24 Valuable Secondary Products from *In Vitro* Culture

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CHAPTER 24 CONCEPTS
SECONDARY PRODUCTS *IN VITRO*

- A wide diversity of natural products can be induced in plant tissue cultures, including edible, medicinal, and industrial compounds.
- Selection and microenvironmental stimulus regulate productivity *in vitro*.
- Cell culture systems facilitate in-depth investigation of metabolism.

INTRODUCTION

The capacity for plant cell, tissue, and organ cultures to produce and accumulate many of the same valuable chemical compounds as the parent plant in nature has been recognized almost since the inception of *in vitro* technology. The strong and growing demand in today's marketplace for natural, renewable products has refocused attention on *in vitro* plant materials as potential factories for secondary phytochemical products, and has paved the way for new research exploring secondary product expression *in vitro*. However, it is not only commercial significance that drives the research initiatives. The deliberate stimulation of defined chemical products within carefully regulated *in vitro* cultures provides an excellent forum for in-depth investigation of biochemical and metabolic pathways, under highly controlled microenvironmental regimes.

Plant-produced secondary compounds have been incorporated into a wide range of commercial and industrial applications, and fortuitously, in many cases, rigorously controlled plant *in vitro* cultures can generate the same valuable natural products. Plants and plant cell cultures have served as resources for flavors, aromas and fragrances, biobased fuels and plastics, enzymes, preservatives, cosmetics (cosmeceuticals), natural pigments, and bioactive compounds.

There is a series of distinct advantages to producing a valuable secondary product in plant cell culture, rather than *in vivo* in the whole crop plant. These include the following:

- Production can be more reliable, simpler, and more predictable
- Isolation of the phytochemical can be rapid and efficient, as compared to extraction from complex whole plants
- Compounds produced *in vitro* can directly parallel compounds in the whole plant
- Interfering compounds that occur in the field-grown plant can be avoided in cell cultures
- Cell cultures can yield a source of defined standard phytochemicals in large volumes
- Cell cultures are a superb model to test elicitation
- Cell cultures can be radio labeled, such that the accumulated secondary products, when provided as feed to laboratory animals, can be traced metabolically

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Secondary products in plant cell culture can be generated on a continuous year-round basis; there are no seasonal constraints. Production is reliable, predictable, and independent of ambient weather. At least in some cases, the yield per gram fresh weight may exceed that which is found in nature. Disagreeable odors or flavors associated with the crop plant can be modified or eliminated *in vitro*. Plant cell culture eliminates potential political boundaries or geographic barriers to the production of a crop, such as the restriction of natural rubber production to the tropics or anthocyanin pigment production to climates with high light intensity. When a valuable product is found in a wild or scarce plant species, intensive cell culture is a practical alternative to wild collection of fruits or other plant materials. Extraction from the *in vitro* tissues is much simpler than extraction from organized, complex tissues of a plant. Plant tissue culture techniques offer the rare opportunity to tailor the chemical profile of a phytochemical product, by manipulation of the chemical or physical microenvironment, to produce a compound of potentially more value for human use.

While research to date has succeeded in producing a wide range of valuable secondary phytochemicals in unorganized callus or suspension cultures, in other cases production requires more differentiated microplant or organ cultures (Dörnenberg and Knorr, 1997). This situation often occurs when the metabolite of interest is only produced in specialized plant tissues or glands in the parent plant. A prime example is ginseng (*Panax ginseng* C.A. Meyer). Since saponin and other valuable metabolites are specifically produced in ginseng roots, root culture is required *in vitro*. Similarly, herbal plants such as *Hypericum perforatum* (St. John's wort), which accumulates the anti-depressant hypericins and hyperforins in foliar glands, have not demonstrated the ability to accumulate phytochemicals in undifferentiated cells (Smith et al., 2002). As another example, biosynthesis of lysine to anabasine occurs in tobacco (*Nicotiana tabacum* L.) roots, followed by the conversion of anabasine to nicotine in leaves. Callus and shoot cultures of tobacco can produce only trace amounts of nicotine because they lack the organ-specific compound anabasine. In other cases, at least some degree of differentiation in a cell culture must occur before a product can be synthesized (e.g., vincristine or vinblastine from *Catharanthus roseus* [L.] G. Don, Madagascar periwinkle). Reliance of a plant on a specialized structure for production of a secondary metabolite, in some cases, is a mechanism for keeping a potentially toxic compound sequestered.

