taxol, camptothecin, colchicine … .

## 3. Plant cell culture for secondary products

Plant cell culture techniques provide a reliable and predictable method for isolating valuable secondary products at high efficiency within a short time comparing to the whole plants *in vivo*. This provides a continuous, stable and economical production of secondary products independent of geography and climate [8].

To stabilize the raw materials for pharmaceutical industry, plant cell culture is emerging as an alternative bioproduction system. This technology offers an attractive potential to produce valuable secondary products such as ajimalicine [9], artemisinin [10], ginsenosides [11], taxol [12], resveratrol [13].

A suspension culture consists of isolated cells and cell aggregates dispersed and growing in a moving liquid medium. It used to be proved as an effective biosystem to produce valuable secondary products for commercialize. However, in most cases, for the large scale production, there are some troubles because of the instability and non-uniformity of the undifferentiated cells in liquid culture.

Adventitious root cultures show a higher constancy in the production of these compounds with more rapid growth than cell suspension cultures [14]. In addition, bioreactor system for root cultures has emerged as a technology with possible commercial applications [15]. In aseptic environment, suitable phytohormone-augmented medium is demanded for adventitious roots formation and proliferation. In another way, hairy roots (transformed roots) derived from the infection of a plant by *Agrobacterium rhizogenes* – can strongly proliferation in medium without

phytohormone, that is a promised biosystem for producing valuable secondary products in large scale [16].

### 3.1 Adventitious root cultures

Adventitious roots are roots that arises from any part of plant other than the radicles or the root axis. The formation of adventitious root needs a combination of a complicated molecular process involving numerous of endogenous and exogenous factors [17]. Adventitious roots appear in response to stress conditions, such as flooding, nutrient deprivation or wounding [18]. *In vitro*, the formation of adventitious roots responses to wounds and exogenous phytohormones, especially auxin (**Figure 3**) [19]. The induction of adventitious roots is promoted by high auxin and low cytokinin levels. There are three phase in adventitious root formation: induction, initiation and extension [20]. Auxin promotes adventitious root initiation but decreases the elongation. Root elongates when auxin concentration decreases. The application of auxins strongly increases the number of roots [21].

IBA (indol butyric acid) is most commonly used for rooting *in vivo* and *in vitro*. The other auxins used commercially are IAA (indol acetic acid) and NAA (naphthalene acetic acid) [22]. 2,4-D (2,4-Dichlorophenoxyacetic acid) is rarely used for rooting but usually used for callus initiation. The commonly cytokinins used are BAP (benzylaminopurine) and kinetin. The appropriate concentration of auxins and cytokinins in rooting depends on species, individuals and organs.

There are many scientific articles related to adventitious root cultures have been published. There are many factors that effect on rooting such as explants (type, age), exogenous phytohormones, light, organic supplements, ... The process of induction and differentiation of rooting can be controlled by changes in endogenous auxin concentrations and exogenous auxins (type and concentration) [23]. The rooting of monocotyledons usually need exogenous auxins only, but dicotyledons need auxins supplemented with cytokinins. Mineral media, source of carbon, light are also important. The requirements of nutrients and exogeneous phytohormones depend on species and physiological age of explants in initiation and proliferation phase. However, the secondary products biosynthesis phase may need a different nutritional and phytohormone requirement.

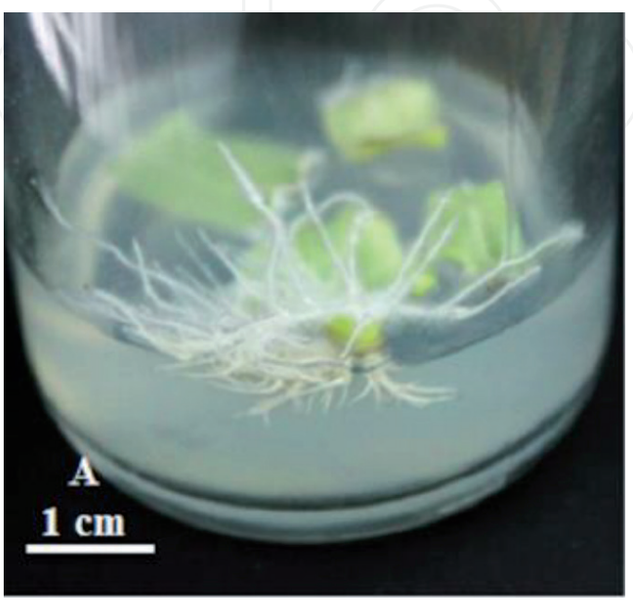


**Figure 3.**
*Adventitious roots from the wounds on Catharanthus roseus leaf explants on MS medium with 0.7 mg/L IBA.*

Adventitious roots formed from all kinds of explants of *Beta vulgaris* seedlings even on free phytohormones medium. The response of root explants with auxins was better than the others. Hypocotyl explants were more suitable than cotyledon explants in adventitious root formation. The numbers of root per explant were different with the different kinds and concentrations of auxins. NAA was suitable for the initiation of roots hypocotyls and cotyledons. Whereas, IAA at various concentrations were suitable for root induction from root explants. Roots on medium with NAA were red with many root hairs, roots with IAA treatments appeared with a thicker shape and brighter red color (**Figure 4**). However, callus could be observed in hypocotyl and cotyledon explants and shoots formed from any treatments in hypocotyl explants [24].

The advances in plant cell, tissue and organ culture have resulted in the production of high amounts of high value secondary products [25]. Due to the rapid growth and stability in secondary metabolites production, adventitious root cultures are considered as the most promising method for biomass production [26]. Root cultures show better biosynthetic ability than plant cell suspension cultures, in a suitable phytohormone supplemented medium, with stable yield of secondary products [27]. So, adventitious roots are interested in order to increase biomass *in vitro* especially medicinal plants to produce bioactive compounds. Plant roots are the main raw materials of herbal drugs (about 60% of herbal medicinal plants applied for ethnomedicine needs). As a result of which, adventitious roots cultures have the potential to be developed as a strategy for large-scale bioactive compound production [28]. Establishing adventitious roots by liquid cultures would accelerate large-scale biomass and conservation in addition to supplementing pharmaceutical products [29].

Secondary products biosynthesis *in vitro* is effected by many factors: phytohormones, carbon sources, mineral elements, light ... In liquid cultures, an important factor that effected on the growth of roots must be tested: initial inoculum density. The initial inoculum density effected on biomass and betalains accumulation of *B.vulgaris* L. roots in liquid culture. The inoculum density 3 g/L seemed be so low that did not sufficiently maintain betalains biosynthesis while 5 g/L and 7 g/L inoculum density almost showed more appropriate for root proliferation as well as betalains accumulation (**Figure 5**) [24].

The optimal condition for initiation and proliferation of adventitious roots from young *Aloe vera* leaves were 0.5 mg/L NAA and 0.2 mg/L BA in Murashige and Skoog (MS) medium. But aloe-emodin concentration was higher on B5 medium (133.08 ± 0.12 μg/g) than on MS medium (3.56 ± 0.26 μg/g) [30].

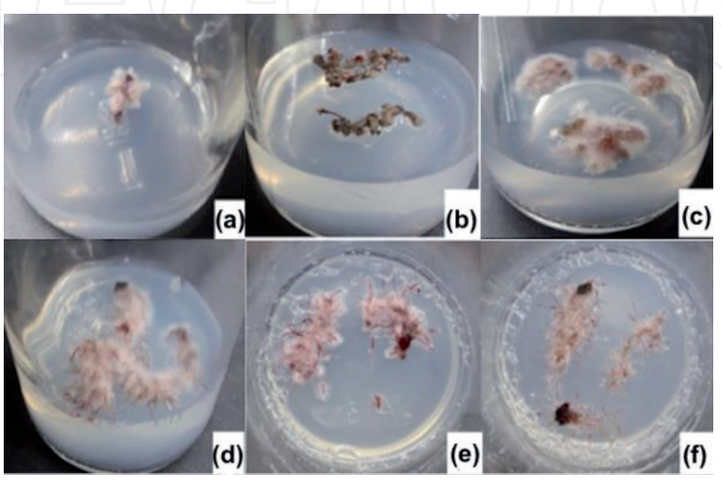


**Figure 4.**
*Adventitious roots from Beta vulgaris root explants after 3 weeks of culture on MS medium with auxins (a) NAA 0.5 mg/L; (b) NAA 1.0 mg/L; (c) NAA 2.0 mg/L; (d) IAA 0.5 mg/L; (e) IAA 1.0 mg/L; (f) IAA 2.0 mg/L.*

