INTRODUCTION TO THE CELL CULTURE SYMPOSIUM

Lawrence Rappaport
Department of Vegetable Crops and Plant Growth Laboratory, University of California, Davis, CA 95616

In many ways the first bloom is off the rosy image of the new cell culture with its promise for early development of new interspecific plants through somatic hybridization, incorporation of useful genes through protoplast fusion and gene transformation, and selection of mutant cells with desirable genetic traits for transmission to regenerated plants. If progress in cell culture has been slower than anticipated, a little reflection will help us to realize that this is not unusual in science. We might examine the satisfaction we feel in our knowledge of the field of intermediary metabolism, but only a little reminiscence leads one to the realization that this satisfaction is the product of leaps of creativity and technological advance, but also of many false starts and a great deal of painstaking effort.

Despite the pace of forward movement in the field, cell culture research is a "growth industry" today. In part, this is due to the remarkable progress being made in the more traditional aspects of regeneration of plants from organs, especially apices. Some species that had been refractory have now yielded to manipulation of the culture medium and can be regenerated reproducibly. Included among these are economic plants, and the ability to regenerate has stimulated interest in recently developed approaches to cell selection and genetic manipulation. For a limited number of species, this activity has provided us with techniques for mass isolation and fusion of protoplasts, and regeneration of the hybrids into plants, the production of haploid plants from microspores and anthers, regeneration of embryoids and plantlets of recalcitrant species from callus and cell suspension cultures, and initial successes in obtaining desirable cell mutations which are expressed in the regenerated plant.

These methodologies have laid the basis for a new "cell genetics" as well as for studies of the physiology and biochemistry of plant cell behavior. This cell culture must be viewed as a field in its infancy which in part derives conceptually from the experience and remarkable success in the area of microbial genetics. Today, workers in the plant cell culture field face many of the same barriers that confront the microbiological workers, and a great many more, because we are dealing with species higher in the evolutionary scale and, therefore, of extraordinary complexity. Moreover, we want not only to learn basic biology of somatic cells, but we expect to have useful results.

My personal view is that major new advances in the field can come with extensive basic research aimed at analyzing each of the rate limiting processes involved. Thus, cell selection for desirable agricultural characteristics is possible, but for broad scale application, penetrating study of each of the barriers in somatic cell selection work – producing haploids, tendency toward polyploidization, problems of cell selection, regeneration of selected cells into plants – is essential. The same applies to other aspects of somatic cell research and the symposium participants were invited because of their high standing in fields of significance to such developments.

It is most appropriate that a symposium on "Cell Culture" is sponsored jointly by the American Society for Horticultural Science and the Plant Growth Regulator Working Group. The subject bridges current knowledge and the possibilities of new advances. It deals with an emerging field which holds promise both of major understanding of plant regulation and, ultimately, of advances in crop improvement, subjects of major interest to both organizations.

CURRENT STATUS OF PLANT CELL AND ORGAN CULTURES

Toshio Murashige[1]
Department of Plant Sciences, University of California, Riverside, CA 92502

In 1902, Haberlandt published on his unsuccessful but pioneering attempt to cultivate cells from leaves of several angiosperms (5). Today, nearly 75 years later, plant cell and organ cultures are established as potent research tools and economical agricultural practices. Cell cultures enabled the demonstration of totipotentiality of plant cells as Haberlandt first suggested (22). Furthermore, they played a critical role in the discovery of cytokinins, an important group of plant hormones (8). Cell and organ cultures provide unique systems to explore organ initiation (17), cell and tissue differentiation (21), and other morphogenetic processes. They furnish new opportunities in somatic cell genetics (2). Organ growth and development can be studied with minimum interference by correlative influences (1, 14, 18, 19, 24).

Perhaps most significant has been the increasing utility of cell and organ cultures in economic applications. They are new sources of pharmaceuticals and other plant constituents. They serve as tools in hybridization and new cultivar development. They are employed to establish pathogen-free stocks and are becoming a standard method of rapid clonal multiplication.

