[*In an intact plant, cells and tissues possess remarkable powers, both to maintain cells in a given physiological state and also to re-organise into definite organs at the appropriate time, in the process of differentiation. Because of the complexity of the living plant, basic studies involving an understanding into the different developmental stages, leading to the formation of differentiated organs is faced with several problems, and consequential slow progress in their solution. A new approach to the study of these processes has been evolved by the establishment of the plant cell and tissue culture technique. The importance of these studies is now evident from the several recent applications of plant tissue culture to many areas of research, in agriculture, horticulture, forestry and plant breeding. These applications have been the direct outcome of the initial discovery that organogenesis using tissue culture can be regulated in cell cultures by the use of chemicals. The present review describes these studies and their involvement in our understanding of the mechanisms of organogenesis.—Ed.*]

PLANT TISSUE CULTURE— ITS ROLE IN STUDIES ON ORGANOGENESIS

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Introduction and History

REMARKABLE progress has been made in the field of plant tissue culture since the first cell cultures[1–3] over 40 years back. Today plant tissue culture, the practice of growing plant tissues on nutrient media, includes successful cultures from a wide number of plants belonging to diverse genera and families and from practically every tissue or organ of the plant. In a large number of species, organised structures can also be manipulated by chemicals[4]. More recent, progress has been in the induction of haploid plants from pollen[5] and in the isolation of mutants[6] and somatic hybrids by protoplast fusion[7]. The impact of all these discoveries has resulted in the exploitation of tissue culture by a growing number of commercial horticultural organisations and to its utilization in programmes of agriculture, forestry and plant breeding. It has also served as an excellent research tool for studying cell, organ and tissue differentiation.

The aim of this review is to examine some of the important developments in the use of plant cell cultures for studying morphogenesis. It is not intended to be a comprehensive bibliography. References have however been made in the text to several important reviews on this subject. Developments regarding the media, technique, etc., have recently been covered by a number of reviews and books[8–12] and have not been dealt with.

Organogenesis *in vitro*

The property of cell cultures to undergo complex developmental sequences by controlled manipulation, with the formation of differentiated organs or even whole plants is defined as organogenesis. The report of White[13] was the earliest on shoot formation from a *Nicotiana glauca* × *N. langsdorfii* hybrid callus, when submerged in a liquid medium. This work was later extended[4, 14, 15] with the finding that the basic mechanism controlling organised development of shoots, roots or plants in tobacco tissue cultures involved a delicate balance of the auxin : cytokinin concentrations in the nutrient medium. This was one of the early demonstrations which indicated that tissue culture could be used for studying the events leading to differentiation. Since this report, there have been several reviews on studies on organogenesis either indirectly involving callus cultures, or directly from primary organised explants or from thin cell layers[16,17].

This review will deal with the main pathways used in tissue culture for studying embryogenesis or organogenesis either directly from free cells or primary explants, or indirectly through an intermediary callus stage. The more recent studies on thin cell layers and some of the recent biochemical work will also be briefly described. However only a few general selected systems, which have been used as models for understanding morphogenesis, have been chosen.

Systems for Studying Differentiation

I. *Free Cells, Primary Explants and Callus Tissues*

Differentiation may occur either *via* (*a*) embryogenesis or (*b*) organogenesis, using free cells, primary explants and callus cultures.