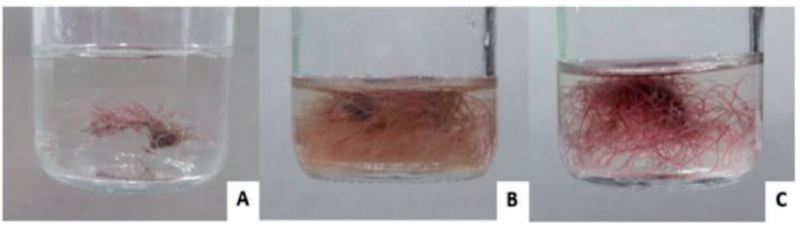


**Figure 5.**
*Beta vulgaris L. adventitious roots in liquid culture. A, B and C: Initial inoculum density at 3 g/L, 5 g/L and 7 g/L respectively.*

*Andrographis paniculata* adventitious roots were induced directly from leaf segments of on solid MS medium with 5.3 μM NAA but grew well and accumulated andrographolide in MS liquid medium with 2.7 μM NAA within four weeks. Fresh biomass increased seven-fold along with 3.5-fold higher andrographolide compared to natural plants [31].

Adventitious roots from *Morinda citrifolia* leaf explant were initiation on medium with 1.0 mg/L IBA. The highest number of roots were induced under red light, followed by blue light and lowest under far-red light. In the other hand, catalase and guaicacol peroxxidase activities were highest under red light, followed by fluorescent light and lowest under red + blue light. Moreover, superoxide dismustase activity was not influenced by light sources [32].

To enhance the production of valuable secondary products from adventitious cultures, many strategies were approached: optimization of medium and physical factors, carbon source, elicitation, precursor feeding, permeabilization and immobilization. Among them, elicitation seems to be the best solution to enhance secondary metabolites productivity in plant cell and organ cultures. Elicitor is a substance which initiates or enhances secondary biosynthesis of a living cell system when introduced in small concentration [33].

In plants, elicitor molecules attach to special receptors located on plant cell membranes. These receptors can recognize the molecular pattern of elicitors and activate intracellular defense via signal transduction pathway (**Figure 6**). The response results are enhancing the synthesis of metabolites which reduce damage and increase resistance to pest, disease or environmental stress [34]. Elicitors can be divided into two types abiotic and biotic according to basic nature. Abiotic elicitors include of substances that are of nonbiological origin, they are grouped in physical (thermal stress, salt tress, drought, osmotic stress) chemical (heavy metals, minerals salts, gaseous toxins) and hormonal (methyl jasmonate, salicylic acid) factors. Biotic elicitors are the biological origin substances of that comprise polysaccharides from

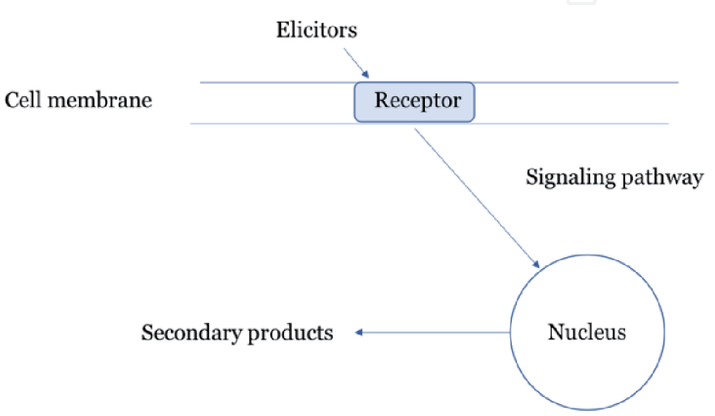


**Figure 6.**
*Model of elicitor signal transduction leading to secondary production.*

plant cell walls (e.g. chitin, pectin, and cellulose), yeast extracts, fungal or bacterial extracts, microorganisms or saliva of insects or herbivores [35]. Methyl jasmonate is a potent elicitor in plant cell, tissue and organ culture for secondary compounds [36].

The effects of elicitors on secondary productivities depend on:

- Elicitor concentration
- Duration of elicitor influencing
- Cell lines
- Time course of elicitation
- Growth stage of culture system
- Phytohormone
- Nutrient composition [37].

Many kinds of elicitor (yeast extract, methyl jasmonate, AgNO3 and sorbitol) were investigated to adventitious roots cultures of *Perovskia abrotanoides* Karel. Biomass and production of cryptotanshinone and tanshinone IIA were estimated. Elicitors had no significant effect on biomass (dry weight). The highest concentrations of cryptotanshinone and tanshinone IIA were achieved with 200 mg/l YE and 25 μM AgNO3, respectively. MJ moderately promoted tanshinone accumulation. Sorbitol was almost ineffective in enhancing tanshinone content. Cryptotanshinone formation was stimulated more significantly by elicitation than tanshinone IIA [38].

Root cultures of *Datura stramonium* were treated with copper and cadmium salts as elicitors. With the concentration at 1 mM, both $Cu^{2+}$ or $Cd^{2+}$ have been found to induce the rapid accumulation of high levels of lubimin and 3-hydroxylubimin (sesquiterpenoid). These compounds were undetectable in unelicited cultures. However, no change was seen in the alkaloid content (tropane alkaloid) of the system when treatment with $Cu^{2+}$ or $Cd^{2+}$ [39].

Adventitious roots of *Glycyrrhiza uralensis* were cultured MS liquid medium for the accumulation of secondary metabolites and salicylic acid has been used as an elicitor. The addition of 1 mg/L salicylic acid significantly enhanced the concentrations of glycyrrhizic acid (0.31 mg/g), glycyrrhetinic acid (0.14 mg/g) and polysaccharide (159.29 mg/g) in the adventitious roots and the contents were 2.58-fold, 1.27-fold, and 2.07-fold respectively over the control. Furthermore, the concentration of total flavonoid (9.40 mg/g) was observed with 2 mg/L salicylic, which was 2.68-fold higher than the control [40].

*Aspergillus niger*, *Alternaria* sp., *Fusarium monoliforme* and yeast extract were added to leaf-derived root cultures of *Datura metel* L., established on B5 medium with 1.2 μ M IAA, to study the influence of biotic elicitors on the growth and production of hyoscyamine and scopolamine. Besides, salicylic acid, $AlCl_3$, $CaCl_2$, NaCl and $Na_2SO_4$ were used as abiotic elicitors. The hyoscyamine and scopolamine concentrations were 1.39 mg/g dw and 0.069 mg/g dw, respectively in control cultures. The highest hyoscyamine (4.35 mg/g dw) and scopolamine (0.28 mg/g dw) accumulation was obtained in cultures treated with 500 μM salicylic acid. 3.17 mg/g dw hyoscyamine and 0.16 mg/g dw scopolamine were observed in treatment with 0.75 g/L yeast extract and 2.49 mg/g dw hyoscyamine and 0.11 mg/g Dw scopolamine were in treatment with 250 μM $AlCl_3$ [41]. Many kinds of elicitors were tested in adventitious root cultures. The effects depended on species and other factors (**Table 1**).

| Species | Elicitors | Secondary products | References |
|---|---|---|---|
| *Datura stramonium* | $Cu^{2+}$, $Cd^{2+}$ | Lubimin, 3-hydroxylubimin | [39] |
| *Capsicum annuum* | Cellulase | Capsidiol | [42] |
| *Datura metel* L. | Salicylic acid, yeast extract, NaCl | Hyoscyamine and scopolamine | [41] |
| *Valeriana amurensis* Smir. ex Kom | Methyl jasmonate, salicylic acid, chitosan | Valtrate | [43] |
| *Morinda citrifolia* (L.). | Chitosan | Anthraquinone, phenolics and flavonoids | [44] |
| *Aloe vera* | Salicylic acid | Aloe emodin and chrysophanol | [30] |
| *Panax ginseng* | Casein hydrolysate | Ginsenoside | [45] |
| *Perovskia abrotanoides* Karel | Yeast extract, $AgNO_3$ | Cryptotanshinone, tanshinone IIA | [38] |
| *Psoralea corylifolia* L | Methyl jasmonate | Psoralen | [46] |
| *Glycyrrhiza uralensis* | Salicylic acid | Glycyrrhizic acid glycyrrhetinic acid polysaccharide | [40] |
| *Glycyrrhiza uralensis* Fisch | Protein fragment of more than 10 kDa | Flavonoids, glycyrrhizic acid, glycyrrhetinic acid and polysaccharide | [47] |
| *Oldenlandia umbellata* L. | Pectins | Anthraquinones | [48] |
| *Gynura procumbens* (L.). Merr | Yeast extract, CuSO4 1 mg/L | Quercetin, kaempferol | [49] |
| *Talinum paniculatum* Gaertn. | Methyl jasmonate | Saponin | [50] |
| *Panax vietnamensis* Ha et Grushv. | Methyl jasmonate | Saponin | [51] |
| *Hybanthus enneaspermus* (L.) F. Muell. | Salicylic acid | L-Dopa | [52] |
| *Hypericum perforatum* | Uv-B 4°C | Hypericin | [53] |