Kinds of plant cultures

The callus is perhaps the most widely cultivated plant tissue (Fig. 1). Its successful culture was first reported in 1939, separately but nearly simultaneously, by Gautheret (4), Nobecourt (15) and White (25). The callus has been used primarily as experimental material in morphogenesis, biochemistry, disease etiology, and other biological studies. After many years' exploration they are now emerging as economic sources of certain plant substances. They are sometimes used as an intermediary in clonal multiplication procedures. Callus cultures can be established quite readily for most plants; indeed, examples are known in various species, from mosses through monocotyledonous angiosperms, and of virtually any plant tissue or organ. One needs only to provide a balanced salt mixture, sugar, one or more vitamins, and the phytohormones auxin and cytokinin. The auxin alone may be sufficient as hormonal addendum, especially when 2,4-D is employed. Sometimes preparations of natural complexes, such as coconut endosperm, yeast or malt extract, fruit pulp or juice, and protein hydrolysates, are provided to obtain additional stimulation. The tissue can be maintained indefinitely through repeated subcultures, but this practice is not always advisable, inasmuch as predominance by polyploids and other genetically modified cells usually results. The callus is simply a wound tissue, and grows as a whitish or cream-colored mass on an agar-gelled medium. It can be transferred to a liquid nutrient and transformed into a suspension of free-living cells and few-celled aggregates (Fig. 2). The dissociation of cells is enhanced by first rendering the tissue friable, by pre-culturing in an agar medium containing high salts, relatively high auxin and casein hydrolysate. The liquid must be agitated vigorously and constantly to maintain the dissociated state. Even then, cultures constituted by predominantly single-cell units are rare.

If desired, callus clones of single cell origin are obtainable. One method involves agar-plating of cell suspension cultures (Fig. 3). It may be necessary to separate the single cells from aggregates by filtering the suspension through a suitable screen. The plating is accomplished by mixing aliquots of the screened suspension with sterile, fluid nutrient agar, the temperature of which should be about 40°C to prevent premature gelling, then pouring the suspension into culture dishes. Other methods of single-cell cloning include the use of microchambers (7) and nurse callus (10).

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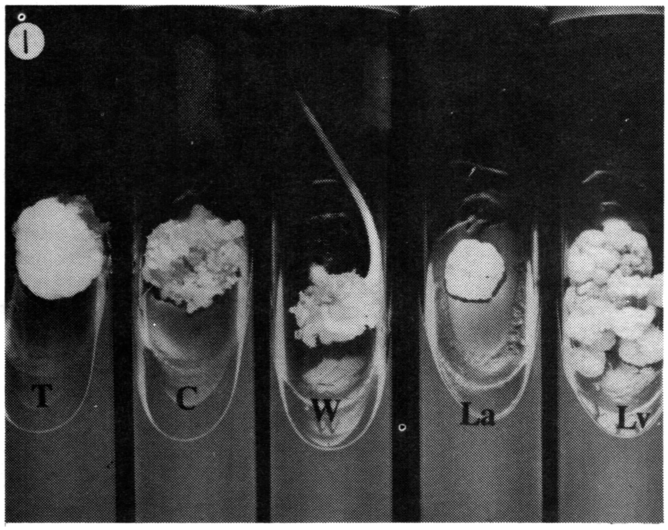

Fig. 1. Callus induced in vitro in tobacco stem (T), carrot root (C), watermelon seedling (W), lemon albedo (La), and lemon juice vesicle (Lv). The medium contained Murashige and Skoog salts, 3% sucrose, 100 mg/liter inositol, 0.4 mg/liter thiamine•HC1, 0.1 mg/liter kinetin, and 0.3 mg/liter 2,4-D.