(i) *Embryogenesis* : The investigations of Steward[18,19] and Reinert[20] established that cultured carrot phloem cells could give rise to embryos, and that embryogenesis can occur from single isolated cells[21]. Based on the results with a carrot cell suspension culture it was postulated that organogenesis *via* embryogenesis could best be studied by isolating a cell in an environment free from the influence of the other surrounding cells[21]. Such free cells can be isolated directly from primary explants taken from practically any part of a plant, *viz.*, embryo, root, shoot, leaf or floral organs or indirectly from callus cultures. This view regarding the necessity of the physiological isolation of cells for studying the organogenetic process was however later contradicted[22]. In somatic embryogenesis from isolated cells, protoplasts[23] or pollen grains[5], it has been observed that the cells in an appropriate medium first undergo a sequence of orderly divisions to give rise to clumps of cells. In these clumps one cell gets structured into an embryo based on its position with the surrounding cells. These somatic embryos during later development follow the same stages that occur during normal zygotic embryogenesis[24]. Since the early work on carrot, in spite of extensive research, plant embryogenesis from tissue culture has been obtained from only a few plants and is still a rare phenomenon as compared to *de novo* bud formation from callus[25]. Several studies have shown that embryogenesis is generally suppressed by the addition of kinetin and 2,4-D, which can be stimulated by its withdrawal in a sequential manner[19,21,26,27]. Addition of cytokinins[28], coconut milk[19], and other sources of reduced nitrogen[29] have been shown to markedly enhance the capacity for embryogenesis in cultures when added at the correct concentrations. Embryoids are readily produced in media containing relatively large amounts of ammonium ions and nitrate but never on medium containing low amounts of nitrate[30]. Recently, precocious flowering has been reported in embryoids derived from mature ginseng root callus cultures on a chemically defined medium[31]. This is a rare example of floral organ induction in the absence of a floral meristem.

(ii) *Organogenesis* : Experimental studies on organogenesis made a significant advance with the demonstration that organised development was the result of quantitative chemical interactions mainly between the auxins and cytokinins with the medium[14]. This concept of an auxin: cytokinin concentration has been confirmed several times[32,33]. Shoot formation is generally promoted with a ratio ranging from 10 to 100 between cytokinin and auxin. Organ formation from primary explants obtained either directly or indirectly *via* callus have now been extensively reported and listed[17]. The primary explants used have included embryos, apical meristems, leaves, flowers, buds, etc., and shoot or root differentiation obtained either from the explants or from callus tissues. Other substances such as adenine have also been shown to modify the auxin: cytokinin interaction in shoot differentiation[14,34].

Organogenesis and plantlet formation by culture of the shoot tips is now being used as an alternative propagative technique in agriculture and also for isolating disease free plants[35]. Working with teak[36] and eucalyptus[37] synergistic effects of a combination of cytokinins for shoot formation and of auxins for rooting was demonstrated.

The extensive data now available on the initiation of organised development, indicate the importance of auxin and cytokinin. Other phytohormones, amino acids and several other chemicals in the medium have also been studied, and found to promote organogenesis[38]. In general gibberellic acid (GA_3) has been shown to repress shoot and root formation in the presence of an otherwise suitable auxin: cytokinin ratio[39,40]. The inhibitory effect of GA_3 in organogenesis in these studies has been linked to a reduction in the accumulation of starch in the early stages of organogenesis. The most dramatic difference between shoot forming and callus forming tobacco tissue was the accumulation of starch in the former[41,42].

In addition to chemical factors the culture environment is also important in studies on organogenesis, the main factors being the physical state of the medium[43], light and temperature[33,35,44].

II. *Thin Cell Layers*

With isolated cells, most of the cell contacts have been lost or drastically changed. A common drawback in the use of the callus system or of plant explants for studies on organogenesis is that only a few cells in the callus mass are directly involved in the process of organ initiation. Both in the use of isolated cells and of callus tissues derived from isolated cells, one cell retains the capacity to callus, or to form buds or roots or embryos. For a cell to express a new morphogenetic response, it has first to be removed from an intact host, and then, to establish a new relationship with its new neighbouring cells[45]. Tran Thanh Van and Dien[48] conducted studies utilising an alternative method between isolated cells and the use of organ fragments or callus, by slicing the organ fragment into thin cell layers consisting of a smaller number of cell types selected from the differentiated cells and without the central cells, *e.g.*, (1) epidermal, (2) epidermal and subepidermal cells, (3) parenchyma cells, (4) cambial and *phloem cells*, (5) medullary cells. These were excised either transversely or longitudinally and cultured alone or reassociated in their previous orderly pattern or in an order, different from that found in organ fragments or in entire plants and on the most simplified media[46]. By the use of such culture media and the right physical factors, the aim of this experimental system was to induce all the morphogenetic patterns normally experienced by a plant like hairs, embryos, buds, roots, flowers or callus, if possible from the same cell layer. Using this system by appropriate manipulations of the media hormones, a visible appearance of meristems was observed on the vegetative or floral explants of tobacco within 14 days, but after 16 days with root explants[46]. This suggested that the biochemical and physiological events occurring

within the cells during the first two weeks o culture are very important and could throw light on our understanding of the mechanism of differentiation and organ development *in vitro*. The use of thin cell layers has revealed the importance of factors which would be masked when using complex and large inocula. This method has also shown that explants taken from the donor plants show a residual effect which greatly influence the response of the explants to their new environment consisting of the medium, explant and culture atmosphere.

