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3. Plant cell and organ cultures as a source of phytochemicals

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Abstract. Plant cell and organ cultures constitute a promising platform for the production of numerous valuable secondary compounds. Currently, *in vitro* culture techniques involve both empirical and rational approaches as suitable strategies to condition high metabolite production and establish competitive plant cell-based bioprocesses. In this context, we have developed hairy root cultures of *Panax ginseng*, and engineered hairy root cultures of *Duboisia*, *Datura metel* and *Hyoscyamus* spp and plant cell cultures of *Centella asiatica* and *Taxus* spp. This chapter describes our work on the development of two different biotechnological systems to improve taxol production in cell suspension cultures of *Taxus* spp and ginsenoside production in hairy root cultures of *Panax ginseng*.

Introduction

Plant secondary metabolites play an important role in plant defense and constitute a source of phytochemicals for human health and nutrition. The three main groups of plant secondary metabolites (alkaloids, polyphenols

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and terpenes) include a wide range of compounds with high added value. Some of them can be synthesized chemically, but due to their highly complex structures most are extracted from the plant. However, sometimes even extraction from the plant is not feasible, due to low production levels, and the risk of endangering the species.

In vitro culture techniques using plant cell and organ cultures are promising tools for the production of numerous valuable secondary compounds. Empirical approaches have long been employed for the development and optimization of plant cell-based bioprocesses, focusing on input (cell line, medium, culture parameters, bioreactors, process operations, etc.) and output factors (cell growth, nutrient uptake, productivity, yield, etc.). In addition, a rational approach, taking into account the molecular and cellular basis of metabolic pathways and their regulation, is currently being used. The successful biotechnological production of phytochemicals with high added value, such as the anticancer compound taxol, shows that plant cell and organ cultures can constitute an alternative to the culture of the whole plant for the production of secondary metabolites with biological activity [1].

The biotechnological production of plant secondary metabolites has several advantages over the culture of the whole plant in the field, including:

- The desired product can be harvested anywhere in the world, maintaining strict control of production and quality.
- It is not necessary to use herbicides and pesticides.
- Climate or ecological problems are avoided.
- Growth cycles are of weeks rather than years.

Our research is based on plant *in vitro* culture and genetic and metabolic engineering techniques with the aim of increasing the production of high-value phytochemicals and obtaining plant stem cells for cosmetic uses (Fig. 1). Working with plant *in vitro* cultures, we are interested in the selection of highly productive genotypes and the micropropagation of aromatic and medicinal plants in order to maintain the most productive ones and, in cases of endangered plants, to help preserve the species. Plant micropropagation also represents the best way to obtain virus-free plants to meet phytosanitary requirements for plant import-export. Another goal is using plant cells and hairy root cultures for the production of phytochemicals.

Another field of our research involves the application of genetic and metabolic engineering techniques to improve the production of target secondary metabolites. In this case, it is necessary to understand the relevant biosynthetic pathways and to know which enzymes catalyze the sequence of

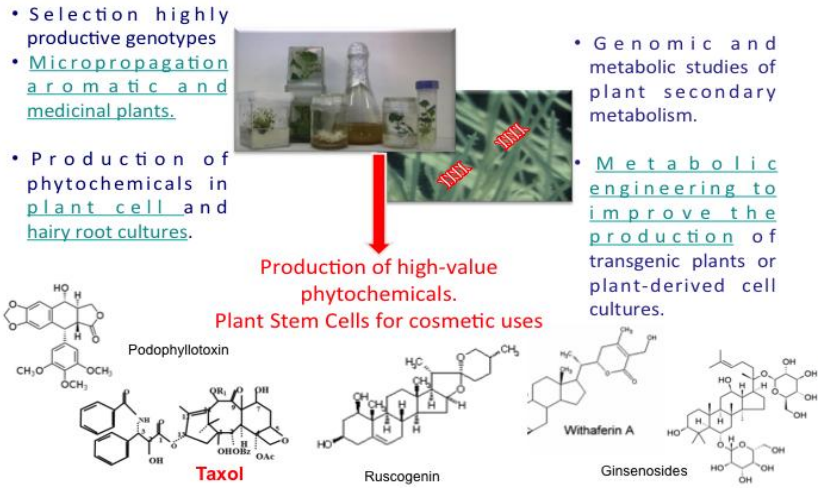

Figure 1. Plant *in vitro* culture and genetic and metabolic engineering techniques as the basis of Plant Biotechnology.

reactions, especially the slow steps, and the genes encoding these enzymes. Using the aforementioned techniques, the plant secondary pathways are studied to detect the flux-limiting steps and improve production.

