Journal of Natural Products
Vol. 48, No. 2, pp. 203-209, Mar-Apr 1985

MILESTONES IN PLANT TISSUE CULTURE SYSTEMS FOR THE PRODUCTION OF SECONDARY PRODUCTS[1]

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ABSTRACT.—This review cites 22 recent review articles considering the production of biochemicals by plant tissue culture. A brief and selective overview is also given of classical and recent texts, plant tissue culture meetings, plant tissue culture research milestones, and some research strategies sometimes used to establish plant systems that produce biochemicals.

It can probably be argued that there are as many gravestones as milestones along the way in developing plant tissue culture systems for the production of secondary products. A recent analysis of commercial biotechnology by the United States Office of Technology Assessment commented that plant cell culture for producing secondary products is necessary only when good farmland is not abundant (1)! Nevertheless, I remain optimistic that in vitro plant tissue culture systems will become more efficient in the production, biotransformation, and discovery of new biochemicals.

Success was recently demonstrated by the Mitsui Petrochemical Industries' (Tokyo) ability to produce shikonin commercially in 750-liter fermentors from *Lithospermum* plant cells. Shikonin is a dye and antibacterial valued at approximately $4000/kg (2). With only one clear commercial success to date, it is obvious that plant tissue culture biotechnology still remains to be exploited (3). The research and development effort required is well worth the investment to achieve the in vitro production of not only specialty biochemicals, but potentially, food, spices, and industrial commodities. Such efforts are needed not only for the production and biotransformation of biochemicals but also for the study of photoautotrophic aseptic/nonaseptic cultures, florogenesis, derepression, i.e., the activation of dormant genes (4), and the somatic hybridization (5) and embryogenesis processes (6). Recently, France, through its Association for Research into Solar Bioenergy (ARBS), stated its intention to develop the photoautrophic production of *cis*-polyisoprene from *Euphorbia characias* cell cultures (7).

The plant tissue culture technique is used commercially to propagate plants rapidly and to develop either improved or new plants. Tissue culture is being used for the micropropagation of ornamentals, oil palms, glycyrrhiza, pyrethrum, pine, eucalyptus, sugar cane, potatoes, and other plants. Selections (8) can be made from plant tissue cultures for cells, and ultimately plants, that are tolerant to stress (i.e., pathogens, drought, salinity, temperature, herbicides) or chemicals (i.e., proteins, oils, pyrethrins, alkaloids). New plants (5) have been made by fusing the protoplasts of the sunflower and frenchbean, tomato and potato, and various *Datura* varieties. Bacterial vectors have introduced foreign genes for kanamycin into tobacco (9) and for proteins into sunflower tissues (10) and then into their redifferentiated plants. As exciting as these developments are for the development of new or improved field plants, we will leave them for the equally exciting developments occurring in the production of secondary products.

MILESTONES IN PLANT TISSUE CULTURE.—Discussions germane to plant tissue culture began more than 100 years ago with Schleiden and Schwann's proposed cell

[1]Presented as a plenary lecture at the "Biotechnology in Natural Products Research" Symposium of the 25th Annual Meeting of the American Society of Pharmacognosy at the University of Texas, Austin, Texas, August 19-23, 1984.

theory (11). Events slowly progressed, and in 1902 Haberlandt postulated that vegetative cells could give rise to artificial embryos (totipotency). Although unsuccessful, serious efforts were made by Haberlandt to grow and divide single plant cells in nutrient solutions (12,13).

It is difficult for one to select milestone events confidently and designate precisely their discoverer and the time of discovery. The list presented is an arbitrary one, given only to provide a perspective. Outstanding botanical tissue culture related events since Haberlandt are the isolation and identification of the plant hormone indoleacetic acid (Kogl, 1934); establishment of viable subcultures of plant cells and organs (White, Gaultheret, 1939); auxin/cytokinin interaction for organogenesis (Skoog and Miller, 1956); cell suspension embryogenesis/totipotency (Reinert and Steward, 1958); protoplast preparation (Cocking, 1960); high concentration inorganic growth medium (Murashige and Skoog, 1962); regeneration of plants from haploid cells (Bourgin and Nitsch); protoplast regenerated plants (Takebe, 1971). Protoplasts are commonly used today for selection and mutation, fusion, and the exogenous uptake of organisms or chemicals. If additional historical information is desired, one should consult the classical texts of Went (14), Gautheret (15), White (16), Steward (17,18), or Street (19).