III. *Biochemical Studies*

As we have seen in the earlier sections different experimental systems have been utilised to study morphogenesis and far more attention has been paid to the nature of the chemical controls that appear to regulate differentiation in culture[17]. From the recent literature on morphogenesis in plant tissue culture, we find a wide variety of physical (light, temperature, pH, physical stress, etc.)[17], biochemical (all types of metabolites, growth substances, minerals, etc.)[47] or molecular factors[48,49,50] which regulate morphogenesis. However, we have still not been able to map the exact morphogenetic events at all levels and also the precise timing and nature of organ determination. Some of these problems may soon be answered by using the thin cell layer system. Although results obtained by the use of biochemical and histochemical techniques have indicated both directly and indirectly, that DNA, RNA and protein synthesis occur during organ formation[48,51,52] and also that the requirements of the newly formed proteins are necessary[53,54], we have not progressed significantly in our understanding of organogenesis.

Recently, by using bud forming and non-bud forming cultures of Douglas fir cotyledons, it was found that bud forming cultures synthesised specific low molecular weight soluble proteins[54,55]. Studying the activities of some hydrolytic enzymes during shoot formation in rice callus, it was found that after 10 days in culture, the activity of α-amylase, ribonuclease, acid phosphatase and catalase was respectively 10, 7, 1·5 and 2·7 times higher in shoot forming regions than in the corresponding callus regions[56]. Similarly working with callus and root cultures of maize and wheat, it was observed that maize and wheat roots had a higher content of malic enzyme and glucose-6-phosphate dehydrogenase and a lower content of pyruvate kinase than the corresponding callus tissues[57].

Thus organogenesis is brought about by mechanisms which control the production of specifically required metabolic patterns at a particular time. However, we still have very preliminary evidence of the biochemical factors that govern the formation of shoots or roots[58].

Conclusions

Street[59] from earlier work drew the following general conclusions regarding totipotency, embryogenesis and morphogenesis *in cultured* cells: (1) the induction of cell division and culture interaction does not unmask the totipotency of cells, (2) the expression of totipotency is dependent on the physiological state of the cells in the explants under the conditions of inoculation, (3) for the development of shoot or root formation or embryos, the conditions of culture should be compatible for the unmasking of totipotency within the individual cells. These reasons could also explain why certain cultures fail to express morphogenesis or cell division even when grown on a wide range of culture media with different levels of hormones and growth regulators. In these cases attention should be directed towards the nature of the explant and also to other physical conditions. By the use of thin cell layers, it has been confirmed that a sequetnial interaction of multiple factors govern the induction of organogenesis, its determination and its expression, and not merely the influence of auxin for roots and kinetin for buds[60]. Morphogenesis also depends on a quantitative equilibrium of more

than one stimulatory and inhibitory factors and not of specific factors. A drawback in the use of thin cell layers is that it is still difficult to decide on which physicochemical changes (DNA, RNA proteins, all metabolic pathways, cell surface, atmosphere, etc.) should be studied[50]. However, one can deal with at least one cellular type and a single cellular pattern at a given time.