In this chapter, we will describe the different *in vitro* techniques being used in our laboratory, with relevant examples of the plant species we have studied. Finally, we will describe our work on the development of a biotechnological system to increase taxol and taxane production using *Taxus baccata* and *T. media* cell suspension cultures and ginsenoside production in hairy root cultures of *Panax ginseng*.

1. Plant *in vitro* cultures

Micropropagation of aromatic and medicinal plants

Micropropagation is a way of obtaining a large number of plants *in vitro* in an asexual manner, ensuring that they are genetically and phenotypically identical to the original plant. It consists of the culture of variably sized plant pieces using a suitable culture medium. In this field, our group has developed specific protocols to micropropagate *Lavandula dentata* [2], *Ruscus aculeatus* [3], etc. Recently our work has focused on the micropropagation of *Centella asiatica* using synthetic seeds and obtaining several virus-free varieties of *Vitis vinifera* plants for exportation (Fig. 2).

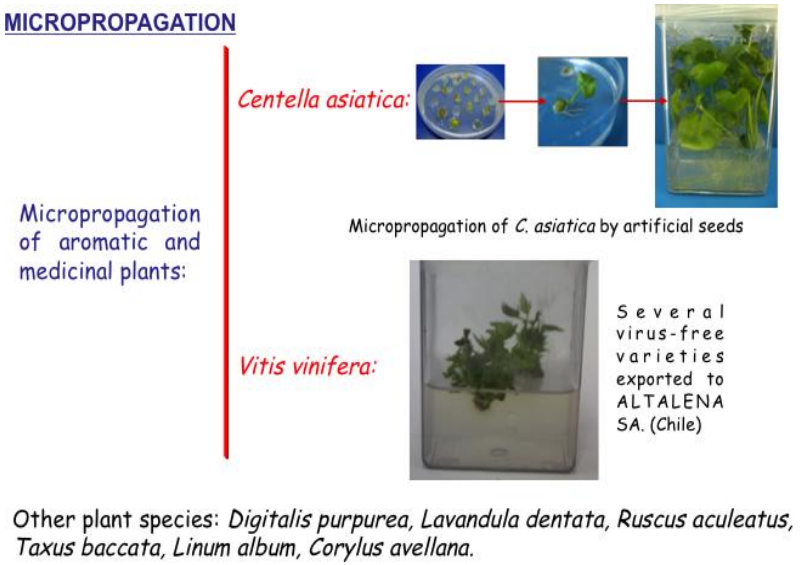

Figure 2. Our Background in Micropropagation.

Plant cell factories and stem cells

Plant cell factories constitute a biotechnological platform for the production of phytochemicals. In this way, cell cultures, also known as plant stem cells, can be utilized as cell suspensions or immobilized cells. Plant stem cells have an unlimited capacity for growth and an ability to produce identical new plants. By means of *in vitro* culture techniques, we are able to obtain plant stem cells with the same biosynthetic capacity as the whole plant. To establish a cell suspension culture that can act as a plant cell factory, it is first necessary to obtain a high callus biomass.

The methodology employed (Fig. 3) involves the following steps: selection of highly productive genotypes of the target plant; induction of calli from the best explant to obtain callus biomass; disintegration of calli by shaking in a liquid medium to obtain cell suspension cultures; optimization of the culture conditions by assaying several basic media, plant growth regulators (PGRs), sugar supplements, addition of elicitors, precursors, etc. to improve the production; and finally, scale-up to bioreactor level. Sometimes immobilization in alginate beads may be necessary to improve the biotechnological production [4].

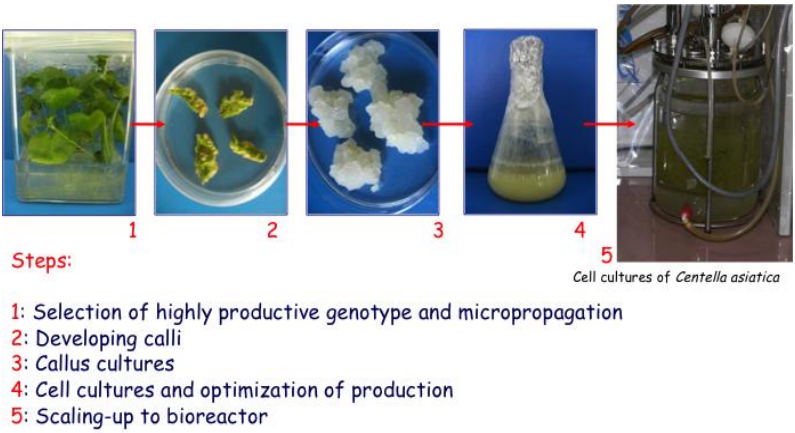

Figure 3. Plant cell factories: methodology

Regarding the development of plant biofactories, our group is working on centelloside production in cell suspension cultures of *Centella asiatica* [5]. We are also working on ruscogenin production in *Ruscus aculeatus* and podophyllotoxin production in *Linum album*, but our main research line is focused on the biotechnological production of taxol and taxanes in cell suspension cultures of *Taxus baccata* and *T.media* [6, 7]. We have previously developed cell cultures for the production of digoxin, digitoxin, tobacco alkaloids and galphimines.

