IN VITRO STUDIES ON THE REGULATION OF ORGANOGENESIS IN CULTURED LEAF DISKS AND EPIDERMAL EXPLANTS OF *NICOTIANA TABACUM* L. cv. WISC. 38

by

© Lise Bentzen

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Department of Biology
McGill University
Montreal, Quebec, Canada

August 1981

Regulation of *in vitro* organogenesis in tobacco culture

to Don

ABSTRACT

The regulation of *in vitro* organogenesis was investigated in two tissue culture systems of *Nicotiana tabacum* L. cv. Wisc. 38, one employing thin epidermal explants excised from superficial tissues of the tobacco stem, the other employing leaf disks excised from fully expanded tobacco leaves. The first system was used to quantitate a gradient of *in vitro* floral organogenesis at three developmental stages of flowering tobacco plants. The second system of tobacco tissue culture was developed for the purpose of this thesis. It was demonstrated that on appropriate media, the leaf disk explants produced a vigorous and rapid organogenetic response consisting of vegetative shoots and propagation nodules. The system was easily manipulated by exogenous hormones, and the leaf disk explants were both highly productive and homogenous in their response. The mode of exogenous hormonal regulation of vegetative bud, root, nodule, and callus formation was established. Also experimentally established and reported in this thesis are the extent to which plant imposed factors affected organogenetic expression, the propagation potential of the leaf disk system, and the morphological development of organogenesis in the system.

Résumé

La régulation de l'organogénèse de Nicotiana tabacum L. cv. Wisc. 38 a été analysée, in vitro, suivant deux systèmes différents: l'un utilisant des explantats épidermiques minces prélevés à la surface des tiges, l'autre utilisant des disques foliaires provenant de feuilles ayant atteint leur taille maximum. Le premier système avait pour but de quantifier un gradient d'organogénèse florale in vitro, à partir de trois stades de développement du tabac en période de floraison. Le deuxième système fut élaboré pour les besoins de cette thèse. Il a été démontré que, sur un milieu de culture approprié, les explantats de disques foliaires produisent une réaction organogénétique rapide et vigoureuse, consistant en pousses végétatives et nodules de propagation. Ce système a été facilement contrôlé par l'apport exogène d'hormones; de plus, la réaction des explantats foliaires fut à la fois très homogène et abondante. Le mode de régulation hormonal exogène, lors de la formation des bourgeons végétatifs, racines, nodules et cals, a été établi. De plus, d'autres faits ont été établis expérimentalement et décrits dans cette thèse: l'importance considérable des facteurs endogènes dans l'expression organogénétique, le potential de propagation du système de disques foliaire et le développement morphologique de l'organogénèse du système.

ACKNOWLEDGEMENTS

I wish to express my gratitude to Dr. William Boll for his advice, guidance, and support throughout this study.

I am especially indebted to Claire Cooney-Sovetts for the considerable amount of time she spent training and helping me with the morphological and photographic work, as well as for her friendship and support, at all times sincerely appreciated.

I am also indebted to Robert Lamarche and Guy L'Heureux of the photographic center for their technical assistance, and to Dr. R. Sattler for the use of his laboratory facilities for part of this research.

Special thanks to Dr. M. Dubuc-Lebreux who translated the abstract, and to all my friends in the McGill Biology Department.

The research for this thesis was funded in part by a grant from the National Research Council of Canada.

TABLE OF CONTENTS

I. INTRODUCTION

The phenomenon of biological development, whether plant or animal, is an extremely complex subject that remains a largely unresolved challenge for modern biologists. Biological development is the result of the interaction of two processes, growth and differentiation. Growth represents the irreversible increase in size of the whole or part of an organism. Differential growth along certain axes, under rigorous control mechanisms, determines the external form of an organ, and ultimately, the morphology of an organism. Differentiation represents qualitative differences between cells, tissues, and organs, as well as the many complex control mechanisms which ultimately determine their functions. The complexity of development arises from the multiple, intricate interactions between control mechanisms involved in growth and differentiation at each level of the organism, from cell to whole organism.

Plant tissue culture has evolved as a simplified approach to the enormously complex study of plant development and organogenetic regulation. Simplification is achieved by dividing the organism into its component organs, tissues, or cells, and growing them in asceptic cultures. Thus, the complexity of development can be reduced through the isolation of certain components of the developmental system, thereby eliminating some of the complex physiological and biochemical interactions between organs, tissues, and cell types, or by eliminating some of the

developmental controls imposed by the whole plant. Since its advent at the turn of the century, plant tissue culture has yielded a wealth of information on biological controls in plant development, especially in the elucidation of factors regulating plant organogenesis and morphogenesis.

The object of this research was to investigate factors regulating *in vitro* organogenesis in two tissue culture systems of *Nicotiana tabacum* L. cv. Wisc. 38; one employing thin epidermal explants from the tobacco stem, the other using disks excised from tobacco leaves.

The first system, the culture of epidermal explants excised from the stem and floral branches of tobacco, was developed by Tran Thanh Van and coworkers (see Literature Review). It was of particular interest since it was reported to show potential for direct floral organogenesis without intermediary callus formation, and furthermore, to show, in a precise quantitative manner, a gradient of *in vitro* floral potential along the tobacco stem. This gradient is expressed in *Nicotiana* by an increasing tendency for stem explants to produce floral buds *in vitro* as the excision locus approaches the floral apex of the flowering tobacco plant, along with a concomitant decreasing tendency for the explants to produce vegetative buds. The system of epidermal stem explants, as described by Tran Thanh Van, seemed to be ideal for a precise, quantitative investigation of some of the factors regulating floral and vegetative organogenesis. Therefore, the

research comprising the initial part of this thesis was focused on quantitative analysis of the floral gradient in tobacco since the gradient itself is, presumably, an expression of factors influencing vegetative and floral organogenesis within the tobacco plant.

However, the research undertaken here on the gradient posed serious and apparently insurmountable problems, since the organogenetic response proved to be unpredictably variable and more complex than anticipated. In most cases the yield of explants showing organogenesis was too low for meaningful analysis. While thousands of explants were excised and plated for these experiments, very low yields of organogenetic explants were achieved (0% in some experiments). The low yield was due to explants necrosing early in culture and/or to lack of any organogenetic response. Hence, it seemed it would be impossible to achieve appropriate numbers of experimental replicates for statistically valid analysis of the subtle physiological factors influencing organogenetic mechanisms. This problem was further augmented by the fact that the system turned out to be considerably more complex than reported by Tran Thanh Van. She reported that the epidermal explants simply showed direct organogenesis from the subepidermal layer of cells (1973, 1974; refer to Literature Review), while the research for this thesis showed that the system apparently involved at least six organogenetic processes.

In spite of the problems encountered in the gradient

research using epidermal explants, some results were obtained. These included a quantitative analysis of the effect of the developmental stage of the donor plant on the gradient, a qualitative and quantitative description of the six organogenetic processes observed in the culture system as well as their relative capacities for expression of the gradient, and the effect of alcohol sterilization and explant excision locus on the capacity for organogenesis. These results are given in Section IV-1 of the thesis.

Because the epidermal explant system proved to be too inefficient and inappropriate for the research into the regulation of organogenesis as envisioned here, a second system was developed. This system employed 12.7 mm leaf disks excised from tobacco leaves. Other than in the choice of explant type, the culture system was similar to the one for epidermal explants. That is, the same type of donor plants, culture media, and culture conditions were retained in the hope of maintaining a situation in which the physiological gradients associated with flowering Nicotiana could still be expressed.

The leaf disk system was chosen for testing since it had, or proved to have, many obvious advantages: 1) Large numbers of explants could easily and quickly be generated using an appropriate disk cutting tool, with complete size uniformity (which was not the case with the epidermal explant system). 2) A tobacco plant could yield several orders of magnitude more explants from its leaf tissue than from its stem tissue, since

the leaf area of a tobacco plant far exceeds the stem surface area. Consequently, much more research material can be generated in limited facilities when using leaf disks as compared to stem epidermal explants. 3) The explants showed 100% viability (i.e. no necrosis). 4) There was an in vitro growth response of 100% (i.e. all the explants produced organogenesis). In other words, large numbers of experimental replicates could very easily be generated with the leaf disk system.

The object of the research using the leaf disk system was similarly to investigate factors regulating in vitro organogenesis, originally in hopes of investigating factors associated with physiological gradients as observed in tobacco stem culture. However, the experiments did not reveal any evidence of physiological gradients in leaf tissue of tobacco, nor any apparent ability for floral organogenesis. Consequently, the experimentation was focused on providing a basic description of this system and evaluating its potential as a research vehicle for further studies on the regulation of in vitro organogenesis.

This thesis therefore reports on exogenous hormonal regulation of vegetative bud, root, and callus formation from tobacco leaf disks, the effect of various plant imposed factors affecting organogenetic expression, the propagation potential of the leaf disk system, and morphological analyses of two of the organogenetic processes observed in the leaf disk cultures. These results are presented in Section IV-2 of the thesis.

For both culture systems, epidermal explants and leaf

disks, an explanation of some of the issues is given along with the relevant results in order to achieve the most appropriate presentation of data.

II. LITERATURE REVIEW

1. Historical Review

Plant tissue culture has its origin in the ideas of Haberlandt, a German botanist who in 1902 predicted the possibility of culturing isolated plant cells (though he was unsuccessful in achieving this himself) which would thus demonstrate the totipotency of plant cells. His ideas were far in advance of his time; some of his predictions were not experimentally tested and proven correct until half a century later. For example, Haberlandt had proposed such things as the use of embryo sac fluids (such as coconut milk) for inducing cell divisions in vegetative cells, the possibility of embryogenesis in vitro from vegetative cells, the potential use of cell culture to investigate the properties of cells, and the use of plant tissue culture as a potential experimental system for investigating "interrelationships and complementary influences to which cells within a multicellular whole organism are exposed" (historical review in Vasil and Vasil, 1972).

Though Haberlandt had in 1902 introduced the concept of plant cell, tissue, and organ culture as experimental systems for investigating various aspects of plant development, it was not until 1934 that the first successful culture was established. This was the culture of excised tomato roots by White, who demonstrated that roots would grow and differentiate in culture

if given the appropriate nutrients. Four years later, carrot phloem was successfully cultured by Gautheret. Both cultures are still being grown and thus have been maintained in vitro for nearly half a century. (Historical review in Vasil and Vasil, 1972; Wareing and Phillips, 1978).

Plant tissue culture has grown enormously in the last forty years. Today there are many plant species in culture and many scientists using plant tissue culture to study the regulation of developmental processes. Just a few brief historical highlights will be given in order to place the research of this thesis in proper perspective.

In 1941 Van Overbeek, a Dutch plant physiologist, discovered that coconut milk acted as a potent growth stimulator, accelerating the development of plant embryos and promoting the growth of plant cells and tissues in vitro. These discoveries hinted at the presence of some type of growth regulator in plants, which, years later, was identified as cytokinin, a class of plant growth regulators which stimulate cell division. Folke Skoog and his coworkers at the University of Wisconsin provided clear evidence for the in vitro requirement for what became called cytokinins with stem segment cultures of Nicotiana tabacum L. Though the stem segments initially grew well in a culture medium containing appropriate vitamins, mineral salts, and sugar, growth soon slowed or stopped. This suggested that some growth stimilus, originally present in the tobacco stem, had become exhausted. Addition of IAA (indole-acetic acid) did not help.

However, addition of coconut milk to the stem cultures did induce the cells to resume division and growth. In the early 1950's work by C.O. Miller, a postdoctoral fellow in the laboratory of F. Skoog, led to the identification and isolation of a degradation product of animal DNA which could replace the effect of coconut milk in stimulating in vitro growth. This product was named kinetin, and is a synthetic member of the cytokinin class of plant growth regulators. A number of other related compounds with cytokinin properties were soon synthesized. As kinetin, they are all similar in structure to adenine, a purine present in DNA. In 1964 the first naturally occurring cytokinin, zeatin, was isolated from corn kernels by D.S. Letham and C.O. Miller (reported 1965).

In 1957 work by F. Skoog and C.O. Miller showed that varying the relative concentration of IAA and kinetin resulted in either root, shoot, or completely disorganized callus growth in cultured tobacco pith tissue.

Concurrent with Skoog's work, research by F.C. Steward on various plant tissue cultures similarly indicated that there are no specific regulating substances in plant development, but rather that regulation is achieved by the appropriate relative concentrations of naturally occurring growth substances or their synthetic substitutes. For example, of particular significance was the discovery by Steward and coworkers of embryogenesis in carrot culture. (The first in vitro embryoids were actually reported by Reinert in 1958, also in carrot cultures.) Steward

found that cultured carrot cells, when appropriately treated with the correct balance of nutrition and growth regulators, would undergo normal embryological development, producing roots, shoots, and eventually mature plants with flowers. Since then in vitro embryogenesis has been observed in many other plant genera and species, such as Nigella (Banerjee and Gupta, 1975b), Panax ginseng (Chang and Hsing, 1980), Nicotiana tabacum (Prabhudesai and Narayanaswamy, 1973), and Antirrhinum majus (Sangwan and Harada, 1975), plus in at least 70 more plant species, described in many excellent reviews such as those by Vasil and Vasil, 1972, and H.W. Kohlenbach, 1978).

Such research using plant tissue culture as an experimental system for investigating the processes of development has provided much evidence to support the hypothesis that the induction and maintenance of orderly development (that is, the control of normal plant growth) is largely a result of the appropriate balance of growth regulators along with the appropriate nutrition (sugar, minerals, vitamins).

However, it must be mentioned that plant tissue culture has provided more than just an experimental system for research into plant development. It has also provided many important horticultural, agricultural, and industrial benefits. These benefits are well described in many excellent reviews such as those by Murashige, 1974; Pierik, 1975; Holdgate, 1977; Murashige, 1977; Narayanaswamy, 1977; Spiegel-Roy and Kochba, 1977; Murashige, 1978; and Zenk, 1978. A few highlights of these

benefits will be given along with some plant examples that were randomly selected for purely illustrative purposes and are not meant to be comprehensive listings.

In plant breeding programs, plant tissue culture can provide a methodological basis for genetic improvement of plants. This can be achieved by providing material for mutation induction and screening of new genotypes faster and more economically than by traditional in vivo methods, by quickly generating large numbers of clones of a desired genotype, and by producing non-chimaeral mutants, haploid plants from anthers, and multiple chromosome-number plants not usually available from traditional in vivo methods (example: Antherrium, Begonia, Boston fern, Citrus, Freesia, Lily, Nicotiana, orchids, Saintpaulia, strawberry).

Other horticultural and agricultural benefits include the rapid clonal propagation of plants when vegetative propagation in vivo is slow, non-profitable, or simply not possible (examples: Boston fern, Gerbera, hyacinth, orchids).

Yet another benefit is the production of plant material free of pathogens, namely viruses, molds and nematodes (examples: Chrysanthemum, Citrus, Dianthus, Freesia, Lily).

Industrial benefits include the potential use of plant tissue culture for mass production of valuable secondary metabolites for medical, industrial, and agricultural usage. These metabolites include plant products such as steroids, morphine, codeine, atropine, caffein, glutathione, and

ginsengoside, just to name a few.

Thus, since its theoretical conception at the turn of the century, plant tissue culture has grown enormously and now has important impact in many fields of research: plant development, biochemistry, physiology and genetics; as well as significant economic impact in horticulture, agriculture, and industry.

2. Regulation of in vitro Organogenesis

Many factors are known to have an effect on *in vitro* regulation of organogenesis. These factors can be broadly grouped into two classes: plant imposed factors and experimentally imposed factors. Naturally these two classes interrelate in their determining effects on organogenesis. Some of the main plant imposed factors are the genetic make-up of the donor plant, its physiological state, the tissue origin of the explant, the cell age of the explant, and inter-tissue and inter-cellular correlations within the explant. Some of the experimentally imposed factors are the composition of the culture medium (*e.g.* nutrition, carbohydrate source, pH, osmotic pressure, and hormones) and the culture environment (*e.g.* culture matrix, temperature, and light). These factors will be briefly discussed below in view of current literature on the subject.

i. Plant imposed factors

The genetic make-up of the donor plant: Organogenetic capacity and factors affecting organogenetic expression in vitro are determined by the genetic composition of the donor plant; so that different species and even different cultivars of the same species show different organogenetic responses under similar culture conditions. Thus, the specific culture conditions optimal for induction of a particular form of organogenesis is very much a function of genotype.

For example, in propagating Freesia plantlets from adventitious buds produced in vitro, Pierik and Steegmans (1975) found different organogenetic responses in all ten cultivars tested.

In testing the morphogenetic potential of callus of stem and leaf origin from two species of tobacco as well as two cultivars of Nicotiana tabacum, Chailakhyan, Bavrina and Konstantinova (1977) found floral buds only on callus derived from explants of the day-neutral cultivar.

On the other hand, workers with Tran Thanh Van found that a day-neutral hybrid of tobacco produced by crossing Nicotiana tabacum L. cv. Samsun with N. sylvestris had reduced flowering capacity, thus ascertaining that total floral organogenetic capacity (i.e. 100% de novo flower formation) is not specific to all day-neutral cultivars of Nicotiana (Tran Thanh Van and Trinh, 1978). This was recently further substantiated by other

hybridization studies in which a long-day hybrid was produced which showed *in vitro* floral potential (Kamate, Cousson, Trinh and Tran Thanh Van, 1981). Together these studies show that the actual photoperiodic requirement of the donor plant is not directly related to the potential for *in vitro* floral expression in *Nicotiana*.

Moreover, there are even more subtle genetic influences present in some tissue culture systems. There are cases where even for the same cultivar some genetic lines show certain organogenetic traits not expressed in other genetic lines of the same cultivar; for example, *Pisum* (Tran Thanh Van and Trinh, 1978, and references therein).

Thus, there must be very subtle genetic factors, as well as the more obvious inter-species genetic factors, which have a determining role in regulation of organogenesis *in vitro*.

The physiological state of the donor plant: In many tissue culture systems it is known that the physiological state of the donor plant has an important determining role in the *in vitro* expression of organogenesis. For instance, for some plant species and genera, floral organogenesis *in vitro* is only observed when the donor plant has previously been florally induced (*i.e.* is in the flowering state). This observation has been reported for several species of *Begonia* by Ringe and Nitsch (1968), for example.

The requirement for donor plants to be in the flowering

state in order to obtain in vitro floral organogenesis has been reported by several workers for Nicotiana tabacum: for example, Chouard and Aghion, 1961, using cultured stem segments of tobacco, and Konstantinova et al., 1974, using tobacco callus cultures. In the laboratory of Tran Thanh Van using thin epidermal explants excised from floral branches of tobacco, it has been shown that for some species of Nicotiana and certain cultivars of N. tabacum that the de novo and direct flower formation is only expressed at a precise physiological stage, namely after fertilization of the terminal bud on the donor plant. Thus, for a given genotype of tobacco, the physiological stage seems to be the determinant for the expression of flower forming ability (Tran Thanh Van, Thi Dien and Chlyah, 1974; Tran Thanh Van and Trinh, 1978).

The tissue origin of the explant: The tissue origin of the explant, with respect to both the tissue type and the excision locus on the donor plant, is known to affect in vitro expression of organogenesis.

It is well documented in many species that the different tissues of a plant show different organogenetic responses under similar culture conditions, or conversely, that the specific culture conditions (in particular, the type and concentrations of growth regulators) optimal for inducing a specific type of organogenesis is a function of tissue type. For example, Banerjee and Gupta (1975a) found that calluses from root, stem,

and leaf tissues of Nigella sativa were all organogenetic, but that the frequency of differentiation and the appropriate media for inducing organogenesis differed for each tissue origin.

The in vitro culture of leaves, petioles and floral stalks from flowering Begonia by Ringe and Nitsch (1968) led to floral organogenesis when appropriate concentrations of cytokinin, adenine and auxin were supplied; however, the optimal concentration of these three substances varied for each organ, and even so the frequency of floral organogenesis differed for each.

It has also been observed that cultured stem sections of Nicotiana glauca did not yield adventitious roots or shoots under the same culture conditions that induced cultures from shoot tips to do so profusely. Similar observations have been made for Petunia stem and shoot tip cultures (Murashige, 1974, and references therein).

Besides the effect on organogenetic expression related to tissue type, there is also an effect in certain species associated with excision locus, namely the position on the donor plant from which the explant originated.

Such a positional effect has been well documented in various stem culture studies on flowering Nicotiana tabacum plants. The positional effect is seen as an in vitro gradient of floral expression, in which the frequency of floral organogenesis increases with increasing proximity of the excision locus to the floral apex of the donor tobacco plant.

This gradient was originally described by Aghion-Prat (1965)

who used entire stem segments, and has subsequently been confirmed by Tran Thanh Van (1973b) using thin epidermal explants and Hillson and LaMotte (1977) using peeled stem segments. This gradient has recently been studied in relation to other physiological and biochemical gradients observed in the stems of flowering tobacco (Tran Thanh Van and Trinh, 1978, and references therein).

The cell age of the explant: Variations in organogenetic characteristics among explants, cell cultures, or callus cultures are sometimes correlated with differences in their physiological age and/or the extent of differentiation among their constituent cells.

For example, Hillson and LaMotte (1977) reported a progressive decline in organogenetic potential down the length of the tobacco stem in their cultures of peeled stem segments, quite apart from the gradient of floral expression noted above. Specifically, they reported that higher excision loci (i.e. closer to the plant apex) showed more vigorous organogenetic production, this possibly being associated with the younger cell age of tissues closer to the apex.

Another example from Nicotiana tabacum tissue culture is the in vitro study by Prabhudesai and Narayanaswamy (1973) using excised petioles. They found that explants from young plants were much more regenerative than those from older or mature plants.

An example in another species is the culture of Echeveria

elegans leaf explants: young leaves produce only roots, old leaves only shoots, and leaves of a median age both roots and shoots (Murashige, 1974, and references therein).

Some callus cultures lose their organogenetic potential with repeated sub-culturing. For example, Banerjee and Gupta (1975b) found that their callus cultures from Nigella sativa root and stem tissues lost the ability for organogenesis after the eighth passage.

Inter-tissue correlations within the explant: As suggested by observation of in vivo adventitious regeneration in plants in which shoot buds generally arise from superficial cells of the aerial part of the plant, whereas roots originate from inner tissues (e.g. perivascular cells), there exist inter-tissue correlations which play determining roles in the regulation of organogenesis in vitro.

Studies in the laboratory of Tran Thanh Van on several plant species have provided evidence for inter-tissue correlations (Tran Thanh Van, Chlyah and Chlyah, 1974; Tran Thanh Van and Trinh, 1978, and references therein). For example, work on various tissues, both singly and in combination, from stem segments of Torenia fournieri showed that the epidermal cell layer had organogenetic capacity that was only expressed when it was in direct or indirect contact with the subadjacent tissue. The sub-epidermal layer formed roots when cultured alone, but formed roots and shoots when cultured in association with the

epidermis. Entire stem segments, which included vascular tissue, also only formed roots when devoid of the epidermal layer (Tran Thanh Van, Chlyah and Chylah, 1974, and references therein). Such results show the existence of both protagonistic and antagonistic correlations among tissues of an organ fragment.

Inter-cellular correlations within the explant: Many in vitro studies have led to the conclusion that a certain relationship between cells (that is, an orderly pattern of inter-cellular organization) seems to be a prerequisite condition for morphogenesis to occur. For example, some authorities believe that single cells in isolation do not develop directly into embryos. Rather, single cells that are potentially embryoidal are differentiated amongst cellular aggregates (which however may arise from divisions of single isolated cells), as evidenced by histochemical changes in the cells. Thus, it seems that some sort of inter-cellular interaction precedes embryogenesis (Narayanaswamy, 1977, and references therein; Wareing and Phillips, 1978, and references therein).

Another very common example of inter-cellular interactions in tissue culture is that, in many cases, survival as well as rate of development in vitro is directly related to the size of the explant (for example, leaf disk explants of tobacco: Mullins, Harsono and Batten, 1976; and Murashige, 1974, and references therein).

Another example of inter-cellular factors affecting in

in vitro organogenesis is provided by work on *Torenia fournieri* in Tran Thanh Van's laboratory. Their results gave evidence for competition among cell division sites in small tissue pieces cultured *in vitro*: the formation of the first buds limited all cellular activity within a certain distance thus preventing the initiation of new primordia as well as the organization of buds from existing primordia within this distance. This particular inter-cellular interaction was probably of a nutritional-hormonal and/or physical type and seemed to be associated with the basipetal transport and accumulation of auxin within the explant tissue (Tran Thanh Van, Chlyah and Chlyah, 1974; Tran Thanh Van and Trinh, 1978).

ii. Experimentally imposed factors

There are two general categories of experimentally imposed factors regulating *in vitro* organogenesis: those associated with the culture environment and those associated with the culture medium. Some of the main factors in each category will be briefly discussed below.

The environmental temperature: In most tissue culture studies the temperature is simply kept at a constant 25 to 27°C. However, there have been studies in which temperature has been shown to affect the expression of *in vitro* organogenesis. For example, in some tobacco callus studies it has been shown that

optimal shoot formation occurs at 18°C (Murashige, 1974, and references therein). The morphogenetic differentiation of unicellular hairs induced in vitro from epidermal cells of the main leaf vein of Begonia rex is temperature sensitive, both with respect to the explant environment and to the environment of the donor plant prior to excision (Tran Thanh Van and Trinh, 1978, and references therein). In plants which show dormancy in vivo, the cultures may require cold treatment to prevent dormancy in the plantlets derived from the cultures; for example, Lilium cultures (Murashige, 1974, and references therein).

The environmental light conditions: In most tissue cultures light is needed to regulate the morphogenetic processes. Both the intensity and duration of light may be factors affecting in vitro organogenesis, though the specific optimal conditions are a function of the types of culture and organogenesis. For example, Tran Thanh Van et al. found that optimal floral bud formation on thin epidermal explants of tobacco occurred with 16 hours of light at 52,000 ergs-$cm^{-2}sec^{-1}$ (Tran Thanh Van, Chlyah and Chlyah, 1974; Tran Thanh Van, 1977). In stem explants of tobacco, increased daylength led to increased bud formation (Hillson and LaMotte, 1977). However, root organogenesis in epidermal explants of tobacco was enhanced by dark conditions (Tran Thanh Van, Chlyah and Chlyah, 1974; Tran Thanh Van, 1977). Another somewhat unusual example is the culture of Cichorium leaf sections in which optimal shoot formation occurred when the

cultures received three days of darkness followed by 37 days of constant light (Murashige, 1974, and references therein).

Other factors associated with the light conditions in tissue culture are photoperiod and wavelength, the latter indicating a probable involvement of phytochrome in morphogenesis (Murashige, 1974, and references therein).

The culture matrix: There are sometimes specific matrix requirements for the production of specific types of organogenesis. The matrix requirement, liquid medium versus agar-solidified medium, is possibly associated with oxygen availability and requirements (Murashige, 1974). In any case, the matrix requirement is definitely a function of the plant species, the explant type, and the desired form of organogenesis. For example, Cousson and Tran Thanh Van (1981) found that thin epidermal explants of Nicotiana tabacum, which produced 100% floral organogenesis on an agar medium, did not produce any flowers (i.e. produced vegetative shoots instead) when floated on a liquid medium identical except without agar.

Liquid media are often more appropriate for shoot tip cultures (Holdgate, 1977); however, shoot tip cultures of Asparagus and Gerbera require initiation on agar media (Murashige, 1974, and references therein).

It has been demonstrated by several workers that Nicotiana callus cultures are induced to form shoots simply by transferring the callus from a solid to a liquid medium (Murashige, 1974, and

references therein).

Nutritional factors in the culture medium: Generally the types and concentrations of inorganic salts and various organic additives (other than growth regulators) as defined by the most common tissue culture media (for example, the Murashige and Skoog medium defined in Materials and Methods of this thesis) fulfill the nutritional requirements of most plant tissue and organ cultures. However, occasionally a specific type of culture may require other additives (e.g. malt and yeast extracts, coconut milk, banana pulp, orange and tomato juices, fish emulsion) to promote growth and differentiation (Murashige, 1974, and references therein). For example, Banerjee and Gupta (1975b) found that they could induce embryogenesis in Nigella cultures by increasing the concentration of casein hydrolysate. Another specific nutritional requirement associated with a specific type of organogenesis is cited by Kohlenbach in a 1977 review on embryogenesis in which he reports reduced nitrogen as a requirement for somatic embryogenesis (Kolhenbach, 1977, and references therein).

The carbohydrate source in the culture medium: Though normally sucrose or glucose at 2 to 3% is considered satisfactory for tissue culture (Murashige, 1974), several studies in the last decade have shown that the concentration and type of carbohydrate used in the culture medium may have a regulating effect on organogenesis. For example, in thin epidermal explants of

tobacco, it was found that, besides a specific auxin/cytokinin requirement, optimal root organogenesis required a lowered (1%) sucrose concentration (Tran Thanh Van, Thi Dien and Chlyah, 1974) or a lowered glucose concentration (Tran Thanh Van, 1977). Using the same system to test four monosaccharides, it was found that optimal floral bud formation required specific combinations of saccharose and glucose or fructose (Tran Thanh Van, 1977).

The pH of the culture medium: Very little work has been done correlating the pH of the medium with the regulation of *in vitro* organogenesis. Generally the pH of culture media fall in the range pH 5 to 6, with pH 4.5 to 7.0 representing the usual limits of pH tolerated by tissue culture growth. However, in a very recent study on thin epidermal explants of tobacco, it was demonstrated that specific pH's of the medium in association with exact auxin-cytokinin concentrations were necessary for floral organogenesis when liquid media were used (Cousson and Tran Thanh Van, 1981).

Osmoregulatory factors in the culture medium: Osmoregulatory factors affecting *in vitro* organogenesis have been suggested by several studies, as reviewed by Thorpe (1978). Thus, the sucrose concentration effect, cited under "carbohydrate source" above, may be partly osmotic in nature. The following examples are from Thorpe's review: Osmotic requirements are known for zygotic embryo culture, cotton fiber growth *in vitro*, and *de novo* shoot formation in tobacco callus. The tobacco callus studies

showed that shoot formation increased with increasing sucrose concentration up to 3% (w/v), but was inhibited above 3%. Mannitol, which is taken up by tobacco cells but not metabolized by them, duplicated the inhibitory effect at 3%, thus supporting the hypothesis that the inhibition was osmotic in nature.

Hormones in the culture medium: There are many growth regulators known to affect organogenesis in vitro, including gibberellins, ethylene, auxins and cytokinins. The latter two are generally considered to be the most important regulating factors, and are certainly the most commonly employed; thus this review will be limited to their actions in vitro. Following the example of Everett et al. (1978), for convenience the term hormone will be used to include natural and synthetic compounds with auxin or cytokinin activity. Generally speaking, the action of plant hormones is the most important regulating factor affecting organogenesis in most tissue culture systems.

Most of the regulatory effects of auxins and cytokinins can be grouped into three broad modes of action, though the exact regulatory mechanism in any given case of in vitro organogenesis is probably very much a function of plant species and explant type. These modes are as follows: i) auxin/cytokinin ratios, ii) the specific type and concentration(s) of auxin and/or cytokinin, and iii) the presence and subsequent removal of auxin. These three alternatives will be briefly described below.

i) auxin/cytokinin ratios: The auxin/cytokinin ratio

hypothesis was initiated by Skoog based on his work on regulation of morphogenesis in tobacco callus cultures. His work showed that the regulation of root and shoot organogenesis in tobacco cultures was a function of auxin to cytokinin ratios and concentrations. Root organogenesis resulted from high auxin to cytokinin ratios, whereas shoot organogenesis resulted from low ratios (Skoog, 1970).

ii) the specific type and concentration(s) of auxin and/or cytokinin: In some tissue cultures, specific auxins or cytokinins at specific concentrations are required for in vitro expression of a certain type of organogenesis.

For example, in cultures from excised axillary buds of Opuntia polycantha, the type of organogenesis was shown to be a function of the type of growth regulator used: NAA(an auxin) induced roots in adjacent tissue, BAP (a cytokinin) induced leaves, and GA (gibberellic acid) induced spines from buds; altering the concentrations led to different types of lateral appendages (Mauseth and Halperin, 1975). A more recent example, typical of in vitro embryogenesis, is the production of plantlets from embryoids of Panax ginseng callus cultures. The production required four stages of tissue culture, each requiring different hormones (or combination of hormones) at specific concentrations (Chang and Hsing, 1980).