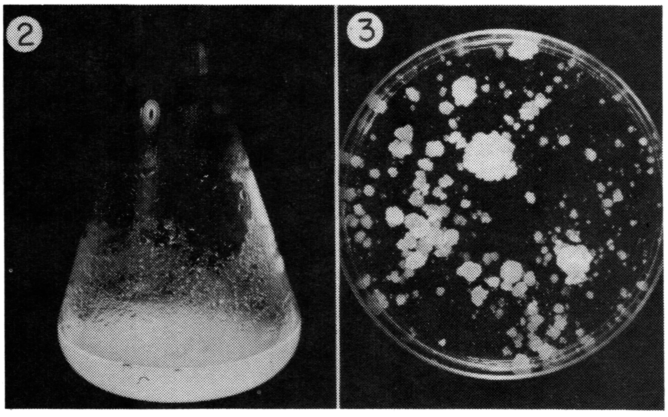

Fig. 2. A liquid suspension culture of tobacco cells. The cells and cell aggregates were maintained in suspension by agitating constantly on a gyratory shaker operating at 150 rpm.

Fig. 3. Regeneration of callus from *Nicotiana glutinosa* cells that have been transferred from liquid suspension to agar-plate culture.

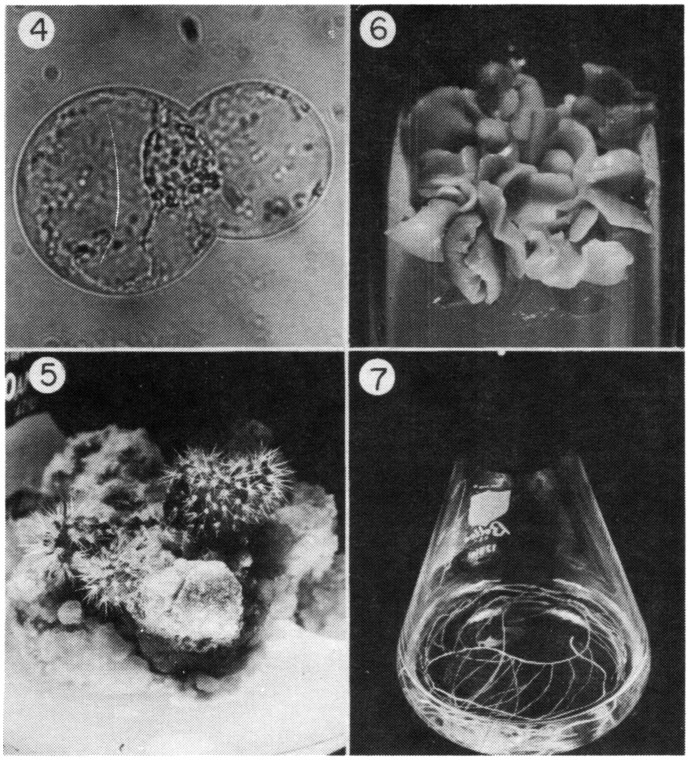

Fig. 4. Initial stage in fusion between 2 tobacco protoplasts.

Fig. 5. Regeneration of *Notocactus* from callus culture, in response to high levels of cytokinin and low auxin.

Fig. 6. Somatic cell embryos in a culture of *Citrus* nucellus.

Fig. 7. A culture of excised tomato roots. The clone is maintainable indefinitely through weekly subcultures of sectors containing 1-2 emerging lateral roots.

Plant cells in culture, whether liquid suspension or callus, are *not uniform* as often claimed. Diversity with respect to morphology, physiology and frequently genotype has been the rule. Furthermore, they are cytologically *differentiated,* but *unorganized.*

The cells suspended in liquid culture serve as an excellent source of protoplasts (Fig. 4). The best cell samples are obtained early during the exponential phase of cell division. The protoplasts are released by incubating the cells in a solution containing cellulase and pectinase preparations and a suitable osmoticum. Fusion among protoplasts is accomplished by employing a high concentration of low molecular weight polyethylene glycol (PEG). The PEG causes the protoplasts to aggregate and fusion occurs during its gradual dilution. Absorption of organelles by protoplasts is also enhanced by PEG.

