to a higher soil temperature than is normally experienced outside. This happened to be in a greenhouse and has been acknowledged by the company.

VICE-PRESIDENT ISHIDA: Our final session this afternoon is on tissue, or meristem, culture and the moderator will be Mr. Richard Maire, Farm Advisor from Los Angeles County. Dick —

MODERATOR MAIRE: Thank you, Henry. I think if we have anything new or exciting in the field of plant propagation, this topic is one of the most exciting. We have two people who are well qualified to cover this subject. Dr. Toshio Murishige from the University of California at Riverside and Dr. Wes Hackett from UCLA. Dr. Murishige, who will speak first, was at the University of Hawaii before he came to Riverside. He has been working in the field of tissue culture for quite some time. OK, Toshio, let's amaze them with some of this "space-age" propagation.

PRINCIPLES OF IN VITRO CULTURE

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Introduction

Increasing use of the *in vitro* approach in botanical investigations and the expanding store of information prohibit a thorough coverage of the subject. This article is intended to simply acquaint the unfamiliar with some highlights of principles. Citations to original research should be viewed only as examples for illustration. More extensive coverage, including the historical development, can be obtained from several reviews and symposium publications now available (4,5,8,11, 13,21,27,28,34,35,39,42,43).

The term "plant tissue culture" has been popularly used indiscriminately to denote cell, tissue and organ culture. It is desirable to distinguish between cell and tissue on one hand and organ on the other, since their behavior and requirements in culture are markedly different. The preferred term which encompasses each of these cultures is "*in vitro* culture" and it is therefore used in this article.

Fundamentals which apply to any *in vitro* culture shall be examined first. This will be followed by some of the more specific aspects of cell, tissue and organ cultures. Finally, we shall consider some applications of the *in vitro* approach in plant propagation.

General Considerations

Whether it be cell, tissue or organ culture, these aspects demand fundamental consideration: asepsis, nutrition, physical environment, and the *in vitro-in vivo* relationship.

Asepsis: Usually the freshly isolated plant part has bacteria, fungi, etc., which must be excluded. If the contaminating organisms are present only externally, they can be easily eliminated by treatment with mild disinfectant. Solutions of sodium hypochlorite, calcium hypochlorite, alcohol, detergents, etc., are commonly used. Internally-contained organisms will require more elaborate exclusion steps, such as culturing with antibiotics and other chemotherapeutic substances (6,22). Viruses are very difficult to eliminate, therefore it is best to use only plant parts known to be virus-free (14).

Nutrient media are rendered free of infectious organisms usually by autoclaving. For volumes of medium normally employed, 15 minutes at 15 lbs/sq. in. is adequate. With heat-labile substances the sterilization must take another course. Concentrated solutions of such substances are passed through microbial filters and then suitable aliquots are combined with the autoclaved, non-labile components.

The surgical and planting manipulations often require special facilities. The primary source of contaminants during manipulations is the air. The preferred practice has been to provide a transfer room or chamber in which filtered air is supplied and in which a positive pressure prevails.

Nutrition: The specific nutritional provision varies with type of culture, plant part, species, and the investigation's objective. Nevertheless, certain characteristics are generally applicable. A supply of inorganic salts, sugar and water is the minimum requirement. Several salt formulae are presently available, e. g., those of White (42), Heller (10) and Murashige and Skoog (20). Sucrose has been generally effective as the sugar, although glucose has been preferred in some cultures of monocot tissue and organs. Demineralized water has been routinely used.

The more specific organic constituents include the B-vitamins, particularly thiamin and inositol; hormonal substances, especially auxin and cytokinin; and reduced nitrogen compounds, such as amino acids or their amides. Coconut milk, yeast extract and other complex addenda of natural origin are also frequently used, although sometimes unjustified.

Physical Environment: Perhaps the most critical of the physical environment is aeration. Satisfactory aeration can be achieved by using an agar-gel medium or, if a liquid is used, by constant vigorous agitation (5,23), bubbling air into the liquid (23), or by providing filter-paper supports (10).

Closures for culture vessels must provide aeration, yet exclude contaminating organisms. Non-absorbent cotton or synthetic sponge plugs and polypropylene or stainless steel covers are commonly employed.