In thin epidermal explants of Nicotiana tabacum and Nautilocalyx lynchei, the same cells were shown to give rise to vegetative buds, roots, or callus (or also to floral buds, for

Nicotiana) depending on the relative concentrations of auxin, cytokinin, and sucrose (Tran Thanh Van, 1973a; Tran Thanh Van, Chlyah and Chlyah, 1974; Tran Thanh, Thi Dien and Chlyah, 1974).

Auxin has been shown to promote in vitro floral organogenesis in Begonia, though there were specific auxin and cytokinin concentrations optimal for floral bud formation (Ringe and Nitsch, 1968).

On the other hand, auxin, specifically IAA, inhibits floral organogenesis in Nicotiana tabacum cultures. Studies by Skoog showed that the floral organogenetic capacity of tobacco stem segments were drastically reduced by IAA, though the inhibitory effect could be counteracted by nucleic acid base analogues (Skoog, 1970). Similarly, it was found that IAA concentrations equal to or greater than 10^{-6}M inhibited floral organogenesis in peeled stem segments (i.e. without epidermis) of tobacco (Hillson and LaMotte, 1977). The inhibition of in vitro floral organogenesis in tobacco cultures by IAA has been linked with an inhibition of synthesis of specific DNA fractions (Skoog, 1970; Wardell, 1977). Moreover, the in vitro gradient of floral expression observed in stem cultures of tobacco (the frequency of floral organogenesis increases from base to apex in flowering tobacco plants) has also been linked with endogenous IAA content (Tran Thanh Van and Trinh, 1978, and references therein; Wardell, 1977; Thorpe et al., 1978).

High concentrations of kinetin (greater than 10^{-5}M) also inhibited floral organogenesis in peeled stem segments of

tobacco; and the relative concentration of kinetin governed the auxin requirement for vegetative organogenesis (Hillson and LaMotte, 1977). Kinetin alone induced the formation of vegetative buds on leaf disks of tobacco cultured on an auxin-free medium (Mullins et al., 1976).

iii) the presence and subsequent removal of auxin: In Kohlenbach's 1977 review of in vitro differentiation and plant regeneration, he concludes that while auxin is definitely required for the induction of embryogenesis in vitro, it hinders its development; thus, development of embryoids requires subsequent lowering or removal of auxin from the medium. A clear example of this is given in studies on stem segment cultures of Antirrhinum majus L. in which it was shown that roots, callus or embryos could be induced depending on the growth regulators used, but that plantlet development from the embryos was dependent on the subsequent removal of auxin from the medium (Sangwan and Harada, 1975).

3. Epidermal Explants of Tobacco

The tissue culture system used in the first part of the research for this thesis was developed by Tran Thanh Van and coworkers at the Laboratoire du Phytotron, Centre National de la Recherche Scientifique, at Gif-sur-Yvette in France. This system uses epidermal explants, consisting of a few superficial layers of cells. They found that such explants, excised from certain

plants and cultured in vitro, had organogenetic potentialities which could be controlled by modification of nutritional and/or environmental factors (Trần Thanh Van and Drira, 1970; Tran Thanh Van, 1973a, 1973b; Chlyah, 1974; Tran Thanh Van, Chlyah and Chlyah, 1974; Tran Thanh Van, Thi Dien and Chlyah, 1974; Tran Thanh Van, 1977; Tran Thanh Van and Trinh, 1978; Kamate et al., 1981). Their studies showed the system to be relatively simple, homogeneous, and yet still capable of reproducing organogenetic potentialities of the plant. Epidermal explants from Nicotiana tabacum showed various types of organogenesis: floral buds or parts, vegetative shoots, leaves, and roots, without any intermediate callus formation. The main advantages to this system were: i) the lack of intermediate callus formation (thus it was a more clearly defined system of organogenesis), and ii) the rapidity of response. Organogenetic structures were well developed within weeks. Many other culture systems might require months for the same degree of development.

The culture system consists of small explants, three to six cell layers thick, of epidermal and subepidermal cells excised from the stems of certain plants and grown on a suitable medium. Applying this system to Nicotiana, Tran Thanh Van found that it was possible not only to obtain mitosis in one specific cellular layer of the explant, with subsequent vegetative shoot or root formation, but also to have mitosis followed rapidly by meiosis, with subsequent flower, anther, or pistil formation (Thi Dien and Tran Thanh Van, 1974; Tran Thanh Van and Thi Dien, 1975). Thus,

different types of organogenesis were obtained in a regulated fashion from the same cell layer. In other words, in this system a small group of homogenous, cytologically differentiated cells were capable of forming directly complex organs such as the flower, the vegetative or floral shoot, or the root. The rapidity of response made the system convenient for studies of organogenetic regulation.

This system was used to quantitate the gradient of floral potential found in Nicotiana tabacum. This physiological gradient in flowering tobacco plants has been well documented in the literature. (Tran Thanh Van, 1973b; Hillson and LaMotte, 1977; Wardell, 1977; Thorpe et al., 1978; and references within these.) Using the system of epidermal explants, Tran Thanh Van quantitatively analysed the gradient of floral potential in Nicotiana tabacum L. cv. Wisc. 38 (Tran Thanh Van, 1973b). Tran Thanh Van's studies revealed two opposing gradients of in vitro organogenesis along the tobacco stem. One was a gradient of increasing floral potential from base to apex, the other was a gradient of decreasing vegetative potential. Tran Thanh Van reported that explants taken from the base of the plant showed 100% vegetative buds and 0% floral buds, explants from the middle region showed 75% vegetative buds and 25% floral buds, explants from the subfloral region showed 60% vegetative buds and 40% floral buds, explants from the floral region showed 38% vegetative buds and 62% floral buds, and explants from the branches of the inflorescence showed 0% vegetative buds and 100%

floral buds. Thus, from base to apex of flowering tobacco plants, floral organogenesis went from 0 to 100%, and vegetative organogenesis from 100 to 0%. Tran Thanh Van also reported a gradient in the number of internodes before flowering in the *in vitro* floral shoots from different explant regions. Floral shoots on middle region explants showed four internodes before flowering, on subfloral explants three internodes, on the floral stem explants two internodes, and on floral branch explants one internode (*i.e.* the floral buds were directly on the explant). All neoformations arose from the subepidermal layer.

These findings indicate two general trends with regards to the floral gradient in tobacco:

i) the existence of a gradient of increasing potential for floral organogenesis from base to apex in *Nicotiana* at the green-fruit stage of development.

ii) a tendency from apex to base to produce increasing amounts of vegetative growth per floral shoot before floral bud formation *in vitro*.

To investigate factors which might regulate *in vitro* floral organogenesis in this system, Tran Thanh Van *et al.* attempted to modify the organogenetic capacity of floral branch explants of the green-fruit stage of tobacco, which under precise conditions expressed only the organogenetic capacity for formation of floral buds (Tran Thanh Van, Thi Dien and Chlyah, 1974; Cousson and Tran Than Van, 1981). Thus, in testing the effect of three physiological stages of the donor plant, Tran Thanh Van found

that whereas explants from plants at the green-fruit stage showed 100% floral organogenesis, explants from plants at the mature-fruit stage showed a reduced capacity for floral bud formation and explants from plants at the full-bloom stage (the earliest stage examined) produced no floral buds at all. This suggested that either floral organogenetic potential was supressed in some way at the full-bloom stage, or that floral organogenesis was not being induced by some endogenous condition that did exist at later physiological stages of the donor plant.

It was also shown that IAA and kinetin were both necessary for floral bud formation; the most favorable concentration being 10^{-6} M with an auxin/cytokinin ratio of one. If the absolute amount of either was altered, even if the ratio remained the same, there was no floral bud formation at all.

As previously mentioned in the literature review, sucrose concentration was another regulating factor: the optimal concentration was 20 to 30 g/L. Light was not an absolute requirement, but those explants grown in the dark had a much reduced capacity for organogenesis. Sucrose concentration only partly compensated for this.

Under conditions standard for floral organogenesis, there was a pronounced polarity in the explants. Floral buds always developed at the basal end of the explants. The polarity was eliminated by increasing the cytokinin concentration to 10^{-5} M; however, this also eliminated the capacity for floral organogenesis (i.e. only vegetative buds appeared).

Root formation was shown to be favored by a high auxin to cytokinin ratio and dark conditions.

Callus formation was induced by the use of the auxin 2,4 dichlorophenoxyacetic acid (24-D), rather than IAA. Callus cultures, even after several years of successive transfers, retained their organogenetic potential. Vegetative structures resulted when the callus tissue was transferred to an IAA medium; however, floral organogenesis was never observed to arise from these callus cultures. (However, the extent of the testing in this capacity was not reported).

A relationship between pH and the capacity for floral expression in these explants was also recently studied, as previously mentioned in this literature review.

Thus, it was evident that the type of organogenesis or callus formation could be induced at will, within the limits of this system. This strongly suggests that in certain well controlled systems such as this one, different cellular states are induced by the growth regulators and nutritional factors provided in the medium. Furthermore, Tran Thanh Van et al. considered that it is the respective quantities of these substances that count, not, generally, their respective special qualities. Since the explants are so small, the amount of endogenous substances they contain are also very small; and, therefore, expression of organogenetic capacities depends heavily on the exogenous substances supplied in the medium. Thus, with such a system it should be possible to more accurately quantify

factors regulating organogenesis.

4. Tobacco Leaf Tissue Culture

There are many reports of leaf tissue culture in the literature, widely establishing its organogenetic capacity. Species in which leaf tissue culture has been especially advantageous include Brassica oleracea, red cabbage, in the clonal propagation of this crop plant (Bajaj and Nitsch, 1975), and Morus alba, mulberry, which being a woody plant is difficult to culture by other methods (Oka and Ohyama, 1981). Examples of the use of leaf disks (as in this thesis) include Streptocarpus nobilis in which floral organogenesis was shown to be regulated by photoperiod (Nitsch, 1972, and references therein) and Begonia (several species) in which the hormonal requirements for floral organogenesis were demonstrated (Ringe and Nitsch, 1968). Regeneration studies using leaf disks have been reported for many species, including tobacco, by Yusufov and Khachumova (1975) in the Soviet Union.

Of particular interest to this thesis are two studies on leaf disk cultures of Crepis capillaris (Brossard, 1977) and Lycopersicon esculentum (Coleman and Greyson, 1977). Both studies used a cytokinin-free medium to show the auxin requirement for root organogenesis on the leaf disks. No vegetative shoot organogenesis was observed.

Compared to the enormous amount of work done on Nicotiana

stem cultures, surprisingly little work has been reported on tobacco leaf tissue culture. As previously mentioned, Chailakhyan et al. (1977) used callus cultures derived from tobacco leaf tissue to show an association between day-neutral photoperiod and the potential for floral organogenesis. Kasperbauer and Collins (1972) and Burk (1975) used tobacco leaf tissue cultures respectively to produce diploid tobacco plants from haploids, and pure diploid and haploid plants from cytochimeras. In both culture studies, organogenesis was associated with the mid-rib of the tobacco leaf.

The first study on organogenetic regulation in tobacco leaf tissue culture was performed by Gupta et al. in 1966. Using small seedling leaves, whole or cut into transverse segments and cultured on modified White's medium supplemented with coconut milk they tested the effect of three growth regulators: kinetin, IAA, and zeatin. They found that vegetative organogenesis from the leaf tissue had an absolute dependence on the kinetin concentration. Optimal bud formation occurred at 10^{-6} and 10^{-5} M kinetin, whereas at 10^{-7}M kinetin or lower there was no bud formation. Kinetin concentrations above 10^{-5} M were not tested. They also found that IAA was not necessarily required for bud formation and that increasing IAA concentrations (10^{-7} to 10^{-5} M) led to decreased bud formation. Zeatin was found to be more potent than kinetin, but otherwise similar in effect.

Further studies on organogenetic regulation in tobacco leaf tissue were reported in 1976 by Mullins et al. Their culture

system consisted of leaf disks excised from young vegetative tobacco plants (Nicotiana tabacum) and cultured on auxin-free Murashige and Skoog medium with a kinetin concentration of 5×10^{-6} M. They stated that, under these conditions, they observed vegetative buds originating externally to the original explant tissue on peripheral callus or internally by localised proliferations of cells in the palisade layers. Vegetative buds were the only form of organogenesis observed (as in Gupta et al.'s studies), and the maximum organogenetic response observed in any of their work was 85% of the disks showing an average of 10 buds per disk. Their studies with this leaf disk system showed that many factors affected the organogenetic response. Disk size was important: optimal bud formation occurred on disks 9 to 13 mm in diameter. The physiological stage of the donor plant also affected the organogenetic response: the greatest response was at the floral bud stage, slightly less at the vegetative stage, and was drastically reduced at flowering and seed-bearing stages. Wounding seemed to adversely affect the organogenetic response, since donut shaped disks showed almost no bud formation, and, in fact, mostly necrosed early in culture. In testing the effect of explant origin within the leaf, they found a gradient of increasing organogenetic capacity from the proximal (basal) to the distal end of the tobacco leaf, which they postulated was correlated with a gradient of endogenous IAA content. In testing the effect of explant orientation during culture, they found an absolute requirement for plating the

disks adaxial side up, with the lower epidermis in contact with the medium. When they plated the disks adaxial side down, the disks turned yellow and died.

Thus, the tobacco leaf disk system developed by Mullins et al. was evidently complex and subject to multiple sources of variation.

Recently (subsequent to the experimentation described in this thesis) results from another study on organogenetic regulation in tobacco leaf explants have become available. This study was by Chih-hung (1978) and coworkers at the Laboratory of Cell Physiology in the Shanghai Institute of Plant Physiology in China. Their culture system consisted of small squares of tobacco leaf (25 mm^2) excised from several cultivars of Nicotiana tabacum in the vegetative phase and cultured on modified Murashige and Skoog medium supplemented with lactalbumin hydrolysate. They tested three cytokinins (BA, kinetin, and zeatin) and two auxins (NAA and 2,4-D). Their results showed that all three cytokinins greatly stimulated bud formation on the leaf explants, whereas auxin (NAA) only promoted root formation. They found that there were no apparent differences between the cultivars of tobacco tested, as well as no effect due to the physiological age of the leaf or donor plant (contrary to the findings of Mullins et al.). They also reported that light was not necessary for bud formation.

Specifically, their cytokinin studies showed that BA greatly stimulated bud formation in concentrations ranging from

10^{-6} M to 5 x 10^{-6} M, but higher concentrations suppressed further bud development and root formation, thus confirming the results of Gupta et al. (1966) while also demonstrating that tobacco leaf tissue did not require coconut milk for expression of organogenesis (as did Mullins et al., 1975). Chih-hung and coworkers also demonstrated that the presence of cytokinin for bud initiation was only required for the first 5 to 6 days of culture, even though buds were not yet histologically evident. In fact, subsequent removal of cytokinin from the culture medium accelerated shoot (and root) development.

In summary, the literature shows that tobacco leaf tissue has in vitro organogenetic potential. In the case of vegetative organogenesis, this potential is expressed in the presence of cytokinin, whereas in the case of root organogenesis, it is expressed in the presence of auxin. Neither direct floral organogenesis nor embryogenesis has been clearly established for leaf tissue cultures of Nicotiana tabacum.

III. MATERIALS AND METHODS

1. Experimental Studies

i. Growth of plant material

Nicotiana tabacum L. cv. Wisc. 38 plants were grown in growth chambers at 24°C continuous temperature with a 16 hour light cycle at approximately 5400 lumens-m^{-2}. Seeds were germinated in vermiculite and irrigated daily with tap water. At approximately the seven leaf stage, the seedlings were transplanted to a soil-vermiculite mixture (3 parts potting soil; 1 part vermiculite). The plants were irrigated five days per week with diluted Hoagland's solution (Table 1) and two days per week with tap water, to flush accumulated salts from the soil. Both the Hoagland's solution and the tap water were dispensed by an automatic irrigation system designed and built by the author. The irrigation system uniformly dispensed either nutrient solution or tap water to each pot once or twice per day as required. Once a day the leaves of the tobacco plants were sprayed with tap water to wash the leaves and to increase the humidity of the growth chamber environment.

Maturation of the plants to the green-fruit stage required 13 ±2 weeks. For each batch of about 30 tobacco plants, the green-fruit stage was reached over approximately a two week period, with the green-fruit stage lasting about two days in an

Stock Solution	Ingredient(s)	Stock Concentration M	Stock Concentration g/L	Stock Volume mL/40 L	Final Concentration mg/L
A	$NH_4H_2PO_4$	1.0	115.04	20	57.5
B	KNO_3	1.0	101.10	120	303
C	$Ca(NO_3)_2 \cdot 4H_2O$	1.0	236.15	80	472
D	$MgSO_4 \cdot 7H_2O$	1.0	246.48	40	246
Micronutrients	H_3BO_3		2.86	20	1.43
	$MnCl_2 \cdot 4H_2O$		1.81		0.90
	$ZnSO_4 \cdot 7H_2O$		0.22		0.11
	$CuSO_4 \cdot 5H_2O$		0.08		0.04
	$H_2MoO_4 \cdot H_2O$		0.02		0.01
Sequestrene				1.4 g/40 L	35

Table 1: Modified Hoagland's Solution

individual plant. By the green-fruit stage, basal senescence had proceeded to the degree that 20 to 25 per cent of the leaves (i.e. 6 to 8 leaves out of a total of approximately 30 leaves) would be totally or mostly senesced.

Various developmental stages of the Nicotiana plants were used during the course of this research. The developmental stage was characterized by the state of the apical meristem. Floral stages were defined by the state of the terminal flower of the main axis. For example, plants at the green-fruit stage were characterized by a terminal fruit containing immature, green seeds.

ii. Preparation and sterilization of explants

In preparation for explanting, the appropriate parts of the tobacco plant (defoliated stem segments for the epidermal explants; leaves for the leaf disk explants) were rinsed with tap water to remove external debris. The plant material was then sterilized by a 60 second immersion in 70% ethanol (v/v), followed by a 10 minute immersion in 0.5% sodium hypochlorite solution (a 10-fold dilution of household bleach which was a guaranteed minimum 5.25% NaOCl), and then rinsed at least three times with sterile, double-distilled water.

Epidermal explants: Epidermal explants were excised from stem segments using a sterile scapel. The explants ranged from 0.1 to 0.4 cm in width and 0.5 to 1.0 cm in length; the explant

size being a function of the excision location. Two factors necessitated this variation in explant size. The small diameter of the floral branch axes limited the size of explants from this location to the smallest dimensions cited above. However, when explants from the lower excision regions (the middle and base of the tobacco stem) were cut to those dimensions, almost total necrosis resulted.

Leaf disk explants: Leaf disk explants were excised from pieces of tobacco leaf (approximately 6 cm x 6 cm) using a sterile, stainless steel disk-cutting tool designed and produced for this research. The explants were uniformly 12.7 mm (0.50 in) in diameter.

iii. Culture media

Epidermal explants: The medium used for culturing the epidermal explants was developed by Tran Thanh Van (1973). It contained macro- and micronutrients by Murashige and Skoog (1962), plus sucrose, myo-inositol, thiamine, kinetin, and indoleacetic acid (IAA) as listed in Table 2. Kinetin and IAA were each present at 10^{-6} molar (M) concentrations.

Leaf disk explants: The medium used for culturing leaf disk explants was the same as the medium for epidermal explants except for the concentrations of kinetin and IAA. The concentration of IAA was 10^{-5} M, unless otherwise specified, while the two most commonly used concentrations of kinetin were 10^{-4} and 10^{-5}

Table 2: Culture Media

Stock soln.	Ingredient(s)	Stock conc. (g/L)	Stock volume (mL)	Final Concentration (mg/L)	(mM)
A	NH_4NO_3	82.5	20	1,650	N 41.2
B	KNO_3	95.0	20	1,900	18.8
C	H_3BO_3	1.24	5	6.2	0.1
	KH_2PO_4	34.00		170.0	1.25
	KI	0.166		0.83	0.005
	Na_2MoO_4 $2H_2O$	0.050		0.25	10^{-3}
	$CoCl_2$ $6H_2O$	0.005		0.025	10^{-4}
D	$CaCl_2$ $2H_2O$	88.0	5	440.0	3.0
E	$MgSO_4$ $7H_2O$	74.0	5	370.0	1.5
	$MnSO_4$ H_2O	3.38		16.9	0.1
	$ZnSO_4$ $7H_2O$	1.72		8.6	0.03
	$CuSO_4$ $5H_2O$	0.005		0.025	10^{-4}
F	Na_2EDTA	3.73	10	37.3	Na 0.20
	$FeSO_4$ $7H_2O$	2.78		27.8	Fe 0.10
Thiamine-HCl		0.02	5	0.1	4×10^{-4} M
Epidermal Explants:					
	IAA 17.5 mg/L		10	0.175	10^{-6} M
	Kinetin 10.8 mg/L		20	0.216	10^{-6} M
Leaf Disk Explants:					
	IAA 175.2 mg/L		10	1.75	10^{-5} M
	Kinetin 2152 mg/L		10	21.52	10^{-4} M
			1	2.15	10^{-5} M
Addenda:	Myo-inositol		100 mg/L		
	Sucrose		30 g/L		
	Agar		10 g/L		

Make to volume; adjust to pH 5.6 to 5.8 (optimum 5.7) with HCl or NaOH; add agar; autoclave 15 min. at 120° C.

molar, as specified.

Media preparation: In large experiments involving hundreds of explants plated in several explanting sessions spaced over a period of one or two weeks, all the media were prepared at the same time using freshly prepared stock solutions. The media were made as a two-fold concentrate and included everything but the IAA, kinetin, and agar. The medium concentrate was then frozen in aliquots until ready for use. The day preceding an explanting session, an appropriate amount of media was thawed, the growth regulator stocks added, the media adjusted to the pH 5.7, and agar added. Thus, the media were always freshly prepared for each explanting session but there was still medium homogeneity within each experiment since the concentrates were always prepared in a single batch. This frozen concentrate method was tested against medium freshly prepared from stock solutions and the same results were obtained with each. For small experiments the medium was directly prepared from stock solutions.

All media were sterilized by autoclaving for 15 minutes at 120°C.

iv. Culture conditions

Both types of explants were plated horizontally on about 25 mL of medium in culture tubes, 25 mm in diameter and 150 mm in length, stoppered with plastic bungs (Canlab: diSPo plugs) and then covered with aluminum foil. The culture tubes were placed in specially designed wire racks (constructed by the author)

which provided maximum use of the growth chamber space and yet allowed free air circulation between tubes.

The explants were cultured at 27°C in growth chambers with continuous 24 hour illumination (unless otherwise specified) at 10800 lumens/m^2, as measured outside the culture tubes.

The explants were harvested at four weeks and either immediately analysed by dissection or fixed in 70% ethanol for later dissection analysis, unless otherwise specified.

2. Morphological Studies

For the morphological analysis, explants were fixed in FAA solution (900 mL 70% EtOh, 50 mL glacial acetic acid, 50 mL 37% formaldehyde solution), rinsed in 70% ethanol for six hours, and then stained overnight with alcoholic acid fuchsin (0.5% acid fuchsin in 95% EtOH). The explants, or buds dissected from the explants, were photographed while immersed in 100% ethanol, following the technique of Sattler (1968).

IV. OBSERVATIONS AND RESULTS

1. Epidermal Explants

i. Description of the experimental system

This experimental system involved the culture of thin epidermal explants excised from superficial cell layers of the stem of *Nicotiana tabacum* L. cv. Wisc. 38 plants, as described in Materials and Methods.

The aim of this research was to investigate factors regulating the expression of the *in vitro* floral gradient observed in *Nicotiana*. Specifically of interest to this research was the relationship between developmental stage of the tobacco plant and expression of the floral gradient. Consequently, the experiments for this research were focused on analysis of the floral gradient in three developmental stages of *Nicotiana tabacum*: green fruit, in which the apical flower was in the process of fructification, full bloom, in which the floral axis terminated in a flower in full bloom, and floral bud, in which the floral axis terminated in a closed flower bud.

The *in vitro* floral gradient in *Nicotiana tabacum* L. was expressed by an increased incidence of floral organogenesis on the explants with increase in proximity of the explant excision locus to the floral apex. Consequently, five excision regions were defined along the tobacco stem (as per Tran Thanh Van,

1973b). These excision regions were:

1) floral branch region, in which explants were excised from the base of floral branches.
2) floral stem region, in which explants were excised from tobacco stem occurring between floral branches.
3) sub-floral stem region, in which explants were excised from the tobacco stem just below the inflorescence. Specifically, this region was defined as the central section of the top third of the vegetative part of the flowering tobacco plant (mathematically determined by node number).
4) middle stem region, in which explants were excised from the central section of the middle third of the vegetative part of the flowering tobacco plant (mathematically determined).
5) basal stem region, in which explants were excised from the central section of the bottom third of the vegetative part of the plant (also mathematically determined).

However, due to the absence of an inflorescence in the earliest developmental stage of tobacco used in this research, only the last three excision regions were available in plants at the floral bud stage (i.e. floral bud plants only had sub-floral to basal stem regions).

This experimental approach was used throughout the epidermal explant research. Since these three developmental stages and five excision regions are frequently referred to in abbreviated form in tables throughout the Results section, a table is given below, summarizing their abbreviations.

Developmental Stage	Abbreviation	Explant Region	Abbreviation
green fruit	GF	floral branch	FB
full bloom	Fl	floral stem	F
floral bud	Bd	sub-floral stem	SF
		middle stem	M
		basal stem	B

Table 3: Summary of Common Abbreviations Used in the Epidermal Explant Results

ii. System analysis: technical appraisal

One of the main concerns in the research using epidermal explants was the production of sufficient experimental replicates for precise quantitative analysis. Early experiments, which were largely aimed at reproducing Tran Thanh Van's precise quantification of the gradient of floral response in green-fruit tobacco plants (1973b), produced low yields (5 to 29%) of explants worthy of analysis due to lack of *in vitro* growth and/or high infection and necrosis rates (up to 100% per set of experimental replicates). Infection, necrosis, and growth response rates for all the epidermal explant experiments are tabulated in detail in Appendix 1 and are summarized in Table 4.

In order to reduce the infection rate, a 60 second treatment with 70% ethanol was added to the sterilizing procedure. The alcohol treatment reduced the contamination rate

Experiment*	Explant Type(s)	Number Plated	% excluding infected explants		
			Infection	Necrosis	No Growth
2: Rack Test	GF:SF	98	21	27	23
3: Gradient	all	918	79	42	
5: Gradient	GF:all	89	37	0	23
7: Gradient	all	900	10	4	15
8: Callus	GF:FB,F	40	0-10	0	0
9: Explant Orientation	GF: FB,F,SF	54	2	6	36
10: Gradient	all	594	14	0	10
12: IAA	GF: FB,F,SF	54	4	2	23
13: Polarity	all	234	1	3	18
14: Gradient	all	468	3	29	24
15a: Gradient	all	468	4	9	9
15b: Alcohol	GF:all	90	1	9	82
	GF:all	90	13	21	77
	Bd:all	54	4	8	25
	Bd:all	54	9	4	31

*Experiments not cited in this table dealt with the growth mode and senescence development of tobacco plants.

Table 4: Summary of Yield Analyses of Epidermal Explant Experiments

% excluding infected explants		Yield % of plated explants	Comments**
Insufficient Development	Organogenesis		
18	31	24	o-p
58		12	s-p
30	46	29	o-p
75	6	5	o-p
callus growth: 100		90-100	o-p/alc
6	53	52	o-p/alc/ep.up
25	66	56	o-p/alc/ep.up
13	62	59	o-p/alc/ep.up
79		78	o-p/alc/ep.up
23	24	24	o-p/alc/ep.up/thin
22	20	19	o-p/alc/ep.up
9	0	0	o-p/ep.up/thin/alc
3	0	0	o-p/ep.up/thin
33	35	33	o-p/ep.up/thin/alc
45	20	19	o-p/ep.up/thin

**Key to abbreviations. o-p: explants excised from open-pollinated tobacco plants; s-p: explants from self-pollinated plants; ep.up: explants plated epidermis up rather than down on the agar; alc: use of alcohol during the sterilizing procedure; thin: explants cut thinner than usual

by 86% without adversely affecting the organogenetic response of the explants. In fact, the alcohol treatment produced a 70% increase in the organogenetic response of the epidermal explants. In general, the use of alcohol resulted in an overall gain of 56% in the experimental yield. These results are summarized in Table 5.

Other concerns in attempting to reproduce Tran Thanh Van's work were explant orientation and explant size and thickness, neither of which were clearly specified in her publications. In the initial experiments of this research the floral response of the explants was much lower than that reported by Tran Thanh Van and the overall organogenetic response less uniform (both issues are reported in detail later under "Gradient Analysis"). It was conjectured that explant orientation and/or thickness may have been contributing factors in the observed differences in explant response.