In conclusion it must be kept in mind that variations in the external factors of the medium alone are not sufficient to produce all the stimuli required for the developmental phenomena seen in tissue culture[16]. Flowering *in vitro* is still a rare phenomenon[50]. There is still need for a greater understanding of the biochemical and molecular events occurring during the early phases of organ development *in vitro*. In the intact plant, every cell is subjected to a complex set of correlations at various levels between organs, tissues and other adjoining cells. When an explant is excised, a part of this environment is brought with it into the new culture medium, and it is now influenced by an interrelationship between the culture medium, the diffusion of some of its metabolites (based on its earlier position in the parent plant and its physiological state) and the new atmosphere around it. A further understanding of all the mechanisms, underlying embryogenesis and organogenesis *in vitro*, may greatly help us in recovering viable plants from a large group which still do not respond to the normal methods of organogenesis. Such studies may help in bridging the gap in our understanding of totipotency and organogenesis in higher plants. This in turn would be of immense benefit for the more universal application of tissue culture to problems of agriculture and plant breeding and could also hasten the progress in the production of a new group of plants, genetically modified through protoplast fusion or by the introduction of foreign DNA.

1. White, P. R., *Pl. Physiol.*, 1934, 9, 585.
2. Gautheret, R. J., *C.R. Hebd. Seanc. Acad. Sci. Paris*, 1934, 198, 2195,
3. Nobecourt, P., *Ibid.*, 1937, 205, 521.
4. Miller, C. O. and Skoog, F., *Am. J. Bot.*, 1953, 40, 768.
5. Guha, S. and Maheshwari, S. C., *Nature*, 1964, 204, 497.
6. Carlson, P. S., *Science*, 1970, 168, 487.
7. —, Smith, H. H. and Dearing, R. D., *Proc. Natl. Acad. Sci.*, 1972, 69, 2292.
8. Street, H. E., *Plant Tissue and Cell Culture*, (ed.) H. E. Street, Blackwell Scientific Publications, 1973.
9. Reinert, J. and Bajaj, Y. P. S., *Plant Cell, Tissue and Organ Culture*, (eds.) J. Reinert and Y. P. S. Bajaj, Springer Verlag, Berlin, 1977.
10. White, P. R., *The Cultivation of Animal and Plant Cells*, The Ronald Press, U.S.A., 1964.
11. Scowcroft, W. R., In *Advances in Agronomy*, 1977, 29, 39.
12. Kurz, W. G. W. and Constabel, F., *Microbial Technology*, 1979, 1, 389.
13. White, P. R., *Bull. Torrey Bot. Club*, 1939, 66, 507.
14. Skoog, F. and Miller, C. O., *Symp. Soc. Exp. Biol.*, 1957, 11, 118.
15. — and Tsui, C., *Am. J. Bot.*, 1948, 35, 782.
16. Gresshoff, P. M., In *Phytohormones and Related Compounds—A Comprehensive Treatise*, (eds.) D. S. Letham, P. B. Goodwin and T. J. V. Higgins, Elsevier/North Holland, Biomedical Press, 1978, 2, 1.
17. Hicks, G. S., *Bot. Rev.*, 1980, 46, 1.
18. Steward, F. C., Mapes, M. O., Kent, A. E. and Holsten, R. D., *Science*, 1964, 143, 20.
19. Steward, F. C., Blakely, L., Kent, A. and Mapes, M. O., *Brookhaven Symp. Biol.*, 1963, 16, 73.
20. Reinert, J., *Planta*, 1959, 53, 318.
21. Bachs-Husemann, D. and Reinert, J., *Protoplasma*, 1970, 70, 49.
22. Steward, F. C., Kent, A. E. and Mapes, M. O., *Ann. N.Y. Acad. Sci.*, 1967, 144, 326.
23. Zapata, F. J. and Sink, K. D., *Theo. Appl. Genet.*, 1981, 59, 265.
24. Steward, F. C., Mapes, M. O. and Mears, K., *Am. J. Bot.*, 1958, 45, 705.
25. Street, H. E. and Withers, L. A., In *Tissue Culture and Plant Science*, (ed.) H. E. Street, Academic Press, London, 1974, p. 71.
26. Rao, P. S., Hondro, W. and Harada, H., *Physiol. Plant.*, 1973, 28, 458.
27. Sengupta, C. and Raghavan, V., *J. Exp. Bot.*, 1980, 31, 247.
28. Rashid, A. and Street, H. E., *Plant Sci. Lett.*, 1974, 2, 89.
29. Wetherell, D. F. and Dougall, D. K., *Physiol. Plant.*, 1976, 37, 97,