We have also recently obtained plant stem cells of *Vitis vinifera* and *Centella asiatica* for cosmetic uses. *C.asiatica* was traditionally used in Ayurveda medicine and due to its dermatological applications, its stem cells are currently an important target for cosmetic purposes in Europe.

Plant biotransformations

Another cell culture application is biotransformation. In this context, we have established a single protocol for extracting α-amyrin from copal resin, which is then added as a substrate to cell suspension cultures of *C. asiatica* for its bioconversion into compounds with high added value, such as centellosides [8]. The results have demonstrated that cell lines are capable of biotransforming a compound with low biological activity but that is abundant in nature, such as amyrin, into other compounds widely used for their pharmacological properties, such as the centellosides (Fig. 4).

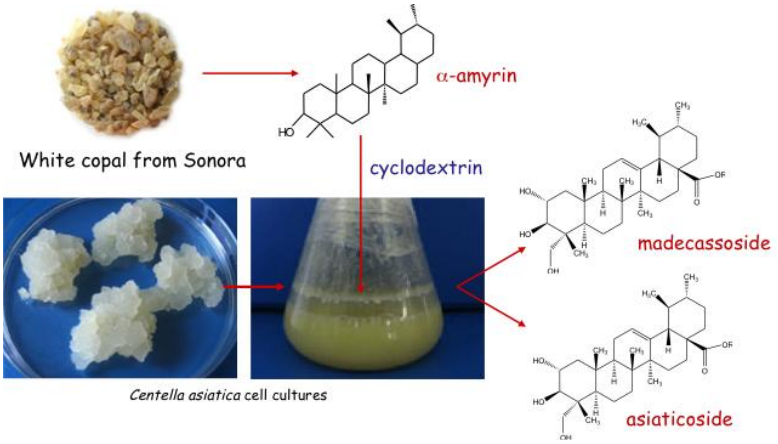

Figure 4. Biotransformation of the substrate α-amyrin into centellosides.

Hairy root cultures

Sometimes the production of plant secondary metabolites requires organized cultures such as roots or aerial shoots. In this context, our group uses the *Agrobacterium rhizogenes* system to genetically transform plant cells and develop hairy root cultures. *A.rhizogenes* is a bacterium that infects plants in nature, transferring a part of its plasmidic DNA, the Transfer-DNA or T-DNA, to the plant cell. The infected plant then develops large roots called hairy roots. Biotechnological processes based on hairy root cultures show a very high biomass production and a metabolic profile similar to the root of the whole plant.

This methodology (Fig. 5) is based on obtaining hairy root lines after infection with *A.rhizogenes*, the isolation and selection of the most productive root lines, optimization of culture conditions for maximum production and eventual scale-up to bioreactors. The scaling up of these cultures is very difficult because bioreactors are generally designed for the culture of microorganisms and need to be specially adapted for the culture of transformed roots.

Our group has worked with hairy root cultures for the production of ajmalicine in *Catharanthus roseus* [9], ginsenosides in *Panax ginseng* [10], tropane alkaloids in *Datura metel*, *Duboisia* sp and *Hyoscyamus* sp. [11,12], withanolides in *Withania coagulans* [13] and lately we have also been working on transformed root cultures of *Linum album* for the production of podophyllotoxin and methoxypodophyllotoxin [14].

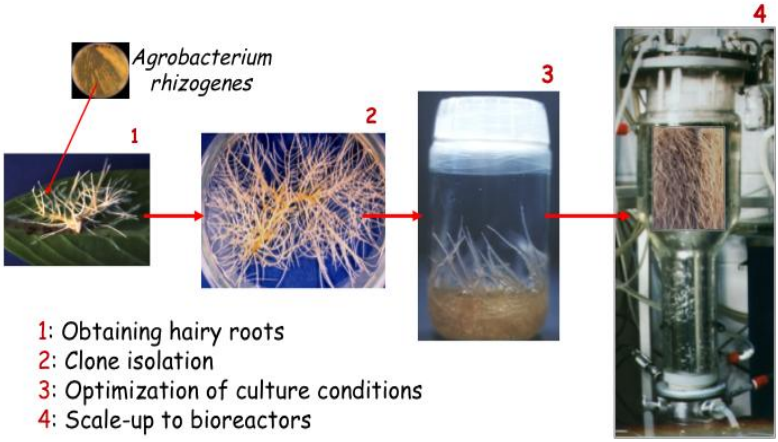

Figure 5. Hairy root cultures of *Catharanthus roseus* scaled up to airlift bioreactor level.