The temperature and light needs should be systematically determined for each cultured material. While constant temperatures in the neighborhood of 25° C has been routine, the best temperature is expected to vary from species to species.

When growth alone is desired, constant darkness seems most favorable for cell and tissue cultures. The induction of differentiation in cells and tissues, however, may necessitate illumination. Organ cultures which involve shoot parts apparently benefit from light, whereas those of root parts may not. The light used is ordinarily of low intensity. Since sugar is provided photosynthesis in the usual sense is not critical, but the synthesis of growth factors may depend on a small quantity of light. Diurnal periodicity with respect to temperature and light is a factor which has not been adequately considered with *in vitro* cultures.

The pH of the nutrient medium is also important. The pH found generally satisfactory has been in the range of 5-6. By providing adequate buffering capacity, changes in pH associated with nutrient utilization can be minimized.

In Vitro-In Vivo Relationships: The primary objective of *in vitro* cultures has been to provide tools with which developmental phenomena can be explored more effectively. Thus, the behavior of a cultured plant part must be reflective of the potentialities *in vivo*. Instances of cultured cells and tissues having undergone polyploidization are not uncommon (25) and, as to be expected the chromosomal change is accompanied by an alteration of cellular behavior (36). It is therefore incumbent on the investigator to examine this possibility in his cultures. The chromosomal change may not be apparent in the gross morphological characteristics, so it will have to be ascertained through nuclear examination.

Specific Aspects

Tissue and Cell Cultures: By far the most extensive investigations have been with cultures of tissue and the most widely cultivated tissue has been callus, or wound tissue. Callus cultures have been established from virtually every part of the plant, including root (3,32,36), leaf (9,41), fruit (30), and even endosperm (33) and pollen (38). Those from the stem are beyond enumeration. Callus cultures have also been obtained of lower plants, such as the fern (15). The callus cultures have been used to explore a range of phenomena, from the basic developmental processes of cell division and tissue and organ differentiation (9,17,18,31 36,37) to the synthesis of secondary biochemical products (5).

Callus is most commonly grown on an agar-gel medium. Once established, it is indefinitely maintainable by periodically subculturing small pieces in fresh medium. A distinction can be made in the nutrition of dicot and monocot tissues. The callus of many dicot species is culturable in media of defined composition. The basal ingredients have been inorganic salts, sucrose, some B-vitamins, and often auxin and/or cytokinin. Monocot callus, with few exceptions, have not been cultured in defined medium and complex addenda such as coconut milk or yeast extract have been routinely used.

Plant cells, in differing from animal cells, tend to adhere together more tenaciously. Thus, the complete dissociation of tissue into unicellular units and their maintenance as such have been virtually impossible. Even the most successful attempts have resulted in cultures containing both single-cells and tissues. Indeed, the delineation into cell culture and tissue culture has been an arbitrary one.

Dissociation of tissue, giving rise to a proportion of unicellular units, is possible through nutritional and mechanical manipulations. Suitable conditions of nutrients have often been found to render plant tissues friable (18). Some cells from such tissues can be either teased free individually (18) or separated en masse in a vigorously agitated liquid medium (23). Through continuous agitation and periodic transfer of aliquots of culture suspension to fresh nutrient solution single cells and few-celled clusters can be maintained indefinitely.

The significant discovery has been that, for yet unestablished reasons, a singly isolated plant cell is incapable of multiplication. Successful induction of cell division has been accomplished by employing a nurse-callus (16), plating a dense suspension of cells (3) or by employing pre-conditioned nutrient medium (12). In the nurse-callus technique a piece of tissue is cultured on nutrient agar, a layer of filter paper is laid over this tissue and the isolated cell is set on the filter paper. The tissue piece provides nourishment to the cell and the filter paper enables movement of substances while preventing a union between cell and tissue. In the plating technique the cells in liquid suspension are simply dispersed onto suitable nutrient agar. The concentration of cells in the suspension to be plated is critical. Moveover, since both cell aggregates as well as free cells are involved, some step must be taken to ascertain which are the single cells. A single isolated cell can also be induced to proliferate into a tissue in the absence of other cells if pre-conditioned medium is used. A pre-conditioned medium is one in which tissue and cells have been previously cultured for a short period.