The three long-standing, classic examples of commercially viable production of a secondary metabolite *in vitro* — ginseng saponines, shikonin, and berberine — each feature products that have diversified uses, including medicinal applications. Ginseng is produced in large-scale root cultures, whereas the other two products are produced in highly colored cell cultures. A tremendous research and development effort has advanced a number of other *in vitro*-derived secondary products to semicommercial status, including vanillin and taxol production in cell cultures. In a myriad of other cases, the *in vitro* processes for secondary metabolite production have fallen far short of expectations and have never approached commercial status. Still, the arena of secondary product formation in cell cultures remains as an industrial pursuit. Engineers and biologists are currently joining forces on a global scale to develop new strategies for streamlining the critical bioprocesses. Research efforts on a broad range of plant cell culture-derived extracts can be cited in each of these major product categories: flavors (onion and garlic, peppermint and spearmint, fruit flavors, chocolate aroma, seaweed flavors, vanilla, celery, coffee, spice, sweeteners, and so on); edible colors for foods and medicines (mainly betalains and anthocyanins); nonfood pigments for cosmetics and textiles (shikonin, berberine, and various other products); several examples of fragrances and essential oils; and bioactive natural insecticides and phytoalexins useful in current integrated pest management programs. Of course, intensive activity has centered on production of natural drugs or chemoprotective compounds from plant cell culture. Some of the most prominent pharmaceutical products in this latter category include ajmalicine (a drug for circulatory problems) from *C. roseus*, and taxol (a phytochemical effective in treatment of ovarian cancer) from *Taxus* species.

SELECTION OF PRODUCTIVE CELL LINES AND STIMULUS OF PRODUCTIVITY

Because different levels of secondary metabolite production can be found within a cell line (e.g., sectoring of colored and uncolored callus in a culture), significant headway in the productivity of plant cell cultures has followed on the heels of intensive selection for high-producing cell lines. Plant cell lines can be recurrently selected to amplify the productivity of the cell culture. Shikonin production *in vitro* at levels over 800 times what is available from plant roots is a case in point.

Similar amplification of yield has followed selection in highly pigmented cell culture lines. Natural pigments including anthocyanins, betalains, shikonin, and other pigmented phytochemicals are conspicuously accumulated in some cell cultures. Some of these products are of interest commercially as replacements for synthetic food dyes that have fallen "under the gun" recently as adverse health and safety concerns have emerged. Negative reports and new medical studies in the popular press have made synthetic additives a target for contention and have created a strong market demand for safe, natural ingredients in foods. As an added bonus, significant chemoprotective benefits (including cardioprotective and anticarcinogenic properties) have been attributed to natural anthocyanin pigments and related flavonoid phytochemicals. These health-enhancing properties are unrelated to the nutritive value of the plants. Even natural nonfood colorants are sought as replacements for environmentally damaging synthetic dyes fabricated with toxic solvents and heavy metals. The myriad of microenvironmental control tools available to *in vitro* production makes this alternative more promising than harvest from the intact plant in nature.