Selections from selected symposia publications of the past 35 years illustrate well the rapid content and growth of plant tissue culture from that of a specialized program on plant growth substances (20) and a forerunner of our international meetings (21) to that of the well-recognized International Congress of Plant Tissue Culture (IAPTC) meetings (22-26).

MILESTONES IN PLANT TISSUE CULTURE BIOCHEMICAL PRODUCTION.—The commodities to be produced from plant tissue culture systems should not be restricted to the pharmaceuticals. Admittedly, the analgesics, antimicrobials, antitumor agents, allergens, CNS active compounds, cardiac and vascular agents, hormones, laxatives, and vitamins are an imposing list of important and often expensive compounds. Nevertheless, the pharmaceutical industry is often not as large as the food industry (bitters, flavors, pigments, spices, sweetners, thickening agents, etc.), specialty houses (cosmetic, enzyme, perfume, tobacco, etc.) or industrial conglomerates (energy-yielding compounds, feed stock, hydrocarbons, insecticides, plant growth regulators, polyaromatic degradation, rubber, silkworm feed, etc.).

A basic list of 22 secondary product review articles published since 1965 is given in the list of references (13, 27-47). Many of these review articles recognize the earlier studies of Gaultheret, Bonner, Tulecke and Nickell, Steward, and Street. In addition, very useful edited texts have been published on plant tissue culture fundamentals (48,49), cell division (50) and genetics (51), secondary product biosynthesis (52-56), and advanced concepts (57-59).

A milestone for the tissue culture production of secondary products cannot simply be based on a higher yield than the parent plant or the discovery of a new, structurally unique compound. In 1982, at least 30 compounds were known to accumulate in plant culture systems in concentrations equal to or higher than that of the plant (46). Commercial milestones can only be counted as those systems that result in profitable processes!

These milestones are events from the past that significantly altered the experimental approach to plant tissue culture secondary product research. They are, in my judgment, Bonner's pioneering effort in 1950 to study rubber biosynthesis in guayule callus; Tulecke and Nickell's early efforts in 1956 to grow plant cultures in multi-liter suspension systems; Street's dedicated efforts in 1971 to control suspension cultures turbidimetrically or as continuous cultures; the development by Steck *et al.* in 1971 of a rapidly growing *Ruta graveolens* cell/organ liquid culture that produced many different

compounds; the semi-batch scale-up of tobacco cells in 20,000 liter fermentors by Kato in 1972; Reinhard and Alfermann's discovery in 1974 that digitoxin was biotransformed to digoxin by digitalis tissue cultures; the importance of selecting producing cells from non-producing cells as was independently demonstrated about 1976 by Tabata and Zenk; and the development by Brodelius *et al.* in 1979 of immobilized plant cell systems.

STRATEGIES FOR PLANT TISSUE CULTURE PRODUCT PRODUCTION.—A traditional or a contemporary research strategy approach can be used to study plant tissue cultures for the production of secondary products.

Traditional research strategies emphasized the importance of the explant, selecting a medium for maximum cell growth, modifying growth regulators or adding precursors to the medium to observe the effect on product formation, and growing the cells in a batch mode often in light (60). There is nothing wrong in employing these strategies (61,62), but they may be self-limiting in the development of an optimal production system. Controversy over the significance of the explant continues (3), but, nevertheless, it appears best to explant the highest-producing tissue from the highest-producing plant available.

Contemporary research strategies consider the tissue culture environment and genetics more comprehensively than did the traditional research strategy. The research goals have also broadened from producing drugs to producing foods and food flavors (cocoa flavor, onion flavor, starch, etc.); enzymes to make alkannin and shikonin, codeine, digoxin, pyrethrins, and so on; and to make new biochemicals. Biological screens have been used to discover new CNS-active indole alkaloids in *Picralima* tissue cultures (63), and tissue culture systems have even been used to screen for antitumor agents (64).