One of the disturbing characteristics of cell and tissue cultures has been their high degree of genetic instability. With few exceptions, every culture which has been critically examined has shown a degree of polyploidization (25). The longer a tissue is kept in culture, the higher is the frequency of polyploid cells; ultimately a completely polyploid population may be attained.

Organ Culture: The object in organ culture has been to obtain development of a plant part *in vitro* which is as nearly as possible comparable to that *in vivo*. It is desired that the same degree of differentiation and organization be retained *in vitro*.

(a). *Root Culture*: The first successful *in vitro* culture of a plant material was that of the excised tomato root by White in 1933. This culture is still being maintained in a vigorous

state now 33 years later. Evidently culturability of isolated roots differs between herbaceous dicot species on one hand and monocot and woody dicot species on the other (4,34). The roots of many herbaceous dicots have been successfully grown in a nutrient solution containing mineral salts, sugar, B-vitamins, and some organic reduced-nitrogen compounds. In contrast, root cultures of monocots and woody dicots have been more difficult and largely unsuccessful.

(b). *Shoot Tip Culture*: The shoot tip of several higher plant species has been successfully cultured, but not the apical meristem. This distinction deserves emphasis, since some investigators have carelessly applied the term "apical meristem culture" to what was correctly "shoot tip culture". The shoot tip, which consists of the apical meristem plus a few subjacent leaf primordia, has been grown to complete plants *in vitro* (1). This has not been the case of the apical meristem itself (2).

(c). *Leaf Culture*: With respect to leaf cuture a distinction in the behavior can be made between ferns and higher plants (7). The youngest leaf primordia of ferns develop into complete plants when isolated and placed in culture, whereas the oldest develop only as leaves. The leaf primordia of higher plants, regardless of degree of maturation when excised, invariably develop into leaves.

(d). *Ovary Culture*: The ovary of many herbaeceous plants has been cultured *in vitro* into mature fruit (24). If the source of ovary is a pollinated flower, development can be obtained in a relatively simple nutrient medium. However, the ovary from an unpollinated flower must be supplied with diverse growth factors, including auxin. Fruits obtained *in vitro* generally have been considerably smaller than those developed *in vivo*. Nevertheless, comparable degrees of differentiation and flavor are retained.

(e). *Anther Culture*: Only limited research has been conducted with anther culture and much of this has been confined to work with the Liliaceae. The available information shows that meiosis can occur and functional pollen can be obtained *in vitro* (40).

(f). *Embryo Culture*: The development in vitro of embryos of higher plants has been achieved in several instances. This development, however, has been possible only with isolates which are at least 50-celled in dimension. No success has been attained by starting with the zygote. Experience with embryo culture has shown that the requirements change as development progresses from the relatively undifferentiated, few-celled structure to a fully differentiated embryo (26). It is thus not appropriate to suggest a culture medium which is generally useful.