2. Genetic and metabolic engineering techniques

To improve the biotechnological production of a secondary metabolite, it is necessary to understand the relevant biosynthetic pathways. It is also necessary to know which enzymes catalyze the sequence of reactions, especially the slow steps, and the genes encoding these enzymes. Using genetic and metabolic engineering techniques, we can study the plant secondary pathways to detect the flux-limiting steps in the biosynthesis of phytochemicals and use engineering techniques to design plants and cell cultures with improved production.

We have used metabolic engineering techniques to improve the production of scopolamine in the species *Datura metel*, *Hyoscyamus muticus* and a *Duboisia* hybrid. The first step was to obtain hairy root cultures of the aforementioned species and then select the most productive ones to be scaled up to bioreactor level (Fig. 6).

The second step was the overexpression, both separately and together, of the genes encoding the enzymes putrescine methyltransferase (PMT), which constitutes the first committed step in the scopolamine biosynthesis, and hyoscyamine 6-β-hydroxylase (H6H), which transforms hyoscyamine into scopolamine [15]. The best results were obtained by overexpressing both genes together in transformed roots of *H. muticus* (Fig. 7).

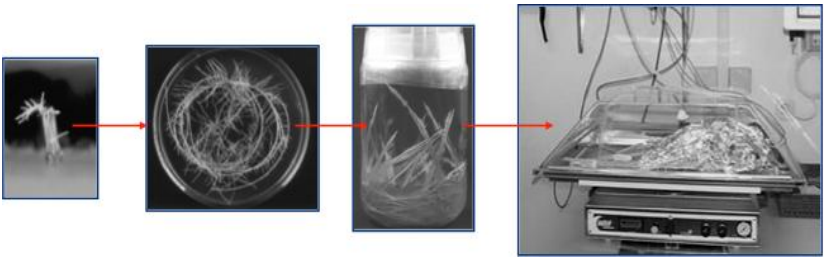

Figure 6. 1st step: Obtaining hairy root cultures of a *Duboisia* hybrid and scaling up to wave bioreactor level.

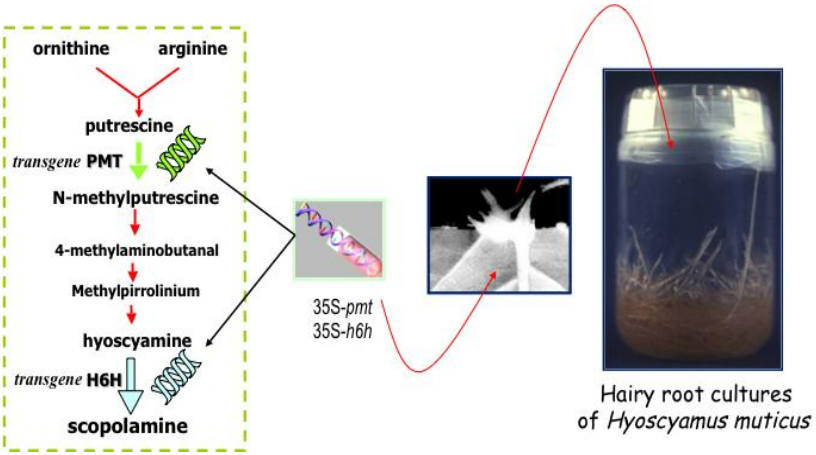

Figure 7. 2nd step: Overexpression of putrescine methyltransferase and hyoscyamine 6-β-hydroxylase.

Thirdly, since scopolamine is an alkaloid of greater commercial interest than the more naturally abundant hyoscyamine, we developed a biotechnological system to biotransform exogenous hyoscyamine into scopolamine based on tobacco transformed roots carrying the hyoscyamine 6-β-hydroxylase gene of *H. muticus* (Fig. 8).

In addition, with the aim of scaling up the system and avoiding the problems of root cultures in bioreactors, we dedifferentiated the roots and established cell suspensions that were also able to biotransform hyoscyamine into scopolamine at a bioreactor level [16].