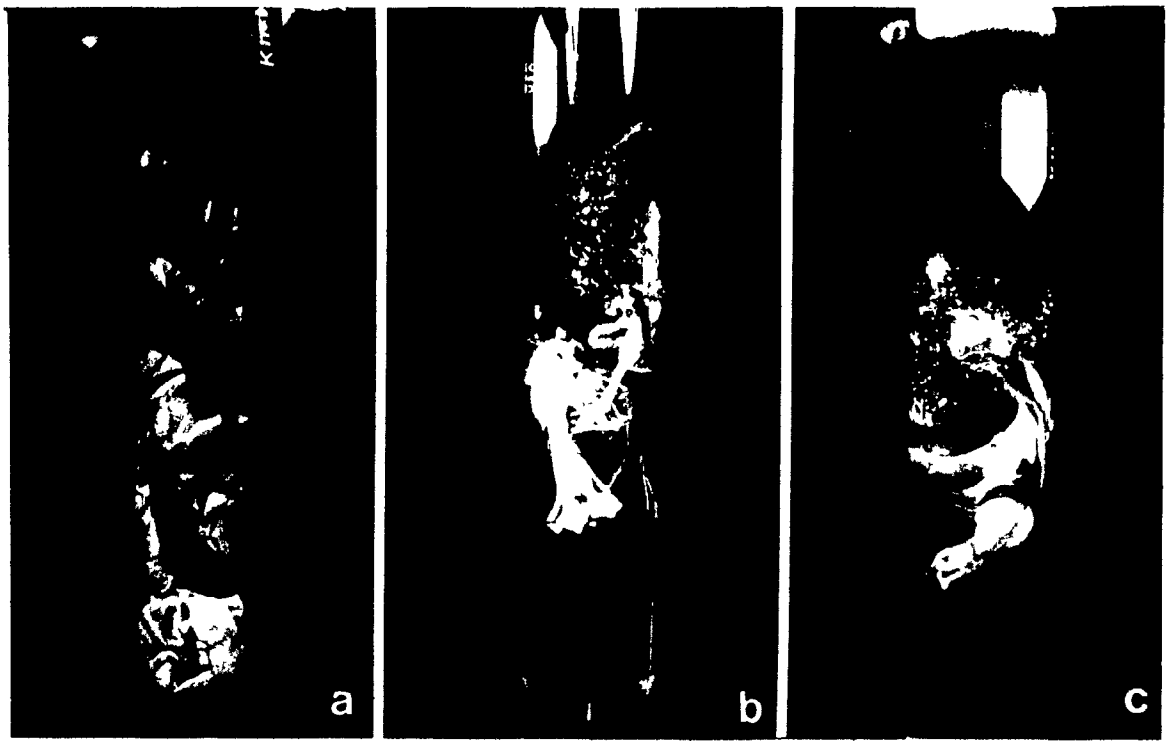
Explant orientation was an issue since it was not known whether to plate the explant epidermal or cortical side in contact with the agar medium; that is, epidermis down or up. (Personal communications sent to Dr. Tran Thanh Van were never answered.) Initially, on the basis of preliminary tests, it was decided to plate the explants epidermal side down. This also seemed to correlate with the reports by Tran Thanh Van that the organogenesis originated in the sub-epidermal layer (1973, 1974). This orientation resulted in many of the organogenetic structures growing down into the agar medium (Figure 1). Such growth added

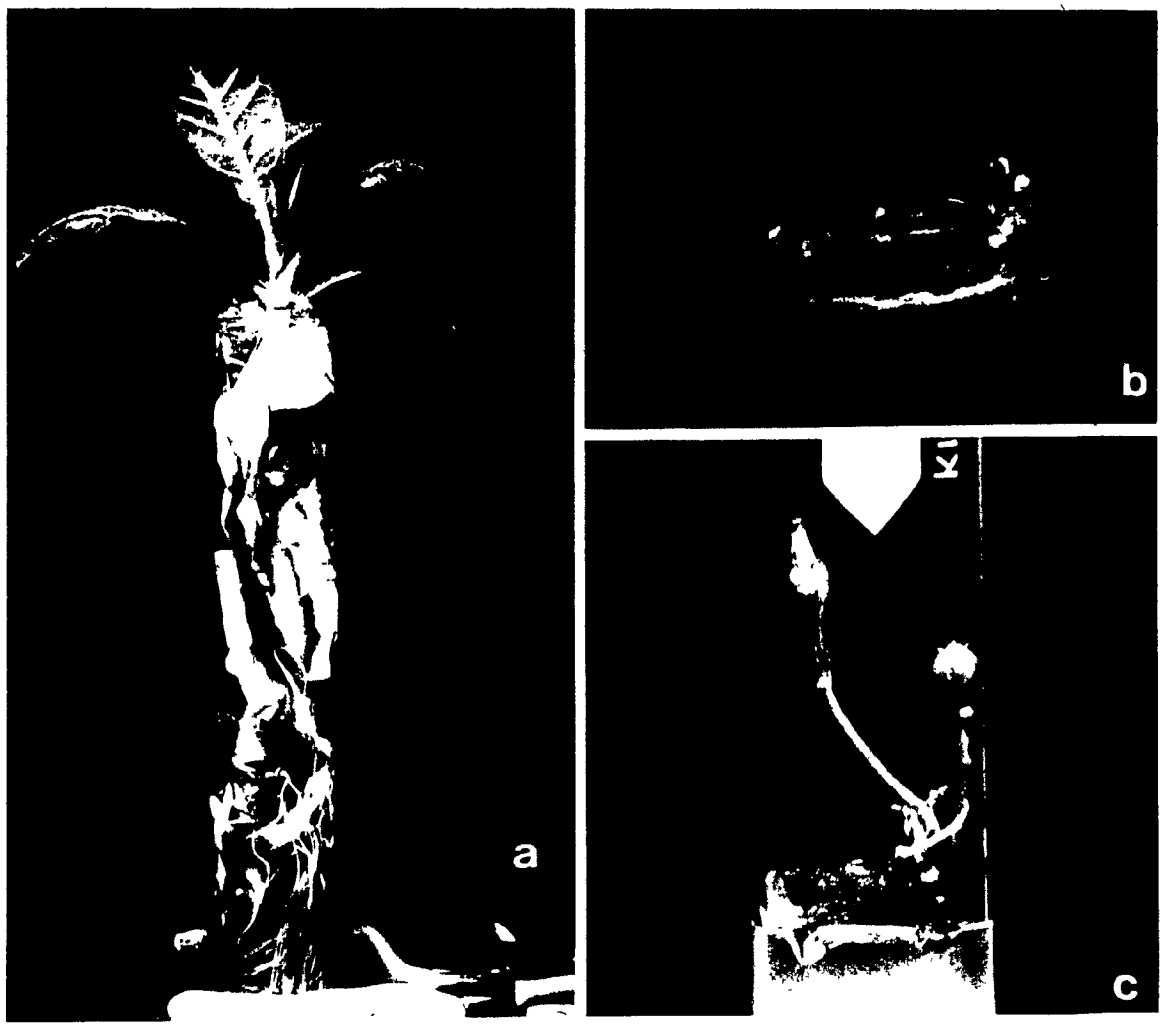
Analysis factor	% Response		Experiments used in analysis
	without alcohol	with alcohol	
average % infection	28	4	all
average % organogenetic explants (excluding infected explants)	26	43	5,7 vs 10,15
average % yield	17	38	"
stimulation of organogenetic response due to alcohol		1.7 1.7	" Exp. 15b

Table 5: Effect of Alcohol Sterilization Treatment on the Infection and Organogenetic Response Rates of Cultured Epidermal Explants. Only comparable experiments were used in the above analyses. Numbers are from Table 4.

Figure 1: Examples of agar oriented organogenesis on epidermal explants plated epidermal side down. a) a F1-SF explant with vigorous vegetative shoot organogenesis (0.75x). b) a GF-F explant with a flower in full bloom in the agar (0.75x). c) a GF-FB explant with flower buds growing in the agar (0.75x).

Figure 2: Examples of the extent of variation in organogenetic response occurring even among replicate epidermal explant cultures. a) a GF-FB explant showing vigorous shoot and root growth (0.75x). b) a GF-FB explant with small buds (2.2x). c) a GF-FB explant with flower buds and callus (1.2x).


a
b
c


a
b
c

another variable to the culture system with possible influence on the in vitro regulation of organogenesis, and, furthermore, was not a phenomenon reported by Tran Thanh Van. Plating epidermis up rather than down did eliminate the problem of agar oriented organogenesis, but otherwise did not quantitatively or qualitatively affect the organogenetic response (Table 4; Appendix 1; and "Gradient Analysis", p. 56). The organogenetic response rate in explants plated epidermis down was 35%, as compared to 56% for those plated epidermis up. The difference was attributed to the effect of alcohol treatment, as reported above.

Explant size was another variable in the system which was difficult to control. The explants were manually excised from stem segments, which introduced some variation in explant thickness (number of cell layers). Also, the five stem regions from which explants were taken differed greatly in diameter and average cell size. For example, the diameter of floral branches of green-fruit tobacco plants averaged less than 5 mm whereas the diameter of the basal portion of the tobacco stem averaged about 2 cm. Achieving explant size uniformity between such dimensionally different plant parts was extremely difficult. Furthermore, basal and middle stem region explants cut to the size of floral branch explants consistently led to complete necrosis of the explants in culture (unreported preliminary investigations). By necessity then, the explants used in this research system showed substantial dimensional differences (see

Materials and Methods). The presence of vascular tissue in some of the explants, unreported by Tran Thanh Van, was thought to be associated with this dimensional variation. In an attempt to decrease the incidence of vascular tissue 'contamination' in the explants, the gradient analysis experiment (Experiment 14, Table 4) was repeated using thinner explants. (This was simply a qualitative attempt to excise thinner, more uniform explants.) The use of thinner explants increased the necrosis rate from 0 to 29% and the 'no growth' (explants remaining green in culture, but showing no in vitro growth) rate from 10 to 24%, thereby decreasing the organogenetic response rate from 66 to 24% (Experiments 10 and 14, Table 4).

The lack of success in producing organogenetic, dimensionally uniform explants from the five excision loci along the tobacco stem led to a final attempt to repeat the gradient analysis experiment using standard excision practices previously employed, simply in order to reproduce and verify previous findings (Table 4: Experiment 15a to repeat Experiment 10). Concomitantly, a controlled experiment was performed using 'thinner' explants with and without the alcohol treatment during sterilization. Since Tran Thanh Van did not employ the alcohol step in sterilizing plant material, it was hypothesized that the alcohol had adversely affected the thinner explants. The results from the alcohol experiment (Experiment 15b, Table 4) revealed that the alcohol treatment did not increase necrosis in the thinner explants, the average necrosis rate being 8% with the

alcohol treatment and 12% without it. Furthermore, the previously noted stimulatory effect of alcohol was evident in this experiment as well. The organogenetic response was 35% with the alcohol as compared to 20% without it. The magnitude of the stimulatory effect was the same as observed in earlier experimentation (about 70%). However, the attempted repetition of the gradient analysis experiment (Experiment 15a) was not successful due to the absence of *in vitro* growth in half of the cultured explants.

iii. System analysis: organogenetic response

An overall analysis of the experiments also revealed certain trends in the *in vitro* organogenetic capacities of epidermal explants. These are summarized in Table 6.

Table 6: Average Organogenetic Response Rates for Epidermal Explants. Numbers are from Appendix 1, Experiments 10, 13, 14, and 15, and represent per cent of explants showing organogenesis.

Developmental Stage	Explant Region					Average for SF, M, B
	FB	F	SF	M	B	
Green-fruit	67	53	50	35	34	40
Full Bloom	72	61	56	33	27	39
Floral Bud	-	-	61	24	36	40
Average	70		56		32	40

The three developmental stages showed similar organogenetic response rates, about 40%. Accordingly, the data do not give evidence for any correlation between inflorescence development of the donor plant and organogenetic capacity.

However, there was a gradient in organogenetic response rates correlating with excision loci in all three developmental stages. The response rate increased from base to apex, being about 32% for middle and basal region explants, 56% for floral and sub-floral region explants, and 70% for floral branch explants. Thus, the data do provide evidence for a correlation between organogenetic capacity and proximity to the plant apex.

iv. Gradient analysis

The gradient of *in vitro* floral expression in *Nicotiana tabacum* L. was clearly evident in all the epidermal explant experiments. Less clear, however, were the means by which to exactly define and quantify this gradient. Therefore, the issues pertaining to this problem, organogenetic definition, numerical analysis, and origin analysis, will be separately reported below, followed by a summary of the gradient analysis results obtained from this research.

a) Organogenetic description and definition

The gradient of floral expression in *Nicotiana* was defined by Tran Thanh Van (1973b) as an increase, on cultured epidermal

explants, in the proportion of floral to vegetative shoots from base to apex of the tobacco plant. Obviously, this necessitated classifying all de novo shoots or buds as either vegetative or floral, but to do this certain issues had to be resolved.

It was found in the work reported here that there was great variation in the degree and quality of organogenetic growth found on the epidermal explants. Even within explant types, organogenesis ranged from a few microscopic buds to vigorous shoot growth exceeding the capacity of the culture tubes (Figure 2). Such variation occurred even among experimental replicates from the same excision locus and donor plant. Besides variation in the number, size, and development of the floral and vegetative de novo growth, there was considerable variation in the amount of callus tissue present, ranging from complete absence to it being the prominent de novo tissue (Figure 3). Similar variation occurred in the amount of root growth present per explant (Figure 4). However, the root growth often represented secondary organogenesis, that is, arising adventitiously from well developed vegetative shoots. When appearing as primary organogenesis, the roots arose randomly on the explant, mostly from callus tissue or non-differentiated outgrowths (further described under "Origin Analysis").

There was great variation even within the floral organogenesis found on the epidermal explants (Figure 5). In most cases, the floral organogenesis consisted simply of microscopic floral apices, discernable only through microdissection. Very

Figure 3: Examples of the variation in callus growth observed on epidermal explants after 4 weeks of culture. a) a F1-F explant with a small amount of callus and floral buds (1.5x). b) a F1-B explant from which the prominent de growth was callus tissue (1.5x).

Figure 4: Examples of the variation in de novo root growth on epidermal explant cultures. a) a GF-F explant with a few roots, as well as some vegetative and floral shoots (1.2x). b) a GF-F explant with much root growth, as well as much vegetative shoot growth (1.2x).

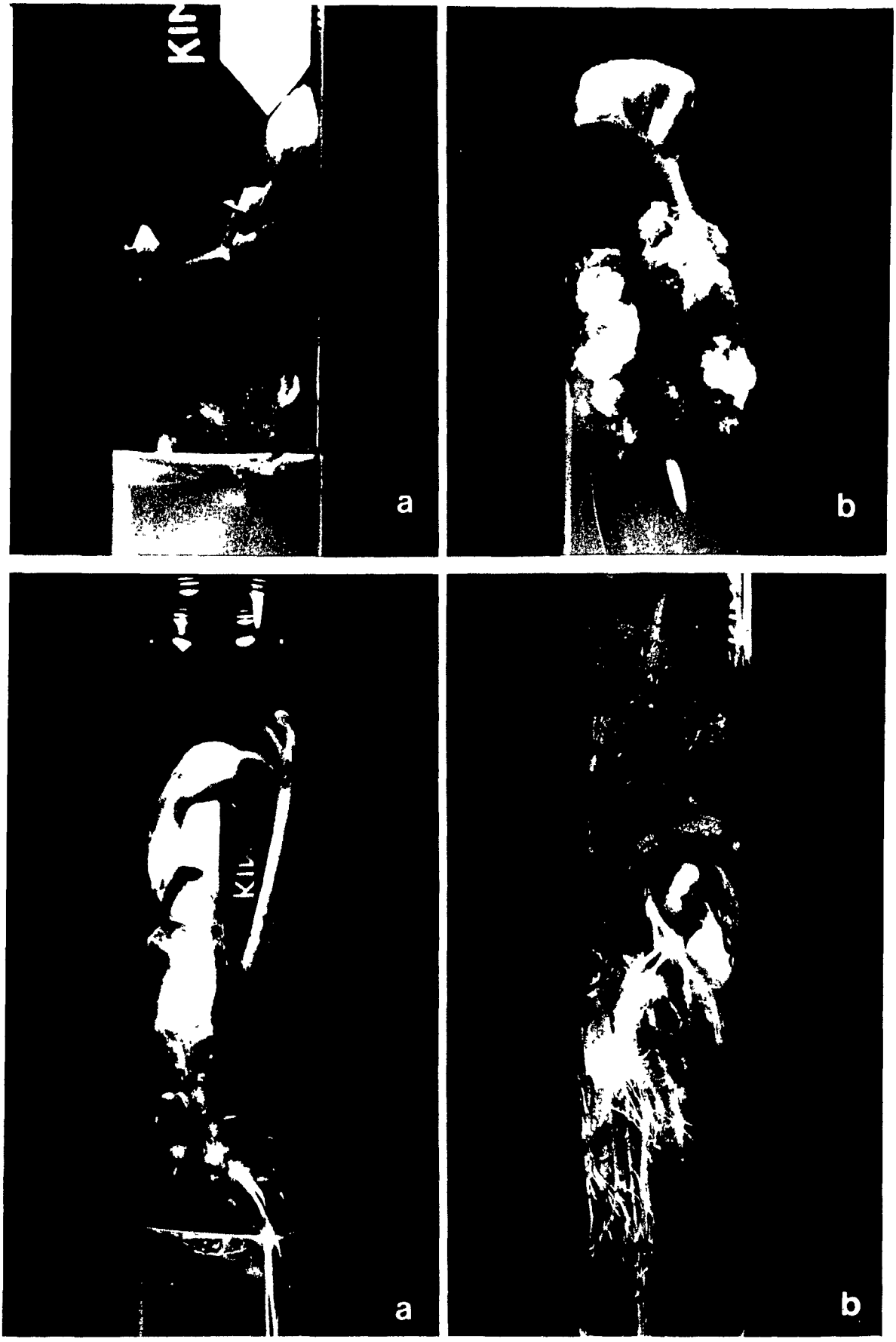
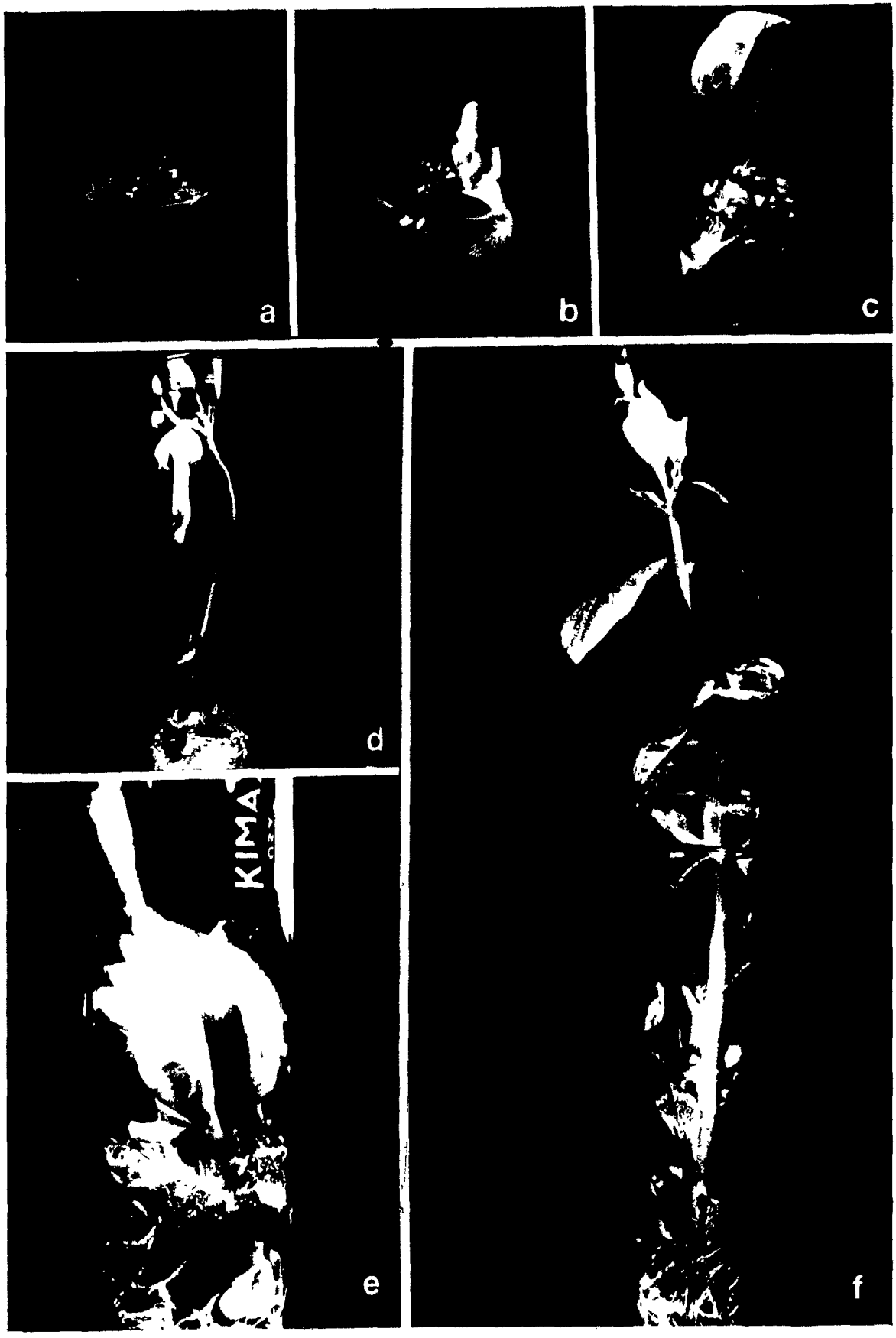

a
b
a
b

Figure 5: Examples of the variation in floral organogenetic response observed even among replicate epidermal explant cultures. Figures 5a to 5f all represent GF-FB explants after 4 weeks of culture. a) an explant with microscopic floral buds (1.0x). b), c) explants with microscopic floral buds and increasing amounts of callus and vegetative shoot growth (1.0x). d) an explant with a well developed inflorescence (0.75x). e) an explant with two full sized flowers and vigorous vegetative growth (1.5x). f) an explant with a floral plantlet (0.9x).


a
b
c
d
e
f

early floral apices were difficult to distinguish from early vegetative apices; in ambiguous cases such buds were discounted. Many of the flower buds, from microscopic to full size, were aborted and necrosed during the course of floral development. As an example, the following is a description of a floral shoot on a green-fruit, sub-floral explant from an early gradient analysis experiment (Experiment 5, as cited in Appendix 1):

The floral shoot had three nodes of vegetative growth, then an aborted flower in which the androecium and gynoecium were incompletely formed, the calyx fully developed, but the petals aborted before cellular enlargement. Following the aborted flower, there were about 13 to 15 further nodes of vegetative growth, then a second floral apex in which the main (apical) bud showed calyx, androecium, and gynoecium in early stages.

In other cases, the floral buds showed abnormal development, often associated with callus growth. The following is an example from the same experiment, but which occurred on a floral branch explant:

The largest floral bud occurred directly on the explant, but was growing into the agar medium and showed abnormal development. The calyx was fully developed but with some callusing. At least two gynoecia were present. The androecium and corolla were very callus-like in quality and caused difficulties in discerning detail.

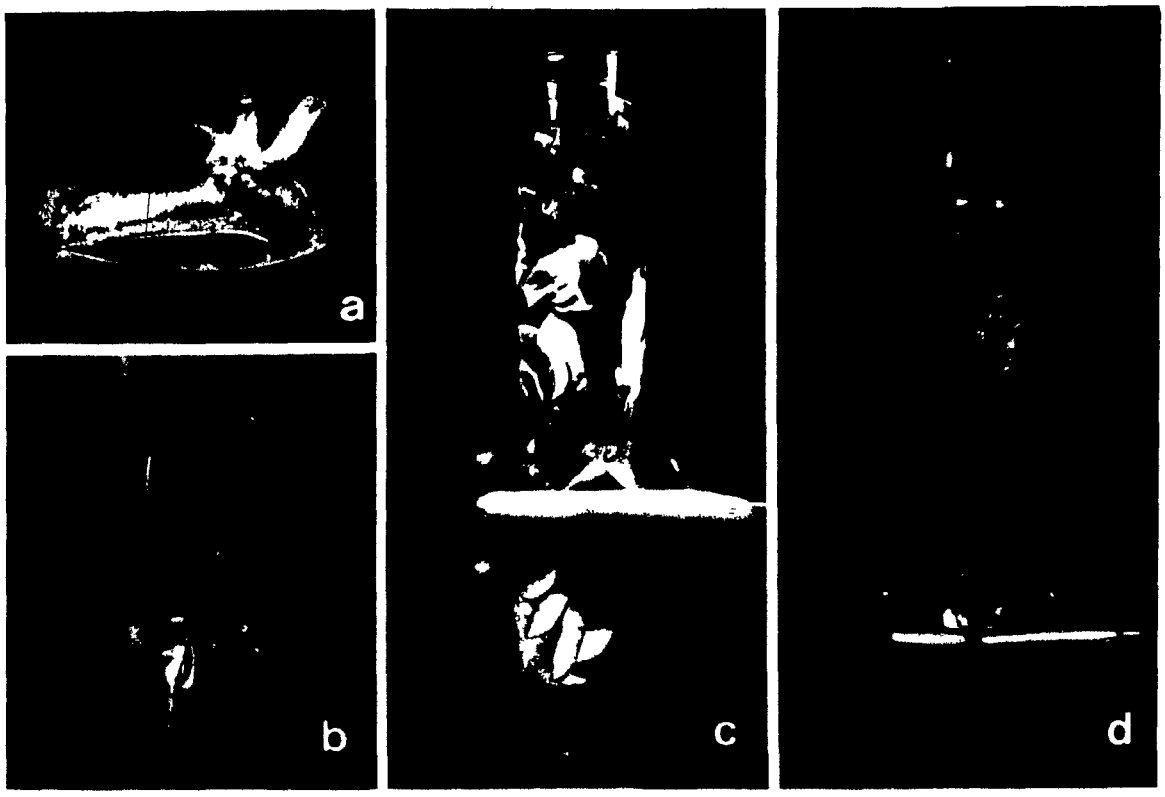
While a normal flower clearly represents a different organogenetic process from an aborted or abnormal flower, both

represent incidences of floral induction. Therefore, all incidences of floral organogenesis, whether normal, abnormal, or aborted, were defined as floral shoots for the purposes of this research. This definition represents, then, all circumstances (intrinsic and external) favorable for floral induction, but not necessarily favorable for floral development. For the purpose of gradient analysis, in which the aim is to quantify the potential of an explant for floral expression, such a definition was deemed adequate and correlations relating to abnormal floral development were not made.

All the explant types produced some vegetative organogenesis. As for the floral organogenesis, there was great variation in the size and development of the vegetative shoots, ranging from microscopic buds to shoots up to and exceeding 70 mm and 30 internodes (Figure 6). The gradient of in vitro floral expression was not only evidenced by an increasing proportion of floral to vegetative shoots from base to apex, but also, according to Tran Thanh Van (1973b), by a decreasing number of internodes per floral shoot from base to apex (i.e. a decreasing amount of vegetative growth per floral shoot). Therefore, an experiment was performed to determine whether there was also a qualitative difference in the vegetative shoots associated with the gradient. In this experiment, cultures from an early gradient analysis experiment using green-fruit stage explants were transplanted to a soil-vermiculite mixture, but were otherwise kept under conditions identical to those for tissue

Figure 6: Examples of the variation in vegetative organogenesis on the epidermal explant cultures. a) a Bd-B explant with vegetative buds (1.7x). b) a Bd-B explant with small vegetative shoots (0.85x). c) a Bd-M explant with vigorous vegetative shoot production (0.75x). d) a Bd-SF explant with a vegetative plantlet (0.65x).

Figure 7: Examples of the types of abnormal organogenetic structures observed on the epidermal explant cultures. a) a GF-M explant with an albino leaf (0.9x). b) a F1-B explant with a peltate leaf (1.3x). c) a F1-F explant with a fasciated production of floral buds (1.6x).


a
b
c
d


a
b
c

culture (27°C, 24 hours light). Only cultures showing well developed vegetative shoots with adventitious roots were transplanted. The results, which are tabulated in Table 7, indicate that there were no apparent qualitative differences between vegetative shoots of the five explant regions with respect to floral potential; that is, there was no gradient effect evidenced by the vegetative shoots. The shoots from all explant regions produced about 37 total nodes of growth, with 1 to 3 floral nodes at the time of analysis. The transplanted shoots developed inflorescences to the green-fruit stage in approximately the same time as tobacco seeds (i.e. the time from explanting onto agar medium to green-fruit development of the transplanted shoot was the same, 2.5 to 3 months, as from seed to green-fruit). The transplant shoots, upon maturity, were proportionally smaller and with less developed inflorescences than seed-grown plants, but otherwise were morphologically very similar to seed-grown plants.

Similar to the definition of floral shoot, all structures with vegetative apices were defined as vegetative shoots, and variations in size and development were not distinguished, with the following exception: all vegetative shoots with fewer internodes than approximately the average observed for floral shoots on that explant type were discounted. That is, only vegetative shoots in which internode development exceeded that expected for floral shoots on that explant were defined as vegetative shoots. This was done in order to avoid classifying

Table 7: Total and Floral Nodes in Transplanted Vegetative Shoots Produced *In Vitro*. Shoots from explants excised from green-fruit stage *Nicotiana* plants.

Explant Type	No. of shoot transplants	Total Nodes Average	Total Nodes Range	Floral Nodes Average	Floral Nodes Range
FB	3	37	35-42	1.7	1-3
F	3	36	33-38	1.7	1-2
SF	4	35	29-41	1.0	1
M	7	40	36-44	2.4	0-3
B	2	36	33-38	2.0	2

as vegetative shoots those which were actually determined to become floral shoots.

Besides variation in size and degree of development, there were also some abnormal structures in the vegetative organogenesis on the epidermal explants. These abnormalities were relatively infrequent, occurring in less than 5% of the cultures, and were apparently not related to the floral gradient in Nicotiana. The abnormal structures included an albino leaf, which occurred only once among the 1100 organogenetic explants analyzed, and more frequently, peltate leaves and fasciation of the explant (Figure 7). Unless a structure could be confidently identified as a vegetative shoot, such abnormal structures were not included in the gradient analysis.

b) Numerical analysis: alternatives in quantification

There were several possible methods by which to quantify the gradient of in vitro floral expression in Nicotiana tabacum L. In two gradient analysis experiments, three methods for quantifying the data were evaluated in order to select the most appropriate for subsequent analysis. These methods were as follows:

1) the percentage of floral shoots per explant, averaged per set of experimental replicates: This method involved careful microdissection of each explant in order to count all vegetative and floral shoots and assess the number of nodes

per floral shoot. The percentage of floral shoots was calculated for each explant and then averaged per set of experimental replicates.

2) the percentage of floral shoots per set of experimental replicates: This method involved the same microdissection analysis for each explant as described above, but the total number of floral and vegetative shoots were calculated per set of experimental replicates, and the percentages then calculated from the total figures.

3) the percentage of floral explants: This method was the simplest of the three since less precise microdissection was required. The number of explants showing any floral organogenesis was ascertained, and the percentage calculated with respect to the total number of analysed explants. While this method was both the most accurate and precise (since there was less chance of microdissection error) this method of quantification did not distinguish between explants with one or a hundred floral shoots.

As detailed in Table 8, Method 3 proved to be the best indicator of the floral gradient. This was quite fortunate since Method 3, besides being the most precise and accurate quantitative approach, was also much less time consuming than Methods 1 and 2. The first two methods, which were based on the actual proportion of floral and vegetative shoots, gave results similar to each other. Both showed about a 10% difference in floral response between floral-branch and sub-floral stem

explants, whereas Method 3, based on the proportion of explants showing floral organogenesis, showed a 30% difference between the same two explant regions. Therefore, both for technical and data analysis reasons, Method 3 was chosen for subsequent analysis.

Another quantitative approach to analysis of the floral gradient, which was used to augment the above types of analysis, was the determination of the average number of nodes per floral shoot for the various explant types. In early experiments, this approach did not yield any clear evidence concerning the floral gradient (Table 8). There was no overall decrease in the number of nodes per floral shoot in explant regions from base to apex of the tobacco plant. In fact, the explant region with the least nodes per floral shoot was the floral stem region, not the floral-branch region as expected. Furthermore, there was great variation in the number of nodes per floral shoot even within single explants. This variation exceeded the observed differences in average node number between explant regions. In other words, on the basis of these first gradient analysis experiments, there was no significant correlation between node number per floral shoot and the *in vitro* expression of a floral gradient in *Nicotiana tabacum* L. However, certain correlations between node number and floral gradient expression were revealed in subsequent experimentation which included analysis of all organogenetic origins involved in the epidermal explant system. These correlations are reported in the next section on origin analysis.

Experiment	Explant Type	Number of explants analysed	% Floral Shoots		% Floral Explants	Nodes/floral shoot	
			Avg./explant	% of total		Average	Range
7	GF-FB	18	45	57	89	3.0	1 - 8
	GF-F	4	28	36	75	2.7	1 - 4
	GF-SF	5	19	33	60	6.3	3 - 8
	GF-M	7	0	0	0	-	-
	GF-B	8	33	42	25	3.3	1 - 6
9	GF-FB	11	37	26	70	4.2	1 - 9
	GF-F	15	44	34	67	2.9	1 - 6
	GF-SF	5	40	37	40	6.0	3 - 9
Average	GF-FB		41	42	80	3.6	
	GF-F		36	35	71	2.8	
	GF-SF		30	35	50	6.2	

Table 8: Comparison of Methods of Quantification of the Gradient of In Vitro Floral Expression in Nicotiana tabacum L.

c) Origin analysis

Early experimentation had revealed that organogenesis originated in several ways on the cultured epidermal explants. Tran Thanh Van had reported only one organogenetic origin, namely the sub-epidermal cell layer of the explants (1973, 1974). Careful analysis of later experiments in the research for this thesis revealed six organogenetic origins which could be separately classified. These organogenetic origins were:

1) the epidermal surface: organogenesis appeared to originate directly at the epidermal surface of the explant.

2) the cortical surface: organogenesis appeared to originate directly at the cortical surface of the explant.

3) polar swelling: organogenesis originated from a basal swelling of the explant tissue.

4) polar outgrowth: organogenesis originated from de novo, nodule-like, basal outgrowths.

5) polar callus: organogenesis originated from de novo masses of non-differentiated cells on the basal end of the explant.

6) non-polar callus: organogenesis originated from de novo, non-polar masses of non-differentiated cells on the explant.

The two most common organogenetic origins in the epidermal explant system were the epidermal surface and polar callus, respectively rated as first and second in frequencies of occurrence(Table 9). 65% of all explants showed some epidermally

Type of Numerical Analysis	Number of Observations	Organogenetic Origins					
		Epidermal Surface	Cortical Surface	Polar Swelling	Polar Outgrowth	Polar Callus	Non-polar Callus
% explants	301	65	23	3	10	54	3
% organogenetic structures	476	41	14	2	7	34	2
Numerical Rating		1	3	5	4	2	5

Table 9: Overall Frequencies of Organogenetic Origins.

Numbers represent the percentages of all explants showing each organogenetic origin and percentages of all organogenetic structures from each origin, as well as a numerical rating of each origin type based on frequency of occurrence.

occurring organogenesis, and 54% showed organogenesis from polar callus. These two origins together accounted for 75% of all observed organogenesis. The third most frequent organogenetic origin was the cortical surface of the explant, followed, in order of organogenetic importance, by polar outgrowths, swellings, and non-polar callus.