Plants can be reconstituted from callus of some genera (Fig. 5). The reconstitution can begin with a protoplast or single cell, but must pass through a callus phase. Most important horticulturally, it is possible to manipulate the organogenetic process by suitably balancing the hormonal contents of the nutrient medium. Another significant occurrence in plant tissue cultures has been the initiation of embryos from somatic cells (Fig. 6). These embryos are identical in basic morphology to those arising from the zygotes; but their origin is different and the basis of their initiation remains obscure.

Organ cultures are equally significant as cell or tissue cultures. As early as 1904, Hannig described the attainment of Cruciferae seedlings by culturing embryos isolated at the torpedo-shaped stage (6). In 1933, White (24) first established an indefinitely maintainable clone of tomato roots (Fig. 7). Horticulturally, the most useful of structures cultivated in vitro has been the shoot tip. Small explants, 0.1-0.2 mm tall, are useful in establishing pathogen-free stock from infected clones. Larger explants, 1-10 mm in height, can be employed to clone many cultivars rapidly. The smaller explant, as recommended for the recovery of virus-free plants, consists of the apical meristematic dome, together with one to a few leaf primordia. The object is to establish a single rooted plant per explant quickly. Each plant is then tested for pathogen content, and those responding negatively are grown to maturity to ascertain their genetic identity. In rapid clonal multiplication the substantially larger shoot-tip explant is chemically stimulated to generate a multitude of shoots (Fig. 8). Escape from virus- and virus-like pathogens is not intended, even though a small proportion of the established plants is often pathogen-free.

Cultures of the true apical meristem, or the apical dome alone, have been achieved (18). They are useful only as investigatory tools. Their survival frequency and rate of development in vitro are too low to be horticulturally practical. Moreover, adequate rates of escape from virus and virus-like pathogens are attainable with the larger shoot-tip explants.

Many reproductive structures have also been cultured successfully. Fruits, with or without seeds, have been obtained from excised flowers (Fig. 9). The unpollinated flowers require nutrient media containing hormonal addenda, particularly an auxin (14). Some unpollinated flowers can be pollinated in vitro through the technique of test-tube pollination or fertilization to recover viable seeds. Ovules from pollinated or unpollinated flowers also can develop as viable seeds in vitro. Rare hybrids might be obtained through ovules of pollinated flowers, especially in instances where embryo cultures have been precluded. Fertilization of ovules from unpollinated flowers has been achieved by applying pollen in vitro. Unfortunately, successful test-tube fertilization, using ovary or ovules, remains confined to a few plant genera.

Embryo culture is now a standard tool in plant hybridization. However, in spite of apparently widespread use, the method is still beset with major problems. Only embryo isolates composed of 50 or more cells have been cultured. Smaller structures can not be

excised without serious injury, nor are the requirements for their development in vitro known. Embryo culture as practiced by plant breeders has generally involved fully differentiated, but undersized, isolates. Such structures are easily cultured in nutrient media of relatively simple compositions. Their development in vitro is primarily a germination process. Thus, any requirements that are peculiar to their seed germination, such as pre-chilling, must be satisfied for normal development to occur.

Anther culturing to obtain haploid plants is a practice that is attracting considerable interest among plant geneticists and hybridizers (Fig. 10). The process of haploid embryogenesis seems unrelated to any unusual nutritional or hormonal requirements. The determining factor has been the stage of microsporogenesis at time of excision. With many plants this stage is the period of first pollen mitosis. Haploid plants from microspores are sometimes obtainable through an intermediary callus. However, this practice often yields a mixture of haploid, diploid and polyploid plants. Development of plants from microspores that have been freed of anther tissue has been possible in at least one instance (13).

Economic applications of plant cell and organ cultures

For simplification and convenience the major economic applications of plant cell and organ cultures can be placed in these categories: 1) production of pharmaceuticals and other constituents, 2) genetics and plant breeding, 3) establishment of pathogen-free stocks, and 4) rapid clonal increase or desired cultivars.

