PLANT CELL TECHNOLOGIES IN SPACE: BACKGROUND, STRATEGIES, AND PROSPECTS

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INTRODUCTION

This section has two points of origin. The first, and earliest, is the long-time effort on the part of many workers in development of methodology for in vitro culture of plant cells or parts, and more specifically, the efforts aimed at understanding how plant cells in culture may be induced to produce chemical products of interest and value. The second point of origin is a recent effort on the part of the writers to develop a system of flight experiment hardware which will support basic science objectives in plant or animal cell culture experiments. These two lines of work have now begun to converge: plant culture technique has progressed to the point that we are able to manipulate cultures to obtain meaningful yields of secondary metabolites; the optimal culture vessel for plant cell experiments approaches the size of a small cell culture bioreactor. The stage is thus set for a serious investigation of the production of plant cell-derived biologicals in space-borne manufacturing facilities.

In this paper, we have attempted to summarize the work which has led us to the present state. We first consider the evolution of concepts and then the general principles of plan tissue culture, and finally, the potential for production of high value secondary products by plant cells and differentiated tissue in automated, precisely controlled bioreactors. The literature on plant tissue culture is voluminous; no attempt will be made to recount in detail. Rather, the general course of its development will be highlighted to convey to the reader a feeling for the major milestones in the path to modern tissue culture as well as the difficulties which had to be overcome. In order to maintain rapid flow of the narrative, specific literature citations will not be included in the text. However, a large bibliography of pertinent and current references is included at the end of this paper.

The Development of the Modern Period

The modern period of plant cell culture began around the turn of the century with attempts by Gottlieb Haberlandt to culture plant cells in isolation. Haberlandt, who was destined to become one of the leading botanical investigators of the 20th century, clearly saw the potentialities inherent in culturing isolated plant cells, but also came to realize that it was necessary to know more about the specific factors required for plant cell division. Because of his lack of success, Haberlandt turned his attention to the study of requirements for cell division. Nevertheless, Haberlandt's paper describing his experiments contains numerous points of principle which recur again and again in any consideration of growing cells in vitro. Other workers contributed importantly to the accumulating knowledge. Erwin F. Smith, the great pioneer of plant technology, saw the possibilities of the application of the aseptic culture methods to the study of relationships between a pathogen and its host as that exemplified by the crown gall tumor system.

A number of workers contributed to the technology through investigations of intact organs, notably roots, in culture. Prompted by contact with B. M. Duggar, a pioneer in plant physiology at Cornell University, first Lewis Knudson, then William J. Robbins, exploited aseptic culture methods in their work on root systems. Knudson utilized aseptically cultured roots to study carbohydrate metabolism, and Robbins from this starting point developed his approach to the culture of roots. These root cultures, however, represented organ cultures and there is a basic difference between the growth of an organ culture, such as that of an excised root in which the root apex is already established as an organized structure before culture has been started, and the culture of isolated tissues or of undifferentiated cells. Nevertheless, the countless investigations which finally established the nutritional and environmental conditions for unlimited growth of selected excised root tips, stem tips, and "tissues" of root and stem, have invaluably aided all those who have attempted to culture other plant materials aseptically for the first time.

Early work with plant "tissue culture" suffered from a number of deficiencies and blind alleys, and much of the early work tended to be tangential to the main thrust. If one uses the early definition that plant tissue cultures are "aseptic preparations of somatic cells and tissues which grow and function without serious physiological derangement, but which at the same time, may not differentiate into distinct organs," Gautheret, Nobecourt, and White probably had the first true cultures which fulfilled this definition. Indeed, during this early period the single criterion of a successful tissue culture was the unlimited capacity for undifferentiated growth. There would

be no point in citing all the pertinent literature of this period, but the well-known works of Philip R. White and Roger Jean Gautheret, which concentrated on the methodology rather than the solving of problems, need not be mentioned, for they developed techniques even though they did not lead to many new concepts. In fact, the unfortunate controversies between White and Robbins tended to focus attention upon the then incompletely understood minutia of technique to the exclusion of the large problems that could be solved by the use of the tissue culture procedures. Similarly in France, Gautheret and Nobecourt became preoccupied with the idea of continued and indefinite cultivation of plant parts and their organs and pursued this line of work even though many of their continuously cultured strains probably deviated very considerably from the tissues from which they originated. Moreover, the methods as developed and adopted by the early workers, such as Gautheret and White, had severe limitations in that they were restricted by the use of large explants on semi-solid media (usually agar). They also seemed concerned with tissue survival and continuous growth by means of callus subculture to the extent that few attempts were made to initiate fresh cultures once a given plant was successfully cultured.

Another very real problem with the technology, as it was then practiced, was that the very definition of a plant tissue culture dictated that it should not undergo organization. This is the very antithesis of what is commonly aimed for now and reflects another of the early limitations.