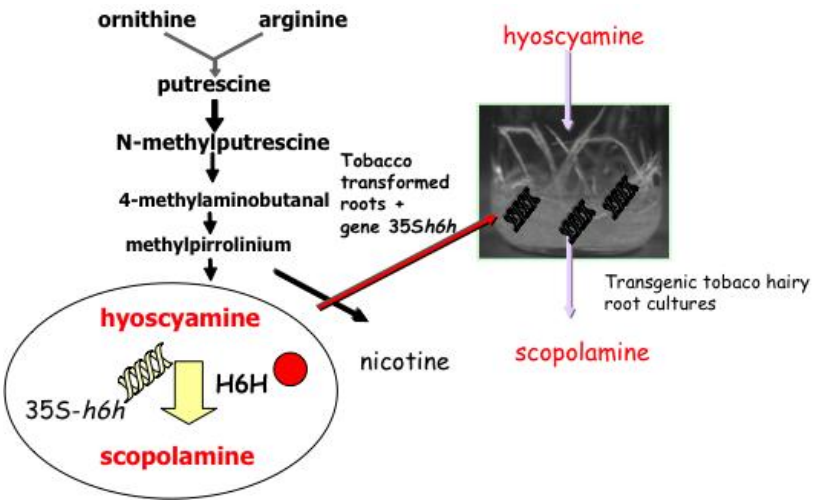

Figure 8. 3rd step: Biotransformation of hyoscyamine into scopolamine in tobacco hairy root cultures.

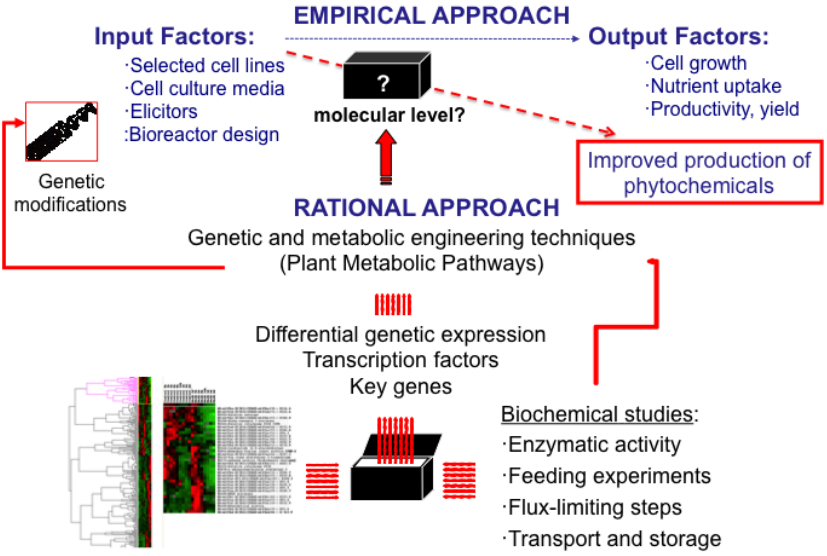

Figure 9. Improving secondary metabolite production using empirical and rational approaches.

To sum up, to improve secondary metabolite production using *in vitro* cultures one can consider two principal approaches (Fig. 9). An empirical strategy involves the modification of input factors, such as the selection of more productive cell lines, optimizing culture media, the use of elicitor treatments, bioreactor design, etc. However, this does not provide information about what is happening in the producer cells at the molecular level. By taking a rational approach based on genetic and metabolic engineering techniques, we can study how the input factors change the metabolic and transcriptomic profile of the target cell cultures.

3. Improving taxol and taxane production in cell suspension cultures of *Taxus* sp.

For some time now, one aspect of our research work has been focused on taxol production using cell cultures of different *Taxus* species. Taxol, a diterpene alkaloid with a very complex chemical structure, is one of the most effective anticancer drugs ever developed. It presents a unique mode of action on the microtubular cell system by inhibiting cell proliferation at the G2 phase of the cell cycle, thus blocking mitosis.

The natural source of taxol is the inner bark of several *Taxus* species. The disadvantages of this source are that taxol accumulates at a very low concentration (0.02% of dry weight), its extraction involves the destruction of yew trees and is very expensive. Moreover, the endangered status of several *Taxus* species excludes this method of obtaining taxol. For these reasons, alternative sources of taxol have been assayed, including its preparation by total synthesis, but this process is not commercially viable. Another possibility is producing taxol semisynthetically from more abundant taxanes, for example, via the conversion of baccatin III found in *Taxus* needles, but the cost and difficulty of the extraction and purification process of these precursors are also very high. An alternative consists of cultivating *Taxus* trees in the field. In fact, in Yunnan province in China, there are conventional *Taxus* crops for the production of taxol, but a harvest can only be obtained after several years and the extraction remains very expensive [17]. Therefore, the most promising approach to a sustainable commercial production of taxol, or other compounds synthesized by plants in similarly small quantities, is provided by plant cell cultures.

