AN INNOVATIVE TECHNOLOGY FOR THE PRODUCTION OF ACTIVE INGREDIENTS FROM PLANTS

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Part One

SECONDARY METABOLITES FROM CULTURES OF PLANT CELLS

The experimentation in the sector of the culture of cells of plant origin illustrated in this article has had the objective of selecting cell lines with high capacities of synthesising active ingredients useful in a pharmaceutical or biomedical context and directed, in particular, at those characterized by a limited availability from the natural sources.

A production method of plant molecules based on cell cultures presents numerous advantages compared to cultures in the field.

In the first place, having a constant supply in time overcomes the problems of the seasonality of the production of secondary metabolites, that of variability due to meteorological conditions and that of the specificity of production linked to the geographical area of growth of the plant.

In the second place, the presence in the extracts of toxic substances, such as herbicides, pesticides or other environmental pollutants, is excluded as culture takes place in conditions of sterility and in the absence of any chemical treatment.

In addition, this technology simplifies the problem of collection, transport and storage of the product as the extraction can be carried out as soon as the culture has reached suitable conditions. Lastly, it allows the protection of the environment as the natural biological equilibrium is not modified due to intensive harvesting of slow growing plants. For the purposes of obtaining consistent quantities of molecules with a scarce production in nature, a further and not negligible advantage presented by this technology is the possibility of carrying out a rapid selection (usually lasting a few months) of the lines with the greatest productivity in the laboratory, whilst the selection in the field carried out on the plant in toto, is subject to the times of growth and development typical of the species (usually in the order of years). The research carried out to date has allowed the identification and the selection of plant cell lines derived from explants of tissue of Catharanthus roseus, Taxus sp, Ajuga reptans, Gardenia jasminoides, Lonicera japonica (Honeysuckle) Olea europaea, Ruta graveolens and others. These lines, characterized by a high productive capacity, are aimed at the production both of molecules used in anti-tumour therapy (e.g. vincristine, vinblastine, taxol, etc.), and of agents with an anti-oxidant activity . The latter include some phenylpropanoid derivatives (PP) such as verbascoside and chlorogenic acid and some secoiridoids including oleuropeine.

The experience matured in the sector has allowed optimising the methods of culture and the large-scale production of these secondary metabolites.

PLANT CELL CULTURES

At present, the glucosides of phenylpropanoids and of secoiridoids are extracted from tissues of plants of various species, but only in small quantities on the laboratory scale. This limit is due mainly to the high reactivity of these molecules with the free radicals of oxygen which are formed in abundance after collection, due to the peroxidase present in the tissues of the plant. For this reason, the active ingredient content is rapidly reduced, in some cases to zero, even a short time after collection. This means that, for these classes of molecules, the transport of plant material from the place of origin and its conservation until processing are extremely critical phases for the purposes of the final titre in active ingredients.

In some cases, an alternative for the production of these molecules could be chemical synthesis. However, in the case of phenylpropanoids and secoiridoids, the structure presents numerous reactive constituents such as aliphatic and aromatic hydroxyls and stereospecific glucoside bonds (in nature the most common are the b-[D]-glycoside ones), which lead to mixtures of stereoisomers and consequently with low final yields. Similar problems of supply exist for the antiblastic taxanes. In this case too, chemical synthesis is not competitive with the natural source and although they are currently extracted in considerable quantities from the bark and needles of yew trees, this entails its intensive exploitation. In fact, the extraction of taxol from yew tree bark entails long periods for the re-growth of the plant (about sixty years), whilst from the leaves of young plants a similar one (10-deacetyl-baccatine III) is mainly obtained as the starting material for numerous processes of semi-synthesis.

All this implies a serious insufficiency of the natural source compared to the requests from the market and the real risk of its exhaustion in nature.

The possible alternative to these problems of supply is represented, as already mentioned, by the technology of in vitro cultures.

The lines of plant cells can be cultivated in the laboratory on a solid medium and selected both by the type of metabolites produced and by their characteristics of production and growth. Subsequently, the selected cell lines can be suspended in a liquid medium and placed in large volume reactors for the production of high quantities of biomass. This passage is necessary for cultivation on an industrial scale of the secondary metabolites. To this end, the rapidity of the selection of new lines characterized by a high content of secondary metabolites as well as their stability in time and the optimisation of the culture mediums for each specific cell line represent stages of fundamental importance for their production on an industrial scale.

WHAT PLANT CELL CULTURES ARE AND HOW THEY ARE OBTAINED

In superior plants, both gymnosperms and angiosperms, there exist tissues characterized by undifferentiated cells in rapid multiplication . These tissues, called meristematic, are characterized by cells of small dimensions, thin walls and the absence of vacuoles. The cytoplasm is rich in ribosomes, protoplasms and mitochondrions, whilst the endoplasmic reticulum is not very well developed. The nucleus is very voluminous compared to the cell dimensions. In superior plants, the meristematic cells, after multiple mitotic divisions, give life to morphologically and physiologically differentiated cells, according to the position and tissue to which they will belong, with a loss of the capacity of active proliferation. Therefore, the meristematic cells can be defined as stem cells of an embryonic type with a potential of "totipotent" differentiation and development.

The original "embryonic" characteristics can again be assumed by the differentiated cells when, for example, the tissue is damaged. In this case, the differentiated cells go back to having an activity similar to meristematic activity and produce an undifferentiated repair tissue commonly called callus.

Normally the phenomenon takes place due to natural causes but, for scientific purposes, the callus tissue is induced by making incisions in the tissues of the plant.

Today's techniques allow cultivating the callus tissue for an indefinite time, preventing its differentiation.