Plant Cell Culture as a Tool

A turning point came when plant physiologists began to use the tissue culture technique not as mere means of growing biological material, but as a means of solving problems. And at this point radical changes in the design of tissue culture strategies occurred. Notable in this connection were the methods developed in the laboratory of F. C. Steward, then at the University of Rochester. As part of the experimental design to study salt and water uptake during growth of plant cells, he recognized the need to have a system in which he could control or initiate cell division. In the course of routinely screening a number of tissue culture systems for potential adaptation to these experimental ends, beet, carrot, and Jerusalem artichoke tuber explants, potato tuber explants, the inner epidermis of bean pods, tissues from the floral receptacle of crab apple, and ovary wall of cherry and basal meristematic areas of Narcisscus leaf and even pistils from unopened flowers of may apple (Podophyllum) were tested in traditional "Latin-square" experiments against a number of potential sources of stimuli such as indole-3-acetic acid, traumatic acid, phenobarbital, 2, 4-D, extracts of fish eggs, yeast extract, stilbesterol (estrogen), and last, but not least, the liquid endosperm of coconut (coconut water, often erroneously referred to as coconut "milk"). The use of coconut water had been suggested by the work of

Van Overbeek, Conklin, and Blakeslee who had shown that heart-stage embryos of Datura that would otherwise abort could be grown to maturity in aseptic culture, using the liquid endosperm of coconut as a supplement to an otherwise standard culture medium.

The work of Blakeslee and Van Overbeek led to an early attempt to find out whether coconut water contained any unique growth promoting substances. Van Overbeek et al. had recognized both heat stable and heat labile substances that had growth-promoting qualities for embryos, and they even recognized a substance that caused embryos to proliferate rather than to grow in an organized way, but attempts to isolate such substances were unsuccessful and led to the notion that growth promoting activity was due to the balance of ordinary known organic and inorganic nutrients rather than to the presence of any exotic and hitherto unidentified substances.

The growth response obtained by Steward and Caplin to the chemical stimili tested ranged from none or negligible to dramatic, in the case of carrot in the presence of coconut water. It was soon realized, moreover, that coconut water was only one of several fluids (e.g., chestnut (Aesculus) and black walnut liquid endosperm, extracts of the female gametophyte of Ginkgo, etc.), being extracts of nutritive fluids for immature embryos, which could produce the same effect. Therefore, cell division factor(s) were not specific to coconut water, but it could not be replaced by any of the wide range of known substances. It did not take long to establish the viewpoint that a number of substances which act in combination was involved -- even to the extent of appreciating a balance between promotion and inhibition. This philosophy involving the chemical basis of growth regulation in higher plants found expression in the aphorism that "no single substance unlocks the door to cell division" and is a view that has maintained itself even as it has become amplified in recent years.

A desire to better understand the chemical nature of, and even to isolate the stimuli for cell division that resided in the naturally occurring fluids led to the refinement of the growing methods and to the eventual development of a bioassay in which uniform growth of fresh tissue explants was promoted in liquid culture. The difficulties of liquid culture were surmounted by culturing the explants in tubes which were rotated slowly (1 rpm) about a horizontal axis, so that they were alternately bathed in liquid and exposed to air. Using explants of carrot root phloem, 4 mgs in weight, with the inner tangential surface of the cylinders one millimeter from the cambium, Steward and Caplin noticed that there was about a 4-day lag after culturing, followed by a very rapid growth rate reaching a compound interest rate of increase in 12 days of 34.1% per day. This amounted to an increase in cell number from about 25,000 to a million and a half in 8 to 10 days! With a system such as the one described, they could study not only the growth promoting, but also the growth depressing activities of a number of substances. Since the

coefficient of variability of the explants was so low even after a prolonged period of growth, the carrot root phloem system was, and still is, used as a bioassay with wide application. The technique was, however, not without its critics who unfortunately failed to recognize the importance of the size of the initial explants in determining growth rates.

THE TECHNIQUES OF PLANT TISSUE CULTURE

Free Cell Culture

Experiments with explants in liquid culture led to a fortuitous discovery. In unreplenished cultures and at the end of about 21 days, the cells in the explants are no longer rapidly dividing and the typical sigmoid growth curve begins to level off, but some of the peripheral cells begin to break away from the parent explant and are freely suspended in liquid medium. Free cells of carrot so liberated in a liquid medium could be transferred as suspensions to fresh media and their growth as free and suspended cells and clusters ensued even in the absence of the parent explants. This technique represented a marked departure from the earlier methods which utilized free cells isolated as such directly from the tissue of their origin or depended on the disruption of proliferated callus cultures by vigorous agitation on rotary shakers. By causing a tissue explant to grow and proliferate in a liquid medium, Steward and his co-workers provided the environment in which cells could rapidly grow and divide.