Source of plant constituents. Although more than 20 years of research have been devoted to achieving feasibility of cell cultures as sources of plant constituents, only now are economic benefits being realized. Some constituents currently produced commercially include camptothecin, proteinase inhibitors, anti-viral substances, and antibiotics (9). Rapid growth in industrial applications of the cell culture method is anticipated (Unpublished survey, Kuhn, P., Laves-Chemie, Frankfurt, Germany).

Genetics and plant breeding. The procedures currently or potentially applicable to genetic investigations are summarized in Table 1. Some are intended simply to enhance the traditional plant breeding practice, e.g., embryo, ovary, and ovule cultures. Cell cultures could be used in mutant induction and isolation. Anther or microspore culture should be helpful in mutagenesis and in obtaining homozygous diploids. Protoplasts will enable hybridization by fusion between somatic cells of selected cultivars. Furthermore, it is hoped that gene transfer among higher plants will be achievable some day through the mediation of protoplasts. The transfer might require plasmids, viruses, or other agents as gene carriers.

Germ plasm reservation through frozen cells and shoot tips should be explored and exploited fully. Recovery of plants from cells or shoot tips that had been subjected to a $-196^{\circ}C$ temperature has been observed with carrot and carnation (3, 16). The use of in vitro cultures to transfer genetic stocks internationally must become accepted. Whereas exclusion of all pathogens is not assurable, tissue cultures are distinctly less hazardous than seeds or other propagules that are currently permitted through international or regional borders.

Recovery of virus-free clones. Any cultivar that has been propagated for substantial periods by traditional asexual methods should be suspected of carrying one or more virus or virus-like pathogens, unless the pathogen has been detected and eliminated. Viruses that cause obvious disease symptoms are easily detected, but the difficulty involves infections that do not result in noticeable morphological modifications. Usually the deleterious effects are manifested gradually and over an extended period. The true potential of the plant can be seen on exclusion of the virus. The available in vitro methods are adequate to enable re-establishment of any cultivar free of all known plant pathogens (11). The common method consists of establishing plants from small shoot tips. Shoot tips of herbaceous genera can be rooted fairly easily in a nutrient medium containing inorganic salts, sugar, one or more vitamins, and low concentrations of auxin and/or cytokinin. With unrootable woody plants the shoot tips from infected sources can be grafted onto seedling root-stocks of a graft-compatible and pathogen-free cultivar (Fig. 11). The graft is allowed to develop in vitro and the resultant plants can be processed further as done with rooted shoot tips. Plants regenerated from callus have shown escape from certain virses (23). The frequency of pathogen-free plants may not be as high as that attainable through rooting or grafting shoot tips, however. All plants obtained in vitro must be tested systematically to insure the exclusion of pathogens, and claims of pathogen-free plants should specify the pathogens for which tests have proven negative. Furthermore, propagation and widespread distribution of plants that have been freed of pathogens should await confirmation of genotype reproduction. Mutants have resulted among clonally propagated plants, especially those derived through tissue culture.

Rapid clonal propagation. The most extensive commercial use of plant tissue culture has been in clonal propagation. Principles for the development of cloning procedures are now established (12). They appear applicable to virtually all plants. Indeed, they are being extended successfully to an increasing number of genera. Many ferns and indoor foliage plants, certain woody ornamentals, and diverse bulbs and other flower crops are being produced in quantity by several commercial laboratories (Fig. 12). Most of the U.S. laboratories have been established as divisions of existing nurseries. In my visits to Taiwan, I have observed tissue culture cloning of diverse food crops, including potato, pineapple, and banana.

Table 1. Cell and organ culture methods that are currently or potentially applicable in plant hybridization.

Culture method	Application
Embryo	Completion of embryo development Precocious germination
Ovule	Test-tube fertilization Embryo development in vitro
Ovary	Test-tube fertilization Embryo development in vitro
Cell & tissue	Rapid clonal multiplication Induction and isolation of mutants Preservation and transport of germ plasm
Anther & microspore	Mutation breeding Hybridization
Protoplasts	Somatic hybridization Gene transfer via plasmids, viruses, etc.