Applications in Plant Propagation

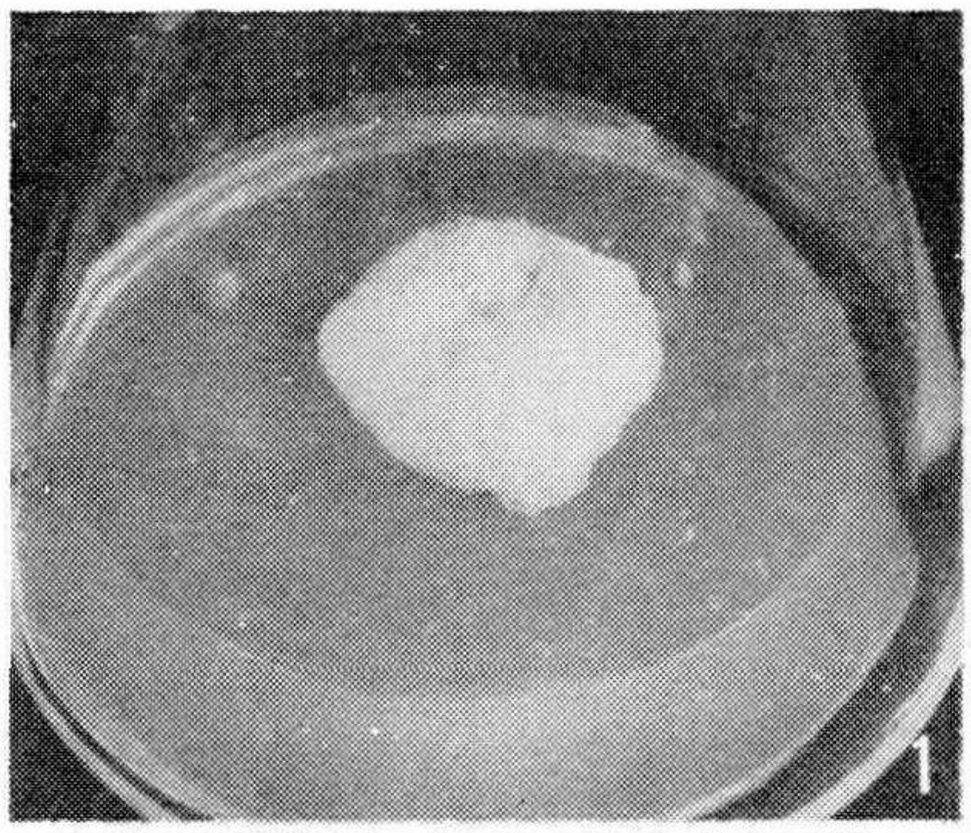
The *in vitro* culture approach has already been applied in plant propagation, but there are still several instances in which this approach might be helpful. Problems of root and shoot

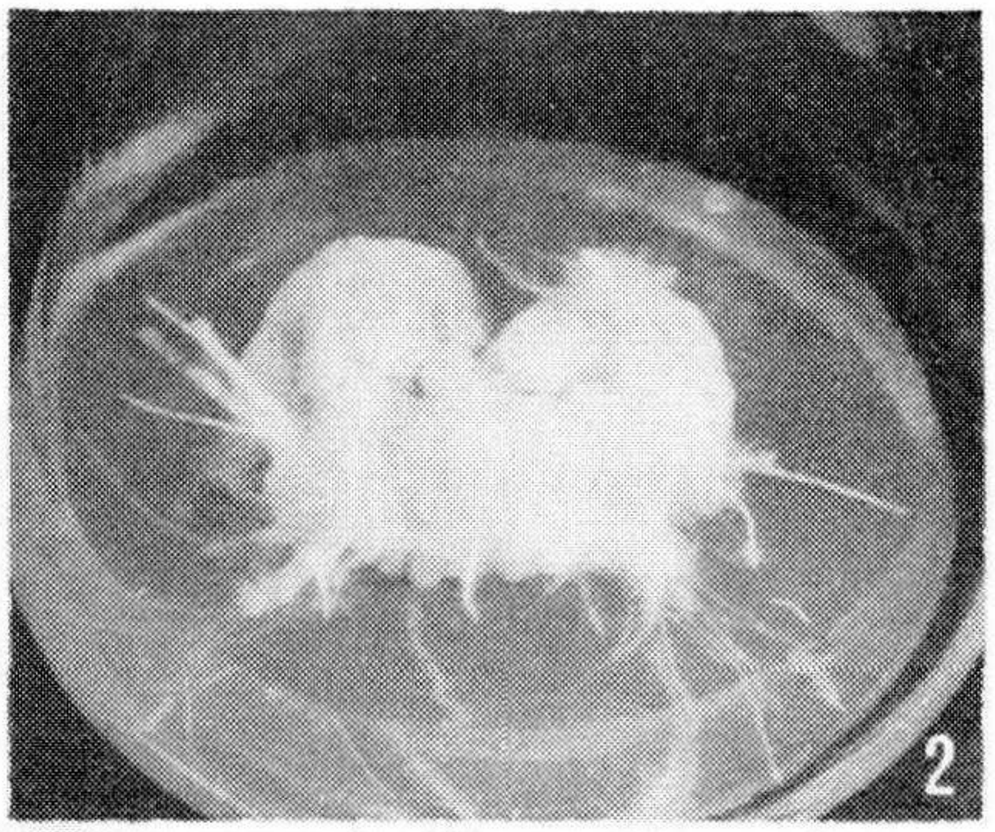
initiation can be explored very effectively. Studies with tobacco callus have disclosed a basic mechanism in the formation of these organs. Root initiation has been related to a high auxin-low cytokinin condition, whereas shoot differentiation has been associated with low auxin and high cytokinin (31). Gibberellin inhibits both root and shoot formation (17).