**Table 1.**
*The application of elicitors on secondary products of adventitious root cultures.*

The regulation of metabolic processes in plants is highly dependent on carbon source, so plant cells and tissue are quite sensitive to sugar concentration in nutrient medium [54]. *In vitro* plant cells are heterotroph, although in many cases they canlive as mixotroph thanks to artificial lighting and chloroplasts. Therefore, the supplement of sugar is necessary. Saccharose is the most common sugar, which accelerates the growth of biomass, which is commonly used in the concentrations of 2 to 5%, but also depends on the purpose of culture [55].

In broccoli (*Brassica olearacea* var. *capitata)* adventitious root cultures, the proliferation of roots enhanced with the increasing of saccharose from 20 to 40 g/L and decreased with saccharose 50 g/L. The color of roots was white with saccharose 20 and 30 g/L and pale yellow with saccharose 40 and 50 g/L (**Figure 7**) [56].

The role of saccharose can be explained by the effect on tubulin, one kind of protein presents throughout the growth and development of the cell. Tubulin controls the cell shape, cell division and intracellular transport via genes *tual* and

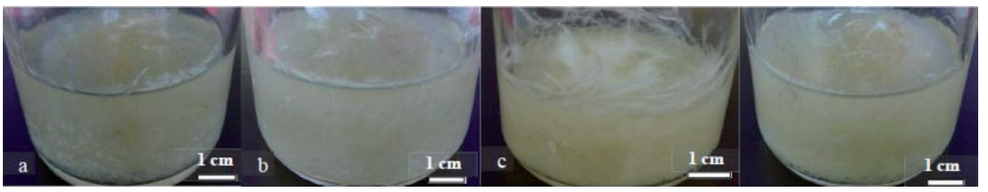


**Figure 7.**
*Adventitious roots from broccoli cotyledons in liquid MS medium with variable saccharose concentration (a) 20 g/L; (b) 30 g/L; (c) 40 g/L; (d) 50 g/L.*

*incw1*. These genes are only exhibited with the presence of saccharose [57]. When the concentration of saccharose in medium is too high, it's difficult for cell to absorb nutrients so the proliferation will decrease.

Beside the role in biomass proliferation, carbon source also effects on secondary products biosynthesis. According to Miao et al., glucose is also an inducer of glucosinolate biosynthesis. Glucosinolate biosynthesis is mediated indirectly by XK1 (hexokinase 1) and/or RGS1 (G1 protein regulatory signal) through MYB28 and MYB29 translation factors, both of them are induced by glucose. As a signaling molecule, glucose can regulate growth, development, metabolism and resistance to environmental stress of cells [58]. Glucose is released from the saccharose during autoclaving as well as by invertase which takes part to glucosinolate biosynthesis [59].

### 3.2 Hairy roots

Hairy roots derived from the infection of plant by *Agrobacterium rhizogenes*, a Gram-negative soil bacterium. Hairy roots can be obtained from a wide variety of plants and be well interested because of the ability of valuable secondary metabolites production. Hairy roots can produce and secrete complex active glycoproteins and organic compounds from a wide variety of plants. Nowaday, hairy roots have positioned as effective biological systems in pharmaceutical industry due to the development of fully controlled large-scale bioreactors [60].

*Agrobacterium* sp. are agents of disease in plants. *Agrobacterium tumefaciens* cause crown gall disease and *Agrobacterium rhizogenes* cause abnormal roots (root-mat disease) in dicotyledonous plants. Hairy roots induced by *Agrobacterium rhizogenes* are very similar to wild-type roots in structure (**Figure 8**) except some characteristics: lateral branching, root hairs are longer, more numerous,

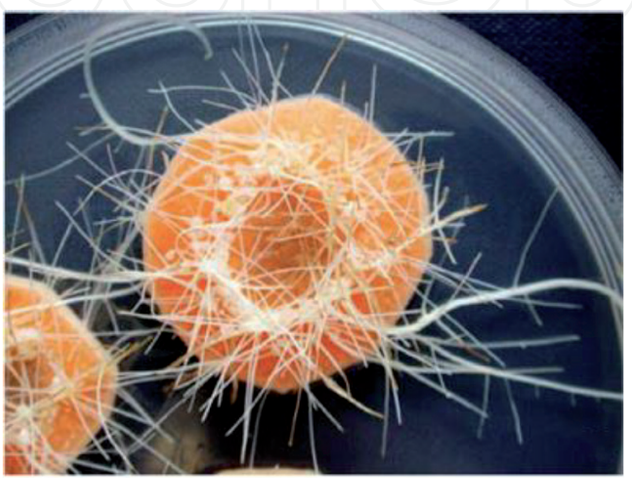

**Figure 8.**
*Carrot root discs after four weeks on mineral medium with Agrobacterium rhizogenes [61].*

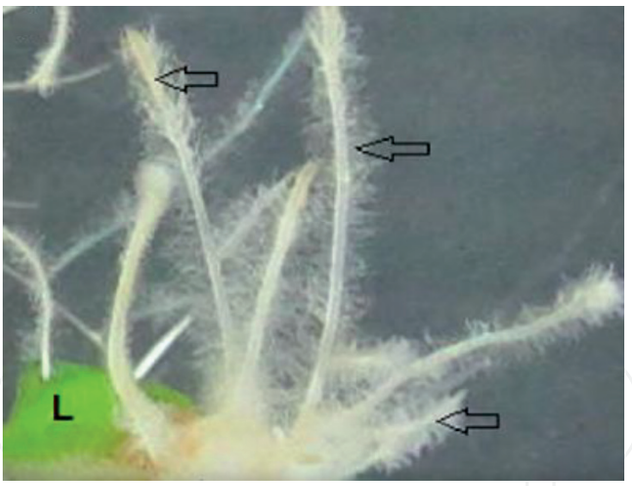


**Figure 9.**
*Transformed roots of Ocimum basilicum with many hairy roots [62].*

have an agravitrpic phenotype and genetic stability (**Figure 9**). In especially, the ability of hairy roots is growing quickly *in vitro* in the absence of exogeneous phytohormones.

*Agrobacterium* are Gram-negative soil, aerobic, rod-shaped (0.6–1.0 x 1.5–3.0 μm) bacteria, of the family *Rhizobiaceae*. They can move by 1-4 peritrichous flagella (**Figure 10**).

The mechanisms for crown gall or hairy root formation are very similar, depend on Ti-plasmid (tumor inducing plasmid) and Ri-plasmid (root inducing plasmid) respectively. In *Agrobacterium*, a portion of Ti-plasmid or Ri-plasmid, T-DNA (region bounded by 25 bp direct oligonucleotide repeats- right border and left border) is transferred to the plant cell, randomly integrated into the host genome and expressed. *Vir* genes are very important to the infection of this bacterium to the plant cell (**Figure 11**).