In order to obtain cell suspensions, first on a small scale and then at bioreactor level, it is necessary to establish fast-growing callus cultures from which the cell suspensions are derived. *Taxus* callus cultures were obtained from young stems of 3-4-year-old yew trees cultured in optimum conditions.

Figure 10 shows the whole process until the establishment of a small-scale cell suspension culture.

To develop a biotechnological system for a high taxol production, it is necessary to optimize the culture conditions by assaying several basic media, plant growth regulators, sugar supplements etc., which requires knowledge of the growth curves of the system. A growth curve represents the biomass production (growth) in relation to the time of culture, and appears as a sigmoid curve with 3 characteristic phases: 1) A lag phase in which the biomass does not increase, when cells are preparing all the machinery for the cell division that will start in the exponential phase. 2) An exponential phase in which cells are dividing continuously by mitosis. All the processes related with mitosis, which are part of the plant's primary metabolism, are now taking place inside the cells, including DNA duplication, and RNA and protein synthesis. In these conditions secondary metabolism is inhibited. 3) A stationary phase in which the biomass of the system remains constant and the number of cells that divide by mitosis is almost equal to the number of dying cells. In these conditions, the cells can accumulate precursors for the biosynthesis of phytochemicals and the machinery of plant secondary metabolism begins to work.

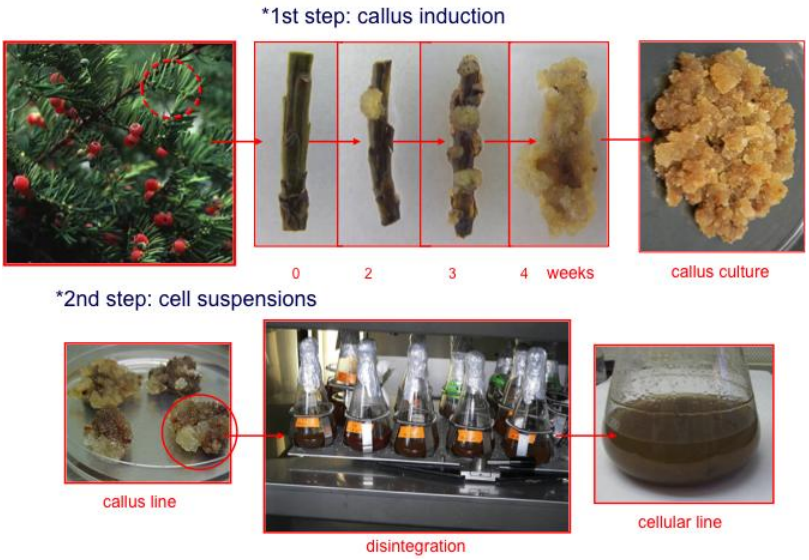

Figure 10. Establishment of *Taxus* cell suspension cultures.

Once the growth curve of a culture is established, we know how long each phase is and when the stationary phase begins, which is the moment for adding precursors, elicitors, permeabilizing agents etc. to improve the production. Knowledge of the growth curve is particularly useful in a two-stage culture, that is, when a separate medium is required to enhance growth and another to increase production. In this case, during the exponential phase the cells are cultured in the growth medium and then transferred to the production medium at the start of the stationary phase.

A two-stage culture system was established for taxol production. Plant cells were first cultured in a medium optimised for their growth, which was then replaced by a production medium that mainly stimulates the biosynthesis of secondary metabolites. This system has an added advantage of permitting the addition of biosynthetic precursors and elicitors in the production medium.

In summary, taxol and baccatin III production was clearly enhanced by the transfer of cells from the growth to the production medium containing the elicitor methyl jasmonate (MeJA) [18]. After optimizing the culture conditions, we scaled up the culture in a stirred bioreactor where the production improved 2.4-fold for taxol and 9-fold for baccatin III.