Maintaining the embryonic characteristics mainly takes place due to the action of hormonal substances called "growth factors" which are capable of keeping the meristematic activity alive. In nature these substances are produced by the plants themselves and can give very extensive and complex physiological responses from the ripening of the fruit, to the induction of the tissue of abscission of the leaves and to the induction of the dormancy of the buds.

In in vitro cultures, the cells are maintained in the undifferentiated state by two groups of substances: the "auxins" such as indoleacetic acid and 2,4-phenoxy-acetic acid and "cytokinins" such as kinetine and benzyladenine.

With the administration of auxins, the induction of the synthesis of the cellulase enzyme has been observed, which determines the elasticity of the cell walls and thus encourages cell stretching growth .

The action of the cytokinins, on the other hand, appears above all at ribosome level, both increasing the synthesis of RNA, and inducing the synthesis of specific proteins.

The combination of the effect of cell growth due to auxins with the effect on cell multiplication caused by the cytokinins is sufficient to have maintenance of the undifferentiated state and of the proliferation activity of the meristematic cells. In addition to the presence of "growth factors", it is necessary to provide the cells with a suitable culture medium and the appropriate environmental conditions. In fact, by varying the composition of the culture medium, it is possible to select cell lines with different biochemical and metabolic characteristics. The culture medium must contain the substances necessary to maintain the cell metabolism, above all a source of organic carbon, which is generally saccharose. The cells in culture lose their capacity of photosynthesis and require an exogenous source of carbon. The macroelements necessary for growth – nitrogen, phosphorus, sulphur and a series of microelements such as iron, manganese, copper, zinc and cobalt as well as some vitamins – must also be provided, both in organic and inorganic forms.

The technique of induction of the callus tissue follows a procedure that is generally confirmed, but which requires adjustment and adaptation to the particular genus of plant and tissue chosen for the generation of the callus. After collection, the plants are washed in running water; leaves, stems and roots are then cut into segments of 2-5 cm and distributed into sterile petri dishes. For sterilization, the plant fragments are treated in succession with EtOH at 70% for 15 minutes, with sodium hypochlorite at 2% for 5 minutes and lastly with $HgCl_2$ at 0.05% for one minute. Between treatments, the segments are washed at least three times with sterile distilled water, all under an extractor with a sterile laminar flow.

Each segment is again cut with a sterile bistoury or forceps into minute fragments (explants) and then deposited in petri dishes containing nutritive medium with the addition of hormones and solidified by the addition of agar.

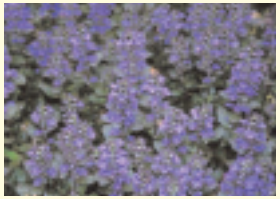
The success of the phase of generation and selection of the plant cell lines depends to a great extent on the number of explants made in this initial phase because, despite the procedure of sterilization, many of them, normally between 70% and 80% will be polluted and will have to be rejected.

After 21 days of preservation in the dark at 28°C, the production of undifferentiated callus tissue can be observed on some explants.

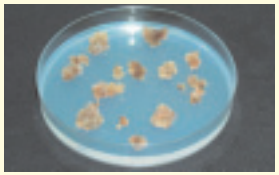
Explant of lamina of Vinca minor with the formation of calluses on the cut edge. The tissue, after washing and chemical sterilization, was placed for three weeks in a nutritive culture with agar in a sterile test tube.

Explants of lamina of Taxus baccata on solid nutritive medium with hormones eight weeks after sowing.

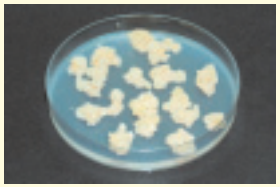
To foster their multiplication and growth, they are transferred on to solid fresh medium and after a further 14 days, the parts of the callus that have developed sufficiently are transferred again (sub-cultivated) to a fresh medium.



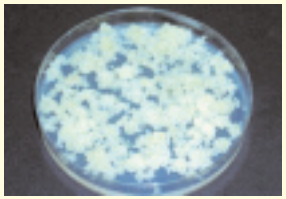
Maquis of Ajuga reptans in blossom. In medieval medicine, extracts of Ajuga reptans were used to sooth and encourage repair of the skin's tissues following wounding by cutting.



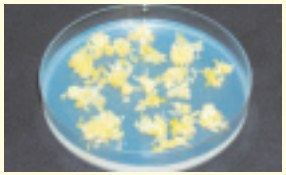
Cell line of Echinacea angustifolia.



Cell line of Taxus baccata



Cell line of Ajuga reptans stabilized after 5 years of selection.

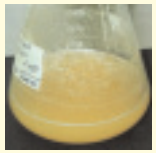


Cell line of Ruta patavina

Cell line of Taxus baccata

To obtain a stable cell line with rapid proliferation, at least about ten sub-cultivations of the initial callus tissue have to be carried out. Once lines in rapid growth, but still extremely variable both as morphology and as productivity, have been obtained, it will be possible to select a high number of lines that are different, but each with characteristics that are constant in time.
For the production of large quantities of metabolites, it is then necessary to transfer the selected lines to a liquid medium (cultures in suspension), first in conical flasks and then in reactors.
Cultures in suspension in laboratory flasks (with a volume of up to 2 L) are kept agitated by orbital shakers. This allows an adequate remixing of the cell mass, guaranteeing the gaseous exchange necessary for the growth of the cells.

To go on to higher volumes, the composition of the medium and the oxygenation (agitation) must be accurately developed for each type of cell cultivated.



Selected cell line of Ajuga reptans in sterile nutritive liquid medium.