Muir et al. had published the first successful attempt to culture individual higher plant cells derived from agar grown callus cultures that had been disrupted in a liquid medium. Single cells so isolated could be induced to grow by placing them on a "nurse" tissue, provided in the form of a vigorously growing agar culture, from which it was separated by a piece of filter paper. This ingenious technique provided the milieu in which single cells could proliferate and form large calluses. But it was neither easy to follow their development nor convenient for growing particularly large numbers of cells. The batch culture methodology, made feasible by directly initiating cultures of carrot in liquid medium, provided the point of departure for rapid develoments, and for some time, the growing of single cells in microchambers became obsolete.

Somatic Embryogenesis in Free Cell Culture

It was early recognized that small carrot root phloem explants only very rarely formed roots in culture, but carrot cell cultures in liquid media formed roots much more frequently. Suspensions of such strains of carrot

cells capable of forming roots were regularly maintained in liquid by continued transfer of small aliquots, but shoots were never observed. When such cultures, bearing roots, were transferred to a semi-solid agar medium, the first shoots and even complete plantlets were obtained.

The aspect of the work that was recognized at the time as being the most significant was that free cells derived from small explants of mature carrot root phloem and in the absence of a cambium, had been induced to grow rigorously in the presence of a natural fluid. And that after substantial number of subcultures, using sparse and dilute inocula, these could readily give rise to plantlets. When such carrot plantlets were later grown to maturity, and after the appropriate biennial growth period, they flowered. The flowers were normal by all visible criteria and contained normal, viable embryos. It then became possible to raise several "generations" of carrot in which the normal reproductive part of the life cycle was entirely by-passed, although the successive generations were limited through cells derived from the phloem of the mature storage root. By explanting plugs of secondary phloem and culturing them, free cells were again obtained which gave rise to rooted nodules which in turn gave rise to new plants. These observations emphasized that a full complement of genetic information must reside in cultured cells, i.e., that the cells were totipotent. Thus, the old idea that genetic material was permanently altered or lost during the process of differentiation had to be discarded, at least in the case of carrot.

The events which led ultimately to controlling the cultured carrot cells and tissues to express their "totipotency" are well documented in the papers of the period and need not be dealt with in detail here. It is in this period of development of modern culture methods that efforts became increasingly canalized towards the study of morphogenesis in cultured tissues. The work of Skoog and his colleagues, dating back to the mid-1940's, is of special significance. Work on the controlled differentiation of tobacco tissues, first from stem explants, and later from callus derived from pith, took a dramatic turn with the discovery that preparations of autoclaved herring sperm DNA could stimulate (in association with the auxin indole-3-acetic acid) cell division. A very active cell division substance named kinetin, N6-furfurylaminopurine, was isolated from such preparations.

The importance of the discovery of kinetin, although it has never been shown to be a naturally-occurring component of plant cells, cannot be overemphasized. It provided the first chemically identified cell division substance. It also led to the dramatic discovery that in combination with auxin, and at least in the case of tobacco, it could either stimulate undifferentiated callus growth, or could lead to the formation of shoots or roots from such callus depending on the relative ratios of exogenously supplied auxin: cytokinin. Higher auxin relative to cytokinin yielded roots; higher cytokinin relative to auxin yielded shoots.

Although we now know that the controls of differentiation and organogenesis are much more complicated than the tobacco pith tissue system implies, the almost simultaneous discovery of the chemical nature of the "first' plant cell division "hormone" and somatic embryogenesis in carrot served as a tremendous impetus to the field of plant tissue culture. The stage was set for a new era in experimental embryogenesis and the study of growth and organizational control in explanted tissues.

Chemical Growth Control for Plant Multiplication

The early investigators working with callus and suspension cultures fully appreciated the significance of their work for multiplication. On more than one occasion it was suggested that mass cell culture methods could perhaps be used for clonal multiplication of horticultural specimens with particularly attractive qualities. Although some efforts were made at implementing culture procedures with practical ends, it became clear that the systems were generally too uncontrollable or seemingly restricted to too few plant species for use on a truly technological level.

Following on the work of early investigators, a great deal of work has been done on evaluating the usefulness of chemically controlling organized growth from a wide range of plant materials. One can reduce the various kinds of observations made on responding systems rather conveniently, however. These responses when seen from the perspective of multiplication, clonal, or otherwise, now permit deliberate strategies to be adopted in the laboratory environment. Table 9-1 lists the bulk of these strategies. It will be apparent that the various routes are largely the outcome of an appreciation, however superficial, that (1) organized growth or indirect organogenesis can be either "husbanded" or fostered in callus systems more or less de novo; (2) organized growth or direct organogenesis can be generated from excised organ or tissue systems without an intermediate and extensive callus stage; (3) somatic embryogenesis can be stimulated to express itself from competent cells, however they may be generated and selected for; (4) various levels of branching or shoot development can be facilitated by releasing certain correlational controls normally present in the intact plant body; (5) plantlets can be generated directly from organs of perennation sometimes precociously inducible in vitro; (6) micrografting procedures performed in vitro can effectively lead to more plants in some cases; (7) ovule culture can rescue or lead to plants that would otherwise be lost; (8) embryo culture can do the same; (9) mega- and microspore culture can similarly yield materials that reflect either of the female or male genotypes, respectively; and (10) infection with a crown gall plasmid genetically altered to stimulate teratoma-like tumors can lead to rescue of multiplied materials.