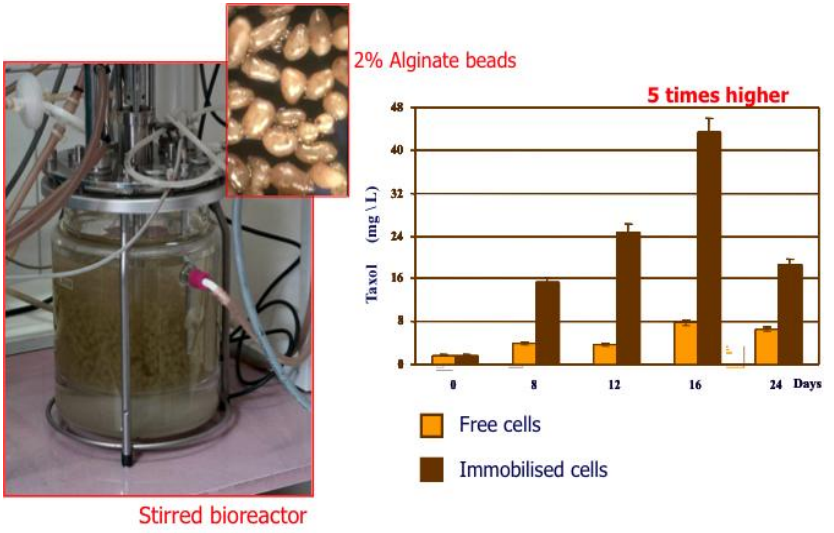

Figure 11. Taxol production in immobilized cell cultures of *Taxus baccata* in a stirred bioreactor.

We continued our study on the improvement of taxol and related taxane production by establishing immobilized cell cultures, first on a small scale and then at bioreactor level. Numerous studies have reported that immobilization of plant cell suspensions enhances the production of valuable plant metabolites. Taxol production clearly improved when cell suspensions were immobilised in 2% alginate beads and cultured in a stirred bioreactor, using the optimum medium for the biosynthesis of taxol. When the taxol production was at its highest, on day 16, its levels were more than 5-fold higher than those obtained by the same cell line growing freely in the same conditions (Fig. 11). The novelty of this work was to demonstrate that immobilization strongly constrains the physiology of *T. baccata* cells and substantially enhances taxane production in bioreactor cultures [4].

4. Ginsenoside production in hairy root cultures of *Panax ginseng*

Ginsenosides are triterpenes with a great variety of properties, being used as tonics, analgesics, antipyretics, stress-beaters, etc.

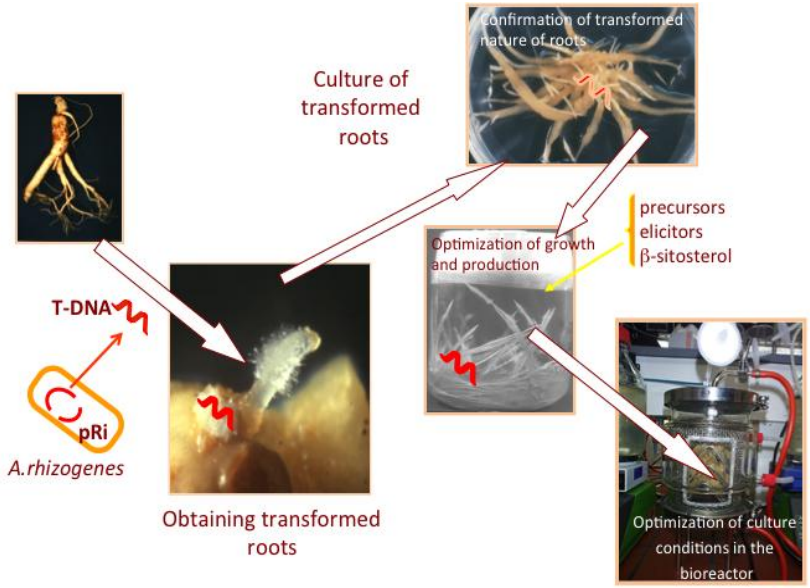

Figure 12. Improving ginsenoside production in hairy root cultures of *Panax ginseng.*

Hairy root cultures were obtained using the following protocol. After infection of the rhizome with *A. rhizogenes*, we obtained hairy roots that were cultured in a solid growth medium and their transformed nature was confirmed. The transformed roots were then transferred to a liquid medium to optimize growth and production, using elicitors, precursors and beta-sitosterol. Finally, we scaled up the culture by assaying different types of bioreactors, optimizing the culture conditions. A two-stage system was not used in this case. Figure 12 summarizes the whole process.

After the infection of the rizome with *A. rhizogenes* we obtained roots with 3 types of morphology: 50% had a typical hairy root morphology (HR-M), 35% a callus morphology (C-M) and 15% a thin morphology (T-M) [19]. The transformed nature of each type of root was confirmed by amplifying the *rol C* gene. The highest growth in solid medium was displayed by C-M roots, although the total ginsenoside content was higher in T-M roots, albeit lower than in the plant rhizome.