The data for organogenetic origins were related both to explant type (Table 10) and to the type of organogenesis (Table 11). There was no apparent correlation between either the developmental stage of the donor plant from which the explants were excised or the explant excision region and the frequencies of the various organogenetic origins. For each explant type, the three most common organogenetic origins were the epidermal surface, polar callus, and the cortical surface, usually in that order. The apparent indication of a relationship between explant excision region and the frequency of the organogenetic origins in Table 10 was simply a consequence of the higher organogenetic capacities of floral branch, floral stem, and sub-floral stem explants, as previously reported (System Analysis: Organogenetic Response).

There were correlations, however, between the type of organogenesis and the type of organogenetic origin, as seen in Table 11. Roots arose almost uniquely from callus origin, with more than 93% of all root organogenesis originating in callus. Floral shoots arose mainly from epidermal and polar callus origins, these two origins accounting for 84% of all floral

Table 10: Frequencies of Organogenetic Origins in Relation to Explant Type. Numbers represent percentages of explants showing each organogenetic origin.

Explant Type	Organogenetic Origin					
	Epidermal Surface	Cortical Surface	Polar Swelling	Polar Outgrowth	Polar Callus	Non-polar Callus
GF-FB	92	12	0	0	100	0
GF-F	79	7	0	24	72	7
GF-SF	77	27	0	4	43	5
GF-M	21	53	15	21	32	0
GF-B	8	33	0	0	42	33
Fl-FB	70	9	0	0	57	4
Fl-F	64	20	0	36	40	0
Fl-SF	67	21	0	12	54	0
Fl-M	44	44	0	11	11	0
Fl-B	38	25	50	0	0	0
Bd-SF	100	22	0	0	91	0
Bd-M	38	15	0	15	62	0
Bd-B	80	5	0	0	50	0
GF avg.	55	26	3	10	58	9
Fl avg.	57	24	10	12	32	1
Bd avg.	73	14	0	5	68	0
FB avg.	81	10	0	0	78	2
F avg.	71	14	0	30	56	4
SF avg.	81	23	0	5	63	2
M avg.	34	37	5	16	35	0
B avg.	42	21	17	0	31	11

Type of Organogenesis	Organogenetic Origin					
	Epidermal Surface	Cortical Surface	Polar Swelling	Polar Outgrowth	Polar Callus	Non-polar Callus
Floral	55	7	2	6	29	.1
Vegetative	32	22	2	5	35	3
Root	0	2	3	3	90	3

Table 11: Relationship Between Types of Organogenesis and Origins. Numbers represent the percentage of each type of organogenesis arising from each organogenetic origin.

organogenesis. There were three significant origins for vegetative shoots, the epidermal and cortical surfaces and polar callus, which together accounted for 89% of all vegetative organogenesis.

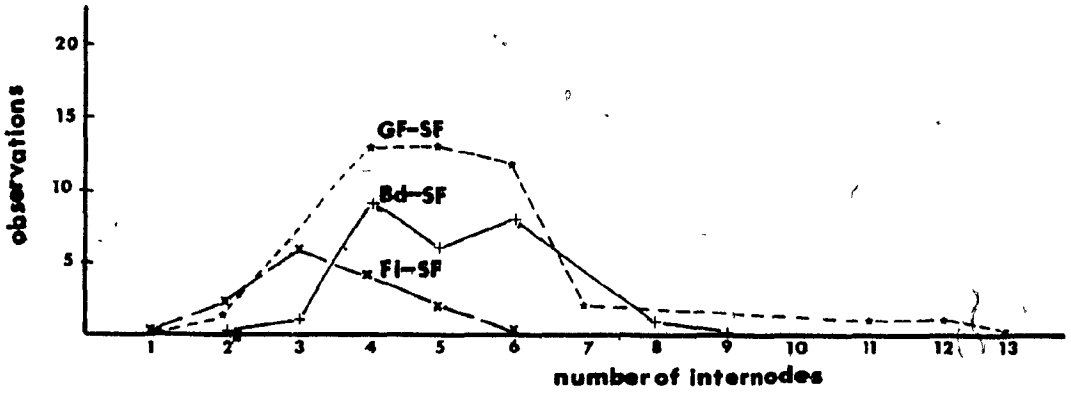
A more detailed analysis of organogenesis in relation to explant type (developmental stage and excision region) and organogenetic origin is given in Table 12. When the organogenetic response data were subdivided by origin, certain organogenetic trends became apparent.

In organogenesis originating at the epidermal surface, the gradient of floral potential was clearly evident. The floral response ranged from 92% (floral branch explants) to 0% (basal explants) for green fruit stage explants; 70% to 12% for full bloom stage explants, and 83% to 15% for floral bud stage explants. The floral gradient was less apparent for the polar callus organogenetic origin, and absent for the cortical surface origin. A similar trend between origin types was evidenced by the node analysis. In epidermal surface organogenesis, it was apparent that the average number of nodes per floral shoot increased from the apical to basal explant regions for both green fruit and floral bloom stage explants (though this was not clear for the floral bud stage explants). This gradient in nodes per floral shoot was absent in the polar callus and cortical surface organogenesis.

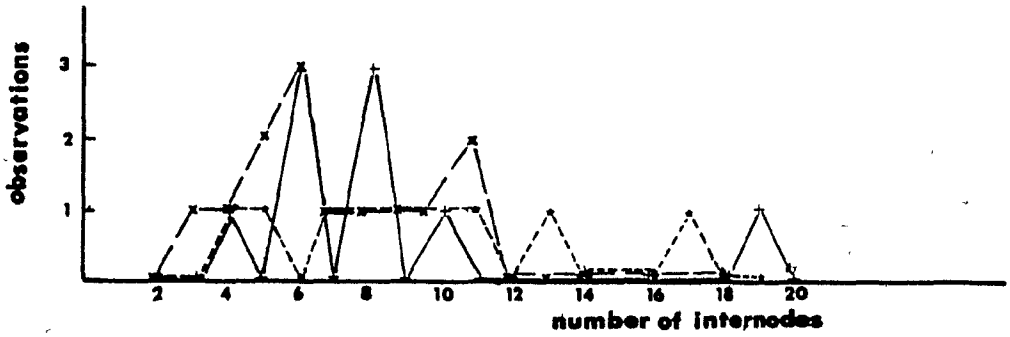
The relationship between nodes per floral shoot and organogenetic origin is further emphasized by Figure 8. Figure 8 (top)

Explant Type	Organogenetic Origins: Epidermal Surface				Cortical Surface				Polar Callus				Overall		
	Floral Shoots	Nodes/ Floral Shoot	Veg. Shoots	Roots	Floral Shoots	Nodes/ Floral Shoot	Veg. Shoots	Roots	Floral Shoots	Nodes/ Floral Shoot	Veg. Shoots	Roots	Floral Shoots	Veg. Shoots	Roots
GF-FB	92	2.2	28	0	12	?	4	0	96	~7	32	68	94	41	62
GF-F	79	2.6	41	0	0	-	3	0	72	6.7	38	55	100	79	62
GF-SF	65	5.3	30	0	5	12.8	27	0	18	9.3	36	33	81	74	36
GF-M	3	6.0	21	0	9	~15	53	0	12	7.0	32	12	38	100	15
GF-B	0	∞	8	0	0	-	33	0	8	~12	33	0	8	92	0
avg: SF,M,B	23	5.6	20	0	5	14	38	0	13	9.4	34	15	42	89	17
GF-all	48	4.0	26	0	5	14	24	0	41	8.4	34	34	64	77	35
Fl-FB	70	2.7	0	0	9	6.0	0	0	48	5.2	17	30	96	29	43
Fl-F	64	2.6	0	0	12	5.3	16	0	28	6.0	28	40	96	59	56
Fl-SF	46	3.3	38	0	4	~7	21	0	29	6.8	25	40	79	62	41
Fl-M	22	~7	22	0	11	~8	44	20	11	~7	0	0	38	81	25
Fl-B	12	5.0	25	0	0	-	25	0	0	-	0	0	25	100	25
avg: SF,M,B	27	5.1	28	0	5	7.5	30	7	13	6.9	8	13	47	81	30
Fl-all	43	4.1	17	0	7	6.6	21	0	23	6.2	14	22	67	66	38
Bd-SF	83	5.2	65	0	9	9.0	17	0	43	8.3	70	78	91	91	78
Bd-M	23	10.3	15	0	8	?	15	0	46	11.7	15	8	77	46	8
Bd-B	15	8.2	75	0	5	10+	5	0	15	8+	40	15	33	95	14
avg.	40	7.9	52	0	7	9.5	12	0	35	9.3	42	34	67	77	33

Table 12: Frequencies of Organogenesis: overall and sub-divided with respect to origin. Numbers refer to percentage of total explants analysed.



Floral Shoots of Epidermal Origin



Floral Shoots of Polar Callus Origin

Figure 8: Frequency distribution of internodes per floral shoot. The data represent observed internodes per floral shoot for all floral shoots of epidermal and polar callus origins on sub-floral stem explants from green fruit, floral bloom, and floral bud stage tobacco plants (from experiment 10, as cited in Appendix 1).

GF-SF------• Fl-SF×——× Bd-SF+——+

graphically demonstrates the existence of a physiological relationship between node number per floral shoot and organogenesis of epidermal origin, as seen by the roughly bell-shaped distribution of nodes (or internodes) for each explant type. This relationship was not evident in floral shoots of polar callus origin, which showed a scatter in their distribution of nodes per floral shoot (Figure 8, bottom).

Thus, this approach through origin analysis indicated that the gradient of *in vitro* floral response in *Nicotiana tabacum* was most clearly expressed in organogenesis arising at the epidermal surface, and that this gradient was weaker or absent in organogenesis from other origins on the explant.

The analysis shown in Table 12 did not reveal any particular trend for vegetative organogenesis, but for root organogenesis it verified previous analyses and observations that roots arose mainly from callus tissue.

d) Final gradient analysis and summary

The analysis in Table 12 simply established the frequency of each type of organogenesis per organogenetic origin. It did not take into account the frequency of each origin. For a complete analysis, a correlation must therefore be made between the frequencies of type and origin of organogenesis. Table 13 relates the number of explants with each type of organogenesis from a particular origin to the total number of explants showing

organogenesis from that origin (i.e. Table 13 relates the information in Tables 9 and 12). The mathematical relationship is as follows:

$$\frac{\text{number of explants with type X organogenesis of type Y origin}}{\text{number of explants with any organogenesis from type Y origin}} \times 100\% = \text{Numerical correlation between frequencies of organogenetic type X and origin Y}$$

For example, to correlate floral response and epidermal origin, the percentage of all explants with epidermally originating organogenesis which showed floral organogenesis of epidermal origin was determined for each explant type.

The analysis in Table 13 clearly established several organogenetic trends in the epidermal explant system.

Gradient of In Vitro Floral Expression: Every experiment in the epidermal explant research confirmed the existence of a gradient of *in vitro* floral expression. This floral gradient was manifested in two ways: 1) by an increasing incidence of floral organogenesis on the epidermal explants with increased proximity of the explant excision locus to the floral apex of the donor tobacco plant and 2) by a concomitant decrease in the average number of nodes per *de novo* floral shoot (though the latter form of floral gradient manifestation was only evident in organogenesis of epidermal surface origin). Specifically then, as seen in Table 13, there was an increase in floral organogenesis from basal, through middle, sub-floral, and floral stem explants to

Explant Type	% Organogenetic Response Per Organogenetic Origin											
	Epidermal Origin				Cortical Origin				Polar Callus Origin			
	Floral	Veg.	Root	Nodes	Floral	Veg.	Root	Nodes	Floral	Veg.	Root	Nodes
GF-FB	100	30	0	2.2	100	33	0	-	96	32	68	7
GF-F	100	52	0	2.6	0	50	0	-	100	52	76	6.7
GF-SF	82	40	0	5.3	20	100	0	12.8	42	83	75	9.3
GF-M	14	100	0	6.0	17	100	0	15	36	100	36	7.0
GF-B	(0)*	(100)*	0	-	0	100	0	-	20	80	0	12
Fl-FB	100	0	0	2.7	100	0	0	6.0	85	31	54	5.2
Fl-F	100	0	0	2.6	60	80	0	5.3	70	70	100	6.0
Fl-SF	69	56	0	3.3	20	100	0	7	54	46	77	6.8
Fl-M	50	50	0	7	25	100	50	8	-	-	-	7
Fl-B	33	67	0	5.0	0	100	0	-	-	-	-	-
Bd-SF	83	65	0	5.2	40	80	0	9.0	59	76	86	8.3
Bd-M	60	40	0	10.3	50	100	0	-	75	25	12	11.7
Bd-B	19	94	0	8.2	-	-	-	10+	30	80	30	8+

Table 13: Correlation Between Organogenetic Response Types and Origins. Numbers represent the percentage of explants with a particular origin that showed a particular type of organogenesis from that origin, except for the node data which represents the average node number per floral shoot. Blanks represent situations in which there were insuffient data for analysis.
*Data based on only one observation in this analysis, but confirmed by other experiments and analyses.

floral branch explants, respectively representing increased proximity to the floral apex of the tobacco plant.

This floral gradient was most strongly expressed in organogenesis of epidermal origin. However, in both the green fruit and full bloom stages, the floral stem explants showed as high floral response (100%, in fact) as floral branch explants. Therefore, in terms of floral shoot frequency, the floral gradient was fully expressed in explant regions from the main axis of the flowering tobacco plant, with a minimum floral response in basal stem explants to a maximum in floral stem explants. While the whole gradient analysis experiment (Experiment 10 as cited in Appendix 1) was not successfully repeated due to high necrosis and lack of de novo growth (Experiments 14 and 15 in Appendix 1), the observation of maximum floral response in organogenesis of epidermal origin of floral stem explants was reproduced in a small experiment using floral branch, floral stem, and sub-floral stem explants from green fruit stage tobacco plants (Experiment 12, Appendix 1). In both experiments the floral response in epidermally originating organogenesis was 100% for both floral branch and floral stem explants.

However, in both experiments the average internode number per floral shoot was higher in floral stem explants than in floral branch explants. Thus using both 'floral organogenetic frequency' and 'average node number per floral shoot' criteria, the floral gradient was indeed more strongly expressed in floral

branch explants than in floral stem explants (i.e. the maximum expression of an in vitro floral potential was only observed in floral branch explants).

In organogenesis of polar callus and cortical surface origins, there was a general trend of increasing floral response from basal to floral branch explants in the green fruit and full bloom stages, but no gradient effect evident in the average node number per floral shoot. Thus, in these origins, the floral gradient was less expressed than in epidermally originating organogenesis.

All three developmental stages of tobacco plant (green fruit, full bloom and floral bud) showed a high potential for floral organogenesis as well as an in vitro gradient of floral expression. These observations were reproduced in experiments throughout this research, though precise quantification was not always obtained. The floral gradient was most clearly defined in organogenesis of epidermal origin for all three developmental stages. The earlier stages, full bloom and floral bud, showed greater potential for floral organogenesis in the middle and basal stem explant regions than did the green fruit stage, in organogenesis of epidermal origin (and possibly of polar callus origin too). As seen in Table 13, the earlier stages averaged 40% floral response for the middle and basal regions, compared to 7% for the green fruit stage. This observation was also confirmed by several experiments.

In other words, the floral gradient was most clearly

expressed in the green-fruit stage (the gradient in response ranging from 0% to 100% floral response), less in the full bloom stage (33% to 100%), and least in the floral bud stage (19% to 83%).

In summary, the strongest expression of a floral gradient occurred in organogenesis of epidermal surface origin on explants from green-fruit stage Nicotiana tabacum L.

Organogenetic Trends in Vegetative and Root Organogenesis: There was also a gradient of in vitro vegetative expression in this system. As shown in Table 13, the vegetative response of the explants generally increased from apical to basal explant regions in organogenesis of epidermal and cortical origins (on the average, vegetative organogenesis increased 76% from the floral branch region to the basal explant region). There was no clear trend expressed in vegetative organogenesis of polar callus origin, however.

Root organogenesis, only originating in polar callus, generally showed a higher response rate in floral branch, floral stem and sub-floral stem explants than in middle and basal stem explants (76% versus 20%).

There was no apparent relationship between the developmental stage of the donor plant and vegetative or root organogenesis.

2. Leaf Disk Explants

i. Description of the experimental system

The leaf disk system consisted of 12.7 mm leaf disks excised from mature leaves of Nicotiana tabacum L. cv. Wisc. 38. The disks were cultured on modified Murashige and Skoog medium at 27°C in continuous light, as described in Materials and Methods. On appropriate media, this system produced 100% yield and an homogeneity in organogenetic response of 100%. The response was both rapid and vigorous. Organogenesis was well developed in 19 to 26 days. Moreover, the system gave an extremely high propagation potential, as evidenced by the hundreds of shoots formed per explant on appropriate medium.

No evidence for polarity in the development of de novo growth on the leaf disk was ever detected in this system.

ii. Hormone manipulation studies

Hormone manipulation studies were performed in view of two main objectives: 1) testing the organogenetic response of tobacco leaf disks to an array of different IAA and kinetin concentrations, and 2) obtaining vigorous callus cultures from leaf disks. The results are reported in detail below.

a) Organogenetic response to different IAA and kinetin concentrations

The culture response of leaf disks to thirty-six different combinations of IAA and kinetin were tested, in which each hormone ranged in concentration from 10^{-8} molar to 10^{-3} molar, with 18 replicates per treatment. Explants were only excised from leaves of the middle portion of green-fruit stage tobacco plants, in order to minimize physiological variation in the tissue. Specifically, leaves 13 to 18 were used from plants averaging a total of 34 nodes each. All the results represent four weeks of culture time.

The results from this experiment indicated that the absolute concentration of kinetin was the most important factor regulating organogenesis in the leaf disk system. Therefore, the results reported here have been grouped according to their kinetin concentration. The results are described in detail below and are summarized on page 106 and in Table 14.

10^{-8} M kinetin cultures

The main characteristic of the 10^{-8} molar kinetin cultures (Figure 9) was small peripheral tufts of brownish, friable callus; 2 to 4 tufts per leaf disk. The tufts were always associated with the cut ends of vascular bundles at the disk periphery. The leaf disks were pale green or partly chlorosed, except for tissue adjacent to vascular bundles, which was

generally deep green. The disks were usually slightly convoluted and enlarged approximately 50% in diameter. The only type of organogenesis observed in the 10^{-8} M kinetin series was that of root formation, which occurred at the highest auxin concentrations (10^{-3} and 10^{-4} M IAA) in correlation with the amount of auxin. 10^{-3} M IAA induced root organogenesis in 60% of the cultures; 10^{-4} M IAA in 20%. The roots were long, fibrous, and completely covered in root hairs.

Specific descriptions of the morphogenetic response to the various 10^{-8} M kinetin media are as follows:

10^{-8} M IAA / 10^{-8} M kinetin: 100% of the explants produced small, peripheral tufts of callus, associated with the vascular bundles. The leaf disks were slightly chlorosed and convoluted, and enlarged about 50% in diameter. (Figure 9a)

10^{-7} M IAA / 10^{-8} M kinetin: 6% (one explant) of the explants showed no apparent meristematic activity; the rest produced small peripheral tufts of callus, as described above. (Figure 9b)

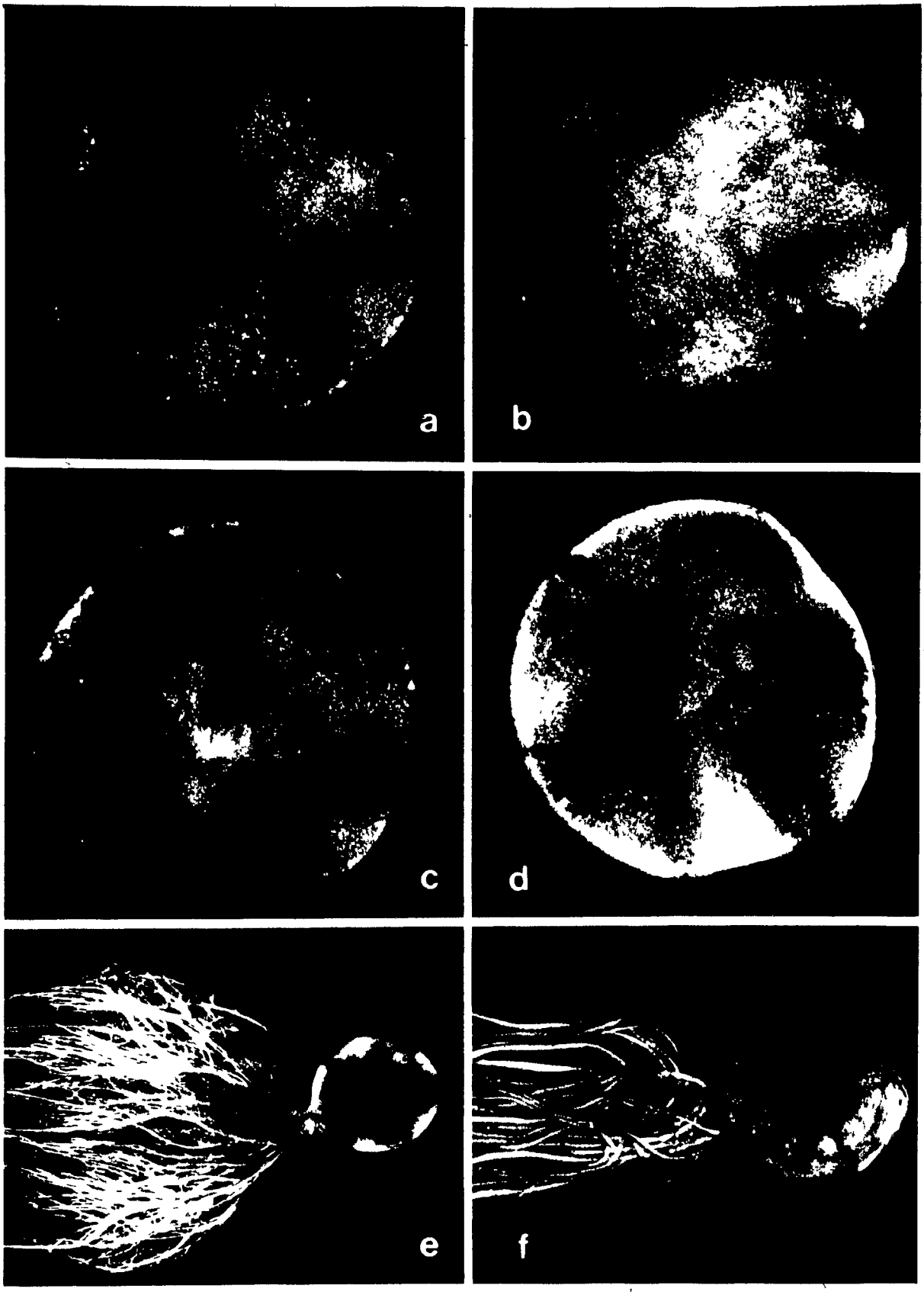
10^{-6} M IAA / 10^{-8} M kinetin: 100% of the explants produced small, peripheral tufts of callus, as described above. (Figure 9c)

10^{-5} M IAA / 10^{-8} M kinetin: 100% callus tufts, as above. (Figure 9d)

10^{-4} M IAA / 10^{-8} M kinetin: 100% of the explants produced small, peripheral tufts of callus; 20% also produced roots. Otherwise, as described above. (Figure 9e)

Figure 9: Tobacco leaf disks cultured for four weeks on medium containing 10^{-8} molar kinetin and various concentrations of IAA.

a) 10^{-8} M IAA (3.4x) b) 10^{-7} M IAA (3.4x)
c) 10^{-6} M IAA (3.4x) d) 10^{-5} M IAA (3.4x)
e) 10^{-4} M IAA (1.7x) e) 10^{-3} M IAA (1.2x)


a
b
c
d
e
f

10^{-3} M IAA / 10^{-8} M kinetin: 40% of the explants on this medium produced small, peripheral tufts of callus, whereas the remaining 60% produced roots. There were no explants producing both callus and roots. The leaf disks were enlarged about 50% in diameter, but showed no convolution. The disks were slightly chlorosed; less so in those explants with roots. (Figure 9f)

10^{-7} M kinetin cultures

The main characteristic of the 10^{-7} molar kinetin cultures (Figure 10), as in the previous culture series, was small, peripheral tufts of callus occurring at the cut ends of vascular bundles. The one exception to this was the 10^{-5} M IAA explants which developed a thick, brown friable callus entirely lining the edge of the leaf disk.

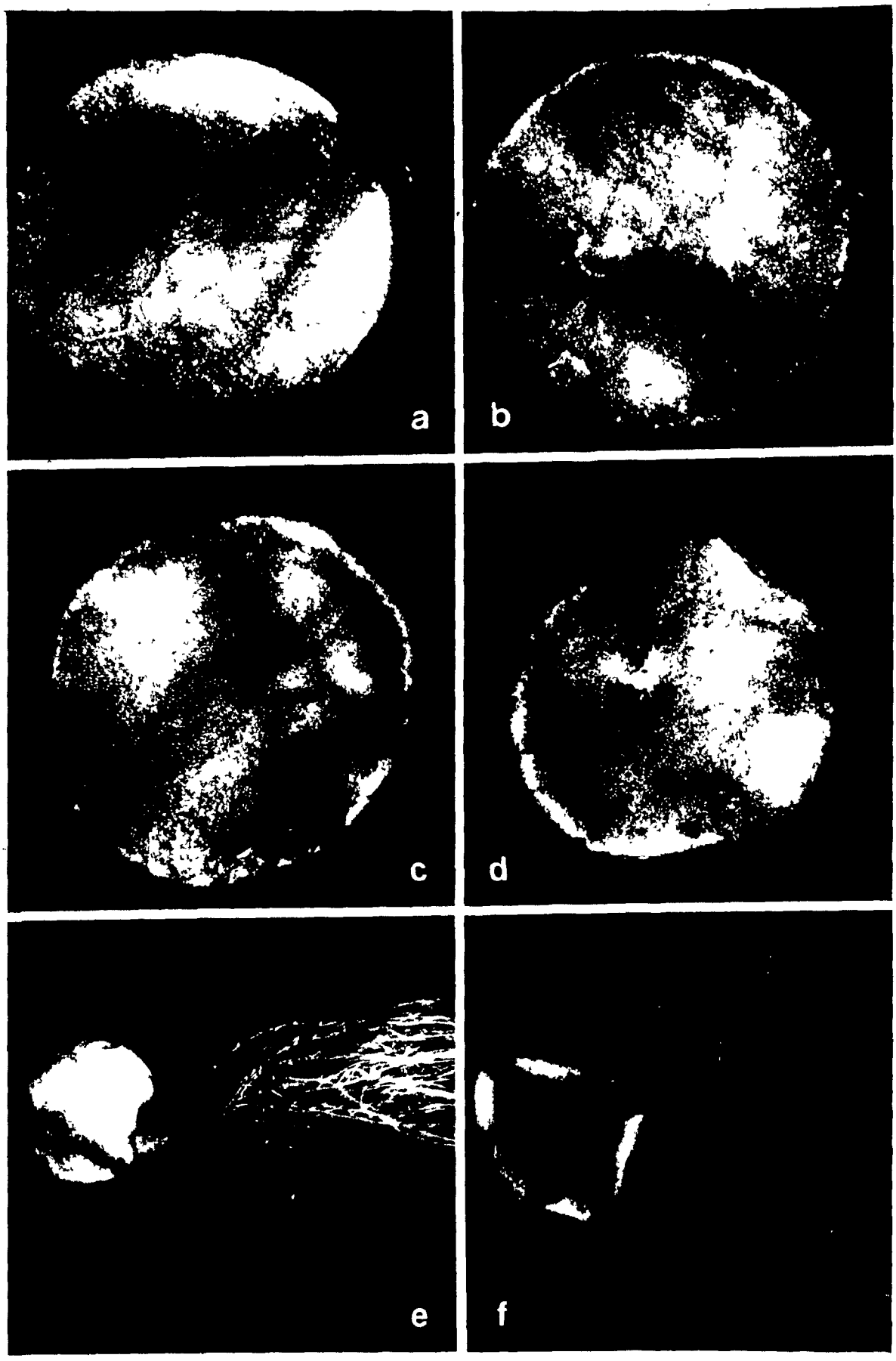
The leaf disks were generally slightly chlorosed, except for tissue adjacent to vascular bundles, which was deep green. The disks were also slightly convoluted and enlarged about 50% in diameter.

As in the previous two series, the only type of organogenesis observed in these cultures was root formation, which only occurred at the highest auxin concentrations in correlation with the amount of auxin. 10^{-3} M IAA induced root organogenesis in 50% of the explants; 10^{-4} M IAA in 10%. The roots were long, fibrous, and covered in root hairs.

Specific descriptions of the morphogenetic response on each medium are as follows:

Figure 10: Tobacco leaf disks cultured for four weeks on medium containing 10^{-7} molar kinetin and various concentrations of IAA.

a) 10^{-8} M IAA (4.5x) b) 10^{-7} M IAA (4.5x)
c) 10^{-6} M IAA (3.7x) d) 10^{-5} M IAA (3.4x)
e) 10^{-4} M IAA (1.4x) f) 10^{-3} M IAA (2.0x)


a
b
c
d
e
f

10^{-8} M IAA / 10^{-7} M kinetin: 100% of the explants produced small peripheral tufts of callus (2 to 4 per explant). The disks were slightly chlorosed and convoluted, and enlarged about 20% in diameter. (Figure 10a)

10^{-7} M IAA / 10^{-7} M kinetin: 6% (one explant) of the explants on this medium showed no apparent meristematic activity. 90% produced small, peripheral tufts of callus, as described above. (Figure 10b)

10^{-6} M IAA / 10^{-7} M kinetin: 100% of the explants produced from a few to many small peripheral tufts of callus. 6% (one explant) also produced roots. The leaf disks were slightly convoluted and chlorosed, and enlarged about 50% in diameter. (Figure 10c)

10^{-5} M IAA / 10^{-7} M kinetin: 100% of the explants on this medium developed a brownish-green friable callus lining the disk periphery. There was no organogenesis of any type. The disks were slightly convoluted and chlorosed, and about 50% enlarged in diameter. (Figure 10d)

10^{-4} M IAA / 10^{-7} M kinetin: 100% of the explants produced tufts of callus along the disk peripheries. 10% also developed roots. Otherwise, as above. (Figure 10e)

10^{-3} M IAA / 10^{-7} M kinetin: 100% of the explants produced peripheral tufts of callus, with 50% also producing roots. Otherwise, as above. (Figure 10f)

10^{-6} M kinetin cultures

The 10^{-6} molar kinetin culture series (Figure 11) was the least consistent in growth response, though to varying degrees all the cultures did produce callus tissue. The highest auxin concentration (10^{-3} M IAA) induced root production in 30% of the explants. Lower auxin concentrations (10^{-5} to 10^{-8} M IAA) induced vegetative shoot organogenesis to varying degrees, the amount inversely proportional to the auxin concentration. Most of the vegetative organogenesis was callused in appearance, with the exception of the 10^{-6} M IAA cultures, which produced well defined shoots. An unexpected finding was the production of roots at the lowest auxin concentration (10^{-8} M IAA); however, the origin of these roots was difficult to ascertain and may have been adventitious. Other than this, roots were only observed in 10^{-3} and 10^{-4} M IAA cultures, regardless of kinetin concentration.

The leaf disks were usually partially chlorosed, slightly convoluted, and enlarged in diameter (8% to 75%).