There are two kinds of Ri-plasmid: agropine and mannopine based on the compounds that are synthesized by the transgenic plant tissue [64]. *Agrobacterium* recognizes some signal molecules (phenolic compounds) excreted by the wound in plant and attached to it. In the Agropine, Ri-plasmids consist of two copies: left T-DNA (TL-DNA) and right T-DNA (TR-DNA), each copy is transferred independently (**Figure 12**). Encoding genes in T-DNA are bacterial origin but they can express in infected plant cells because of eukaryotic regulatory. Genes of auxins synthesis are ascribed to the TR-DNA. The right T-DNA of Ri-plasmid contains two

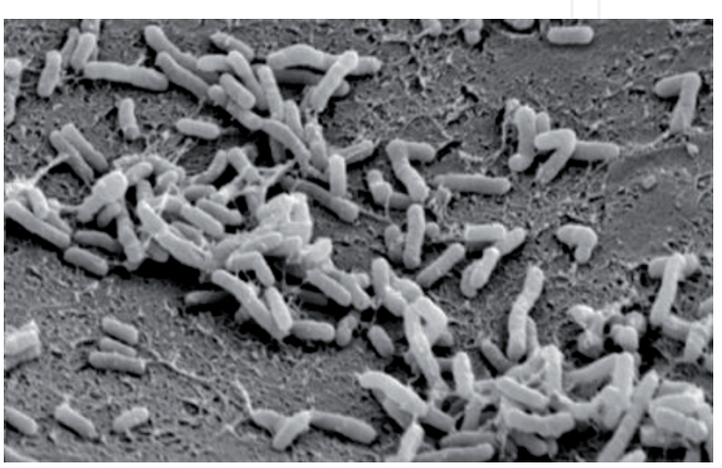

**Figure 10.**
*Agrobacterium rhizogenes [63].*

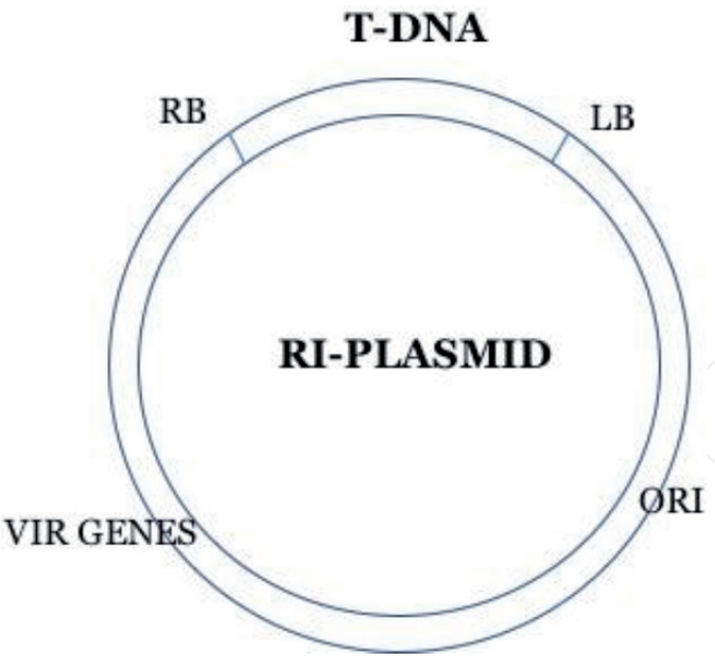


**Figure 11.**
*Ri-plasmid of Agrobacterium rhizogenes. T-DNA: transfer DNA, RB: Right T-DNA border (25 bp), LB: Left T-DNA border (25 bp), Vir genes: Virulence genes, ori: Origin of replication.*

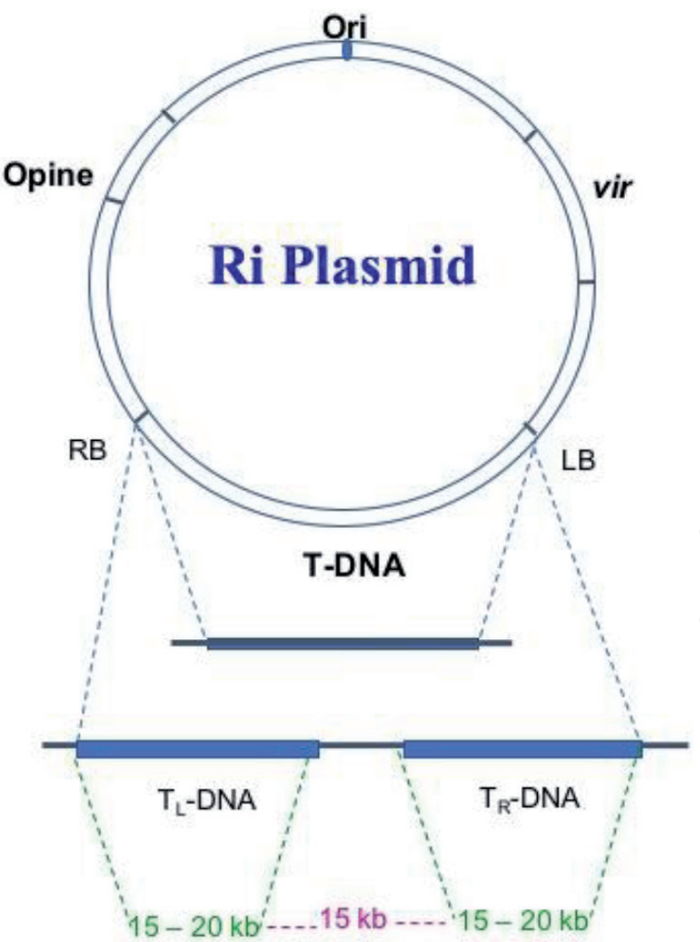


**Figure 12.**
*Ri plasmid (T-DNA with two copies: Left T-DNA and right T-DNA).*

genes in the role of auxin synthesis referred to as *tms1* and *tms2* (*aux1* and *aux2*). The TR-DNA also contains genes for agropine synthesis (*ags*). The TL-DNA has been sequenced and a total of 18 open reading frames (ORF1–ORF18) have been identified

[65]. For the formation of the hairy roots, four *rol* genes (*rolA*, *rolB*, *rolC*, and r*olD*) are very important. These genes correspond to reading frames ORF10, ORF11, ORF12 and ORF15 [66]. The products of *rol* genes have specific functions in the hairy roots formation; among them, *rolB* gene seems to be the most relevant in the induction. Also the rol-genes have a big influence on the phenotype of hairy roots [67]. RolA protein is suggested as a transcription factor that has been proposed to participate in the metabolism of gibberellins. The *rol*A gene is also reported to be responsible for changes in polyamine metabolisms. The *rolB* gene is important in the mechanism of adventitious root formation in plants. The adventitious roots induced by the *rolB* gene produce lateral roots in cell plant cultures, that indicates that the rolB protein has an effect on the formation of both lateral and adventitious roots. The *rolC* gene effects on the plant size and architecture, these include height decreasing, internode elongation, male fertility, apical dominance and increasing number of flowers. Other effects are the changes in leaf size, color and shape that increasing their ornamental value. The *RolD* is suggested to exert a positive effect on flowering by inducing a striking earliness in the flowering process and increasing the number of flowers [68].

Hairy roots grew more rapidly and produce higher levels of secondary products than the adventitious root obtained by hormonal control. One of the final goals of hairy root cultures is to produce valuable plant secondary products in large-scale bioreactors [69].

Hairy roots have different shapes depends on the *Agrobacteroum rhizogenes* strain that infected. Hairy roots were established by the infection of six different *Agrobacterium rhizogenes* strains to two varieties of *Catharanthus roseus*. Fourty seven hairy root clones were recorded. Growth rate and morphological appearance of hairy roots were wide showed (**Figure 13**) [70].

Hairy roots from root discs of *Panax ginseng* C.A. Meyer were obtained after the infection of *Agrobacterium rhizogenes* A4. Hairy roots displayed three phenotypes (three lines): the first lines showed the characteristic traits of hairy roots (HR-M), the second were callus-like (C-M) and the third were thin, without branching (T-M) (**Figure 14**). HR-M and C-M root phenotypes presented the highest biomass. The highest ginsenoside production was achieved by HR-M root lines, followed by C-M and the lowest yield was found from T-M root phenotype [71].