TABLE 9-1. STRATEGIES FOR MULTIPLICATION OF HIGHER PLANTS IN VITRO

Shoots from terminal, axillary or lateral buds

- apical meristems (no leaf primorida present)
- tips (leaf primordia or young leaves present)
- buds
- nodes
- shoot buds on roots

Direct organogenesis

- adventitious shoot and/or root formation on an organ or tissue explant without an intervening callus

Indirect organogenesis

- adventitious shoot and/or root formation on a callus

Somatic embryogenesis

- direct formation on a primary explant
- indirect formation from cells grown in suspension or semi-solid media

Direct plantlet formation via an organ of perennation formed in vitro

Micrografting

Ovule culture

Embryo rescue

Mega- and microspore culture

Infection with a genetically altered crown gall plasmid

These topics are not as peripheral to the subject of this paper as it may seem because cells can be the starting points to generate plantlets de novo and then via a combination strategy, the techniques outlined in table 9-1 can be brought to bear on the propagules according to a defined need. Even so, no purpose is served in extensively discussing each of these strategies here. Many comprehensive works are now available which address the specifics. Several are cited in the extensive bibliography at the end of this work.

Hormones in Multiplication Technology

Of more direct concern to the subject of bioreactor culture technology is the rationale for hormone application that underlies the currently used strategies for somatic embryogenesis and for induction of adventitious shoots and roots on such unorganized callus cultures as tobacco. It is a strategy widely used commercially in the multiplication of plants from preformed or organized propagules, and it is understood in relatively simplistic terms.

It stands to reason that good familiarity with the biology of any given plant is the first of several prerequisites to successful manipulation in aseptic culture. Botanists appreciated, long before axenic culture techniques came into laboratory practice, that the development of the higher plant body involves the suppression of many, and the development of relatively few, actual or potential primordia. Produciton of new growing regions, tissues, organs and even new plants were clearly held in check by correlative influences and inhibitions. Applied auxin in the form of indole-3-acetic acid or indole-3-butyric acid was early used to induce root formation on cuttings ever since auxin was chemically identified.

The discovery in the 1960's that explanted shoot meristems and shoot tips of the orchid Cymbidium grown in aseptic culture produce protuberances which resemble normal protocorms and can grow into plantlets provided the most dramatic impetus for the further development of procedures for multiplying and maintaining plants in aseptic culture. Shoot tip and meristem-containing cultures from many other plants have since been exploited in the obtaining, maintaining, and multiplying of stocks. In some cases, a single plant is generated from one cultured shoot tip or lateral bud explant while in others, multiple shoots can be stimulated to develop.

In each of these cases, it is often helpful to force precocious (multiple branches usually in numbers greatly in excess of normal) axillary branches to form, so these can be separated and rooted. Axillary shoots or laterals, can produce additional axillary branches, theoretically in perpetuity, as each newly formed shoot or node explant is subcultured. The method has been applicable to a great variety of species ranging from herbaceous foliage plants to bulbous moncotyledons, and also to woody species.

Micropropagation was originally defined as any aseptic culture procedure involving the manipulation of plant organs, tissues, or cells that produces a population of plantlets bypassing the normal sexual process or non-aseptic vegetative propagation. Stem tips and lateral buds have been, to date, the most commonly used starting point or origin of the primary explant. For this and other reasons, the use of callus, free cells or other of the more demanding approaches covered in the earlier section, rarely comes to mind when the word micropropagation is used. The key feature of micropropagation via

precocious axillary branching, however, directly arose out of the early work on kinetin. Wickson and Thimann were taken by the possibility that kinetin might well exert an effect on the development of buds, not just their initiation as had been suggested by the work of Skoog and Miller. The essential point is that cytokinin can antagonize the inhibitory effect of auxin on lateral bud elongation. One is essentially releasing the tendency for a shoot apex to exhibit apical dominance over the lateral buds.

Murashige and others have found it helpful to segregate the sequence of events associated with the multiplication process as follows:

Stage I - the initiation or establishment stage. Here the initial or primary culture is established.

Stage II - the shoot multiplication or multiplication stage. Here the goal is to multiply shoots.

Stage III - the rooting or pre-transplant stage. Here the goal is to produce a self-sustaining plant that can survive transplantation to soil, greenhouse, or growth chamber condition.

Frequently, specific media or aseptic culture conditions are associated with each of these stages and can be helpful in developing a strategy, or attempting interpretation. However, one ought to avoid the implication that these stages are always temporally discrete and separable. They may not be. Murashige initially reduced the stages of in vitro propagation to three: I, II, and III. But a fourth stage, IV or final transfer to the natural environment stage, is also seen as an integral part of the procedure as well. Moreover, an initial stage "0" has also been added. This stage "0" involves selection of the mother plant and selection of a program of pretreatment to render the strategy to be adopted workable.