When *in vitro* manipulation results in accumulation of pigments in cell cultures, the result is a superb model to investigate control over anthocyanin, betalain, or other production by introducing gene constructs using regulatory genes to the cell culture tissues. For example, when excessively high anthocyanin levels interfere with cell culture growth, gene constructs may use an inducible promotor system. In this case, the bright red pigment is an obvious marker for research on manipulating flavonoid production and modifications in the simple cell culture system. These lines of research would be complex and unwieldy in whole plant models due to the restrictions of organ-specific product accumulation and interactions with other plant tissues. In many ways, natural plant pigments are ideal target compounds for laboratory classroom or demonstration experiments on metabolite production, because their accrual *in vitro* is quick, visible, easy to detect, and fairly simple to quantify. In practice, and for validation of research results, pigment accumulation is typically measured by extraction, then quantification and separation with thin layer chromatography (TLC), high performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR), or absorbance (spectroscopy). However, in a classroom, when traditional methods of pigment quantification are prohibited by the lack of sophisticated laboratory analytical equipment and insufficient student expertise, the production of a pigment *in vitro* can still be very easily detected and quantified by visual means alone or by coupling visual observation with very simple absorbance measurements using a spectrophotometer.

Many of the best-known *in vitro* pigment production systems are actually difficult to demonstrate in a classroom setting, because they require cultures derived from specific, hard-to-initiate explants, or because the high yielding models described in research reports have only been achieved after years of laborious repetitive subculture and selection of only highly pigmented sectors. One exception to the general rule is the *Ajuga in vitro* pigment production system, which requires no selection and is readily adaptable to classroom research. *Ajuga reptans* N. Tayl. (bugleweed) and the related species *A. pyramidalis* L. "Metallica Crispa" are both widely distributed groundcover plants that grow rapidly and spread via stolons. *Ajuga* tolerates adverse conditions so well that it is considered an intractable weed when it oversteps its boundaries in the landscape, and stock plants can be maintained long term in a greenhouse with minimal care. *Ajuga* cultivars are typically propagated by division of mother plants. A few select cultivars of *A. reptans*, which feature purple

or bronze foliage, include "Purpurea," "Giant Bronze," and "Burgundy Glow." The latter cultivar has a characteristic chimeral green, creamy white, and dark pink variegated foliage, but produces unicolor bronze, albino, or green variants from adventitious buds *in vitro*. *A. pyramidalis* "Metallica Crispa," which is frequently confused with purple forms of *A. reptans* in the trade, is characterized by savoy-type, glossy, bronze-purple foliage.

For a number of reasons, selections in the genus *Ajuga* are particularly well suited for use in plant tissue culture exercises. Like many members of the mint family (*Labiatae*), microplants flourish in culture and may be held in cold storage successfully for up to a year, which helps cut back on the need for repetitive subculture maintenance between semesters. *Ajuga reptans* proliferates readily *in vitro* from axillary or adventitious buds; exhibits classic, predictable responses to gradient increases of cytokinin concentration; and is a good, rapidly growing model plant on which to illustrate stages in an *in vitro* production cycle through acclimatization within a single semester.

The purple or bronze leaves that distinguish some varieties of *Ajuga* are also readily expressed in microcultured plants, and even undifferentiated callus can exhibit pigment expression. These suspensions produced anthocyanin pigments in the dark and pigment expression responded to changes in the light regime and to chemical medium treatments. In our alternative approach, vegetative disks are directly explanted from *Ajuga* foliage and induced to produce callus, which rapidly acquires a vivid purple hue when exposed to illumination (Madhavi et al., 1996). Intense, uniform pigment expression is routinely achieved without selection and friable callus colonies can be explanted into liquid suspension culture. Cell division and pigment accumulation are not mutually exclusive processes; both cell biomass and pigment accumulation increase steadily throughout each subculture cycle. The growth and pigment expression are so predictable and consistent that this system was used as a model to test the application of video image analysis to regulate bioprocesses by visual characterization of cell size, shape, aggregation, and color intensity.