Hairy roots were induced from *Rhaponticum carthamoides* leaf explants by the transformation of *Agrobacterium rhizogenes* strains A4 and ATCC 15834. A4 strain was more appropriate than ATCC 15834 in the formation of transformed roots. Hairy roots systems were established in liquid media (WPM, B5, SH) with full and

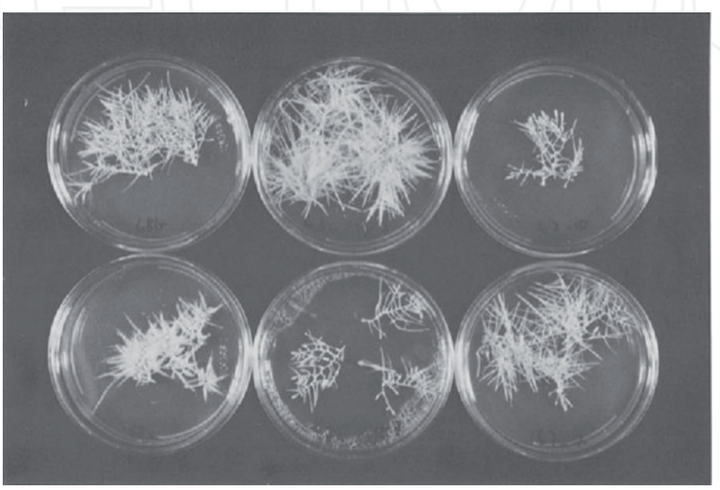

**Figure 13.**
*Hairy root cultures of Catharanthus roseus showing the diversity in the growth between different clones derived from the same variety.*

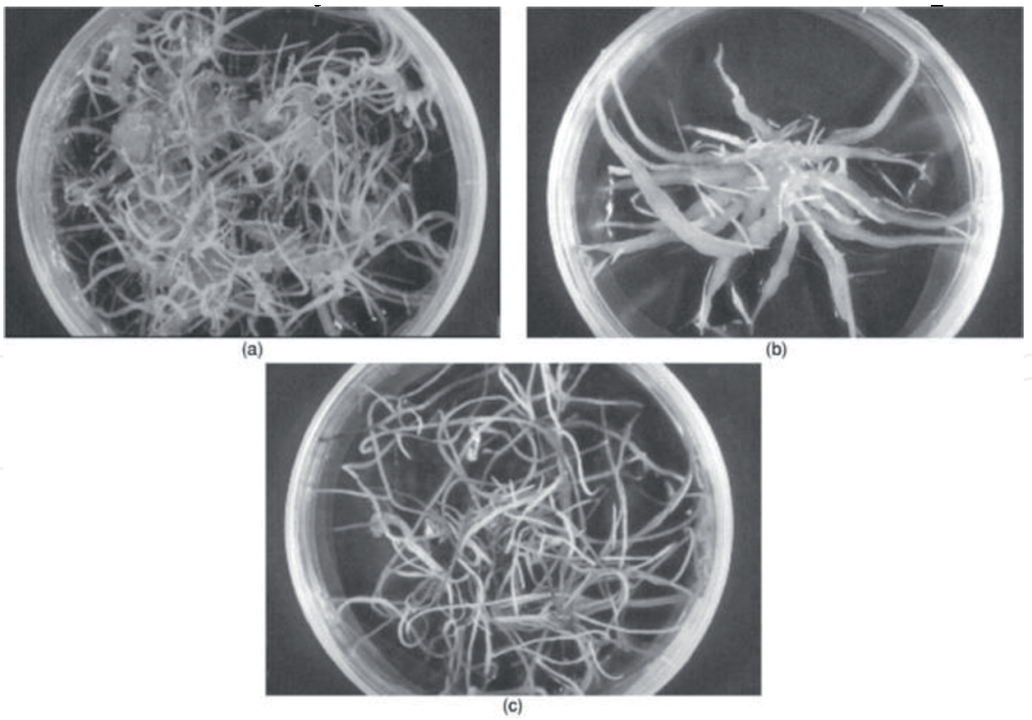


**Figure 14.**
*Three phenotypes of Panax ginseng C. (a). Meyer hairy roots. Hairy root morphology (HR-M), (b) callus morphology (C-M), (c) thin morphology (T-M).*

half-strength concentrations of macro- and micronutrients. Two different lighting conditions (light or dark) were tested on the biomass of hairy root line (RC3). The highest biomass was obtained in WPM medium under periodic light. The content of caffeoylquinic acid and their derivatives was raised in hairy roots grown in the light. Besides, the biosynthesis of flavonoid glycosides such as quercetagetin, quercetin, luteolin, and patuletin hexosides was detectedin the light. Chlorogenic acid, 3,5-di-*O*-caffeoylquinic acid and tricaffeoylquinic acid derivative were found as the major compounds present in the transformed roots [72].

Hairy roots from petiols of *Isatis tinctoria* L were induced by *Agrobacterium rhizogenes* strain LBA9402 to investigate eight bioactive flavonoid constituents (rutin, neohesperidin, buddleoside, liquiritigenin, quercetin, isorhamnetin, kaempferol and isoliquiritigenin). Many basal salt media were used (Chu (N6), Nitsch & Nitsch (NN), Gamborg (B5), Schenk & Hildebrandt (SH), White, (Murashige & Skoog) MS and ½ MS) for the biomass and flavonoid accumulation. Other factors were studied such as: carbohydrate sources and initial pH. ½ MS medium, 3% sucrose and pH 5.8 were suitable for either biomass or flavonoid accumulation as the results. The total flavonoid concentration after 24 days of culture (438.10 μg/g DW) was higher than 2 year-old natural plants (341.73 μg/g DW) [73].

The efficiency of transformation depends on many factors: type and age of explant, the strain, density and growth stage of *Agrobacterium rhizogenes*, aceto-syringone concentration, the pre-culture time, the infection time...

Plant secondary production by hairy roots process:

1. Hairy roots induction and proliferation.

2. Hairy roots in liquid phase: nutrient medium optimization, several strategies can be used to improve the yields of target compounds.

3. Bioreactor stage: batch / fedbatch or continuous culture. Optimization airflow rate, temperature, pH....

To improve the yield of valuable secondary products in hairy root cultures, elicitation seems to be the most effective strategy. Hairy root cultures are preferred for the application of elicitation because of their stable genetics and biosynthesis and high growth rate in non-phytohormone medium. Elicitors act as signals that were recognized by elicitor-specific receptors on the plant cell membrane and stimulate defense responses during elicitation. The results are the increasing of synthesis and accumulation of secondary metabolites. The effects of elicitation depend on elicitor type, concentration, duration of exposure and treatment schedule (**Table 2**).

*Panax ginseng* C.A. Meyer hairy roots from roots, stems, and leaves induced by the infection of *Agrobacterium rhizogenes* (KCTC 2703) were propagated in 5-liter cone type bubble bioreactors containing MS media supplemented with 2.0 mg/L NAA and 30 mg/L sucrose. Jasmonic acid in various concentrations was added to the culture system after 30 days of culture to increase ginsenoside concentration. Total ginsenoside concentration increased with the increasing of jasmonic acid concentration, but the root growth was inhibited with high concentration. Total productivity was greatest at 2.0 mg/L jasmonic acid but there was the difference in groups of ginsenoside. Ginsenosides in the Rb group mainly increased, while those in the Rg group did not. High concentrations (5 and 10 mg/L) of jasmonic acid decreased Rg1 content but significantly increased the Rb1. In the Rb group, the Rb1 content increased more than Rb2, Rc, and Rd. [88].

| Species | Elicitors | Secondary products | References |
|---|---|---|---|
| *Azadirachta indica* A. Juss | Jasmonic acid, Salicylic acid | Azadirachtin | [74] |
| *Silybum marianum* (L.) Gaertn. | $Ag^+$ | Silymarin | [75] |
| *Plumbago indica* | Jasmonic acid | Plumbagin | [76] |
| *Glycyrrhiza inflata* | Chitosa<br>Methyl jasmonate, Yeast extract | Glycyrrhizin | [77] |
| *Artemisia annua* L. | Methyl jasmonate, fungal elicitors (*Alternaria alternate, Curvularia limata, Fusarium solani*, and *Piriformospora indica*) | Artemisinin | [78] |
| *Valeriana officinalis* L | $CaCl_2$ | Valerenic acid | [79] |
| *Salvia miltiorrhiza* | Salicylic acid | Tanshinone | [80] |
| *Astragalus membranaceus* | Methyl jasmonate | Isoflavonoid | [81] |
| *Rauwolfia serpentina* and *Solanum khasianum* | NaCl, cellulase from *Aspergilus* and mannan from *Saccharomyces cerevisiae* | Ajmaline, solasodine and α-solanine | [82] |
| *Psoralea corylifolia* | *Methyl jasmonate* | Daidzin | [83] |
| *Datura metel* | *B. cereus* and *S. aureus* | Scopolamine | [84] |
| *Panax quinquefolium* | Yeast extract | Ginsenosides | [85] |
| *Ocimum tenuiflorum* L | Yeast extract, Methyl jasmonate, Salicylic acid | Ursolic acid and eugenol | [86] |
| *Scutellaria bornmuelleri* | Methyl jasmonate + chitosan | Chrysin, wogonin and baicalein | [87] |

**Table 2.**
*The application of elicitors on secondary products of hairy root cultures.*

In another experiment, peptone and jasmonic acid were used as elicitors to promote ginsenosides accumulation in *Panax ginseng* C.A. Meyer hairy roots induced by the infection of *Agrobacterium rhizogenes* (KCTC 2703) to root explants. Root system was cultured in phytohormone-free Murashige and Skoog liquid medium. Jasmonic acid in the range 1.0–5.0 mg/L strongly improved total ginsenoside production. Peptone (300 mg/L) showed good effects on ginsenoside concentration but weaker than that of jasmonic acid. The Rb group of ginsenoside content was increased remarkably by jasmonic acid, while Rg group ginsenoside content changed slightly compared to controls. However, jasmonic acid also strongly inhibited hairy root growth [89].