30. Tazawa, M. and Reinert, J., *Protoplasma*, 1969, **68**, 157.
31. Chang, W. C. and Hsing, Y. I., *Nature*, 1980, **284**, 341.
32. Gresshoff, P. M. and Doy, C. H., *Aust. J. Biol. Sci.*, 1972, **25**, 259.
33. Hendre, R. R., Mascarenhas, A. F. and Jagannathan, V., In *Regulation of Growth and Differentiated Function in Eukaryote Cells*, (ed.) G. P. Talwar, Raven Press, N. Y., 1975, p. 17.
34. Nitsch, C. and Nitsch, J., *Planta*, 1967, **72**, 355.
35. Murashige, T., *Annu. Rev. Plant Physiol.*, 1974, **25**, 135.
36. Gupta, P. K., Nadgir, A. L., Mascarenhas, A. F. and Jagannathan, V., *Plant Sci. Lett.*, 1980, **17**, 259.
37. —, Mascarenhas, A. F. and Jagannathan, V., *Ibid.*, 1981, **20**, 195.
38. Hill, R. H., *In Vitro*, 1976, **12**, 216.
39. Coleman, W. K. and Greyson, R. I., *Ann. Bot.*, 1977, **41**, 307.
40. Maeda, E. and Saka, H., *Proc. Crop. Sci. Soc. Japan*, 1973, **42**, 442.
41. Thorpe, T. A. and Murashige, T., *Can. J. Bot.*, 1970, **48**, 277.
42. — and Murashige, T., *Science*, 1968, **160**, 421.
43. Mascarenhas, A. F., Pathak, M., Hendre, R. R. and Jagannathan, V., *Indian J. Exp. Biol.*, 1975, **13**, 103.
44. Mutashige, T., In *Plant Tissue Culture and its Biotech. Application*, (eds.) W. Barz, E. Reinhard and M. H. Zenk, Springer-Verlag, Berlin, 1977, p. 392.
45. Tran Thanh Van, K. and Trinh, H., In *Frontiers of Plant Tissue Culture*, (ed.) T. A. Thorpe, Publ. Int. Assoc., P.T.C., Canada, 1978, p. 37.
46. Halperin, W., *Can. J. Bot.*, 1973, **51**, 1901.
47. Thorpe, T. A., In *Frontiers of Plant Tissue Culture*, (ed.) T. A. Thorpe, Publ. Int. Assoc., P.T.C., Canada, 1978, p. 49.
48. Tran Thanh Van, K. and Dien, N. T., *Can. J. Bot.*, 1975, **53**, 533.
49. Halperin, W., *Ann. Rev. Plant Physiol.*, 1978, **29**, 239.
50. Tran Thanh Van, K., In *Adv. Biochem. Engg.*, 1980, **18**, 152.
51. Kovacs, E. L., *Acta Bot. Acad. Hung.*, 1971, **17**, 391
52. Vassent, J., *C.R. Acad. Sci. Paris, Ser. D*, 1972, **275**, 2865.
53. Syono, K., *Plant Cell Physiol.*, 1965, **6**, 371.
54. Hasegawa, P. M., Yasuda, T. and Cheng, T. Y., *Plant Physiol. Suppl.*, 1977, **59**, Abstr. No. 2, p. 1.
55. Yasuda, T. and Cheng, T. V., *Ibid.*, 1978, **60**, Abstr. No. 246, p. 45.
56. Saka, H. and Maeda, E., *Proc. Crop. Sci. Soc. Japan*, 1973, **42**, 307.
57. Mascarenhas, A. F., "Metabolism of plant cells grown *in vitro*," *Ph.D. Thesis*, University of Poona, 1971.
58. Thorpe, T. A., In *Propagation of Higher Plants Through Tissue Culture*, Symp. Univ. Tennessee, April 16–19, 1978.
59. Street, H. E., In *Cell Genetics in Higher Plants*, (eds.) D. Dudits, G. K. Farkas and P. Maliga, 1973, p. 7.