Specific descriptions are as follows:

10^{-8} M IAA / 10^{-6} M kinetin: 6% (one explant) of the explants on this medium were necrosed after four weeks in culture; all the rest produced some callus tissue. 50% of the viable explants produced only nodule-like callus tufts, while the rest also produced some organogenetic structures (i.e. shoots and/or roots). Of the explants showing organogenesis, approximately half produced only shoots, and half

both shoots and roots. All the shoots were indistinct in morphology, with thick fleshy leaves (the tissue had a callus-like quality, being somewhat friable, and lacking a smooth surface). The leaf disks were partly chlorosed, slightly convoluted in shape, and about 20% enlarged in diameter. (Figure 11a)

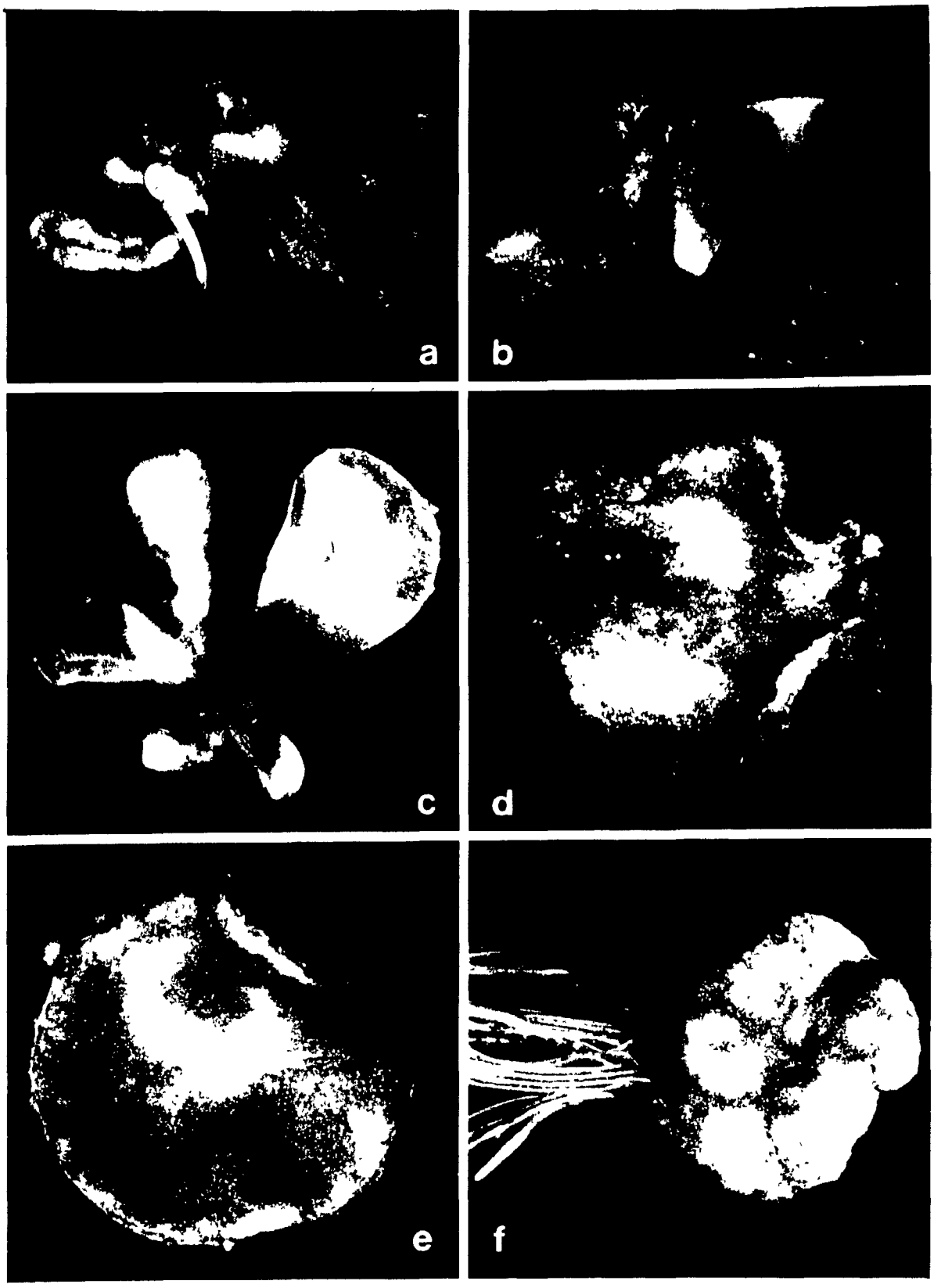
10^{-7} M IAA / 10^{-6} M kinetin: 70% of the explants on this medium produced somewhat nodule-like callus tufts. The tufts were green, but had a brownish, slightly necrosed surface. 30% of the explants also produced some peripheral vegatative shoots that were slightly callused and fleshy in form. The leaf disks were partially chlorosed, and very slightly convoluted and enlarged in diameter (about an 8% increase in disk diameter). (Figure 11b)

10^{-6} M IAA / 10^{-6} M kinetin: 100% of the explants produced small tufts of callus along the disk peripheries. 20% produced one or two vegetative shoots per explant. These shoots originated in the callus and were well developed, 0.5 to 1.0 cm in height, and had 6 to 11 nodes of growth. The leaf disks were slightly convoluted and about 30% enlarged in diameter. (Figure 11c)

10^{-5} M IAA / 10^{-6} M kinetin: 100% of the explants on this medium produced friable, brownish-green peripheral callus. 10% also showed vegetative organogenesis consisting of a small vegetative shoot (or what appeared to be a single leaf, in some cases) originating in callus. The vegetative organogenesis was

Figure 11: Tobacco leaf disks cultured for four weeks on medium containing 10^{-6} M kinetin and various concentrations of IAA.

a) 10^{-8} M IAA (1.3x) b) 10^{-7} M IAA (3.4x)
b) 10^{-6} M IAA (2.0x) d) 10^{-5} M IAA (3.1x)
e) 10^{-4} M IAA (4.0x) f) 10^{-3} M IAA (2.4x)


a
b
c
d
e
f

very callus-like in appearance. The leaf disks were convoluted and enlarged in diameter by about 75%. (Figure 11d)

10^{-4} M IAA / 10^{-6} M kinetin: 100% of the explants produced the typical peripheral tufts of callus. There was no differentiated growth on this medium. The leaf disks were somewhat enlarged (about a 30% increase in diameter), slightly chlorosed, and convoluted in shape. (Figure 11e)

10^{-3} M IAA / 10^{-6} M kinetin: 100% of the explants on this medium produced callus growth in form of small peripheral tufts growing in association with vascular bundles. 30% also produced long, fibrous roots. The leaf disks were slightly chlorosed, convoluted, and about 50% enlarged in diameter. (Figure 11f)

10^{-5} M kinetin cultures

The 10^{-5} molar kinetin cultures (Figure 12) produced the densest, most vigorous shoot organogenesis, the best response occurring at 10^{-5} M IAA. With all the IAA concentrations except 10^{-3} M, the response was the production of hundreds of vegetative shoots per explant. Most of these shoots were only a few millimeters in height, and consisted of only a few nodes (less than 10 leaves visible by eye) of growth. On each explant, however, there were ten to twenty shoots which were much larger than the rest. The larger shoots ranged up to 15 nodes (15 leaves visible by eye) and two centimeters in height. Growth was predominantly oriented down into the agar, which in this experiment corresponded to growth from the abaxial surface of the

leaf disk. The organogenetic origin of the shoots was mostly obscured by the density of growth. However, nodules were evident in all the cultures. The leaf disks were deep green and greatly convoluted. Though the disks were obviously enlarged in diameter, the extent of enlargement could not be assessed due to the density of shoot growth.

Specific descriptions of the 10^{-5} M kinetin cultures follow below:

10^{-8} M IAA / 10^{-5} M kinetin: 100% of the explants produced a dense mass of vegetative shoots (hundreds of shoots per explant). The largest shoots were about 2 cm long and exceeded 15 nodes. All the vegetative shoots were well developed and distinct in form (no callus-like quality). Some callus was evident in the compacted interior of each culture's mass of de novo growth. The vegetative growth completely filled several centimeters of each culture tube. Nodules bearing shoots were evident. The leaf disks were deep green, convoluted, and enlarged in diameter. (Figure 12a)

10^{-7} M IAA / 10^{-5} M kinetin: As above. (Figure 12b)

10^{-6} M IAA / 10^{-5} M kinetin: 100% of the explants produced dense vegetative growth, as described above, except that much of the growth was callused in appearance. In general, the smaller shoots were not clearly defined in form: leaves were often thick and friable, and shoot apices were indistinct from callus-like growth. Much actual callus tissue was present on each explant. However, there were several larger shoots (e.g. 1 cm high with

Figure 12: Tobacco leaf disks cultured for four weeks on medium containing 10^{-5} molar kinetin and various concentrations of IAA.

a) 10^{-8} M IAA (1.5x) b) 10^{-7} M IAA (1.3x)

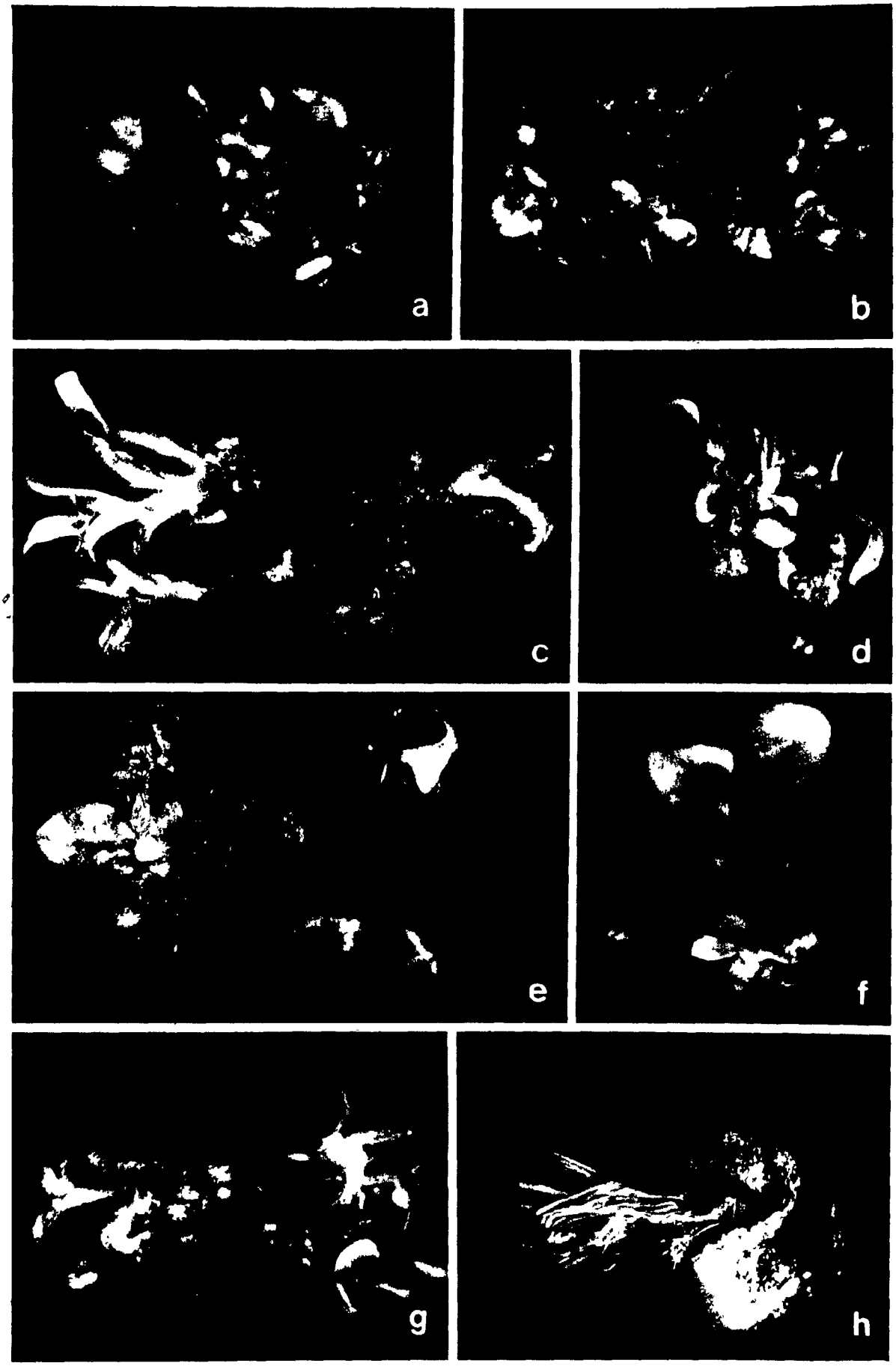
c) 10-6 M IAA, side view (1.6x)

d) 10-6 M IAA, top view (1.2x)

e) 10-5 M IAA, side view (1.9x)

f) 10-5 M IAA, top view (1.7x)

g) 10^{-4} M IAA (1.2x) h) 10^{-3} M IAA (1.9x)


a
b
c
d
e
f
g
h

leaves up to 2 cm long) that were clearly defined in form; that is, did not have a callus-like quality. Organogenetic origins were obscured by the density of growth. (Figures 12c,d)

10^{-5} M IAA / 10^{-5} M kinetin: 100% of these explants were covered in a profusion of young shoots and shoot apices. This medium produced the densest, most vigorous growth of all the media tested. The growth per explant fully occupied two to three centimeters of culture tube space. Several hundred shoots were present per explant. The origin of the organogenesis was largely obscured by the density of growth, though nodules were definitely present and could be identified as the origin of some of vegetative organogenesis. The leaf disks were deep green and very convoluted in shape. (Figure 12e,f)

10^{-4} M IAA / 10^{-5} M kinetin: 100% of the explants showed dense vegetative organogenesis with hundreds of shoots per explant. Some callus growth was evident and much of the organogenesis had a callus like-quality, though well defined, normal shoots were evident. The largest shoots were 1 to 2 centimeters tall. Nodules were also evident, though callus-like in appearance. The leaf disks were deep green and convoluted. (Figure 12g)

10^{-3} M IAA / 10^{-5} M kinetin: 100% of the explants produced a thick peripheral ring of callus. The callus was green, large-celled and friable. The leaf disks themselves were deep green, very convoluted, and about 75% enlarged in diameter. 60% of the explants showed differentiated growth, either vegetative shoots

or roots. No explant developed both shoots and roots. 50% of the explants produced shoots, while 10% produced roots. Both forms of organogenesis were well defined and several centimeters in length. (Figure 12h)

10^{-4} M kinetin cultures

The organogenetic response in the 10^{-4} molar kinetin cultures (Figure 13) consisted mainly of green nodules and small shoots lining the explant edge. These small shoots were termed 'microshoots' for this thesis, in order to emphasize their small size (less than 5 mm) and uniform lack of development beyond a few leaves. New shoot apices were continuously being produced on the nodules. However, if the cultures were maintained longer than the regular 4 week culture period, the number of microshoots greatly increased, but none developed further than a few nodes or exceeded 5 mm in height.

Nodules occurred on the explant periphery and on the bottom surface (abaxial surface) of the leaf disks, but these very seldom gave rise to shoots and, therefore, were not given further attention in this section of the results. The peripheral nodules always greatly outnumbered the shoots formed per explant. The nodules were of two general types: smooth, green, translucent nodules and whitish-green, opaque nodules usually with epidermal hairs (trichomes). Both types gave rise to shoot buds, though more commonly the latter. (The translucent nodules were possibly an immature form of the opaque nodules.) Since shoots

only arose from nodules, and never directly from the disk surface, the nodules were termed 'propagation nodules', though often abbreviated simply to 'nodules' in this thesis. New nodules, as well as shoots, developed on propagation nodules.

There was a pronounced increase in disk size in the 10^{-4} M kinetin cultures, with the greatest increase occurring in the 10^{-5} M IAA cultures. The increase in disk diameter ranged from 40% to 70%.

All the leaf disks became convoluted in culture. Generally the greatest amount of organogenesis occurred along those portions of the explant edge which were lifted away from the agar by convolution of the leaf disk. All the cultures were a healthy green colour, although the leaf disks were often pale green at the center and deep green at the periphery.

There was a general gradient of increased size and degree of development of the microshoots with increasing concentrations of IAA.

Detailed descriptions of the morphogenesis occurring on each medium in this culture series are as follows:

10^{-8} M IAA / 10^{-4} M kinetin: 100% of the explants showed peripheral propagation nodules, microshoots and shoot apices. Both types of nodules were present: hairless, translucent green nodules, and haired, whitish-green, opaque nodules. Shoots most frequently occurred on the latter. Nodules outnumbered shoots by at least 5 to 1. The leaf disks were mainly deep green, convoluted, and about 40% enlarged in diameter. (Figure 13a)

10^{-7} M IAA / 10^{-4} M kinetin: 100% of the explants on this medium showed peripheral propagation nodules, shoot apices and microshoots. There were about 100 to 150 nodules per explant, and about 25 to 50 microshoots and shoot apices. The leaf disks were deep green with yellowish patches, convoluted, and about 40% enlarged in diameter. (Figure 13b)

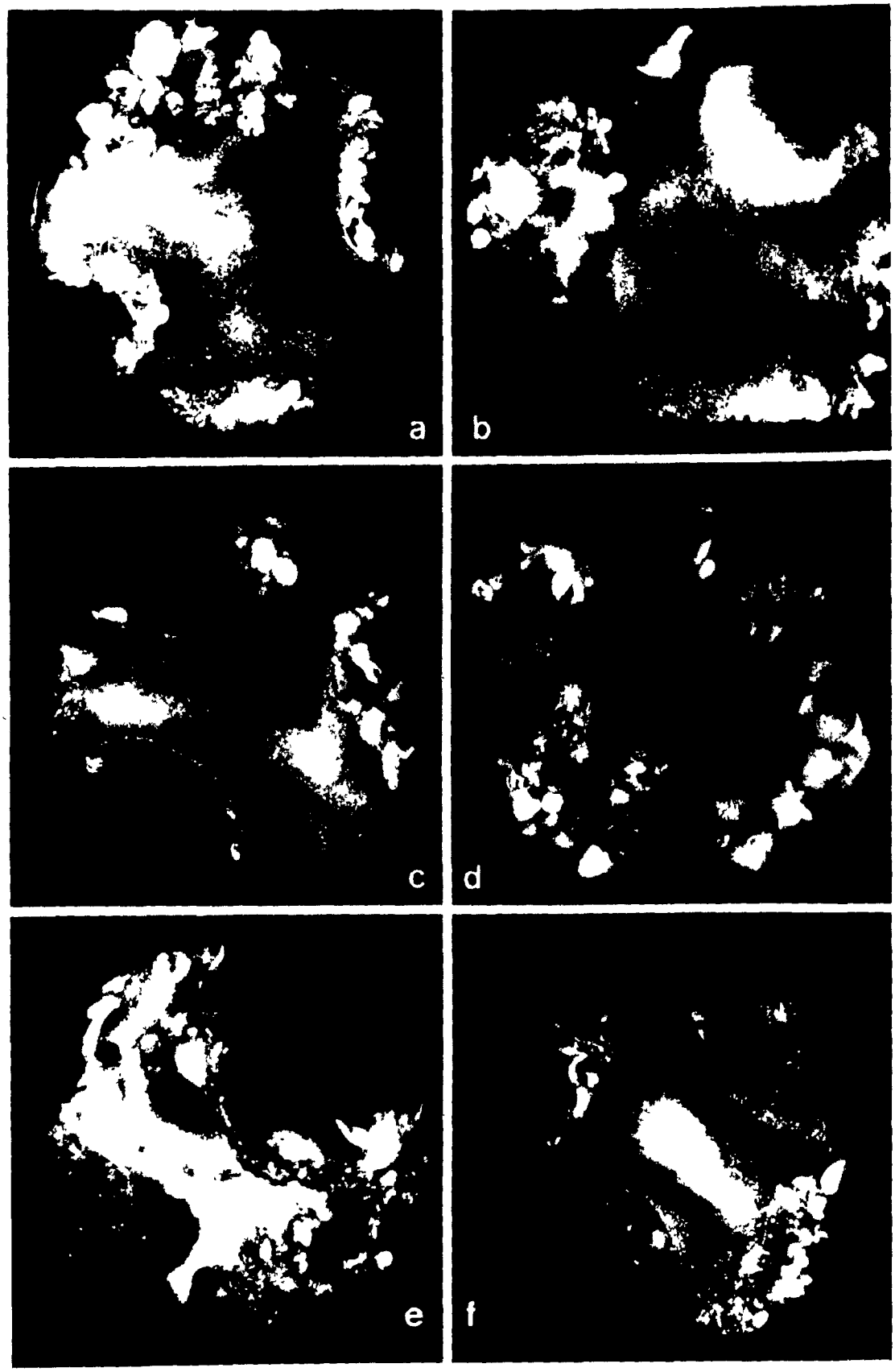
10^{-6} M IAA / 10^{-4} M kinetin: 100% of the explants were peripherally lined with propagation nodules. A few of these bore microshoots and shoot apices. The number of nodules per explant ranged from 100 to 150, but there were only about 10 shoot apices. The explants were slightly chlorosed at the centres, but deep green at the peripheries, and showed about a 40% increase in diameter. (Figure 13c)

10^{-5} M IAA / 10^{-4} M kinetin: 100% of the explants showed a profusion of propagation nodules, young vegetative shoots (microshoots) and shoot apices along the explant edge. There was a 60% to 70% increase in disk diameter, and all the disks were convoluted. The explants averaged 50 to 70 readily visible shoots per explant, and an estimated 100 to 200 propagation nodules. Thus, there was at least a 2:1 nodule to shoot ratio. (Figure 13d)

10^{-4} M IAA / 10^{-4} M kinetin: 100% of the explants had propagation nodules, shoot apices and microshoots lining the explant edge, the largest microshoot a few millimeters high. The explants were deep green, convoluted, and 50% to 60% enlarged in diameter. (Figure 13e)

Figure 13: Tobacco leaf disks cultured for four weeks on medium containing 10^{-4} molar kinetin and various concentrations of IAA.

a) 10^{-8} M IAA (3.6x) b) 10^{-7} M IAA (3.6x)
c) 10^{-6} M IAA (3.4x) d) 10^{-5} M IAA (3.6x)
e) 10^{-4} M IAA (3.6x) f) 10^{-3} M IAA (3.3x)


a
b
c
d
e
f

10^{-3} M IAA / 10^{-4} M kinetin: 100% of the explants had propagation nodules, shoot apices and microshoots lining the explant edge. The largest shoots were about 8 mm tall with well developed leaves. Generally, but not always, the shoots came from nodules with hairs. The explant edges were thickened from cell growth and division. Explant diameters were increased by 40% to 50%. The leaf disks were convoluted and deep green. (Figure 13f)

10^{-3} M kinetin cultures

The organogenetic response in the 10^{-3} molar kinetin cultures (Figure 14), the highest of the kinetin concentrations tested, consisted predominantly of nodule-like structures. These structures were tiny (diameters less than 1 mm), green, translucent globes so termed to distinguish them from propagation nodules. The globes were smaller than nodules and never gave rise to vegetative shoots. The globes occurred near the periphery of the leaf disks (but not directly on the explant's edge, as in the case of propagation nodules) on the upper, adaxial surface.

Only the 10^{-3} M IAA cultures gave rise to propagation nodules and shoot apices.

At concentrations of IAA lower than 10^{-3} M, the high cytokinin concentration was obviously antagonistic to tissue viability. This was indicated by necrosis of the peripheral cells, which were in the most immediate contact with the agar

medium due to down-curling of the leaf disk edges. The explants also showed various degrees of chlorosis. The amount of chlorosis diminished with lower concentrations of IAA.

Specific details follow for each medium:

10^{-8} M IAA / 10^{-3} M kinetin: 20% of the explants on this medium showed no apparent meristematic activity; 80% produced minute, green globes, as described above. All the explants were slightly chlorosed, with down-curled edges and necrosed peripheral cells. (Figure 14a)

10^{-7} M IAA / 10^{-3} M kinetin: 60% of the explants showed no apparent meristematic activity; 40% produced globes. All the explants were partially chlorosed (tissue pale green with yellow patches) with down-curled edges and necrosed peripheral cells. (Figure 14b)

10^{-6} M IAA / 10^{-3} M kinetin: 80% of the explants showed no apparent meristematic activity; 20% developed globes. All the explants were chlorosed with edges curled downwards and peripheral cells necrosed. (Figure 14c)

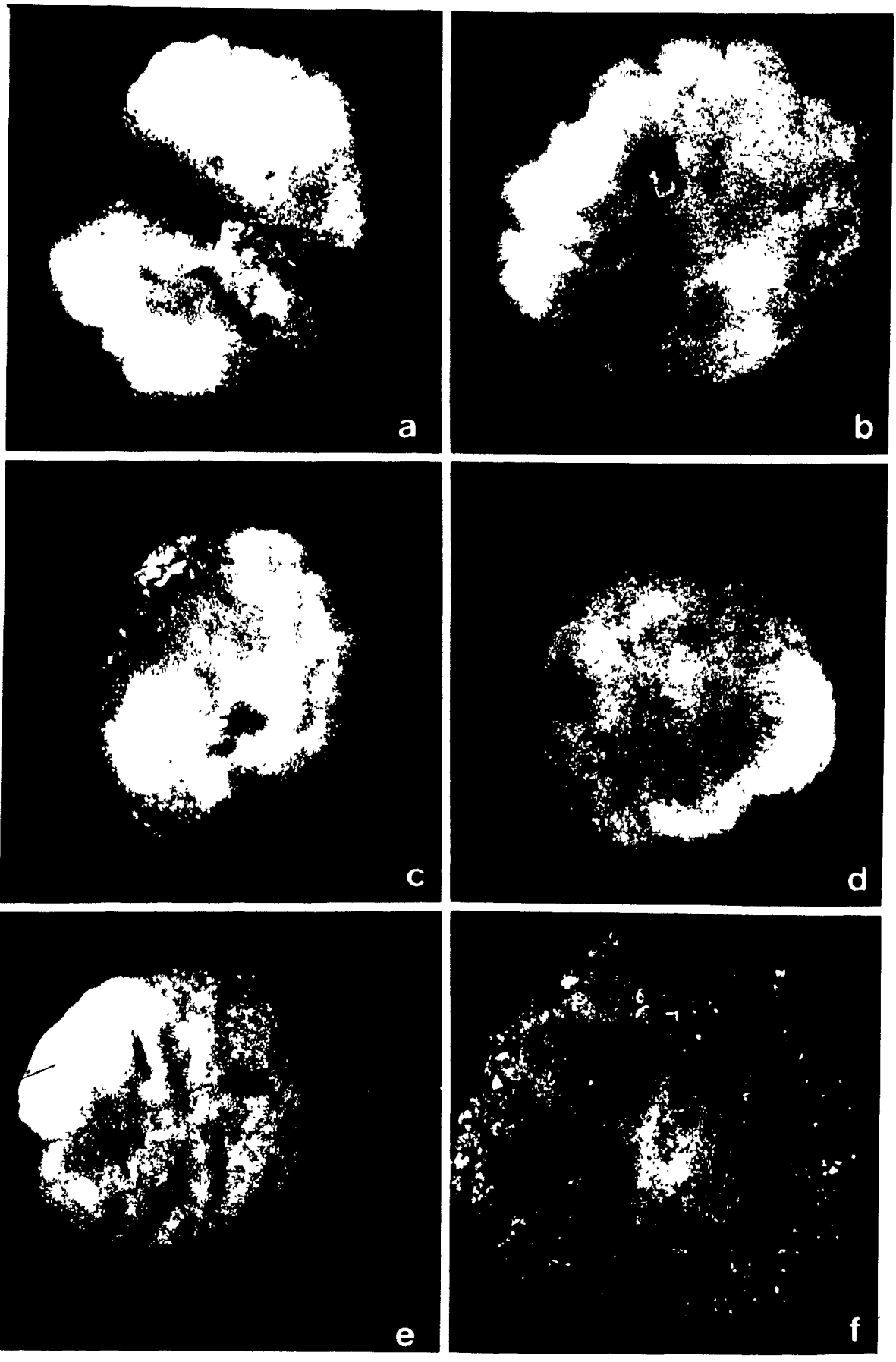
10^{-5} M IAA / 10^{-3} M kinetin: 90% of the explants showed no apparent meristematic activity; 10% produced nodules. Explant appearance, as above. (Figure 14d)

10^{-4} M IAA / 10^{-3} M kinetin: 10% of the explants showed no apparent meristematic activity, whereas 90% produced globes. Explant appearance, as above. (Figure 14e)

10^{-3} M IAA / 10^{-3} M kinetin: The upper, adaxial explant edge was covered in small, green, translucent propagation nodules.

Figure 14: Tobacco leaf disks cultured for four weeks on medium containing 10^{-3} molar kinetin and various concentrations of IAA.

a) 10^{-8} M IAA (6.5x) b) 10^{-7} M IAA (6.5x)
c) 10^{-6} M IAA (5.5x) d) 10^{-5} M IAA (5.5x)
e) 10^{-4} M IAA (6.5x) f) 10^{-3} M IAA (5.5x)


a
b
c
d
e
f

Some of the nodules had epidermal hairs, unlike the globes formed at lower IAA concentrations. Some vegetative shoot apices were apparent originating from nodules. The shoot apices had one or two well formed leaves and several leaf primordia. Nodule-like structures were also present at the peripheries of necrosed patches occurring in the central area of the leaf disk. (Figure 14f)

A number of these explants were left in the culture environment to determine whether the vegetative apices (which were less developed than the microshoots occurring on 10^{-4} M kinetin nodules) present on the cultures would develop further with longer culture times. After nine weeks of culture, the apices were no more developed than after four weeks of culture time. However, the number of apices per explant had greatly increased. At nine weeks of culture, the explants were covered in apices over the entire upper, adaxial disk surface; however, these apices had no more than eight nodes of growth including all visible primordia (as determined by microdissection).

Thus, the lesser degree of shoot development on the 10^{-3} M kinetin nodules, as compared to the microshoots on the 10^{-4} M kinetin nodules, was not due to slower growth rates, but to apparent inhibition of development beyond the formation of about 8 primordia.

Summary of the leaf disk response to different IAA and kinetin concentrations

The above results, which are also summarized in Table 14 and in Figures 15 and 16, showed that the most important factors regulating vegetative organogenesis and callus production in the tobacco leaf disk system was the absolute concentration of kinetin. 10^{-8} and 10^{-7} molar kinetin cultures were characterized by small, peripheral tufts of callus.

10^{-6} molar kinetin cultures were characterized by peripheral tufts of callus and some vegetative shoot production. At this kinetin concentration only, the amount of vegetative organogenesis decreased with increasing IAA concentration (Figure 15). Thus, only at 10^{-6} M kinetin, was the auxin/cytokinin ratio a possible factor regulating organogenesis (Figure 16).

10^{-5} molar kinetin cultures were characterized by dense vegetative shoot growth and propagation nodules. 10^{-4} M kinetin cultures were characterized by the prolific production of propagation nodules as well as small vegetative shoots (microshoots). 10^{-3} M kinetin cultures were characterized by small green translucent globes except at the highest concentration of auxin (10^{-3} M IAA) at which nodules and vegetative apices were produced.

Thus, vegetative organogenesis only occurred at kinetin concentrations greater than or equal to 10^{-6} M, and callus production at kinetin concentrations less than or equal to 10^{-6} M, with the one exception of the 10^{-3} M IAA / 10^{-5} M kinetin

	10^{-8} M IAA	10^{-7} M IAA	10^{-6} M IAA
10^{-8} M kin	100% callus tufts	100% callus tufts (-6% no growth)	100% callus tufts
10^{-7} M kin	100% callus tufts	100% callus tufts (-6% no growth)	100% callus tufts -6% roots
10^{-6} M kin	100% callus tufts 50% veg. shoots 38% shoots and roots	100% callus tufts 30% veg. shoots	100% callus tufts 20% veg. shoots
10^{-5} M kin	100% vegetative shoots and nodules	100% vegetative shoots and nodules	100% vegetative shoots and nodules (callus-like quality)
10^{-4} M kin	100% nodules and microshoots	100% nodules and microshoots	100% nodules and microshoots
10^{-3} M kin	100% globes (20% no growth)	100% globes (60% no growth)	100% globes (80% no growth)

Table 14: Leaf Disk Response to Different IAA and Kinetin Concentrations. Numbers refer to the percentage of viable explants showing a particular response. Numbers in parentheses represent the percentage of explants showing no in vitro growth.