The fact that many papers have been written on each of these stages or features of in vitro multiplication should be indication that the approach is now well established.

Multiplication Via Organs of Perennation Formed in Aseptic Culture

Some plants form organs of perennation in vitro. When this occurs, one has the means for multiplication at another level and it may well turn out that direct planting or germplasm storage of plants can be implemented by this means. Potatoes can form miniature tubers, gladiolas can form cormlets, bulbils have been encountered in certain lilies, onion, narcissus, hyacinth, Dioscorea, etc. and, of course, protocorms are produced by orchids. None of

these, other than the orchid protocorm system, has been so controllable that it has been seriously adopted as a means of multiplication.

Micrografting

Micrografting is perhaps best seen in the context of providing virus-free or virus-indexed material for further conventional multiplication. In Citrus, the technique involves grafting, in vitro, of a shoot tip, usually with two leaf primordia, onto a disease-free (zygotic) seedling root stock. Because Citrus seedlings are generally free of virus and hence are sued as the stock, and because the shoot apex of the stem which is to be grafted onto the root stock is presumptively pathogen-free, the system offers a unique opportunity to produce mother block plants which are virus-free. It is not particularly important to have high efficiency in the micrografting since conventional grafting can be carried out once specific pathogen-free mother plants are generated. It is standard practice to re-graft onto indicator plants for evaluation of cryptic viruses prior to final large scale nursery grafting in the field. Micrografting has been carried out with such commercial species as apples, camellias, and cinchona, as well as the citrus indicated above.

There is no evidence that exogenous growth regulators above and beyond those used in maintenance of shoot tip cultures play a major role in the micrografting technique.

Embryo or Spore Culture

It was shown as far back as the 1920's that one could sometimes stimulate growth, of certain embryos otherwise unobtainable or erratic, in aseptic culture. In some cases, embryos with poorly developed food reserves do not germinate because they are very dependent upon external nutrient sources. For instance, the seeds of orchids contain a very small embryo comprised only of a simple mass of several hundred cells. The embryo is totally dependent, for germination, upon exogenous organic foods such as sugar. In nature, this sugar is provided by a symbiotic mycorrhizal relationship. Another well known example of failure to germinate involves the formation of inhibitors in the seed. Here, embryos can often germinate only after an appropriate period of dormancy. In some plants (e.g., Iris), one can eliminate both the dormancy requirement and the effect of germination inhibitors present in the seed of some hybrids by excising embryos and rearing them in aseptic culture until they reach a size sufficient for transplanting to soil. Aseptic culture has become a widely used and routine procedure for rescuing embryos that would not normally grow into plantlets. In the strict sense of the word multiplication, this in not truly a process of multiplying stock, but considering

that germplasm would otherwise be lost without the technique, then it is appropriate to include it in the list of strategies for multiplication.

The late Emerita de Guzman and her associates at the University of the Phillippines at Los Banos had considerable success in the growing of the coconut sport called "Makapuno". Under natural conditions, the endosperm of "Makapuno" seed rots and hence deprives the developing embryo of necessary nutrients. But by removing individual embryos from the seed and providing them with an apropriate nutrient medium, they were able to rescue (and grow seedlings) to a size sufficient for field planting.

Splitting embryos longitudinally is yet another possible means of multiplication. In this case, the plant "halves" are identical, or clonal, of course.

In certain orchids, seeds can be aseptically germinated, and these in turn may develop even in the absence of exogenous growth regulators into protocorm masses, which can be subdivided and multiplied. Populations may thus derive from a single embryo and this is, of course, a clonal population. In cases such as the rare epiphytic or endangered terrestrial orchids, where only seeds may be accessible, this is an reasonable strategy for clonal multiplication.

Rearing of ferns from spores sown aseptically on nutrient medium has been carried out for years as a way of increasing success in multiplication. In recent years, prothallia have been generated from spores and these divided to provide a source of gametophytic tissue. This gametophytic tissue, in turn, can be broken down using extreme procedures such as chopping in an electric blender, and in time sporophytes are formed. Ferns have long been favorite subjects for the study of apogamy - i.e., the development of a sporophytes from gametophytic tissue. What is significant from the perspective of this treatment of growth regulators in tissue cultures is that all this is achieved without use of exogenously supplied hormones.