Culture Medium.—After appropriate cell growth, the growth medium may be changed to a production medium that induces the biochemical(s) desired. The nitrogen, microelements, and carbohydrate source in the medium can markedly effect product yield (65). Photoautotrophic cultures can produce different compounds than their nonphotoautorophic culturer (7). The tissues photosynthetic state is significantly affected by the medium, gas phase, and light source (66). These nutrient and environmental factors, as well as chemicals or microorganisms added to the medium, or genetic manipulations, are sometimes viewed as an induced plant stress. Such stress effects have recently been reviewed (67), as well as those induced by fungal elicitors, on the secondary metabolism of cultured plant cells. Lastly, if the medium is made biphasic (68) or has added to it solvents, such as DMSO, products may be leaked and accumulated.

Culture Systems.—Batch and semibatch suspension cultures that are controlled turbidimetrically or semicontinuously have often been studied. It is well recognized that plant systems have a slow growth rate and that their cell and tissue chemical transport system is unique and different from that of typical microorganisms (69). Their air or oxygen requirements are lower than those of microorganisms and may amply be supplied by air-lift fermentors (70). Recently, multistage continuous (71) and hollow-fiber reactors (72) have been suggested for plant cell suspensions.

Attention has recently been given to immobilizing plant cells to prolong their production phase and their total product yield (73). The product would have to be leaked from the plant cells, unless their immobilized protoplasts (74), organelles (75,76), or cell-free enzymes (77) were active.

Organized plant tissues such as embryoids (6), roots (78), shoots (79), and plantlets (80) have been grown as liquid cultures. There is a need to develop plant systems to grow organized plant cultures more efficiently, perhaps even nonaseptically, and to be able to induce flowers predictably. Organized plant tissues and cells are sometimes

grown with other microorganisms to induce biochemicals or plant organs, to provide conditions for genetic selection, or to incorporate genetic material.

Unorganized Tissues.—It is well-recognized that a number of unorganized cell cultures are genetically variable, containing both product producing and nonproducing cells. It is possible to select from that heterogenous population cell lines that produce the product higher than the normal cell population and in the plant. For example, cell lines of *Lavendula vera* produce seven times more biotin than that found in the unselected culture and 4.5 times more than in the plant leaves (81). Yamada and his co-workers were also able to fuse berberine producing *Coptis japonica* protoplasts with anthocyanin producing *Euphorbia milli* protoplasts to make hybrid cells with high yields of both products (82). Unfortunately, selected plant tissue culture strains are often unstable.

Unusual metabolites, such as azulenes and volatile terpenes, are produced in the volatile oil (2.0-3.3%) from unorganized *Calypogeia* liverwort tissue cultures (83) and monoterpenes in unorganized *Perilla* suspension cells (84). Such unorganized cells may have different stages of differentiation. As examples, it is now known that high-auxin-containing cells may accumulate starch (85) and that some *Catharanthus* suspension cells can differentiate into storage cells and their vacuoles accumulate alkaloids (86). Cells and tissues can be grown to and held at a growth stage (e,g, stationary phase by semicontinuous culture) to induce differentiation and product formation (e.g., phenolics).

Organized Tissues.—Although unorganized tissues adapt to conventional fermentation systems better than organized tissues do, they frequently do not produce the biochemicals desired or their strains lose that ability. Organized tissues have the ability to produce the products desired in amounts similar to that of the plant and are genetically more stable. Selection can also be done from organized tissues to increase product yields, as, for example, scopolamine from *Hyoscyamus niger* root suspension cultures (87) and pyrethrins from pyrethrum shoot cultures (88).

The situation is not always clear regarding the unorganized or organized cell and tissue conditions needed for product production. For example, digitalis cells produce insignificant amounts of cardenolides, but embryogenic strains (89) and shoot tissues (79) do produce cardenolides. Morphinane alkaloids are sometimes produced in unorganized tissues (90,91) and sometimes not (92), but they are consistently produced in organized tissues (93,94).

CHALLENGES.—In summary, the broad challenges facing those using the plant tissue culture technique are (a) to understand better how to de-repress cells or activate dormant genes, (b) to determine how cells are interdependent upon each other, (c) to know how to stabilize genotypes, and (d) how to grow uniform cell and organ biomass economically.

ACKNOWLEDGMENTS

The author wishes to thank Mr. Mark W. Staba for his assistance in preparing the literature cited.

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