10^{-5} M IAA	10^{-4} M IAA	10^{-3} M IAA	
100% callus tufts	100% callus tufts 20% roots	40% callus tufts 60% roots	10^{-8} M kin
100% callus (lining explants)	100% callus tufts 10% roots	100% callus tufts 50% roots	10^{-7} M kin
100% callus (lining explants) 10% veg. shoots	100% callus tufts	100% callus tufts 30% roots	10^{-6} M kin
100% vegetative shoots and nodules	100% vegetative shoots and nodules (callus-like quality)	100% thick callus 50% veg. shoots 10% roots	10^{-5} M kin
100% nodules and microshoots	100% nodules and microshoots	100% nodules and microshoots	10^{-4} M kin
100% globes (90% no growth)	100% globes (10% no growth)	100% nodules and shoot apices	10^{-3} M kin

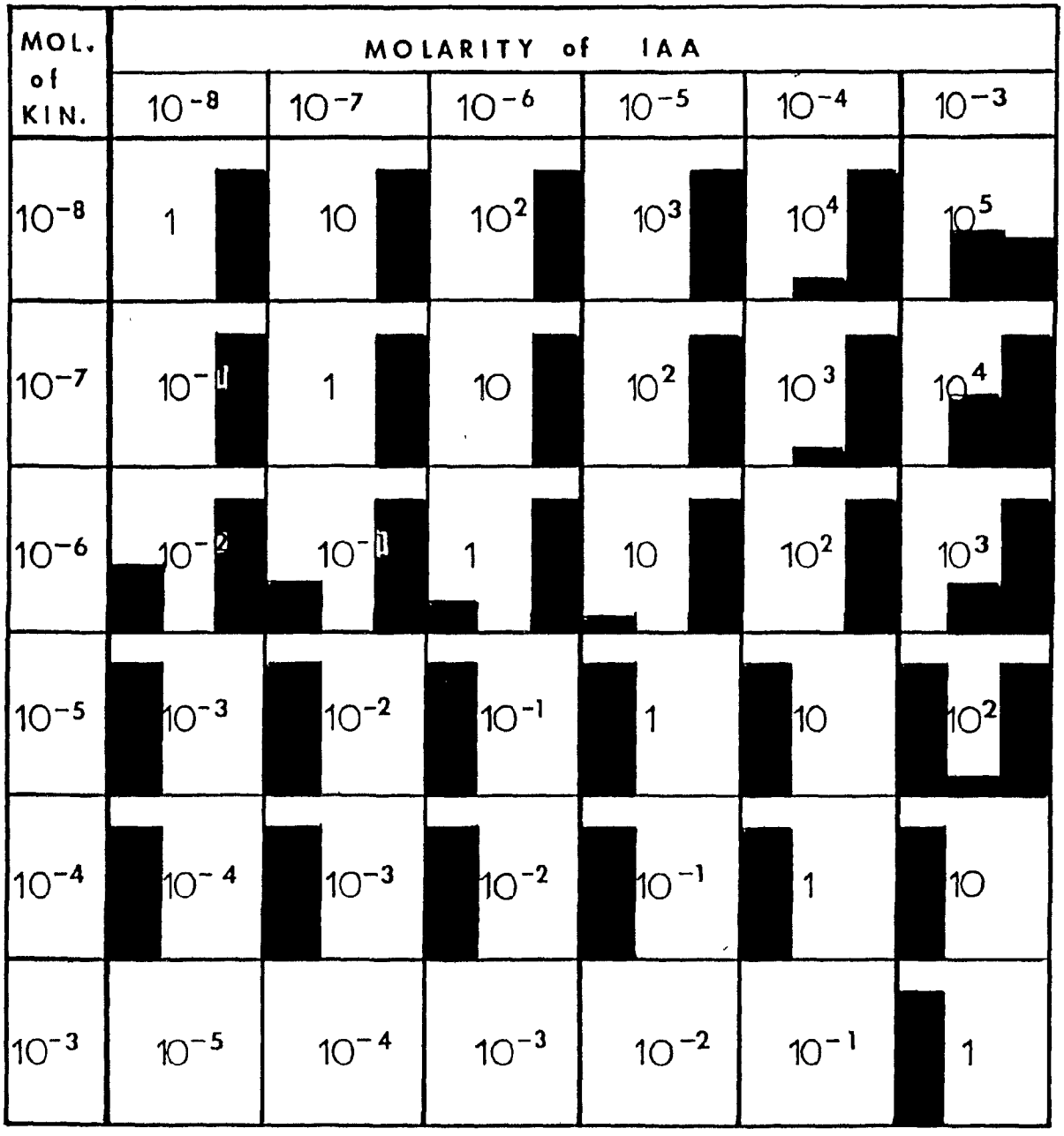


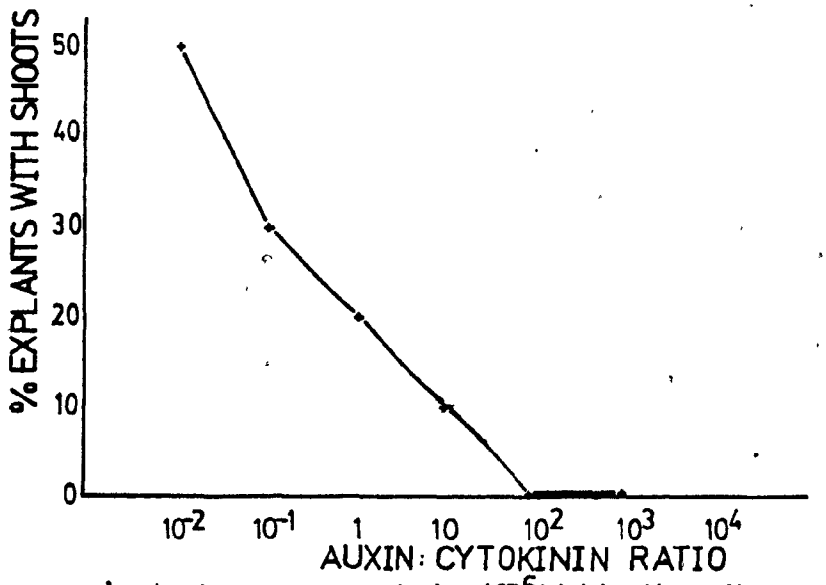
FIGURE 15

LEGEND:

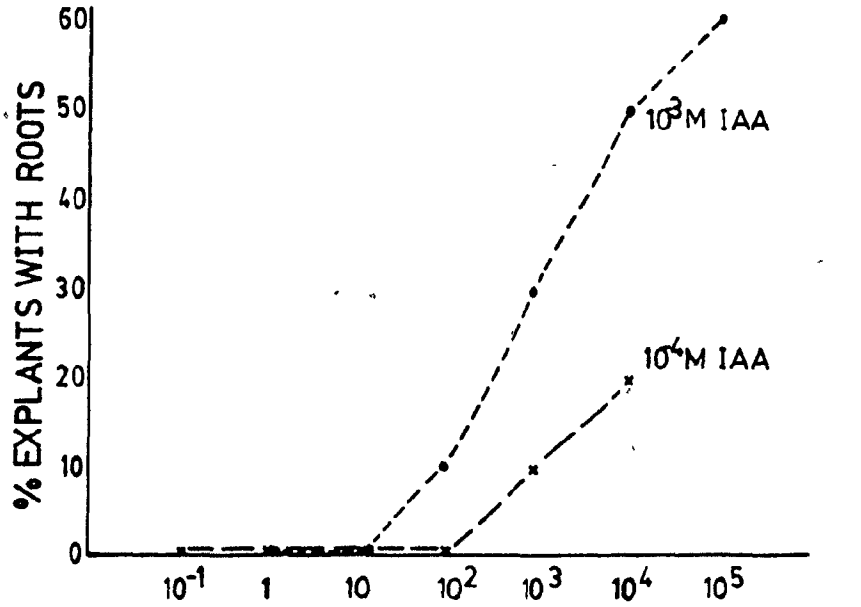
callus
roots
shoots

Histographic Representation of Leaf Disk Response to Different IAA and Kinetin Concentrations.

The vegetative shoot, root, and callus production for each medium is shown.
The number in each square represents the auxin / cytokinin ratio.



a) shoot organogenesis in 10^{-6}M kinetin cultures



b) root organogenesis in 10^{-4} and 10^{-3} M IAA cultures

FIGURE 16: SHOOT AND ROOT ORGANOGENESIS AS A FUNCTION OF AUXIN: CYTOKININ RATIO.

medium (Figure 15). Propagation nodules only occurred at kinetin concentrations greater than or equal to 10^{-5} M (Table 14).

Root organogenesis occurred only at the highest auxin concentrations, 10^{-4} and 10^{-3} M IAA, and even then only at the highest auxin/cytokinin ratios (Figures 15 and 16).

b) Physico-chemical and biological response to high IAA concentrations

An interesting, although incidental, observation from the preceding experiment was the appearance, in the 10^{-3} M IAA media, of a photochemical product which vigorously stimulated root growth.

In the 10^{-3} M IAA cultures of the preceding experiment, a brown band formed in the medium, starting 7 to 8 mm below the surface of the agar and extending for 15 mm through the medium (Figure 17). The brown band had a gradient of colour intensity: it was deepest brown at the top and just barely visible 15 mm lower. The intensity of colour was also inversely related to the kinetin concentration; the lower the kinetin concentration, the deeper the brown colour (Figure 18a).

The brown band appeared to act as a root stimulator, in that there was vigorous root growth and extensive branching within the limits of the brown band (Figure 18b,c,d). As the roots approached the lower limits of the concentration of brown product there was an apparent inhibition of root growth in that none of the roots extended beyond the limits of brown colour and

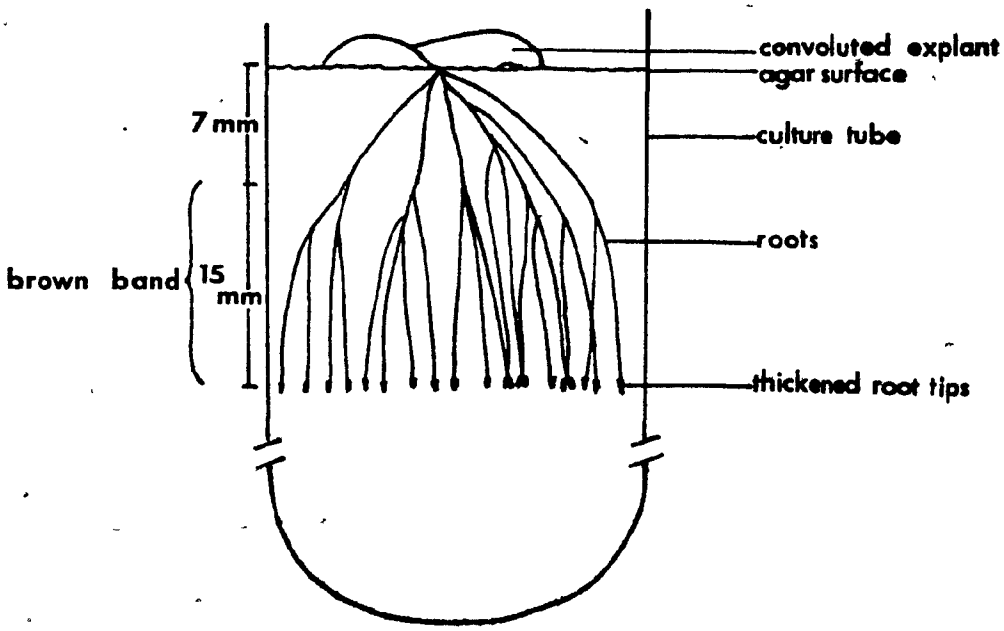


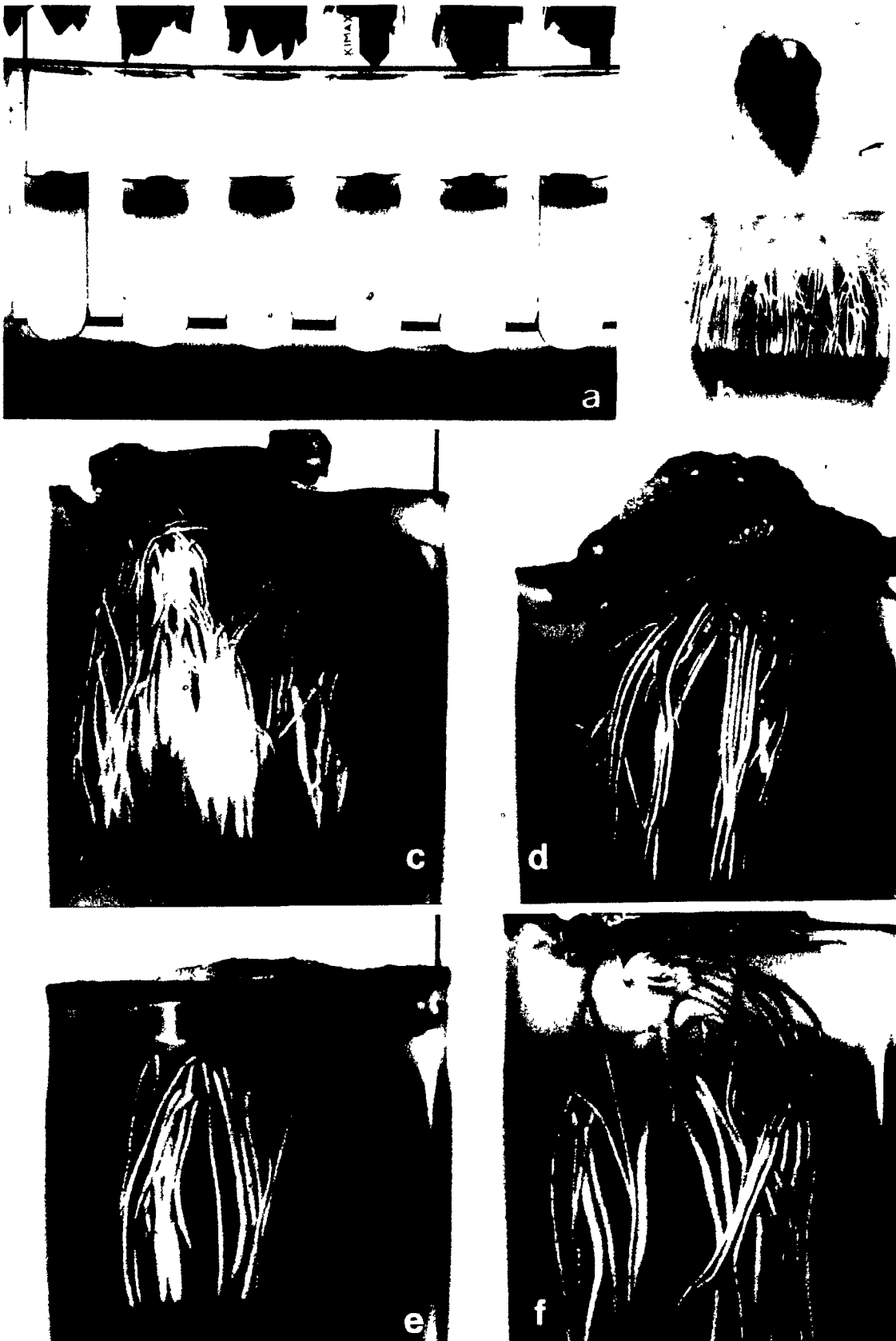
Figure 17: Diagram of root growth from tobacco leaf disk in relation to brown photochemical product appearing in the 10^{-3} molar IAA media.

Figure 18: Tobacco leaf disks cultured on 10^{-3} molar IAA media. Figure 18a represents 1 week cultures, and 18b to 18f represent 4 week cultures.

a) The appearance of the brown band in 10^{-3} M IAA media after 1 week of culture. The band was only a few mm wide and deep brown in colour. The culture tubes represent, from left to right, duplicates containing 10^{-3}, 10^{-4} and 10^{-5} M kinetin. The intensity of colour in the band increased with decreasing kinetin concentrations. (0.4x)

b) to d) Examples of extensive root growth and branching within the brown band (band not clearly reproduced by black and white photography) in media containing 10^{-3} M IAA and, respectively, 10^{-8}, 10^{-7} and 10^{-6} M kinetin. (b: 1.4x; c and d: 2.7x)

e) and f) Examples of root growth with thickened root tips in media containing 10^{-3} M IAA plus, respectively, 10^{-7} and 10^{-8} M kinetin. (e: 2.7x; f: 2.8x)


a
b
c
d
e
f

consequently, all the roots were unusually uniform in length (Figure 18, b to f). Also, the root tips were abnormally thickened, often an indication of inhibition of root growth (Figure 18e,f).

To ascertain whether this brown band was a consequence of photochemical, physico-chemical, or biological processes an experiment was performed in which 10^{-3} M IAA media in combination with various kinetin concentrations (0, 10^{-8}, 10^{-6}, 10^{-4} M) were cultured under total light or total dark conditions, with and without leaf disk explants. The results are reported in Table 15.

These results indicated that the brown bands were a consequence of a photochemical process and were definitely not due to the explants. The experiment also confirmed the observation that the intensity of brown colour was related to the kinetin concentration (as evaluated by eye, the brown bands in the kinetin-free medium were slightly darker than those in 10^{-4} M kinetin medium).

The results from this experiment also indicated that both light (possibly due to the brown photochemical product) and kinetin were required for root organogenesis. No roots were formed in the dark on any of the media tested (Table 15). Also, no roots formed on the medium without kinetin, but did form on the media with 10^{-8} and 10^{-6} M kinetin (Table 15).

The photochemical product, likely related to indole, was not identified. The following type of physico-chemical process

Regime	0 M kinetin	10^{-8} M kinetin	10^{-6} M kinetin	10^{-4} M kinetin
with explants				
Light	-explants viable but no growth -brown band	-callus + roots -brown band	-callus + roots -brown band	-vegetative shoots -brown band
Dark	-explant necrosis -no band	-explant necrosis -no band	-some callus -no band	-explant necrosis --no band
without explants				
Light	-brown band	-brown band	-brown band	-brown band
Dark	-no band	-no band	-no band	-no band

Table 15: The Physico-chemical and Biological Effects of 10^{-3} M IAA.

The physico-chemical effect of 10^{-3} M IAA on media at various concentrations of kinetin in light and dark regimes, with and without the presence of explants. The biological effect of IAA on leaf disk explants also noted. 10 replicates per treatment.

would explain this phenomenon: The brown band could be the result of a photochemical, partial oxidation reaction requiring a specific oxygen concentration. This photochemical product is diffusible through the agar but is bleached at higher oxygen concentrations as found near the surface of the agar. This would explain why the band appeared as a gradient of decreasing concentration of brown product and why there was no coloured product above the band (in the top 7 mm of agar).

As this was simply an incidental observation in the course of the research for this thesis, no further efforts were made to elucidate the brown band phenomenon and this subject will not be referred to again in this thesis.

c) Callus production from leaf disks

Various media were tested in which the cytokinin (kinetin) and auxin (IAA or 2,4-D) concentrations were varied in order to obtain vigorous callus production from leaf disks of tobacco.

Results previously reported in this thesis indicated that 10^{-5} M IAA in combination with 10^{-6} M kinetin was the best callus producing medium when using IAA as the auxin. Therefore, this medium was tested against a series of media using 2,4-D as the auxin. The concentrations tested were 10^{-6} M, 5×10^{-6} M and 10^{-5} M for 2,4-D, and 10^{-7} and 10^{-6} M for kinetin. There were 18 replicates for each auxin-cytokinin combination and the cultures were grown for 39 days.

The results for this experiment are summarized on page 117.

10^{-5} M IAA / 10^{-6} M kinetin: This was the control medium for the experiment. 100% of the explants produced callus, but the callus growth was not extensive. The callus was generally about 3 mm in thickness, growing along a few scattered sections of the explant edge.

90% of the explants on this medium showed either shoot or root organogenesis, after 39 days of culture. The same medium in the previous experiment had shown organogenesis in only 10% of the cultures, after 28 days of culture. Thus, it is possible that a longer culture period leads to an increase in the incidence of organogenesis.

The explants with the greatest degree of callus growth also showed the greatest degree of vegetative growth. The callus tissue was brownish with the edges of each callus tuft necrosed.

10^{-6} M 2,4-D / 10^{-7} M kinetin: 100% of the explants produced callus tissue which was greenish-brown and friable. The explants were pale green, convoluted, and lined with 7 to 8 mm of callus tissue. There were some dense, pale green and whitish tissue clumps in the callus, as well as small white, elongated projections associated with fine hairs.

5×10^{-6} M 2,4-D / 10^{-7} M kinetin: 100% of the explants produced small tufts of callus tissue along the explant edges, but the callus tissue was totally necrosed by 39 days of culture, as was most of the original explant tissue.

10^{-5} M 2,4-D / 10^{-7} M kinetin: As above, except necrosis

was more extensive.

10^{-6} M 2,4-D / 10^{-6} M kinetin: This medium produced the most vigorous callus tissue of all the media tested. 100% of the explants produced vigorous callus growth generally greater than 1.5 cm in thickness. The callus was mostly translucent green and friable, with some pale green or whitish hard, dense areas (much like cauliflower heads in appearance).

The callus tissue also showed some organized growth. This consisted of small translucent or whitish elongated, nob-like projections. These formations were often associated with delicate tufts of white hairs, usually growing from the base of the projections.

5×10^{-6} M 2,4-D / 10^{-6} M kinetin: 100% of the explants produced a thick callus growth about 10 mm thick. However, the callus was deep brown and almost totally necrosed by the time of analysis. The amount of growth was not sufficient for nutrient depletion to be the cause of necrosis. There were some small patches of white callus among the necrosed tissue. Thus, this medium apparently induced a vigorous rate of callus growth, but with premature necrosis.

10^{-5} M 2,4-D / 10^{-6} M kinetin: 100% of the explants produced callus tissue which was deep blackish-brown and necrosed by the time of analysis. The explants (i.e. the leaf disks) were pale yellow, enlarged in diameter, convoluted in shape, and lined with about 5 mm of callus tissue. As in the previous case, the medium induced callus growth, but with premature necrosis.

Summary of callus production from leaf disks

2,4-D was superior to IAA in the production of vigorous, green callus tissue from leaf disks. In testing various concentrations of 2,4-D (10^{-6} M, 5 x 10^{-6} M, 10^{-5} M) and kinetin (10^{-6} M, 10^{-7} M), optimal callus production occurred on medium containing 10^{-6} M concentrations of both 2,4-D and kinetin. Higher concentrations of 2,4-D induced callus growth, but with early necrosis of both the explant and callus tissues.

The callus produced on 10^{-6} M 2,4-D media showed some potential for organized growth. The organogenetic structures consisted of small nob-like projections usually associated with tufts of white hairs. These structures were thought to be root apices.

iii. Descriptions of leaf disk response on optimal nodule- and shoot-producing media

Two media were selected for all subsequent experimentation reported in this thesis. These were chosen from the previously reported experiment on leaf disk response to various IAA and kinetin concentrations. The selected media were: the optimal medium for producing propagation nodules and microshoots, and the optimal medium for producing vegetative shoots. The IAA concentration in both media was 10^{-5} M; the concentration of kinetin was 10^{-4} M in the nodule-producing medium and 10^{-5} M in the shoot-producing medium.

Since the two media were used in all subsequent research for this thesis, it is appropriate at this point to give a more detailed description of explant morphology, after culture on these media, than that previously offered in Section IV-2-ii-a of this thesis.

Culture response on nodule-producing medium: Figure 19 shows two typical leaf disks after four weeks of culture on the nodule-producing medium (10^{-4} M kinetin / 10^{-5} M IAA). The main characteristic of leaf disks cultured on this medium was the great number of propagation nodules formed along the disk periphery and on the underside (Figure 19,b to e). The nodules were chlorophyllous, organized structures. The peripheral nodules were mainly of two basic types: smaller, green, translucent nodules, and larger, whitish-green, opaque nodules which often had epidermal hairs. Cross-sections of the latter type of nodule under the dissecting microscope revealed that the nodule interior was composed of green, translucent cells, while the exterior was composed of whitish-green cells that were loose and friable in appearance.

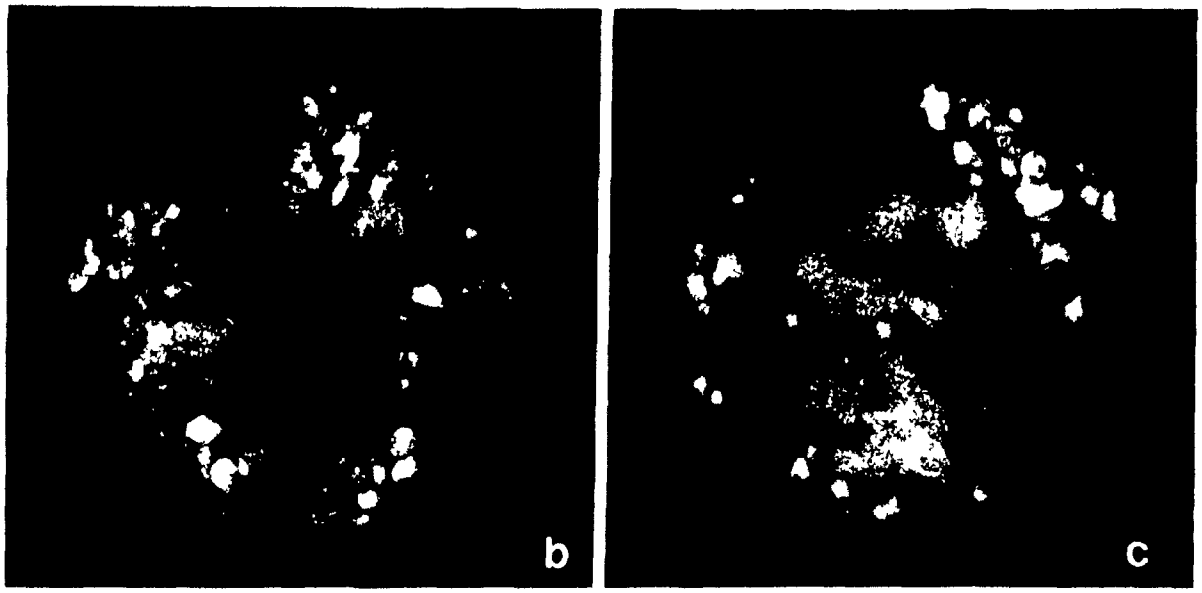
The nodules on the underside of the disk (i.e. in contact with the agar medium) were apparently randomly scattered over the disk surface (Figure 19c,e) in that the nodules were neither associated with vascular bundles in the leaf disk nor polarly located. These nodules were generally larger than peripheral nodules and had whitish or brownish exteriors. The nodules on the disk underside seldom developed shoot buds. The ones that did show differentiation of apices were those exposed to air and

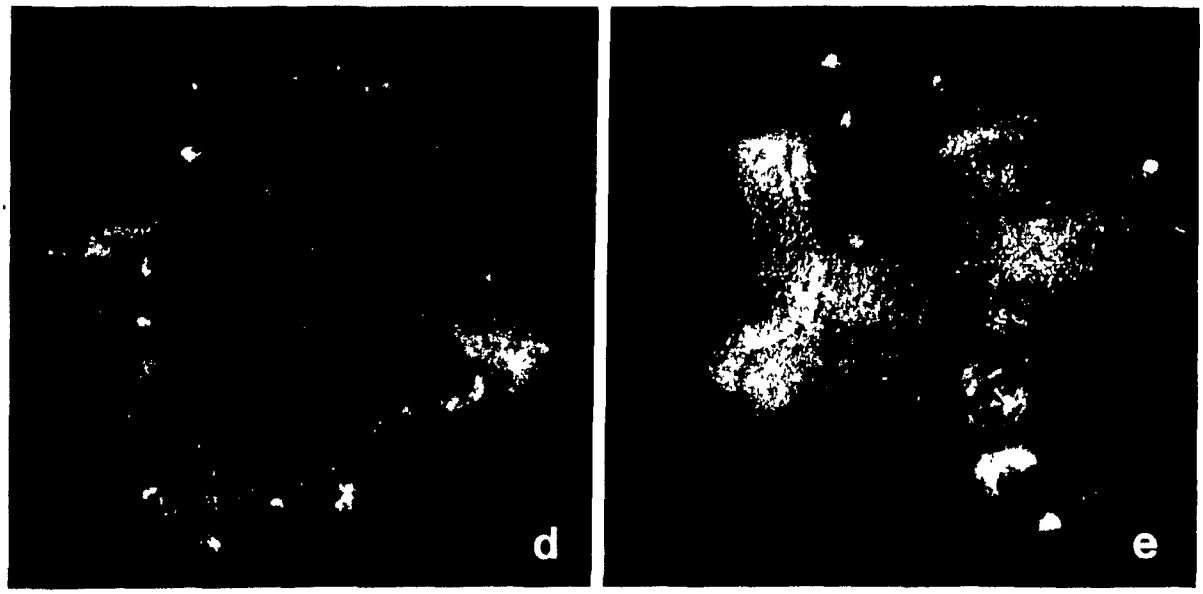
Figure 19: Two typical leaf disk explants after four weeks of culture on nodule-producing medium (10^{-4} M kinetin / 10^{-5} M IAA). Figures 19a to c represent one explant; 19 d and e, the second.

a) An in situ view. (3.7x)

b) and d) The top (adaxial) surface of the explant. (b: 2.6x; d: 3.3x)

c) and e) The bottom (abaxial) surface of the explant. (c: 2.6x; e: 3.3x)


b
c


d
e

were greener than most of the underside nodules.

There were several hundred nodules and about 50 to 100 shoot buds per explant. The shoot buds (shoots and shoot apices) always arose from propagation nodules and never directly from the leaf disk. Propagation nodules also gave rise to other propagation nodules.

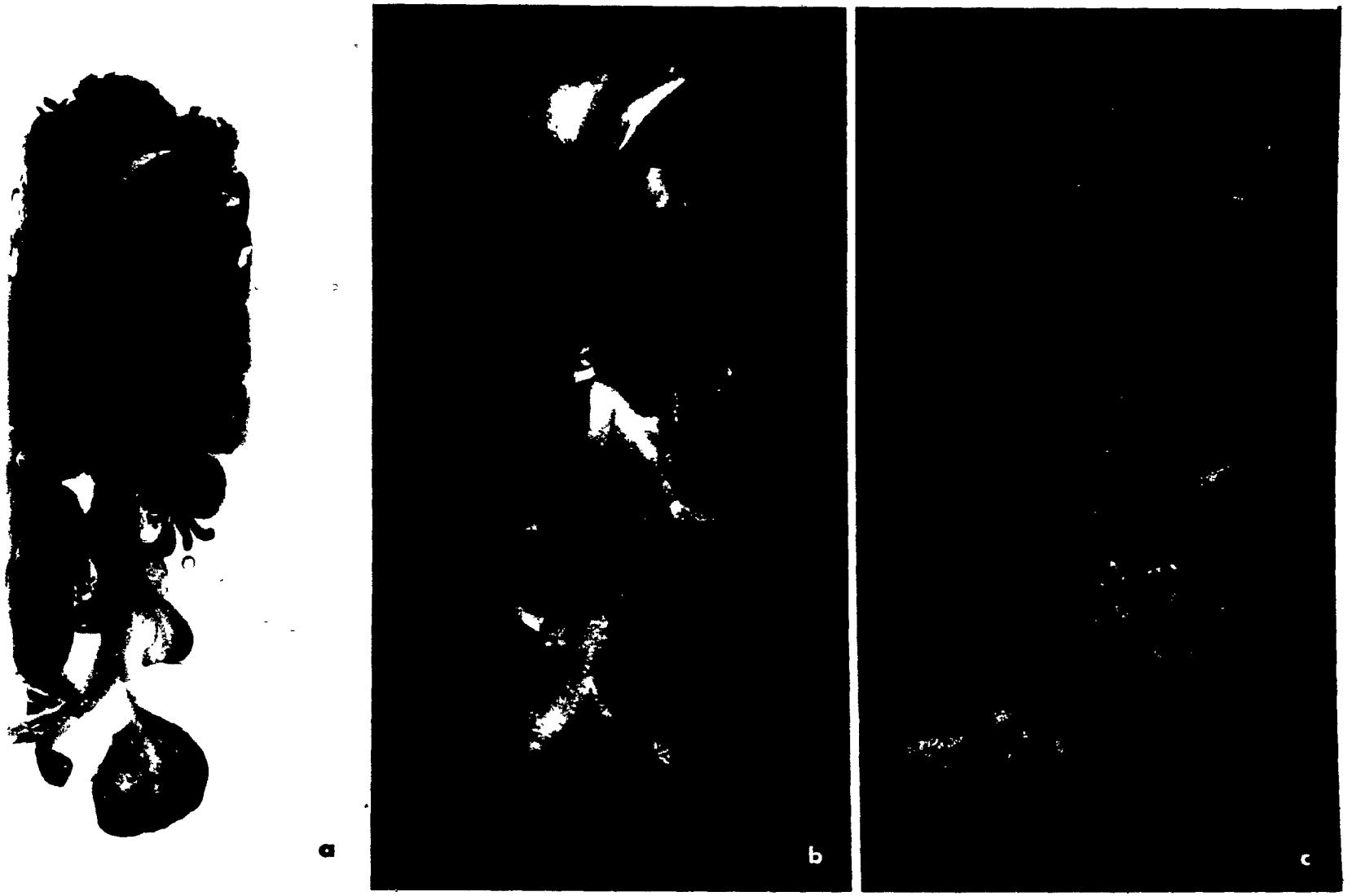
The diameters of the leaf disks increased in culture by some 60 to 70%. The disks also became convoluted in shape, as shown in Figure 19. All the original explant tissue and all de novo tissue were chlorophyllous, although a slightly paler green in the center of the disk than at the periphery.