Node explants of *Vitis vinifera subsp. sylvestris* were used as materials for the hairy root induction by *Agrobacterium rhizogenes* ATCC 15834. Hairy roots were immerged in ½ B5 medium without phytohormone. Methyl jasmonate and other elicitors were used to enhance resveratrol biosynthesis of hairy roots. The result showed that the resveratrol production of hairy roots was higher than natural roots. Especially, the production of resveratrol increased with the present of elicitors. There was a significant difference in inducing resveratrol production between the elicitors. The treatment with 3 mM acetic acid led to the highest resveratrol content and methyl jasmonate seemed to be less effective than the others [90].

## 4. Conclusion

Adventitious roots and hairy roots are promising materials for the production of valuable secondary compounds of plants which are used in pharmaceutical, food and cosmetic industry. The chemical characteristics of these compounds are the same as that in natural plants but the yields are proved higher. Furthermore, there are many investigations which focused on improving bioreactor for root cultures to raise their quality and productivity.

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## References

[1] Farnsworth N.R. and Morris R.W., 1976. Higher plants-the sleeping giant of drug development. *American Journal of Pharmacy and the Sciences Supporting Public Health*, 148(2):46-52.

[2] Xiao-Tian and Fang, 2006. *Medicinal chemistry of bioactive natural products*. John Wiley & Sons, Inc.

[3] Paul M. Dewick, 2009. *Medicinal natural products – A biosynthetic approach*, 3rd edition, A John Wiley and Sons, Ltd.

[4] Taiz L. and Zeiger E., 2002. *Plant Physiology*, 3rd edition. Sinauer Associates.

[5] Wenqiang Y., Xu C., Yanli L,, Shaofen G., Zhen W. and Xiuling Y., 2020. Advances in pharmacological activities of terpenoids. *Natural Product Communications,* 15(3): 1-13.

[6] Lin D., M. Xiao, J. Zhao, Z. Li, B. Xing, Xi. Li, M. Kong, L. Li, Q. Zhang, Y. Liu, H. Chen, W. Qin, H. Wu and S. Chen, 2016. An overview of plant phenolic compounds and their importance in human nutrition and management of type 2 diabetes. *Molecules*, *21*: 1374.

[7] Noureddine Bribi, 2018. Pharmacological activity of alkaloids: A review. *Asian Journal of Botany,* 1.

[8] Sandra Goncalves and Anabela Romano, 2018. Production of plant secondary metabolites by using biotechnological tools - *Secondary Metabolites - Sources and Applications.* IntechOpen.

[9] Ten Hoopen H.J.G., Vinke J.L,, Moreno P.R.H., 2002. Influence of temperature on growth and ajmalicine production by *Catharanthus roseus* cell suspension cultures. *Enzyme Microb Tech*, 30: 56-65.

[10] Baldi A. and Dixit V.K., 2008. Yield enhancement strategies for artemisinin production by suspension cultures of *Artemisia annua*. Bioresource Technol,99: 4609-4014.

[11] Jeong C., Murthy H.N., Hahn E. and Paek K., 2008. Improved production of ginsenosides in suspension cultures of Ginseng by medium replenish ment strategy. *J Biosci Bioeng*, 105: 288-291.

[12] Li S. W., Xue L., Xu S., Feng H., An L., 2009. Mediators, genes and signaling in adventitious rooting. *Bot Rev.,* 75(2): 230-247.

[13] Cai Z., Knorr D. and Smetanska I., 2012. Enhanced anthocyanins and resveratrol production in *Vitis vinifera* cell suspension culture by indanoyl-isoleucine, N-linolenoyl-l-glutamine and insect sativa. *Enzyme Microb Tech*, 50: 29-34.

[14] Sivakumar G., 2006. Bioreactor technology: a novel industrial tool for high-tech production of bioactive molecules and biopharmaceuticals from plant root. *Biotechnol J* 12: 1419-1427.

[15] Paek K.Y., Murthy H.N., Hahn E.J. and Zhong J.J., 2009. Large scale culture of ginseng adventitious roots for production of ginsenosides. *Adv Biochem Eng Biotechnol* 113:151-176.

[16] Laura Pistelli, Annalisa Giovannini, Barbara Ruffoni, Alessandra Bertoli and Luisa Pistelli, 2010. Hairy root cultures for secondary metabolites production, *Bio-Farms for Nutraceuticals: Functional food and safety control by biosensors.* Landes Bioscience and Springer Science + Business – Media.

[17] Sorin C., Bussell J.D., Camus I. et al., 2005. Auxin and light control of adventitious rooting in *Arabidopsis* required Argonaute1. *The Plant Cell* 17: 1343-1359.

[18] Bianka St. and Amanda R., 2016. The physiology of adventitious roots. *Plant Physiology*, 170: 603-617.

[19] Vo Thanh Phuc, Nguyen Minh Trung, Huynh Tri Thien and Le Thi Thuy Tien. Proliferation and Ajmalicine Biosynthesis of *Catharanthus roseus* (L). G. Don Adventitious roots in self-built temporary immersion system. *4th International Conference on Chemical Engineering, Food and Biotechnology, ICCFB 2017*, *Vietnam, Malaysia.*

[20] Li Y. and Tao W. 2009. Paclitaxel producing fungal endophyte stimulates the accumulation of taxoids in cell suspension cultures of *Taxus cuspidata*. *Sci Hortic-Amsterdam*, 121: 97-102.

[21] Liu, J. H. and Reid, D. M., 1992. Adventitious rooting in hypocotyls of sunflower (*Helianthus annuus*) seedlings. IV. The role of changes in endogenous free and conjugated indole-3-acetic acid. *Physiol. Plant*. 86: 285-292.

[22] Geert-Jan De Klerk, Wim Van Der Krieken and Joke C. De Jong, 1999. Review: The formation of adventitious roots: new concepts, new possibilities. *In vitro cell. Dev. Biol. Plant* 35: 189-199.

[23] Praveen N., Manohar S.H., Naik P.M., Nayeem A., Jeong J.H. and Murthy H.N., 2009. Production of androghrapholide from adventitious root cultures of *Andrographis paniculata*. *Curr Sci* 96: 5-10.

[24] Thuy Tien T. Le, Cam Tu H. Nguyen, and Minh V. Tran. The induction of *Beta vulgaris* L. adventitious roots in *in vitro* culture for betalains, 2019. 4th International *Conference on Chemical Engineering, Food and Biotechnology, ICCFB Vietnam, Malaysia.*

[25] Gantet P., Imbanlt N., Thiersault M., Doireau M., 1998. Necessary of a functional octadecanoic pathway for indole alkaloid synthesis by *Catharanthus roseus* cell suspensions cultured in an auxinstarved medium. *Plant Cell Physiol* 39: 220-225.

[26] Carvalho E., Curtis W.R., 1998. Characterization of fluid-flow resistance in root cultures with a convective flow tubular bioreactor. *Biotech Bioeng* 60: 375-384.

[27] Kevers C., Jacques Ph., Thonart Ph. and Gaspar Th., 1999. *In vitro* root culture of *Panax ginseng* and *Panax quinquefolium*. *Plant Growth Regul* 27: 173-178.

[28] Sudha C.G. and Seeni S., 2001. Establishment and analysis of fast-grown normal root culture of *Decalepis arayalpathra*, a rare endemic medicinal plant. *Curr Sci* 81: 371-374.

[29] Baskaran P., Chukwujekwu J.C., Amoo S.O. and Van Staden J., 2014. Anticholinesterase and mutagenic evaluation of in vitro regenerated *Agapanthus praecox* grown ex vitro. *In Vitro Cell. Dev. Biol. Plant* 50: 271-275.