Androgenesis, or the production of plantlets from anthers as sources of haploid cells has been recognized since the late 1950's - early 1960's. Cultures can be initiated from anthers containing immature pollen grains - actually microspores - prior to the development of pollen grain or mature male gametophyte. In tobacco, for instance, the vegetative nucleus divides to give rise to the proembryo while still within the original wall of the pollen grain. In still other cases, it has been possible to induce isolated pollen grains to form somatic embryos. Plants produced by these means are likely to be dissimilar to their parents and, therefore, the means is not strictly applicable to clonal multiplication, but there are some instances where this indeed results in cloning of the individual. As in the case of fern spores, the culture media that are most often used to achieve

adrogenesis omit exogenously added hormones. On the other hand, gynogenesis, the process whereby haploid plants are procuded in vitro by induction of haploid tissues from the female gametophyte, usually employs a more complex medium.

PLANT CELL CULTURE FOR PRODUCTION OF HIGH VALUE BIOLOGICALS

The emphasis given above to discussion of micropropagational technology should not be taken to mean that work on cells in suspension culture came to a halt during the period of the most rapid emergence of multiplicaion strategies using shoot tips, etc. It is true, however, that the greatest amount of publicity was given to them becuase there was clear-cut potential for short-term commercialization. As mentioned earlier, it was recognized from the outset of plant cell culture studies that there were possibilities for clonal multiplication using free cells. These ideas took time to mature, however, even as the techniques were being worked out. Similarly, it was appreciated in the mid-1950's that plant cells grown in culture might well be usable for production of specific biochemicals (figure 9-1). As a direct consequence of some of the early work, several industrial research programs were initiated to explore the reasibility of growing cells in bulk. As it turned out, however, most of those attempts led to the conclusion that it is not easy to simulate accurately and at will in unorganized cell cultures otherwise organ-specific or tissue-specific biosyntheses. In those days, the controlled course of metabolism and biosynthesis, which is often linked to morphogenetic complexity, severely limited the economic use of unorganized cell culture systems for biosynthetic purposes. The doctoral dissertation work of one of us (A.D.K.) on that precise problem, initiated in 1959, led to the conclusion that our ability to evoke in culture any feasible feature of normal growth, form and composition of cells in situ was very often a measure of our ability to apply external stimuli that could successfully emulate the genetically based signals that cells must receive during their organized development.

Because the yields of components producible by cultured cells were relatively low when compared to the amounts extractable from intact plant parts, attention was necessarily diverted by most investigators interested in this field to other, more approachable, fundamental problems of differentiation and growth. The advances in our understanding of plant cell nutrition, cell division, differentiation, biochemistry and biochemical genetics has once again made the field approachable and there has been a dramatic rise in resumed interest. The theoretical possibility of using virtually any plant part to generate competent or totipotent cells equivalent to the zygote from which they were derived by a series of equational divisions, makes it all the more reasonable to believe that such cells should contain the full biochemical capacities of the whole organism, if these can be evoked.