Culture response on shoot-producing medium: Figure 20 shows two typical leaf disks after four weeks of culture on the shoot-producing medium (10^{-5} M kinetin / 10^{-5} M IAA). As seen in Figure 20, this medium produced vigorous shoot organogenesis. Hundreds (500 plus) of chlorophyllous shoots were formed per explant. Most of the shoots were only a few millimeters in height with three to four leaves per shoot. However, on each explant there were ten to twenty shoots greater than a centimeter in length. These larger shoots had up to 15 well formed leaves and were up to three centimeters in length. Most of the de novo growth on this medium arose from the abaxial surface of the leaf disk and was oriented down into the agar medium (Figure 20a). The de novo growth was very dense, filling 3 to 4 centimeters of culture tube space. Some of the shoot tissue was callus-like in quality, especially in the central portions of the culture mass.

Figure 20: Two typical leaf disk explants after four weeks of culture on shoot-producing medium (10^{-5} kinetin / 10^{-5} M IAA). Figures 20a and b represent one explant; 20c the second.

a) An *in situ* view. (1.6x)

b) and c) Side views of the explants removed from the culture tubes. (b: 1.7x; c: 2.0x)


a
b
c

The callus-like quality apparent by four weeks of culture (Figure 20b,c) was not apparent at earlier stages when the de novo growth was not yet so dense in the culture tubes.

Shoot bearing nodules were evident although the density of growth obscured most of the origins of organogenesis.

The leaf disks increased in size during culture. However, accurate measurements of the extent of the increase could not be made because of the density of de novo growth which displaced much of the original explant tissue. The disks were deep green and convoluted.

iv. Analysis of plant imposed factors affecting organogenesis

Several experiments were performed to determine the extent to which plant imposed factors affected organogenesis in the leaf disk system. These experiments tested the effect of excision locus, leaf position, orientation of the disks in culture, and developmental stage of the donor plant on the organogenetic response of tobacco leaf disks cultured on nodule-producing medium (10^{-4} M kinetin) and on shoot-producing medium (10^{-5} M kinetin), as described in the previous section.

The definitive experiments are reported below. All results describing leaf disk response to a particular experimental condition represent essentially homogenous results occurring in 100% of the leaf disk cultures. Therefore, statistical substantiation was not required.

a) Effect of explant excision locus

The effect of excision locus on the organogenetic response was tested by culturing leaf disks excised from four specific regions of the tobacco leaf, as shown in Figure 21. The leaf lamina was sectioned into a rectangular shape excluding the petiolar and leaf tip regions. This rectangle was then evenly divided in thirds, sequentially identified from leaf tip to base as regions A, B, and C. The side margins of the leaf were identified as region D. As always, the disks were excised from leaf tissue avoiding main vascular bundles.

Basically, the experimentation on excision locus was done in two parts which were chronologically separated. The first part of the experimentation tested the effect of excision locus at various early floral stages of the donor tobacco plant (this being the only plant material available at the time). The floral stages used were: 1) early floral bud, in which the floral apex of the tobacco plant was only detectable through microdissection, 2) floral bud, in which the floral apex had a terminal closed flower bud, and 3) full bloom, in which the floral apex had a flower in bloom. For this part of the experimentation only the nodule-producing medium was used. Explants were excised from leaf #13 of each donor plant and there were 18 replicates per explant type.

The second part of the experimentation used green-fruit donor plants, in which the terminal flower was in the process of

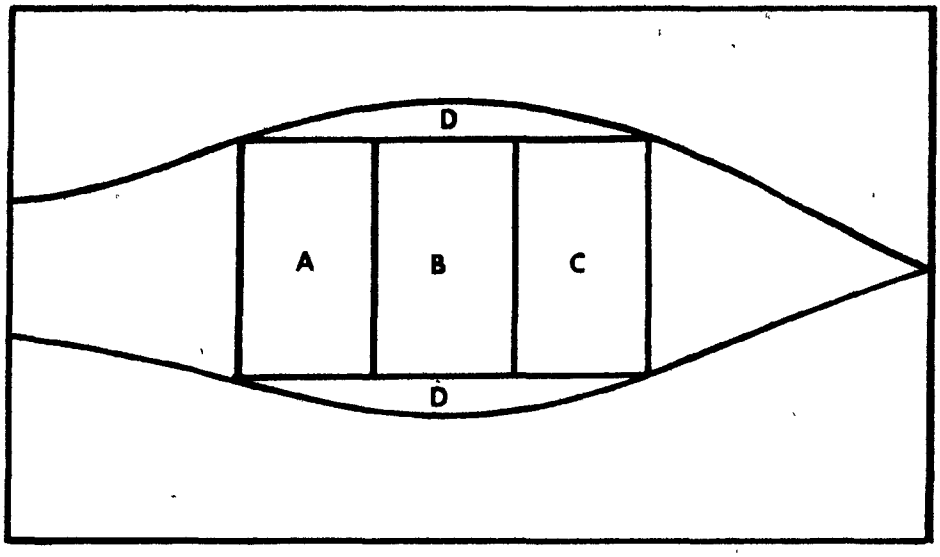


FIGURE 21

Excision Regions of the Tobacco Leaf

Diagram of the four excision regions (A,B,C,D) used in experiments testing the effects of excision locus on the organogenetic response of cultured tobacco leaf disks.

fructification. The green-fruit stage was the developmental stage used in the experiments on hormonal regulation of organogenesis (Section IV-2-i of this thesis) and thus was required for valid comparisons with previous work.

In this experiment, both the nodule-producing and shoot-producing media were tested. Explants were excised from leaves #24 to 27, since these leaves were of a comparable size and green colour to the leaves from the donor plants of earlier floral stages which had been used in the first part of this experimentation.

Results: The results from these experiments revealed that the excision locus on the donor leaf had no effect on the organogenetic response of the leaf disks. On nodule-producing medium, explants from all the developmental stages tested (i.e. green-fruit, full bloom, floral bud, and early floral bud) produced the same organogenetic response in culture regardless of excision locus. Similarly, on shoot-producing medium, there was no difference in the culture response of leaf disks from the four excision loci on the tobacco leaf. In fact, in all cases the morphogenetic response was entirely typical for the media, as described in Section IV-2-iii of this thesis.

Fresh weight analysis of the explants cultured on nodule-producing medium revealed a greater number of cultures from the marginal excision region of the leaf (region D, Figure 21) with fresh weights more than two standard deviations below the mean for the developmental stage. No attempt was made to

statistically correlate growth rates with excision locus, since several experimental variables were implicated. The standard procedure for sterilizing the plant tissue, in which 6 x 6 cm leaf pieces are consecutively sterilized by alcohol and bleach solutions (see Materials and Methods), was not applicable in the case of marginal tissue. As can be seen in Figure 21, the marginal region (D) of the tobacco leaf was much narrower than the interior regions (A,B, and C) as defined for these experiments. Thus, possibly lower growth rates in explants from the leaf margin could well be attributed to the effects of alcohol and/or bleach penetration during sterilization, rather than to intrinsic tissue factors associated with locus. In any case, the use of disks from the leaf margin was avoided in subsequent experiments.

b) Effect of leaf position

The effect of leaf position was tested by culturing leaf disks from three different regions along the stem of green-fruit stage tobacco plants. These regions corresponded to the floral, subfloral, and middle explanting regions, as defined for the epidermal explant system reported in Section IV-1-i of this thesis. (Note: The basal leaves could not be used for explanting due to partial or total senescence of the leaf tissue.) Explants were cultured for 4 weeks on both nodule-producing and shoot-producing media (10^{-4} and 10^{-5} M kinetin, respectively).

There were 18 replicates per experimental condition.

Results: On both media there were no differences in the organogenetic response of explants from leaves of the three defined regions along the tobacco stem. The response was typical of the medium in each case, as described in Section IV-2-iii of this thesis, and not related to leaf position.

In other words, there was no gradient of organogenetic response in the leaf disk system, as seen in the epidermal explant system for Nicotiana tabacum.

c) Effect of disk orientation

The effect of disk orientation on the organogenetic response of leaf disks was tested by culturing leaf disks with either their adaxial or abaxial surfaces in contact with the agar medium. Both nodule- and shoot-producing media were used in these experiments; for each medium 36 replicates were plated with adaxial contact, and 18 control replicates were plated with abaxial contact (the standard for all previous experimentation). The explants were excised from leaves #16 to 24 of a green-fruit tobacco plant with 34 nodes.

Results on shoot-producing medium: There was no apparent difference in the organogenetic response of the leaf disks whether they were cultured with adaxial or abaxial contact with the medium (Figure 22a,b). The test explants, plated with adaxial contact, showed the same type and amount of shoot

production as the controls, which were plated with abaxial contact. Thus, both sides of the leaf disk were equally responsive to the 10^{-5} molar kinetin medium. In both cases, growth was predominantly from the disk edges and underside (whether it be the adaxial or abaxial surface). Also, in both cases, the response was entirely typical for the shoot-producing medium as previously described in Section IV-2-iii.

Results on nodule-producing medium: On 10^{-4} M kinetin medium, in contrast to the above, disk orientation did influence the culture response (Figure 22c to g). The response of the control explants, which had abaxial contact with the agar medium, was typical of the organogenetic response induced by the nodule producing medium (Figure 22d,e) as previously described in Section IV-2-iii.

The test explants, plated so that the adaxial surface had contact with the agar medium, produced comparably vigorous organogenesis on the lower side but no organogenesis or other form of growth on the upper side (Figure 22f,g). The leaf disks were rolled up as shown in Figure 22c, which resulted in most of the bottom side being removed from medium contact. The adaxial nodules in most direct contact with the agar medium were brownish (that is, the superficial tissue was slightly necrosed) and had very few apical centers (Figure 22g). The rest of the adaxial nodules, which were not in direct contact with the medium due to the rolled conformation of the leaf disks, were green and often bore small leafy shoots a few millimeters high (Figure 22f).

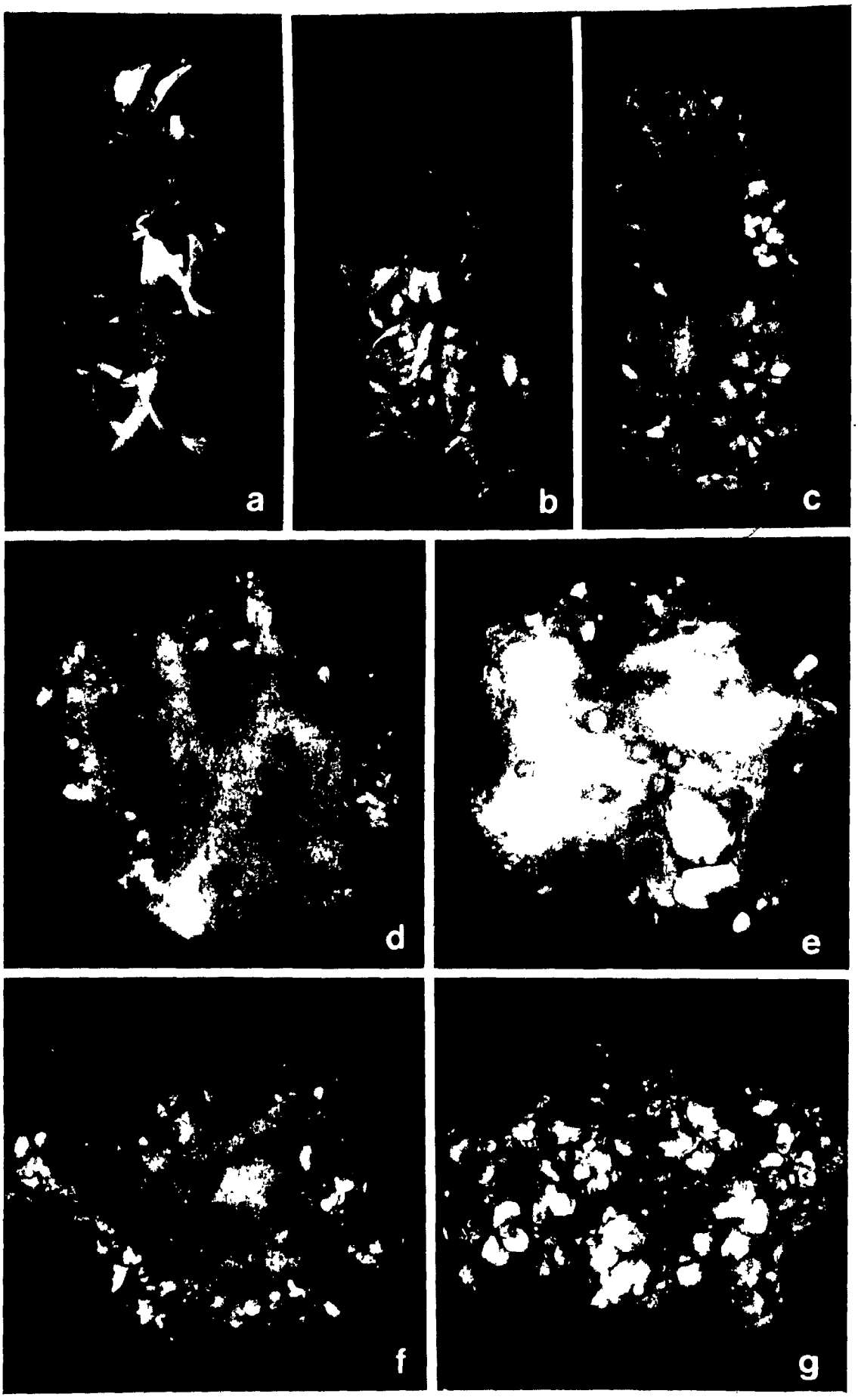
Figure 22: The effect of disk orientation on the organogenetic response of leaf disks cultured with either their abaxial or adaxial surfaces in contact with the agar medium.

a) and b) Leaf disk explants cultured on shoot-producing medium; a) with abaxial contact, b) with adaxial contact. (1.1x)

c) The rolled conformation typical of leaf disk explants cultured with adaxial contact on nodule-producing medium (3.8x)

d) and e) A leaf disk explant cultured with abaxial contact on nodule-producing medium; d) top (adaxial) surface, e) bottom (abaxial) surface. (3.1x)

f) and g) A leaf disk explant cultured with adaxial contact on nodule-producing medium; f) top (abaxial) surface, g) bottom (adaxial surface. (3.1x)


a
b
c
d
e
f
g

The organogenetic growth from the disk underside was more extensive than organogenesis on the control explants; that is, there were more nodules, more shoots, and more advanced shoot development. There was also some organized growth along the disk edges, but these nodules were smaller and bore fewer and less developed shoot apices than edge nodules on the control explants.

Summary: The results from this experiment are summarized in Table 16. The orientation of the leaf disk on the agar medium only affected the culture response on nodule-producing medium. There was no morphogenetic effect on the shoot-producing medium. On the latter medium, organogenesis occurred on the surface having the most immediate contact with the agar medium, and both sides of the leaf disk were equally responsive. Thus, the orientation of the disk was irrelevant to morphogenetic response on the shoot-producing medium.

On the nodule-producing medium organogenesis also appeared on the surface having the most immediate medium contact, but the abaxial and adaxial surfaces of the leaf disk were not equally responsive. On this medium, more numerous and developed organogenetic structures appeared when the adaxial side of the disk was in contact with the medium. Moreover, there was a difference in the production of peripheral nodules related to disk orientation; more edge nodules occurred on disks plated abaxially down.

response characteristic	nodule-producing medium		shoot-producing medium	
	control	test	control	test
	abaxial contact	adaxial contact	abaxial contact	adaxial contact
organogenesis on upper disk surface	none	none	none	none
organogenesis on bottom disk surface (medium contact)	some	much	much	much
organogenesis on disk edge	much	some	some	some
predominant type of organogenesis	nodules	nodules	shoots	shoots
relative degree of development	+	++	++++	++++
relative amount of de novo growth	+	++	++++	++++

Table 16: Summary of Disk Orientation Results

A summary of qualitative results from the experiment on leaf disk orientation in culture. Tobacco leaf disks were cultured for 4 weeks on nodule-producing medium and shoot-producing medium, with either the abaxial or adaxial surfaces in contact with the agar medium. '+' signs indicate relative quantities.

d) Effect of developmental stage of the donor plant

The possible effect of developmental stage of the donor plant on the culture response of leaf disks was tested in two experiments in which leaf disk cultures from plants of various developmental stages were compared with control cultures from green-fruit stage tobacco plants. The developmental stages tested were three different floral stages and a vegetative stage. The definitive experiments on developmental stage are as follows:

Leaf disks from floral stages of tobacco: In this experiment, aspects of which were previously reported since it was also designed to investigate the effect of excision locus (see Section IV-2-iv-a), the organogenetic response of leaf disks from tobacco plants at various floral stages was compared with the response of disks from green-fruit tobacco plants. The floral stages tested were early floral bud, floral bud, and full bloom. Three plants of each developmental stage were used for explanting. The plants were carefully chosen for developmental similarity. In this experiment only nodule-producing medium was used.

Results: The morphogenetic response of leaf disks from the various floral stages were comparable, and were consistent with the response of the controls. The response was typical of leaf disks cultured on nodule-producing medium, as previously described (Section IV-2-iii). Thus, development of the floral apex from shortly after floral induction to fruiting had no

apparent effect on the culture response.

Leaf disks from a vegetative tobacco plant: The culture response of leaf disks from a totally vegetative tobacco plant was tested against control explants from a green-fruit plant. The vegetative plant had a total of 29 nodes; leaves #11 to 17 were used for explanting. The apex of the plant was dissected to confirm that the plant was vegetative. This particular stage of vegetative tobacco plant was chosen because it had leaves of a size comparable with leaves of the green-fruit control plant. Both nodule- and shoot-producing media were used. For each medium there were 36 test and 18 control explants.

Results: As before, the morphogenetic response of test and control explants were comparable. It was impossible to distinguish between control and test explants cultured on shoot-producing medium. The culture response was typical of explants on this medium, as previously described (Section IV-2-iii). On nodule-producing medium there were minor differences apparent between the control and test explants. Some of the explants from the vegetative tobacco plant were paler green and more convoluted than the control explants from the green-fruit plant. The test explants also had fewer nodules and less shoot growth. These differences are pictured in Figure 23. However, in many cases on this medium it was impossible to distinguish between control (green-fruit) and test (vegetative) explants. Thus, in spite of the minor differences observed, essentially the same type and

Figure 23: The effect of the developmental stage of the donor plant on the culture response of leaf disks from green-fruit stage and vegetative tobacco plants, when cultured on nodule-producing medium.

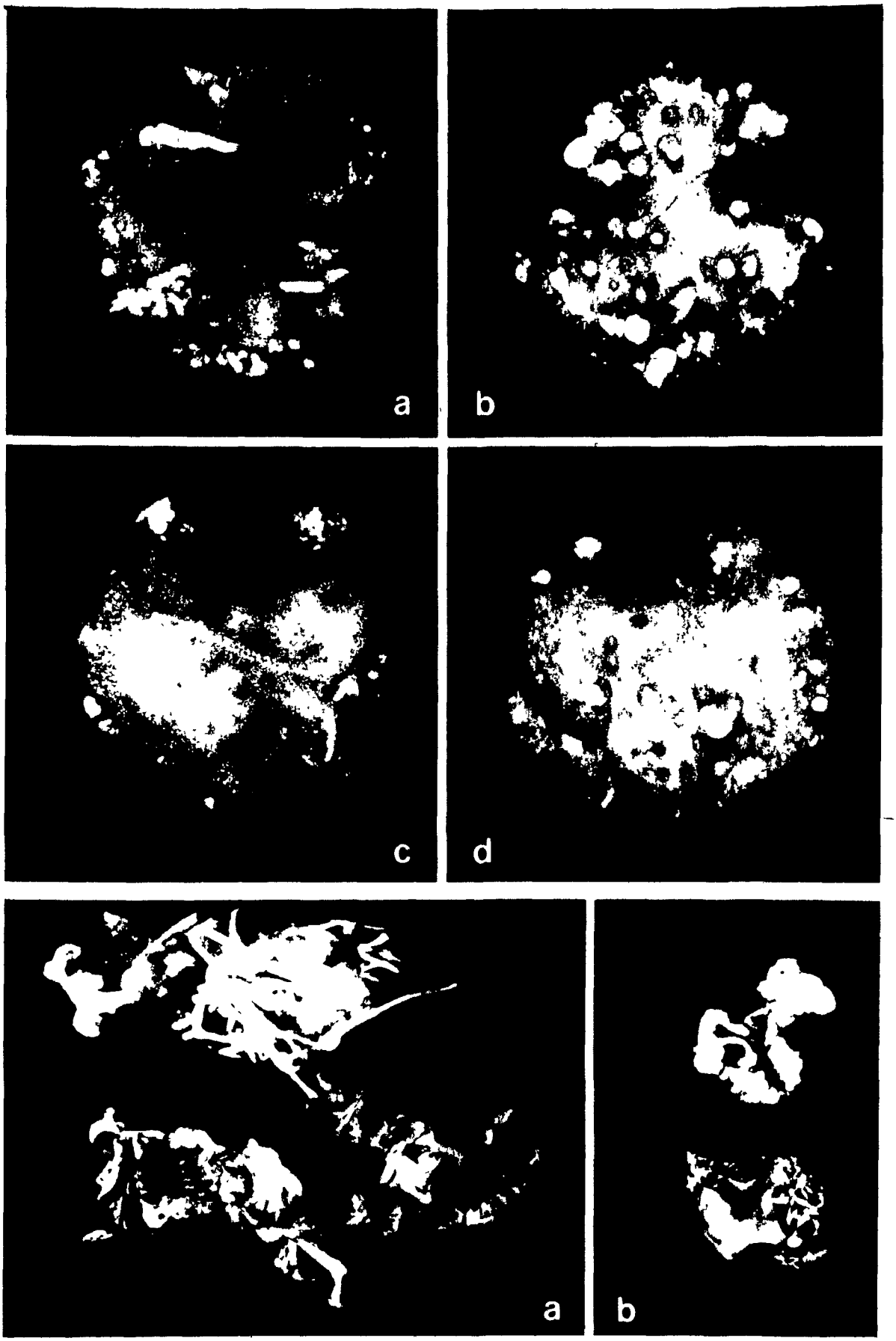
a) and b) A leaf disk explant from a green-fruit stage plant; a) the top (adaxial) surface, b) the bottom (abaxial) surface. (2.4x)

c) and d) A leaf disk explant from a vegetative tobacco plant; c) the top (adaxial) surface, d) the bottom (abaxial) surface. (2.4x)

Figure 24: Dark and light grown leaf disk cultures.

a) Leaf disk explants cultured for six weeks on shoot-producing medium in either total darkness (top) or total light (bottom). 1.0x)

b) Leaf disk explants cultured for six weeks on nodule-producing medium in either total darkness (top) or total light (bottom). (1.0x)


a
b
c
d
a
b

pattern of organogenesis resulted on explants from green-fruit and vegetative stage tobacco plants.

Summary: The developmental stage of the donor tobacco plant, from vegetative to green fruit, did not influence the organogenetic response of leaf disks cultured on either nodule- or shoot-producing media.

e) Summary of analyses of plant imposed factors affecting organogenesis

In summary, there was a large degree of intrinsic homogeneity in the organogenetic response of tobacco leaf disks cultured on nodule- and shoot-producing media (respectively, 10^{-4} M kinetin / 10^{-5} M IAA and 10^{-5} M kinetin / 10^{-5} M IAA). No effects were observed due to excision locus, leaf position, or developmental stage of the donor plant. In each case, the type, origin, and distribution of organogenetic structures were similar. While the orientation of the leaf disk on the agar medium influenced morphogenesis of the explants on nodule-producing medium (though not on shoot-producing medium), the types of organogenetic structures were still similar to the organogenetic response typical of that medium. The difference in response evoked by disk orientation was not a difference in organogenetic processes (e.g. nodule to shoot formation) but rather a difference in organogenetic responsiveness between the adaxial and abaxial surfaces.

Thus, plant imposed factors had little effect on the regulation of organogenesis in the leaf disk system as it is defined in this research.

v. Light requirement for the induction and development of organogenesis

A number of experiments were performed to assess the light requirement for organogenesis on cultured leaf disks.

To test the light requirement for organogenetic development, organogenesis on leaf disks was initiated in the light and then transferred to the dark. Specifically, the explants received 3 weeks of the light regime (shoot apices just apparent) followed by 18 days of dark regime. In this experiment only nodule-producing medium was used. There were 36 test explants and 18 light-maintained control explants.

To test the light requirement for organogenetic induction, leaf disks were placed directly into the dark regime and cultured for 4 weeks. Again only the nodule-producing medium was used. There were 72 test explants and 36 light-maintained control explants.

A third experiment was performed to confirm the results from the previous two experiments, as well as to extend the observations to shoot-producing medium. Therefore, in this experiment both nodule-producing and shoot-producing media were used. Leaf disks were placed directly into the light (control)

or dark (test) regime and cultured for about 6 weeks. There were 9 replicates per experimental condition.

In all three experiments, explants were derived from green-fruit tobacco plants, and, other than the light regime, all culture conditions were identical.

Results: The results from these experiments showed that light was definitely not required for either the induction or development of organogenesis on leaf disks cultured on either nodule- or shoot-producing medium (Figure 24). As always in the leaf disk system, 100% of the leaf disk cultures in each experimental condition produced an homogenous response indicative of that condition. On both media, the culture response in the dark regime was similar to that of the light grown controls. Organogenesis in the form of nodules and shoots was initiated even in dark conditions. Also, the cultures developed to an extent quite comparable with the light grown controls, as seen in Figure 24. Most of the morphological differences apparent in the dark grown cultures were those typical of light deficiency. All de novo growth in the dark was achlorophyllous and the shoots were very etiolated in cultures grown on shoot-producing medium.

Apart from etiolation and lack of chlorophyll, the dark grown cultures on nodule-producing medium produced larger and more numerous nodules than the light grown controls and less shoot development (Figure 24b). In other words, dark conditions promoted nodule growth but retarded shoot growth. The dark grown cultures on shoot-producing medium produced more callus

growth and less leaf development than the light grown controls (Figure 24a). In this case, the dark conditions promoted callus growth and retarded leaf growth. In the experiment where light induced cultures were transferred to dark conditions, some abnormal morphology was evident. The most common abnormal structure was a shoot apex on an elongated base, much like a celery stalk in appearance.

In summary, the results demonstrated conclusively that light was not an absolute requirement for induction and development of organogenesis in the leaf disk system, although light was required for optimal de novo growth (i.e. the production of healthy, normal shoots).

vi. System subculture potential

The subculturing potential of the leaf disk system was tested by subculturing leaf disk cultures on both nodule- and shoot-producing media for respectively four and two passages, and by testing the organogenetic capacity of the subcultures on nodule-producing medium at both the second and third passages.

One aim of these experiments was to determine whether propagation nodules could be selectively passaged with retention of organogenetic capacity in order to 1) increase the cloning potential of the leaf disk system and 2) assess the possibility of conveniently generating cultures through subculture to be used in studies on in vitro regulation of organogenesis thus

eliminating or reducing the constant need for growing tobacco plants. The second aim of these experiments was to determine whether the highly organogenetic cultures produced on the shoot-producing medium could be passaged with retention of organogenetic capacity, for reasons as previously stated.

The definitive experiments and results are described below.

a) Propagation through subculture

To test the subculture potential of the leaf disk system; disks were excised from leaves of green-fruit tobacco plants and cultured on either nodule-producing or shoot-producing medium, under standard culture conditions (see Materials and Methods). After four weeks the cultures were subdivided into 9 sections each, and each section plated onto medium of the appropriate type and cultured for four weeks. This process was repeated two more times for the subcultures on nodule-producing medium. Thus, there were a total of two passages on shoot-producing medium and four passages on nodule-producing medium. There were 36 replicate cultures per passage per medium. All the cultures were visually examined and representative cultures were removed for dissection after each passage.

Results on nodule-producing medium: The leaf disk cultures which were initiated and maintained on nodule-producing medium were successfully cultured for four passages with no decrease in growth rate or in the organogenetic capacity for nodule and

microshoot production.

In the first passage, the leaf disks showed the typical response of producing hundreds of nodules and a large number of microshoots. However, on subculturing (passage #2) the growth rate actually increased and microshoots quantitatively predominated over nodules as the main organogenetic structure (Figure 25, a and b compared with d and g). The subcultures produced hundreds of small leafy shoots (450 to 600 per subculture explant) of uniform size (a few millimeters in height) growing in all directions (Figure 25d,g). Distinct nodules were seldom identified due to the extreme density of growth, but some nodules could be detected in the subcultures. The subcultures easily fell apart into smaller clumps of shoots. These clumps were spherical with shoots radiating in all directions (Figure 25c). While the number of de novo shoots per culture increased with longer culture periods, the shoots never exceeded a few millimeters in size. Passages #3 and 4 were identical in appearance to passage #2, and showed no change in organogenetic development or growth rate (Figure 25c).

Results on shoot-producing medium: Leaf disk cultures initiated on shoot-producing medium produced identical results after subculture on the same medium. There were hundreds of shoots per subculture explant, and the overall morphology was typical of the culture response on this medium (as described in Section IV-2-iii). There was also no change in growth rate upon subculture.