One often cited drawback of reliance on secondary product accumulation in plants — whether in the field or in an *in vitro* production system — is an observed lack of reproducibility. This problem is a consequence of the variable nature of plant secondary metabolism, since the enzymatic pathways for synthesis of secondary compounds are highly inducible. For example, an alkaloid or bioflavonoid product extracted from plant cells may exhibit a sharp decline in potency or level of accumulation over time. Various stress factors in nature related to climate or pathogen insult or physical stress clearly impact on the qualitative and quantitative accumulation of valuable secondary products in nature, and similarly, deliberate introduction of stress agents (elicitation) is a strategy which can regulate secondary product recovery from cell cultures and allow for development of reliable, predictable production systems. Elicitors (compounds that "stress" cells, leading to formation of a secondary product) from biotic and abiotic sources can be added directly to culture media to stimulate production. Fungal filtrates, methyl jasmonate, chitosan, sodium acetate, beta glucan, metal ions (iron and copper, for example), and a wide range of other biotic and abiotic additives have effectively stimulated and intensified productivity in cell culture regimes. Another related approach is biotransformation — the deliberate feeding of metabolic precursors to a cell culture that already contains the necessary enzymes to convert them to product.

Another target for research has been the development of improved instrumentation for cultivation of plant cells *en masse*. For example, improved bioreactors have been specifically designed to permit light to reach plant cells during division and product accumulation. In many cases, the infiltration of light at optimum intensities and spectral qualities is prerequisite to successful synthesis and maximal expression of secondary metabolites. Modifications in aeration, agitation, and nutrient supply have similarly led to enhanced plant product yields in recent years.

UTILITY OF CELL CULTURE SYSTEMS FOR IN-DEPTH INVESTIGATION OF METABOLISM

One of the most important categories of plant secondary compounds, and one that has captured the attention of the general public, is the category of bioactive, medicinally important plant phytochemicals. Throughout history, plants have been formulated in potions and powders and routinely used to cure, to diagnose, and to prevent diseases (as well as related conditions like sleeplessness, impotence, infertility, hangovers, and the like). Current consumers have readily embraced the concept of being proactive about maintenance of their own health. Instead of being dependent on a physician's recommendations and synthetic drugs, they are receptive to having natural plant-derived products available in the marketplace. These natural additives have strong consumer and political support, and face substantially fewer international trade restrictions. As noted earlier, these biologically active compounds can be extracted from field-grown plants or from their cell culture counterparts, the latter usually with greater ease and reproducibility.

However, one of the drawbacks to the use of "natural" plant-derived medicinal substances is that, in fact, relatively little is known about their mechanisms of action or metabolism in the human body. Almost all evidence for their efficacy is based on anecdotal or epidemiological evidence. Two reasons contribute to this: first, plant compounds most commonly work in active mixtures, not as single compounds, which makes it very difficult to track their transport after ingestion; and second, plant compounds break down metabolically in the human body, which further complicates our ability to trace progress in the body or determine organ accumulation or absorption of certain metabolically active plant substances.

Because plant cell cultures can be induced to accumulate complex bioactive compounds and mixtures of compounds in predictable cell cultures using defined substrates, they can also provide a partial solution to the investigative challenge cited above. Recently, radio labeled ^{14}C substrates have been introduced to plant cultures, resulting in the incorporation of traceable labels into the bioactive compounds of interest. These compounds, once introduced to experimental animal models, will soon allow scientists to conclusively determine the mode of action, timing, and metabolic clearance of valuable, medicinally important plant secondary metabolites, which will be a further step towards more effective diagnosis and recommendations for dosage and treatment. These natural additives have strong consumer and political support, and face substantially fewer international trade restrictions.

SUMMARY

Experimental models that result in obvious, measurable accumulation of secondary metabolites are highly amenable to individualized, student-directed experimentation with a vast range of physical or chemical microenvironmental parameters (treatments) that can be deliberately varied to determine the effects on metabolite accumulation. The experimental results also help students to appreciate the value of chemical analytical methods for measuring metabolite production as an adjunct to strict visual gauges for pigment production in a callus or suspension culture.

LITERATURE CITED

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