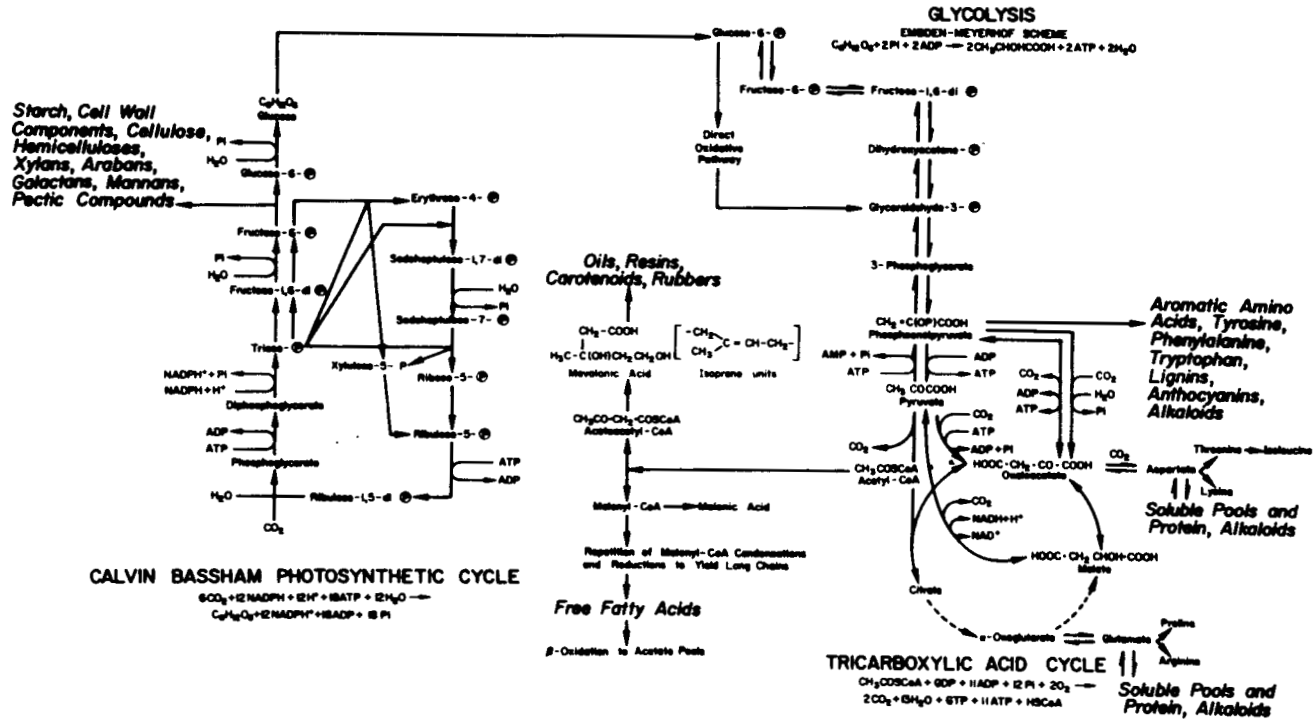

Figure 9-1. General scheme of higher plant metabolism showing biosynthetic pathways leading to secondary product synthesis and accumulation. Note the tight linkage of secondary product synthesis to primary functions.

The aim of much research has been to determine and define those conditions which permit cultured higher plant tissues and cells to express their innate biosynthetic potential. Strategies have included selection of plant species that can serve as models with which to assess the range of problems that must be addressed before full and routine expoitation of plant cell systems can be achieved for specific biosyntheses. One of the strategies adopted has concentrated on the control of, or release of, the suppressed biochemical potential without the need to do this via a complete new ontogenetic cycle of development.

Selection of particular genotypes or "chemovars" of plants intended for culture has been more carefully addressed than ever before. It is well recognized that strains within a species may vary widely in their inherent capacity for production of secondary compounds. There is little hope for achieving high level synthesis from a system limited by its genetics and heritage. Efforts have also been made to decide which plants are best investigated or justified from an economic view. Some estimates have been made that suggest no product producible by cell culture technology is ever going to be economically viable unless it can sell for at least $80 a gram or $36,320 a pound. Other estimates are considerably lower, but it is clear that such products cannot be inexpensive.

The availability of appropriate cell lines which are producers is, of course, central to the entire theme. It has long been recognized, however, that procedures must be available to maintain cell lines in a culture bank, either lyophilized or cryopreserved, if industrial application is to be a reality. As it turns out, and primarily as a result of a research initiative directed at maintaining genotypically stable materials for germplasm storage, dramatic progress has been made in the area of cryopreservation.

The use of biological materials or cell types even more precisely selected from a wider range of possible genotypes is now possible through use of protoplasts produced by techniques of enzymatic digestion or mechanical maceration of plant tissue. Such cells may be "plated" out on selective media or under selective environmental conditions in much the same manner as microbial cells. Immunofluorescence-based and monoclonal antibody procedures can also play a role in facilitating the selection of appropriately "turned-on" cells. The rationale here is that those cell cultures which have derived from cells which are not appropriately "programmed", are not going to perform, whereas those that are programmed can be rescued from a larger population of heterogeneous cells, as in a leaf where many of the individual cells having potentially useful characteristics are never "allowed" to express themselves.

In still other cases, brute force has been the instrument whereby cell lines have been screened for production potential. This is easier to do if the substances sought are colored, or fluorescent or have other readily recognizable characteristics. Long-term strategies adopted by Japanese industry to analyze production of shikonin, a red dye from Lithospermum erythrorhizon used in pharmaceuticals, involved such varied approaches as optimizing environmental conditions, selection of high-producing strains, derivation of variants, addition of elicitors, addition of precursors, testing biotransformation and morphological differentiation and immobilzation of cells. In the final analysis, the production involved a two-stage process. The first involved growth and the second involved transfer to a setting in which the medium was changed to permit synthesis of the product. This first commercialization, albeit on a small scale in comparison to those routinely used by the antibiotic or fermentation industries, is rightly seen as a pioneering step and is sure to have a heuristic effect on the field.

Additional strategies to stimulate production of secondary substances and metabolites include utilization of the principles of stress physiology as a probe to understand and manipulate intermediary metabolism. These biotic elicitors are an excellent means of confirming the biosynthetic potentialities of cultured plant cells. There is, therefore, an increasing body of literature and knowledge which shows that in select circumstances, and with a great deal of patience and work, sufficiently controllable systems can be achieved for industrialization of higher plant cells in culture.

Certainly, problems remain. Higher plant cells grow relatively slowly in culture in comparison to microbes. Subculture periods are much longer. The secondary products are generally not produced in logarithmic phase, but in stationary phase. Also, products are not generally excreted into the culture medium. Cells must be extracted to yield products. Whereas cryopreservation methods are being worked on and progress is good, we are nowhere near the level of competence reached by industrial microbiology in terms of the technology. This is all the more worrisome since there is a great deal of evidence that change can and does occur in higher plant cells as they remain in culture. Prospects for genetic engineering of cells to produce substances will be best achievable in those cases where simple enzyme systems coded for by single genes are in question. But the fact is that there are not many known examples of such control of high-value, low market volume products. In all cases, the economics will be the "driver" in addition to the biological and engineering feasibilities.