[30] Lee Y. S., Yang T. J., Park S. U., Baek J. H., Wu S. Q. and Lim K.B., 2011. Induction and proliferation of adventitious roots from *Aloe vera* leaf tissues for *in vitro* production of aloe-emodin. *Plant Omics* 4(4): 190-194.

[31] Praveen N., Manohar S.H., Naik P.M., Nayeem A., Jeong J.H., Murthy H.N., 2009. Production of andrographolide from adventitious root cultures of *Andrographis paniculata*. *Curr Sci* 96: 694-697.

[32] Baque M. A., Hahn E. J. and Paek K. Y., 2010. Induction mechanism of adventitious root from leaf explants of *Morinda citrifolia* as affected by auxin and light quality. *In Vitro Cell Dev Biol Plant* 46: 71-80.

[33] Sarfaraj Hussain, Sheeba Fareed, Saba Ansari, Akhlaquer Rahman, Iffat Zareen Ahmad and Mohd Saeed, 2012.

Current approaches toward production of secondary plant metabolites. *J Pharm Bioallied Sci*. 4(1): 10-20.

[34] Bektas Yasemin and Eulgem Thomas, 2015. Synthetic plant defense elicitors. *Frontiers in Plant Science*, 5: 804.

[35] Poornananda M. Naik and Jameel M. Al–Khayri, 2016. Abiotic and biotic elicitors–role in secondary metabolites production through *in vitro* culture of medicinal plants. Abiotic and biotic stress in plants - *Recent advances and future perspectives*. Intech Open.

[36] Repka Vladimir, Fischerová and K. Šilhárová, 2004. Methyl jasmonate is a potent elicitor of multiple defense responses in grapevine leaves and cell suspension cultures. *Biologia Plantarum* 48(2): 273-283.

[37] Patel Heena and Ramar Krishnamurthy, 2013. Elicitors in plant tissue culture. *Journal of Pharmacognosy and Phytochemistry* 2(2): 60-65.

[38] Zaker Arehzoo, Christina Sykora, Florian Gössnitzer, Seyed Hadi Mousavi, Javad Asili, Parvaneh Abrishamchi and Christoph Wawrosch, 2015. Effects of some elicitors on tanshinone production in adventitious root cultures of *Perovskia abrotanoides* Karel. *Industrial Crops and Products*, 67, 97-102

[39] Judith M. Furze, Michael J. C. Rhodes, Adrian J. Parr, Richard J. Robins, Ian M. Withehead and David R. Threlfall, 1991. Abiotic factors elicit sesquiterpenoid phytoalexin production but not alkaloid production in transformed root cultures of *Datura stramonium*. *Plant Cell Reports,* 10: 111-114.

[40] Li Jing, Juan Wang, Jinxin Li, Jianli Li, Shujie Liu and Wenyuan Gao, 2016. Salicylic acid induces the change in the adventitious root of *Glycyrrhiza uralensis* Fisch.: bioactive compounds and antioxidant enzymes. *Research on Chemical Intermediates* 42: 1503-1519.

[41] Ajungla L., Patil P. P., Rajkumar B. Barmukh and Nikam Td., 2009. Influence of biotic and abiotic elicitors on accumulation of hyoscyamine and scopolamine in root cultures of *Datura metel* L. *Indian Journal of Biotechnology* 8(3).

[42] Martha Patricia Chávez-Moctezuma and Edmundo Lozoya-Gloria, 1996. Biosynthesis of the sesquiterpenic phytoalexin capsidiol in elicited root cultures of chili pepper (*Capsicum annuum*). Plant Cell Reports 15(5): 360-366.

[43] Cui Lei, Zhen-Yu Wang and Xiu-Hua Zhou, 2012. Optimization of elicitors and precursors to enhance valtrate production in adventitious roots of *Valeriana amurensis* Smir. ex Kom. *Plant Cell Tissue and Organ Culture* 108(3): 411-420.

[44] Baque A., Shiragi H.K, Lee Eun J. and Paek Kee Y., 2012. Elicitor effect of chitosan and pectin on the biosynthesis of anthraquinones, phenolics and flavonoids in adventitious root suspension cultures of *Morinda citrifolia* (L.). *Australian Journal of Crop Science* 6(9): 1349-1355.

[45] Petr Marsik, Lenka Langhansova, Marcela Dvorakova, Petr Cigler, Michal Hruby and Tomas Vanek, 2014. Increased ginsenosides production by elicitation of *in vitro* cultivated *Panax ginseng* adventitious roots. *Med Aromat Plants*, 3: 1.

[46] Siva Govindarajan, Sivakuma Subiramani, Prem Kumar G., Thiruppathi Senthil Kumar and Jayabalan Narayanasamy, 2015. Enhanced production of Psoralen through elicitors treatment in adventitious root culture of *Psoralea corylifolia* L. *International Journal of Pharmacy and Pharmaceutical Sciences* 7(1): 146-149. January 2015.

[47] Li Jianli, Juan Wang, Jing Li, Jinxin Li, Shujie Liu, Wenyuan Gao,

2016. Protein elicitor isolated from *Escherichia coli* induced bioactive compound biosynthesis as well as gene expression in *Glycyrrhiza uralensis* Fisch adventitious roots. *RSC Advances* 6: 112.

[48] Saranya Krishnan S.R. and Siril E.A., 2018. Elicitor mediated adventitious root culture for the large-scale production of anthraquinones from *Oldenlandia umbellata* L. *Industrial Crops and Products,* 114: 173-179.

[49] **Faizah Hanik, Mulyadi Tanjung, Hery Purnobasuki** and **Yosephine Sri Wulan Manuhara, 2018.** Biomass and flavonoid production of *Gynura procumbens* (L.). Merr. adventitious root culture in baloon-type bubble-bioreactor influenced by elicitation. *Asian Journal of Plant Sciences* 17(2): 107-119.

[50] Ahmad Faizal and Anggita Veningtia Sari, 2019. Enhancement of saponin accumulation in adventitious root culture of Javanese ginseng (*Talinum paniculatum* Gaertn.) through methyl jasmonate and salicylic acid elicitation. *African journal of biotechnology*, 8(6): 130-135.

[51] Nguyen Thi Nhat Linh, Le Kim Cuong, Ho Thanh Tam, Hoang Thanh Tung, Vu Quoc Luan, Vu Thi Hien, Nguyen Hoang Loc and Duong Tan Nhut, 2019. Improvement of bioactive saponin accumulation in adventitious root cultures of *Panax vietnamensis* via culture periods and elicitation. *Plant Cell, Tissue and Organ Culture*. 137: 101-113.

[52] Sathish Selvam, Venkatachalam Vasudevan, Sivabalan Karthik, Dhandapan Elayaraja, Gadamchetty Pavan, Chandrasekaran Ajithan and Markandan Manickavasagam, 2020. Elicitors induced L-Dopa accumulation in adventitious root cultures of *Hybanthus enneaspermus* (L.) F. Muell. *Vegetos* 33: 304-312.

[53] Farahnaz Tavakoli, Mohammad Rafieiolhossaini, Rudabeh Ravash & Morteza Ebrahimi, 2020. Subject: UV-B radiation and low temperature promoted hypericin biosynthesis in adventitious root culture of *Hypericum perforatum*. *Plant Signaling & Behavior*, 15: 7.

[54] Elena L. et al., 2001. Why and how do plant cells sense sugars? *Annals of Botany* 88: 803-812.

[55] Yong-Ling R., 2012. Signaling role of sucrose metabolism in development. *Shanghai Molecular Plant* 5(4): 763-765.

[56] Nguyen Minh Nhut and Le Thi Thuy Tien, 2017. Proliferation and glucosinolates accumulation of broccoli adventitious roots in liquid medium. *AIP Conference Proceedings.*

[57] Datta R. and Chourey P. S., 2001. Sugar regulated control of tubulin in maize culture. *Plant Cell Rep*. 20 (3): 262-266.

[58] Miao H. et al., 2013. Glucose signalling positively regulates aliphatic glucosinolate biosynthesis. *Journal of Experimental Botany*, 64 (4): 1097-1109.

[59] Ramon M., Filip Rolland and Jen Shen, 2008. Sugar sensing and signaling. *The Arabidopsis book*. American society of plant biologists.

[60] Le Flem-Bonhomme V., Laurain-Mattar D. and Fliniaux M.A., 2004. Hairy root induction of *Papaver somniferum* var. *album*, a difficult to transform plant, by *Agrobacterium rhizogenes* LBA 9402. *Planta* 218: 890-893.