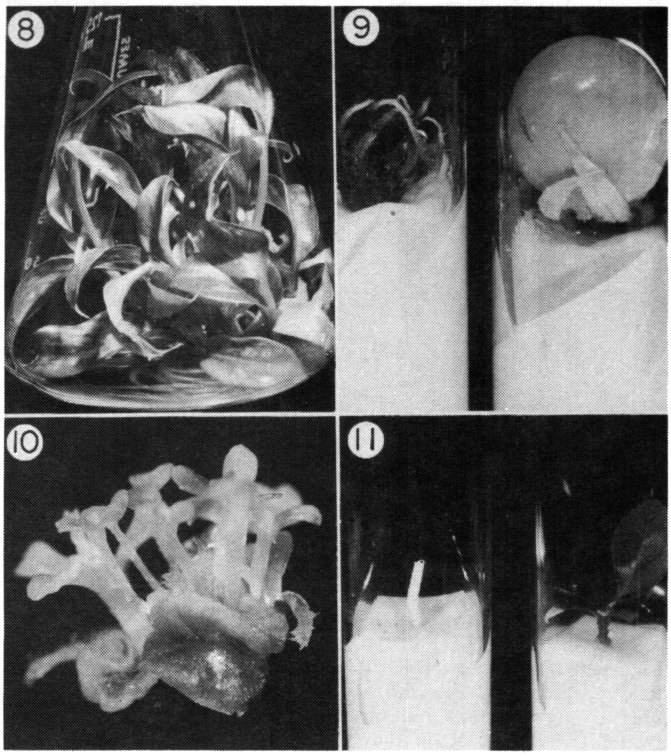

Fig. 8. Multiplication of *Dieffenbachia* through shoot tip culture.

Fig. 9. Tomato fruit obtained *in vitro* from excised flower buds. Medium contained no auxin. left, and 10^{-6}M CPA, right.

Fig. 10. Haploid tobacco plants arising through embryogenesis of microspores in a cultured anther.

Fig. 11. Pathogen-free *Citrus* plant obtained through shoot-tip graft *in vitro*. Freshly performed graft on left, established plant on right.

Fig. 12. Boston ferns obtained through tissue cultures. Photograph courtesy of Transplant Nursery, Oxnard, California.

The nutrient media and the culture environment play significant roles in tissue culture applications. But the principal limiting factor has been the explant, or the initially cultured tissue. For successful tissue culture, the explant's characteristics with respect to organ source, age and degree of juvenility, and dormancy requirements should be assessed. Stem tips, lateral buds, and sections of leaves, bulb scales, corms, tubers, roots, etc., should be compared. Success with tree genera may be restricted to explants from juvenile growth. Such growth is sometimes found as sprouts at the base of the tree. Severing the tree at its base, provided the tree had originated as a seedling, could stimulate juvenile out-growths from the stump. Reversion from adulthood to juvenility is often observed, and sometimes it can be hastened through hormonal treatments, graftage, etc.

Two points need emphasis with respect to plant cloning through tissue cultures. First, a frequency of genetically aberrant plants should be anticipated. Cultivars of known instability should not be subcultured indefinitely, since the frequency increases progressively with each passage. Separation of chimeral components is also possible, resulting in modification or loss of variegation patterns. Second, pathogen exclusion is not intended in most rapid cloning methods. Only through prescribed tests can freedom from disease be ascertained.

Conclusions

Plant cell and organ cultures are characterized by great diversity of kind and utility. Many fundamental biological problems can be resolved through their employment. But it must not be assumed that the in vitro phenomenon accurately reflects that which occurs naturally. It is also important that users of plant tissue cultures understand clearly the basic concepts and, particularly, the limitations.

Tissue cultures have become significant economically. Soon they will be common practice in agriculture, especially in rapid clonal multiplication and in establishing pathogen-free stocks. Hopefully, we will be storing and transporting plant germ plasm routinely through in vitro cultures. Perhaps the development of new varieties will rely on genetic modification techniques that employ protoplasts, microspores, single cells, and others.

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