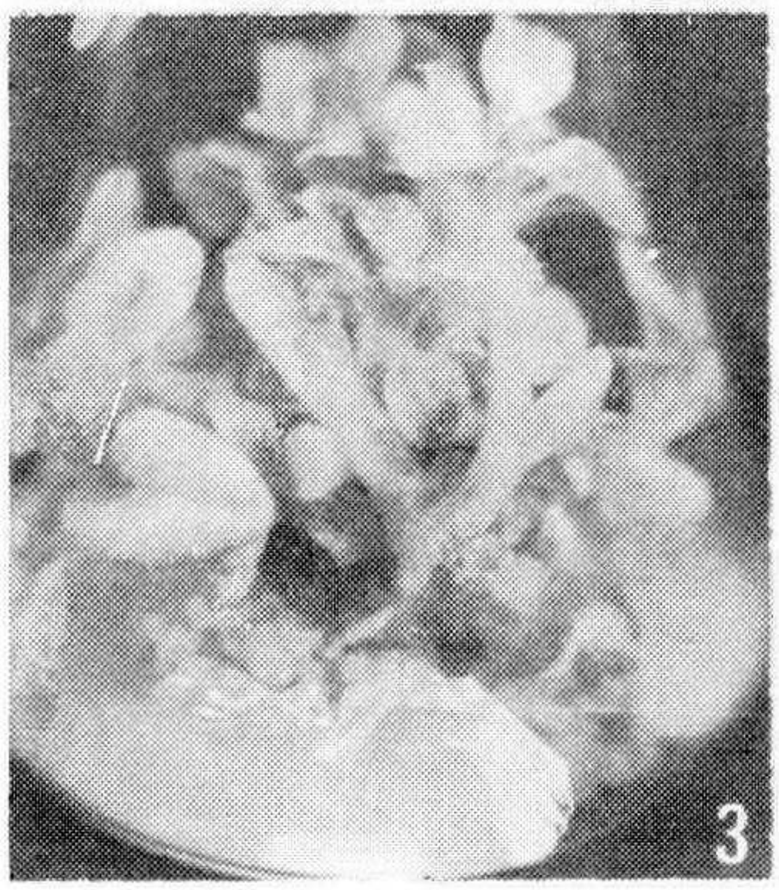
Asexual propagation of many species has been enhanced by the *in vitro* approach. Unlimited supply of orchid plantlets has been obtained from cultures of the excised shoot apex (29). In tobacco (19). carrot (9,32), endive (41), and others, a similar increase in plantlets can be achieved through callus cultures derived from stem, root and leaf.