[61] Veena Veena and Christopher G. Taylor., 2007. *Agrobacterium rhizogenes*: recent developments and promising applications. *In Vitro Cellular & Developmental Biology – Plant*, 43: 383-403.

[62] Shivani Srivastava, Xavier A. Conlan, Alok Adholeya & David M. Cahill, 2016. Elite hairy roots of *Ocimum basilicum* as a new source of rosmarinic

acid and antioxidants. *Plant Cell, Tissue and Organ Culture,* 126: 19-32.

[63] Gelvin Stanton B., 2018. Agrobacterium Biology - From Basic Science to Biotechnology – *Current topics in Microbiology and Immunology*. Springer, 2018.

[64] Frank F. White, Brian H. Taylor, Gary A. Huffman, Milton P. Gordon and Eugene W. Nester, 1985. Molecular and genetic analysis of the transferred DNA regions of the root-inducing plasmid of *Agrobacterium rhizogenes*. *Journal of Bacteriology*, 164(1): 33-44.

[65] Vilas P. Sinkar, Frank F. White, Ian J. Furner, Mitchell Abrahamsen, Francois Pythoud and Milton P. Gordon, 1988. Reversion of aberrant plants transformed with *Agrobacterium rhizogenes* is associated with the transcriptional inactivation of the TL-DNA genes. *Plant Physiol.* 86: 584-590.

[66] White, F. F., Garfinkel, D. J., Huffman, G. A., et al., 1983. Sequence homologous to *Agrobacterium rhizogenes* T_DNA in the genome of uninfected plants. *Nature*, 301: 348-350.

[67] Sevón, N., Dräger, B., Hiltunen, R., and Oksman-Caldentey, K.-M., 1997. Characterization of transgenic plants derived from hairy roots of *Hyoscyamus muticus*. *Plant Cell Rep*. 16: 605-611.

[68] Pistelli L., A. Giovannini, B. Ruffoni, A. Bertoli and L. Pistelli, 2010. Hairy root cultures for secondary metabolites production. *Adv Exp Med Biol*: 698:167-184.

[69] Noemi Gutierrez-Valdes, Suvi T. Häkkinen, Camille Lemasson, Marina Guillet, Kirsi-Marja Oksman-Caldentey, Anneli Ritala and Florian Cardon, 2020. Hairy root cultures—A versatile tool with multiple applications. *Frontiers in Plant Science*, 11(33): 1-11.

[70] Toivonen L., Balsevich J. and Kurz W., 1989. Indole alkaloid production by hairy root cultures of *Catharanthus roseus*. *Plant Cell, Tissue and Organ Culture* 18: 79-93.

[71] Mallol Anna, Rosa M. Cusidó, Javier Palazón, Merce Bonfill, Carmen Morales and *M. Teresa* Piñol, 2001. Ginsenoside production in different phenotypes of *Panax ginseng* transformed roots. *Phytochemistry* 57(3): 365-371.

[72] Ewa SkaBa, Agnieszka Kicel, Monika A. Olszewska, Anna K. Kiss and Halina WysokiNska, 2015. Establishment of hairy root cultures of *Rhaponticum carthamoides* (Willd.) Iljin for the production of biomass and caffeic acid derivatives. *BioMed Research International*, 2015: 1-11.

[73] Gai Q.Y., Jiao J., Luo M., Wei Z.F., Zu Y.G. and Ma W., 2015. Establishment of hairy root cultures by *Agrobacterium rhizogenes* mediated transformation of *Isatis tinctoria* L. for the efficient production of flavonoids and evaluation of antioxidant activities. PLoS ONE 10(3): e0119022

[74] Ramesh K S., Devanand P. F. and Susan E., 2007. Enhanced production of azadirachtin by hairy root cultures of *Azadirachta indica* A. Juss by elicitation and media optimization. *Journal of Biotechnology* 128(2):281-289.

[75] Masoumeh K., Tahereh H. and Seyyed K.K.T., 2010. $Ag^{+}$ enhanced silymarin production in hairy root cultures of *Silybum marianum* (L.) Gaertn. *Plant Omics Journal* 3(4):109.

[76] Gangopadhyay, M., Dewanjee, S., and Bhattacharya, S. (2011). Enhanced plumbagin production in elicited *Plumbago indica* hairy root cultures. *J. Biosci. Bioeng.* 111: 706-710.

[77] Putalun W., Wongwicha W., Tanaka H. and Shoyama Y., 2011. Enhanced glycyrrhizin production in *Glycyrrhiza inflata* hairy roots cultures using elicitation. *Planta Medica* 77(12).

[78] Ahlawat S., Saxena P., Alam P., Wajid S. & Abdin M. Z., 2014. Modulation of artemisinin biosynthesis by elicitors, inhibitor, and precursor in hairy root cultures of *Artemisia annua* L. *Journal of Plant Interactions*, 9(1): 811-824.

[79] Mohammad R. D. T., Naser A., Morad J. and Afsaneh S., 2014. Elicitation of valerenic acid in the hairy root cultures of *Valeriana officinalis* L (Valerianaceae). *Tropical Journal of Pharmaceutical Research* 13 (6): 943-949.

[80] Hao, X., Shi, M., Cui, L., Xu, C., Zhang, Y., and Kai, G. (2015). Effects of methyl jasmonate and salicylic acid on tanshinone production and biosynthetic gene expression in transgenic *Salvia miltiorrhiza* hairy roots. *Biotechnol. Appl. Biochem*. 62, 24-31.

[81] Gai, Q. Y., Jiao, J., Luo, M., Wang, W., Gu, C. B., Fu, Y. J., et al. (2016). Tremendous enhancements of isoflavonoid biosynthesis, associated gene expression and antioxidant capacity in *Astragalus membranaceus* hairy root cultures elicited by methyl jasmonate. *Process Biochem*. 51, 642-649.

[82] Mrinalini S., Swati S. and Pratibha M., 2016. Elicitation based enhancement of secondary metabolites in *Rauwolfia serpentina* and *Solanum khasianum* hairy root cultures. *Pharmacognosy Magazine*, 12(46): 315-320.

[83] Zaheer, M., Reddy, V. D., and Giri, C. C. (2016). Enhanced daidzin production from jasmonic and acetyl salicylic acid elicited hairy root cultures of *Psoralea corylifolia* L. (Fabaceae). *Nat. Prod. Res*. 30: 1542-1547.

[84] Zahra S., Mehrnaz K. and Mustafa G., 2017. Biotic elicitation for scopolamine production by hairy root cultures of *Datura metel*. *Mol Biol Res Commun*. 6(4): 169-179.

[85] Ewa K., Piotr S., Łukasz K. , Anna L. and Grażyna S., 2017. Yeast extract stimulates ginsenoside production in hairy root cultures of American Ginseng cultivated in shake flasks and nutrient sprinkle bioreactors *Molecules 22*(6), 880.

[86] Sharan S., Sarin N.B. and Mukhopadhyay K., 2019. Elicitor-mediated enhanced accumulation of ursolic acid and eugenol in hairy root cultures of *Ocimum tenuiflorum* L. is age, dose, and duration dependent. *South African Journal of Botany*, 124: 199-210.

[87] Zahra G., Khadijeh B., Hossein D. and Ali S., 2020. Enhanced flavonoid production in hairy root cultures of *Scutellaria bornmuelleri* by elicitor induced over-expression of *MYB7* and *FNSΠ2* genes. *Plant Physiology and Biochemistry*, 148: 35-44.

[88] Yu K.W., Hahn E.J. and Paek K.Y., 2003. Ginsenoside production by hairy root cultures of *Panax ginseng* C.A. Meyer in bioreactors. *Acta horticulturae* 597: 237-243.

[89] Yu Kee-Won, Wen-Yuan Gao, Sung-Ho Son and Kee-Yoeup Paek, 2000. Improvement of ginsenoside production by jasmonic acid and some other elicitors in hairy root culture of Ginseng (*Panax ginseng* C. A. Meyer). *In Vitro Cellular & Developmental Biology. Plant* 36(5): 424-428.

[90] Bahare Hoseinpanahi, Bahman Bahramnejad, Mohammad Majdi, Dara Dastan and Morahem Ashengroph, 2020. The effect of different elicitors on hairy root biomass and resveratrol production in wild *Vitis vinifera*. *J Appl Biotechnol Rep*. 7(1):25-31.