THE SPACE ENVIRONMENT AS A VEHICLE FOR INTEGRATION OF NEW BIOTECHNOLOGIES

While the problems may be rather simply stated, the operational solutions may be quite complex. The duplication in culture with external stimuli of the genetically-based signals that plant cells use to direct their pattern of growth is more art than science and the technology is still in its infancy. It is likely to remain so unless certain innovations are made in the technology. The only feasible way of defining the nature, qualitatively and quantitatively, of the necessary stimuli and conditions is through tedious trial and error, which at this point can only be accomplished at the expense of considerable time and skilled manpower.

An innovation necessary to exploit the full potential of the cultured plant cell is an apparatus capable of large scale screening and automated testing of plant cells against complex programs of exposure to chemical and environmental regimes. There are good reasons for planning at the outset to utilize the space environment as an adjunct to the system. In a micro-g or hypo-g space environment where there is no buoyancy, no convection, no stratification of layers, and where surface tension dominates, we can anticipate major impacts on metabolism that will be reflected in biosynthetic potentials of cultured cells and protoplasts. There are also significant advantages of such a system for a 1-g micro-environment. For example, (1) cell cultures can be grown and maintained under controlled conditions with respect to nutritional and environmental requirements. Such a situation would allow establishment of conditions for optimal cell growth or maximum secondary product formation, and for the selection of high producing genotypes; (2) the cell culture methods would permit location of production facilities in any place without dependence on a region with certain anticipated or required climatic conditions; (3) cultured cells would allow biochemical

production to occur around the year in a reliable manner without interruptions due to agronomic practice, to season, or to other environmental factors or even political factors; (4) biomass production by cells in rapidly growing cultures can be considerably more than in cells in situ; and (5) production in cell suspension culture should be automatable and this can lead to a significantly improved biotechnology; it also provides the basis for disclosing principles which can lead to a still fuller understanding of the entire process of growth, metabolism, and differentiation.

The generally large size of plant cells subjects them to shearing in conventional industrial fermentors. Instrumentation of the sort provided by the bioreactor under development at Johnson Space Center in Houston by Dennis Morrison and his colleagues will provide the means whereby all the technological parameters may be systematically examined with a maximum of precision. We may anticipate relatively few modifications to the apparatus necessary to render it useful to plant cell biologists and biochemists.

We have seen in this broad overview the many possibilities associated with the manipulation of plant cells that are morphogenetically competent. Availability of a bioreactor to generate and grow cells in space and to expose them in a controlled manner and even to generate somatic embryos and plantlets provides a powerful means of providing valuable germplasm for experimentation and for use in a CELSS-type controlled environment life support system. The feature of being a renewable and regenerable resource means that small amounts of initial inocula have the potential to produce huge amounts of material. Having generated plantlets, these in turn provide the opportunity to implement the alternative strategies for multiplication outlined in table 9-1.

One cannot of course reliably predict the outcome of such efforts to draw ever closer the problems of basic plant physiology and biochemistry and those of biomaterials and processing inspace. But one can be assured that anything that facilitates realization of better understanding of controls is sure to pay off. All of the new biotechnologies as they relate to plants depend upon reliable and controllable tissue, and cell and protoplast systems. A bioreactor will go far towards providing a state-of-the-art instrument which can meet the challenge (table 9-2). This, coupled with all the unexpected interrelationships that the space environment can provide and the reactions it can elicit, are sure to give us the wherewithall to move to ever increasing levels of sophistication. It is difficult to conceive of an area of research which can yield as much new and dependable primary data and insight into the inner workings of one of nature's most impressive chemical factories -- the higher plant cell.

TABLE 9-2.- TEMPORAL AND PRIORITIZED RANKING OF RESEARCH OBJECTIVES WHICH CAN BE SERVED BY AUTOMATED CULTURE APPARATUS

Research with Potential for Near-Term Impact

- Rapid multiplication of select specimens
- Elimination of virus and specific pathogens
- Virus indexing
- Germplasm introduction and evaluation
- Germplasm collection, preservation, and management
- Production of polyploids, haploids, somaclonal variants for new crop production and use in breeding, etc.
- Elimination of certain breeding barriers
  - in vitro fertilization in ovulo
  - embryo rescue and/or storage
  - androgenesis
  - gynogenesis

Research with Intermediate Impact

- All of the above in more recalcitrant species, plus
- Selection for complex traits such as tolerance to stress
  - biotic - diseases and pests
  - abiotic - temperature
    salt
    herbicides
  - In vitro mutation breeding
  - Cyropreservation

Research with Long-Range Implications

- All of the above in more recalcitrant species, plus
- Genetic engineering
  - transformation by selectable genes, etc.
  - organelle transfer
  - wide crosses - somatic hybridization
- Understanding controls in developmental and physiological processes

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