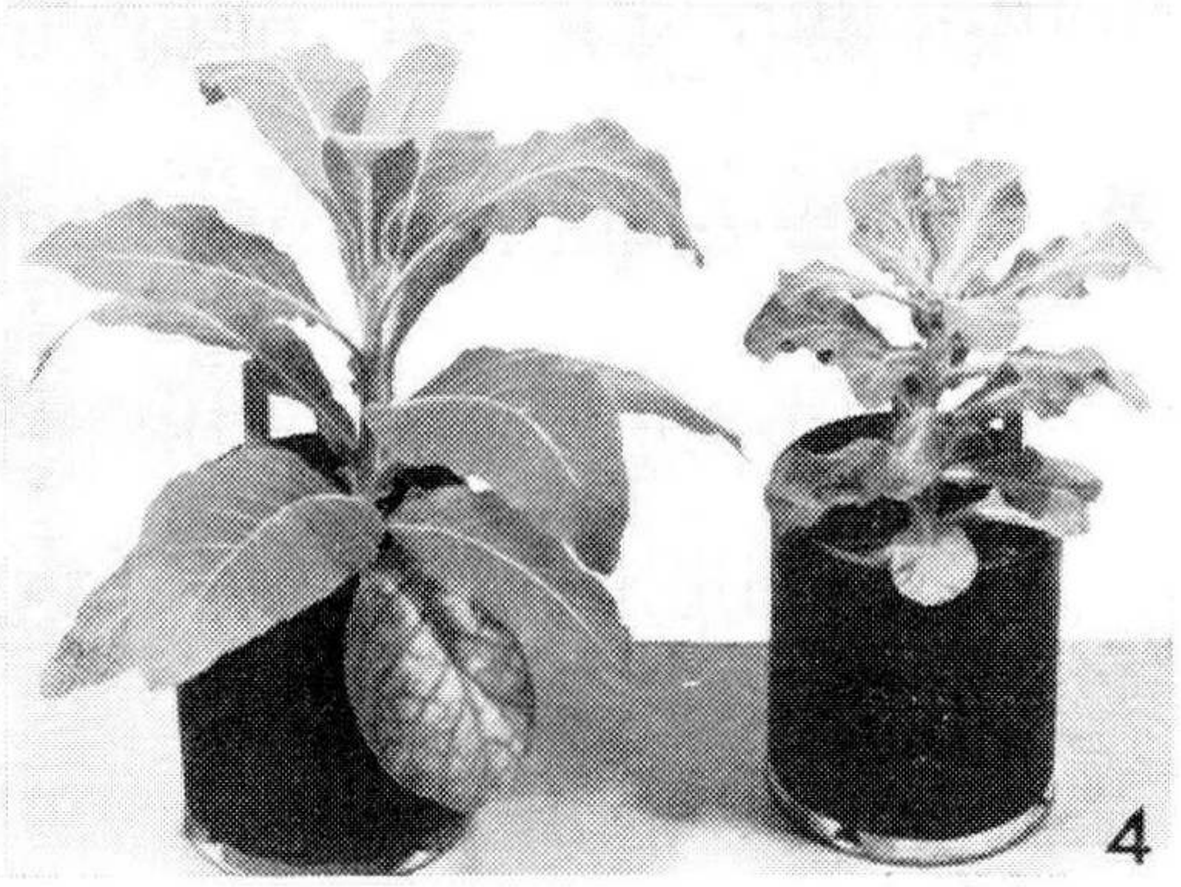
Embryo culture has long been used to obtain seeds of many plant hybrids (27).

Cultures of shoot tips are often employed to obtain disease-free plants from infected plants (14). Disease-free plants might also be obtained by starting with single cells and reconstituting the plants.









Figs. 1-4. Morphogenic manifestations of a tobacco cell *in vitro*. — Fig. 1. Undifferentiated callus obtained through nurse culture of a single pith cell isolate.—Fig. 2. Roots resulting in a culture medium of high indoleacetic acid and low kinetin. — Fig. 3. Formation of stems and leaves as result of high kinetin-low indoleacetic acid provision. —Fig. 4. A tetraploid plant, right, reconstituted in vitro from a tetraploid pith cell; pith cell was isolated from a diploid plant similar to the one on left.

Finally, another potential application of *in vitro* culture is in obtaining polyploid plants. Polyploid cells may be found in either the plant or tissue culture. These cells might be isolated and cultured to give rise to polyploid plants. This has been achieved with tobacco (19).

Summary

This article considers some principles of plant cell, tissue and organ cultures. Generalities with respect to asepsis, nutrition, physical environment, and *in vitro-in vivo* relationship are first examined. These are followed by some characteristics more specifically associated with each of cell, tissue and organ culture. Finally, a few applications of the *in vitro* approach in plant propagation are considered.

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MODERATOR MAIRE: Isn't that exciting! It just — I don't know — you let your mind go wild just thinking about the possibilities that may develop from all of this sort of thing we are calling "tissue culture".

Anyhow, now we're going to go on and find out what are some of the possibilities that this type of tissue culture — or meristem culture — offers. We now have Dr. Wes Hackett who has done considerable work on this subject. He received