After transferring the roots from the solid to the liquid medium to optimize the growth and production, the best growth was achieved by the C-M roots, followed by the HR-M roots, as occurred in the solid medium. It should be emphasized that all the roots grew better in liquid medium.

Regarding the ginsenoside production, the best results were obtained with HR-M roots in liquid medium, in contrast with the solid medium where the best production was obtained with T-M roots.

We then optimized the culture conditions by assaying several elicitors (chitosan, methyl jasmonate, vanadyl sulfate), precursors (mevalonic acid, squalene, farnesol) and beta-sitosterol (which bioregulates the synthesis of ginsenosides). The best result was obtained with MeJA, which increased the production of ginsenosides in all the root types [20]. The precursors and beta-sitosterol did not yield any significant results. Compared with a 4-year-old *Panax ginseng* plant rhizome, the ginsenoside production of a transformed HR-M root grown in liquid medium with MeJA for 4 weeks was almost 2-fold higher.

Finally, we optimized the process at a bioreactor level. We first selected the most productive hairy root line (HR-M), which was cultured in the liquid medium optimized on a small scale in 3 types of bioreactors: airlift, spray and wave. After 28 days of culture without changing the medium, the best growth was obtained in the wave bioreactor, but the best ginsenoside production was achieved in the spray bioreactor. However, if we determine the productivity, that is, the ginsenoside production per liter and per day, the best results were obtained with the wave bioreactor in the same culture conditions: after 28 days without changing the media. This confirms that the

productivity of a culture does not only depend on the accumulation of the target compound but also on its growth capacity.

In summary, after 28 days of culture without changing the media, we obtained the best production in a spray bioreactor but the best productivity in a wave bioreactor. Optimum growth was also obtained in the wave bioreactor.

We next determined the biomass and ginsenoside production in 2 bioreactors (spray and wave) after 28 days of culture but this time changing the culture medium at day 15. In these conditions, the biomass production increased in both bioreactors, and was therefore higher in the wave bioreactor. Regarding the ginsenoside production, the results again showed more production in the spray bioreactor, but when we determined the productivity, the roots in the wave bioreactor were the most productive, as before.

Finally, we cultured the roots (HR-M) in the two bioreactors (spray and wave) but now for 56 days and changing the medium every 15 days [21]. The highest productivity of the roots was again achieved in the wave bioreactor, being almost 2-fold higher than in the small flasks (Fig. 13).

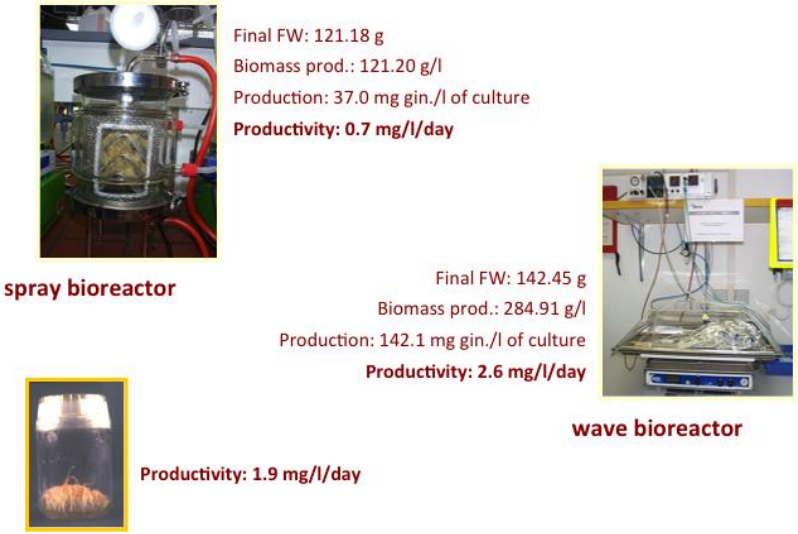

Figure 13. Ginsenoside productivity of typical hairy roots (HR-M) grown in small flasks, spray and wave bioreactors on a 56-day culture with change of media every 15 days.

5. Conclusion

We have established two biotechnological systems (cell suspension cultures of *Taxus* sp. and hairy roots of *Panax ginseng*) to improve the production of two high-value target compounds (taxol and ginsenosides) using an empirical approach that considers input and output factors (Fig. 9). Taxol production was improved employing a two-stage culture system, elicitation with MeJA, immobilization in alginate beds and scaling up in a stirred bioreactor. Ginsenoside production was improved using selected hairy root lines of three different morphologies, elicitation with MeJA, changing the medium during the culture, assaying three bioreactor designs and taking into account production and productivity. These results can be improved in future studies taking a rational approach.

Acknowledgements

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