Figure 25: Leaf disk subcultures:

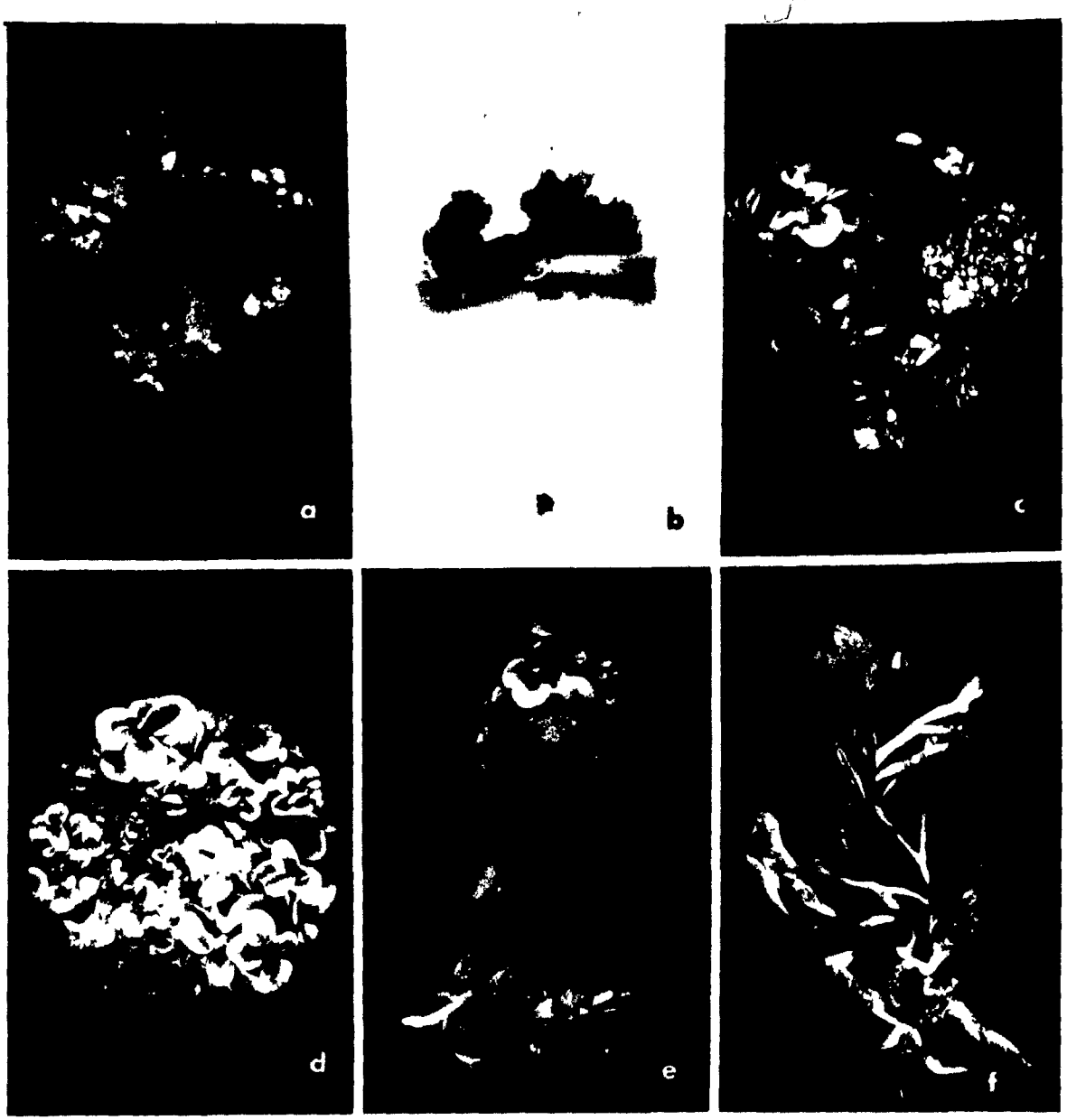
a) and b) Leaf disk explants cultured for 1 passage on nodule-producing medium; a) explant removed from culture tube, top view (1.9x), b) explant *in situ*, side view (1.5x).

c) A leaf disk subculture after 4 passages on nodule-producing medium (1.5x)

d) and g) A leaf disk subculture after 2 passages on nodule-producing medium; d) culture removed from culture tube, top view (1.9x), g) culture *in situ* (0.9x).

e) and h) A leaf disk subculture on shoot-producing medium after 1 prior passage on nodule-producing medium; e) culture removed from culture tube, side view (0.9x), h) culture *in situ* (0.9x).

f) and i) A leaf disk subculture on root-producing medium after 1 prior passage on nodule-producing medium; f) culture removed from culture tube, side view (0.9x), i) culture *in situ* (0.9x).


a
b
c
d
e
f


g
h
i

Further passages on this medium were not attempted due to the success in subculturing on the nodule-producing medium. For the purposes of long term maintenance of leaf disk cultures through repeated subculturing, it appeared much more convenient to do so on nodule-producing medium which produced slower and more uniform growth.

b) Organogenetic capacities in the subcultures

To test the capacity for producing organogenetic structures, other than microshoots and nodules, subcultures from the nodule-producing medium were further subcultured in the second and third passages onto shoot-producing medium and a medium optimal for root production.

The shoot-producing medium was the standard one as defined for this research (10^{-5} M kinetin with 10^{-5} M IAA). The root-producing medium contained 10^{-7} M kinetin with 10^{-3} M IAA (as determined in Section IV-2-ii of this thesis). Subculturing was performed as previously described and each passage represented 4 weeks of culture. There were 36 replicates in each case.

Results: Subcultures passaged onto shoot-producing medium after one or two passages on nodule-producing medium produced cultures identical in appearance to leaf disks plated directly on shoot-producing medium (Figure 25e,h). The subcultures produced vigorous vegetative organogenesis in the form of hundreds of shoots per culture which ranged up to several

centimeters in length. The culture response after either one or two prior passages on nodule-producing medium was entirely typical for the shoot-producing medium as described in Section IV-2-iii.

Subcultures passaged onto root-producing medium after one or two passages on nodule-producing medium produced cultures with greater root production than leaf disks plated directly on this medium. When leaf disks were plated directly on the root-producing medium, 100% produced callus tufts and 53% roots. When subcultures, previously subjected to one passage on nodule-producing medium, were plated on the root medium, 97% of the cultures produced callus, 88% roots, and 83% shoots (Figure 25f, i). When subcultures, previously subjected to two passages on nodule-producing medium, were plated on the root medium, 100% of the cultures produced roots, shoots, and callus.

Thus, there was an apparent increase in organogenetic capacity expressed on a root-producing medium associated with repeated prior subculture on nodule-producing medium.

c) Summary of system subculture potential

The leaf disk system demonstrated a large potential for subculture in that repeated passaging produced no decrease in organogenetic vigour as expressed both by growth rates and organogenetic capacity. In fact, in some instances there were increases in growth rates (e.g. subculture on nodule-producing

medium) and in organogenetic capacities (e.g. as expressed by subcultures on root-producing medium).

vii. Clonal propagation of tobacco

An effective and efficient method of cloning tobacco plants through leaf disk culture was developed both to establish the cloning potential of this system and to ascertain that the system could indeed produce mature, fertile tobacco plants. This method is briefly described below.

Most of the steps in cloning any plant species are quite straightforward once shoot-producing cultures have been established. Therefore, the only step for the cloning procedure which had to be established experimentally for this system was how to best induce roots on the de novo vegetative shoots. Six media were tested in this capacity. The largest vegetative shoots from leaf disk cultures on shoot-producing medium were subcultured onto these six potential rooting media, with one shoot per culture and 36 replicates per medium.

The rooting media were:

1) culture medium with 10^{-3} M IAA and 10^{-5} M kinetin,
2) culture medium with 10^{-4} M IAA and 10^{-5} M kinetin,
3) culture medium with 10^{-3} M IAA and 10^{-6} M kinetin,
4) culture medium with 10^{-4} M IAA and 10^{-6} M kinetin,
5) culture medium without IAA and kinetin, and
6) solidified Hoagland's solution (see Materials and Methods).

Optimal plantlet formation occurred on the culture medium without hormones. 100% of the vegetative shoots developed extensive root systems and the resulting plantlets were healthy and bushy. The other media produced callus growth or de novo shoots (rather than plantlets) or were simply slower in producing plantlets.

An effective method for cloning tobacco plants from leaf disk cultures (as established in this research) was, therefore, as follows:

Step 1: Vegetative shoots were produced on shoot-producing medium (10^{-5} M IAA and kinetin). The culture period was about 3 to 4 weeks.

Step 2: Individual shoots were subcultured onto rooting medium (no IAA or kinetin) to induce root formation. The culture period was about 3 weeks.

Step 3: Plantlets were transplanted to vermiculite and grown for about 2 weeks to establish more extensive root systems.

Step 4: The young plants were transplanted to a soil-vermiculite mixture (as per Materials and Methods). Subsequent growth time until the green-fruit stage was about 5 to 6 weeks.

Thus, the total time elapsed from leaf disk to the green-fruit stage was about 14 weeks, which is comparable with the time required to produce a green-fruit tobacco plant from seed. The clones were definitely capable of seed production.

The cloning potential of this system is enormous. For example, even without subculture multiplication, 5400 clones, by

modest estimates, can be produced from a single tobacco plant (assuming 10 clones per leaf disk, 36 leaf disks per tobacco plant, and 15 potentially usuable leaves per plant). With subculture multiplication, there is essentially infinite cloning potential.

viii. Morphological development in culture

A photographic study was made on the morphological development of leaf disk cultures on nodule- and shoot-producing media during the course of the culture period. In order to produce an appropriate developmental series for morphological analysis, 108 leaf disks were plated onto each medium and 15 harvested from culture every four days and placed in FAA solution. Later the explants were stained with acid fuchsin, dissected, and photographed (see Materials and Methods). The development of cultures on shoot-producing medium was followed for 28 days, while the development on nodule-producing medium was followed for 35 days. A description of the morphological development of cultures on each medium is given below.

a. Developmental series on nodule-producing medium

4 days: After 4 days in culture, no meristematic activity was apparent yet. The edges of the leaf disks were slightly curved upwards, off the agar medium, and were lighter green than the central tissue.

8 days: After 8 days in culture, some small edge nodules were detectable. Early nodules, appearing more like meristematic centers at this stage, were also scattered over the bottom surface of the disks (Figure 27a).

12 days: After 12 days in culture, edge nodules were more frequent, both right on the disk edge and on the outer millimeter of the disk's upper surface (Figure 26a). The nodules occurring on central portions of the bottom surface were larger and more numerous than at 8 days (Figure 27b). The leaf disks were now somewhat convoluted in shape.

16 days: After 16 days in culture, the edge nodules were larger and more numerous, so that the disk periphery was now lined with propagation nodules. The first few shoot apices (less than 5 per leaf disk) were just becoming apparent on the edge nodules (Figure 26b). The nodules on the bottom surface were also larger in size (Figure 27c), but otherwise showed no distinguishing characteristic. The leaf disks were even more convoluted than at 12 days.

20 days: After 20 days in culture, the leaf disk explants were now extremely convoluted in shape with up to a centimeter's rise in the convolution. The edge nodules were larger and so numerous that indiviudal nodules could seldom be distinguished (Figure 26c). Many shoot apices were apparent on the edge nodules. The nodules on the bottom surface of the disk were larger, but no more numerous than at 16 days (Figure 27d).

24 days: After 24 days in culture, some leafy shoots were

Figure 26: Development of peripheral nodules on leaf disks cultured on nodule-producing medium. Figures 26a to g represent sections of leaf disk edge after various lengths of time in culture.

a) After 12 days in culture: nodules were actually separating the upper and lower epidermal layers. (13x)

b) After 16 days in culture: nodules completely lined the disk edge and were almost confluent. Shoot apices were becoming apparent on the nodules. (13x)

c) After 20 days in culture: the disk edge was thickly lined in nodules and shoot apices were frequently apparent. (13x)

d) After 24 days in culture: leaf development was beginning on the shoot apices, so that the first microshoots were now visible by eye. (13x)

e) After 28 days in culture: leafy microshoots were frequently apparent on the peripheral nodules. The nodules are more numerous and larger, and there were several shoot spices per nodule (as seen on the nodule to the extreme right in Figure 26e). (13x)

f) and g) After 35 days in culture: many microshoots were evident (f), and several shoot apices were apparent on most nodules (g). (f: 13x; g: 27x)

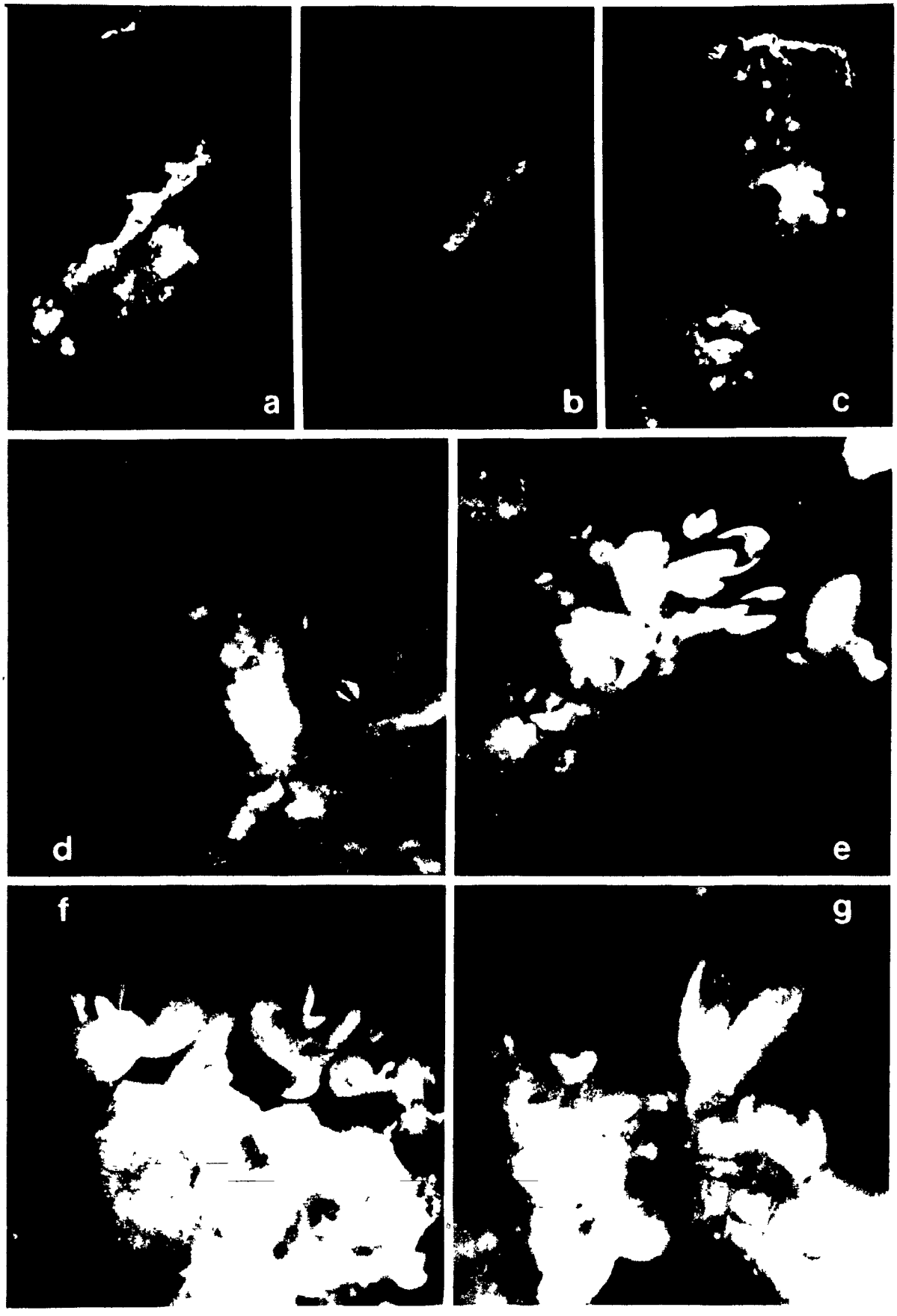

a
b
c
d
e
f
g

Figure 27: Development of nodules on the bottom surface of leaf disks cultured on nodule-producing medium. Figures 27a to f represent sections of the bottom (abaxial) surface of the leaf disk after various lengths of time in culture.

a) After 8 days in culture: the nodules were evident as centers of meristematic activity scattered over the bottom surface of the disk. (15x)

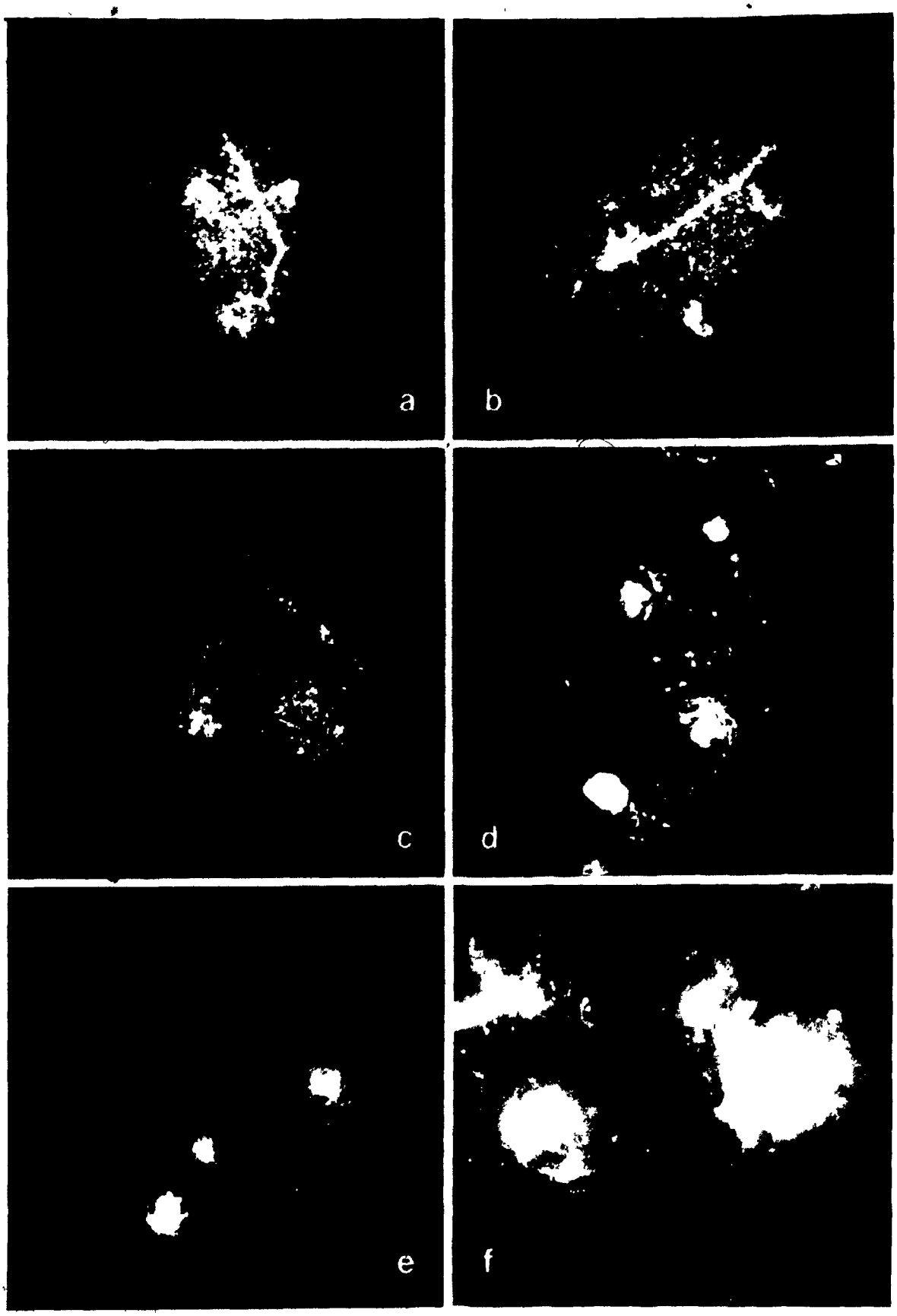
b) After 12 days in culture: the meristematic centers were beginning to assume a nodule shape. (15x)

c) After 16 days in culture: the nodules were larger and more numerous on the disk underside. (15x)

d) After 20 days in culture: the nodules now had a distinct nodule morphology. (15x)

e) After 24 days in culture: the nodules were larger but otherwise showed no further morphological development. (15x)

f) After 28 days in culture: detail of two bottom surface nodules. (27x)


a
b
c
d
e
f

evident on the edge nodules. There were many more shoot apices present, and more numerous and larger nodules (Figure 26d). The nodules on the bottom surface were somewhat larger than at 20 days days, but were obviously developing much slower than the peripheral nodules (Figure 27e).

28 days: After 28 days in culture, the edge nodules were still increasing in size and number and there were both more leafy shoots (microshoots) (Figure 26e) and more shoot apices evident. The nodules on the bottom surface of the disk showed no further growth or development than at 24 days (Figure 27f), except for an occasional shoot apex.

35 days: After 35 days in culture, there was even further increase in the number of peripheral microshoots, shoot apices, and nodules (Figure 26f,g) which together produced a thick ring of organogenetic growth around the explants.

b. Developmental series on shoot-producing medium

4 days: After 4 days in culture, no morphological development was apparent.

8 days: After 8 days in culture, meristematic centers were apparent all over the explant underside (Figure 28a).

12 days: After 12 days in culture, the meristematic centers had taken on a nodule-like appearance and were more numerous (Figure 28b). There was no edge growth at this stage.

16 days: After 16 days in culture, edge growth was not

Figure 28: Morphological development of organogenesis on leaf disk explants cultured on shoot-producing medium.

a) After 8 days in culture: a meristematic center on the leaf disk underside, typical of this stage of culture. (18x)

b) After 12 days in culture: a portion of the convoluted bottom surface of the leaf disk bearing numerous nodules. (8.7x)

c) to f) After 16 days in culture:

c) detail of bottom surface nodules. (18x)

d) a portion of leaf disk edge showing an early shoot apex on almost confluent nodule growth. (18x)

e) top view of a 16 day leaf disk explant showing the convoluted form of the explant and the extent of edge growth. (1.4x)

f) bottom view of a 16 day explant showing the nodules scattered over the bottom surface. (1.4x)

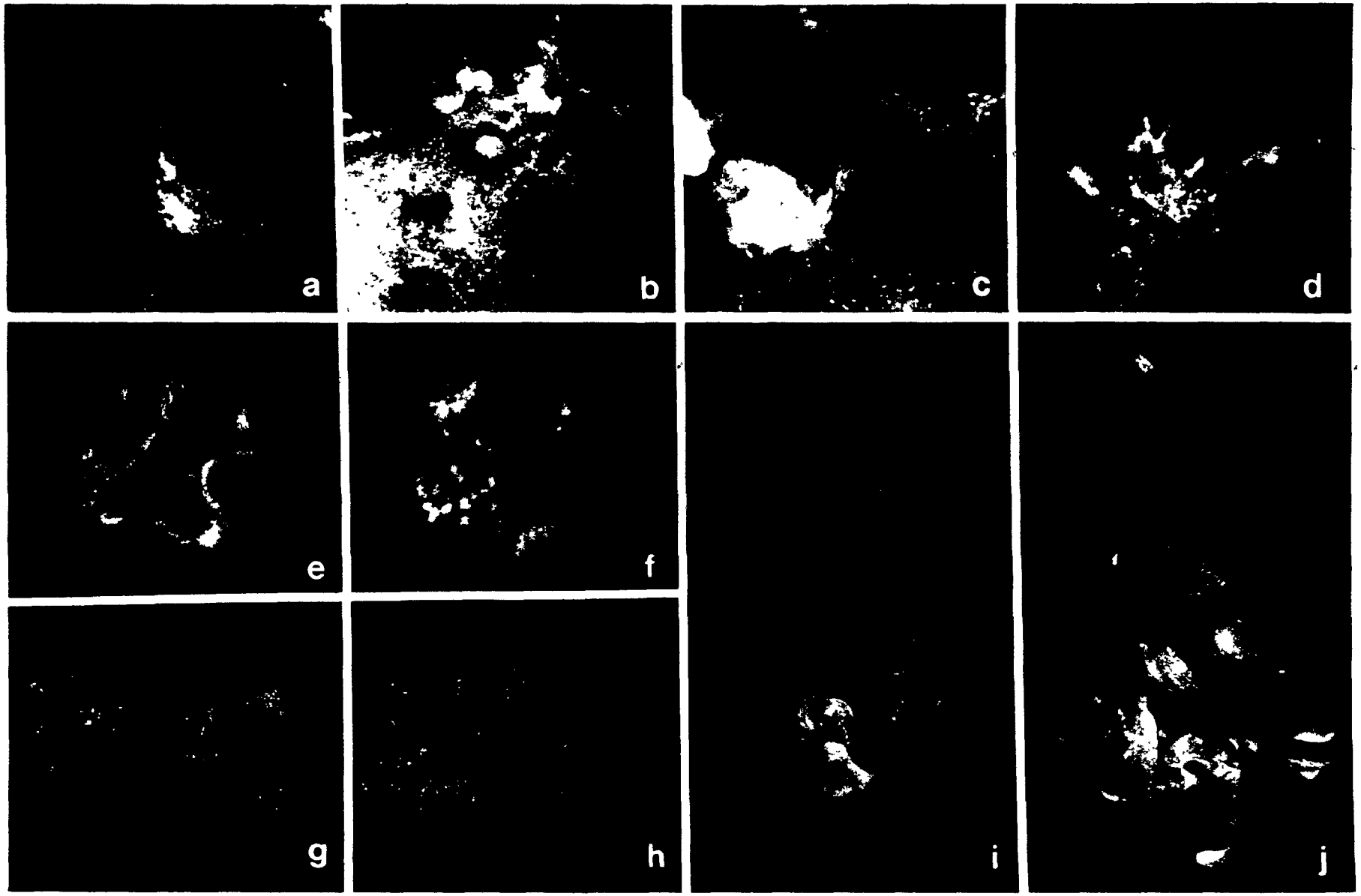
g) and h) After 20 days in culture:

g) top view of an explant. (1.4x)

h) bottom view of an explant. (1.4x)

i) After 24 days in culture: side view of a leaf disk culture showing the downward shoot development. (1.4x)

j) After 28 days in culture: side view of a mature leaf disk culture on shoot-producing medium. (1.4x)


a
b
c
d
e
f
g
h
i
j

only apparent but more developed than the growth on the disk underside (Figure 28e,f). The edge growth consisted of nodules and a few early shoot apices (less than 5 per disk) on these nodules (Figure 28d). The nodule-like structures on the bottom surface of the explant were larger and more numerous than at 12 days, but showed no apical differentiation (Figure 28c,f). The explants were now very convoluted in shape, and de novo growth along the disk edge was generally confined to those sections of the edge not lifted away from the medium by convolution (Figure 28e).

20 days: After 20 days in culture, the leaf disk explants were covered in a profusion of shoots (Figure 28g,h). Hundreds of shoots were evident per explant, and explant volume had increased 5 to 10 fold in the previous 4 days of culture.

24 and 28 days: After 24 to 28 days of culture, the explants had increased in size and had larger de novo shoots (Figure 28i,j). However, there was some callus tissue present, apparently associated with the extreme density of de novo growth.

c. Summary of morphological development

Thus, on both nodule- and shoot-producing media, the same developmental process was taking place: that of nodule formation followed by shoot initiation and development. However, shoot development was arrested at an early stage on the nodule-producing medium.

V. DISCUSSION

In this thesis, the regulation of organogenesis was investigated in two tissue culture systems of Nicotiana tabacum L. cv. Wisc. 38: one employing thin epidermal explants excised from the stem and floral branches of various floral stages of tobacco, the other using disks excised from tobacco leaves.

The research involving the first system, which used epidermal explants, was focused on the quantitative analysis of the gradient of in vitro floral expression known to exist in flowering Nicotiana tabacum L. (as described in the Literature Review, Section II-3). Specifically, the research was intended to reproduce and extend the work of Tran Thanh Van (1973a,b). Tran Thanh Van had described the system as simple and rapid, with organogenesis occurring directly and uniquely from the subepidermal layer of the explants. Tran Thanh Van reported an extremely precise expression of the floral gradient, in which the floral branch explants produced only floral organogenesis (under appropriate conditions) and that this organogenesis was direct, de novo organogenesis from subepidermal cells (see the Literature Review, Section II-3). That is, the flower buds were formed directly without prior vegetative growth.

The results reported in this thesis did not reproduce the above observations by Tran Thanh Van, namely, the precise and simple expression of the floral gradient in Nicotiana. As described in Section IV-1, the organogenetic response was

unpredictably variable and more complex than anticipated. Contrary to the reports by Tran Thanh Van, in the research for this thesis organogenesis did not uniquely originate in a subepidermal layer of the explant. As determined by microdissection and visual analysis, there were as many as six organogenetic origins. These were, in order of frequency of occurrence, the epidermal surface, polar callus, the cortical surface, polar outgrowths, non-polar callus and polar swellings. Possibly, these were not six discrete origins representing six discrete organogenetic processes. Rather, some of these might simply have been various stages of a particular process of development. For example, perhaps polar callus on the explants was the result of a transition from polar swelling to polar outgrowth to polar callus. It is also possible that some of the apparent origins of organogenesis actually represented a secondary form of morphogenesis induced by secondary growth of vascular connections between de novo shoots formed on the explants. These suggestions are purely conjecture and there is no evidence to substantiate them. They are simply intended to illustrate the possible extent of the complexity of the epidermal explant system as observed in the work for this thesis.

Even assuming that the classification system used for the organogenetic origins was valid, there was probably a certain amount of error inherent to the classification. For example, an epidermally originating shoot might subsequently have become engulfed by callus tissue and hence be erroneously classified as

of callus origin.

By quantifying the gradient of *in vitro* floral expression in green-fruit stage *Nicotiana* through origin analysis, it revealed that the gradient was most clearly expressed in organogenesis arising at the epidermal surface (Tables 12 and 13). The floral gradient was weaker or absent in organogenesis from other origins. However, the origin analyses also revealed gradients of *in vitro* vegetative shoot and root organogenesis. There were gradients of vegetative shoot production in organogenesis of both epidermal and cortical surface origins (Table 13). The vegetative shoot gradient was opposite in direction to the floral gradient; that is, vegetative shoot production increased from apex to base. The gradient of root organogenesis was in the same direction as the floral gradient, but was only expressed in organogenesis of polar callus origin. Thus, it was clear that there were different organogenetic processes associated with the various origins observed in this research. Consequently, it is evident that the system of thin epidermal explants of *Nicotiana tabacum* L. used in this research produced a far more complex expression of organogenesis than reported by Tran Thanh Van for apparently the same system.

It is possible, however, that the organogenesis occurring specifically at the epidermal surface in this research was equivalent to the subepidermal organogenesis reported by Tran Thanh Van. Although histological analysis to determine the exact cellular origin of organogenesis was not done in this research,

it was reported by Tran Thanh Van that the subepidermal organogenesis appeared, by visual inspection, to originate directly at the epidermal surface. Even so, the type of quantified expression of the floral gradient reported by Tran Thanh Van was not reproduced in the work for this thesis, as shown in Table 17. However, both sets of results clearly indicate the existence of a gradient of *in vitro* floral potential in green-fruit tobacco plants.

Tran Thanh Van also reported a concomitant gradient in vegetative potential expressed by the epidermal explants. This was observed in the research for this thesis as well, although it was observed in organogenesis originating at the cortical surface as well as the epidermal surface. Since organogenesis at the cortical surface did not also show a gradient in floral potential, the results from this research imply that the floral and vegetative gradients are not necessarily concomitant, which is contrary to the implication in Tran Thanh Van's work. Thus, the work for this thesis indicates that *in vitro* gradients of floral and vegetative expression in tobacco probably represent two distinct, but interrelating physiological gradients present in the stem of flowering *Nicotiana tabacum* L.

Moreover, the research in this thesis also demonstrates the presence of another type of physiological gradient in tobacco. This is a gradient of increasing organogenetic vigour from base to apex in the tobacco plant. That is, there exists a correlation between organogenetic capacity and proximity to the

excision region	results reported by Tran Thanh Van		results reported in this thesis		
	% explants with floral buds	nodes per floral shoot	% explants with floral buds*	nodes/floral shoot avg.*	nodes/floral shoot range*
FB	100	1	100	2.2	1 - 4
F	62	3	100	2.6	1 - 5
SF	40	4	82	5.3	2 - 7
M	25	5	14	6.0	6
B	0	-	0	-	-

* of epidermal origin only; results from Table 13.

Table 17: Quantification of the Floral Gradient in Tobacco by Tran Thanh Van and Bentzen

A comparison of the results reported in this thesis with those reported by Tran Thanh Van (1973b) on the quantified expression of floral organogenesis in thin epidermal explants derived from the stem of green-fruit Nicotiana tabacum L.

plant apex (Table 6). This gradient has also been suggested to be a function of cell age (Hillson and LaMotte, 1977; also see Literature Review). Tran Thanh Van did not report this gradient in her research, and therefore presumably did not correct for it when quantifying the floral gradient. This may explain some of the difference between her quantification of the floral gradient and that reported in this thesis.

Another significant difference between Tran Thanh Van's data and that reported in this thesis is that of node number per de novo floral shoot which is another type of expression of the floral gradient (Table 17). The gradient in node numbers is much more distinct and simple in Tran Thanh Van's data than in the data obtained in this research. For example, Tran Thanh Van reported that floral branch explants only showed organogenesis in the form of floral buds occurring directly at the epidermal surface (i.e. only 1 node per floral "shoot"). In the work for this thesis, every excision region produced a range in node numbers per floral shoot, as indicated in Table 17. In the case of floral branch explants, the range was 1 to 4 nodes, with a mean of 2.2 nodes per floral shoot. Thus, even in terms of node numbers, the epidermal explant system expressed a greater complexity in this research than in that reported by Tran Thanh Van.

In a 1974 publication, Tran Thanh Van reported that whereas floral branch explants from plants at the green-fruit stage showed 100% floral organogenesis, explants from plants at the

full bloom stage produced no floral buds at all (Tran Thanh Van, Thi Dien and Chlyah, 1974). The results reported in this thesis do not confirm this observation by Tran Thanh Van. The capacity for floral organogenesis was clearly present at both the full bloom and floral bud stages of Nicotiana tabacum (Table 13). In fact, floral branch explants from plants of the full bloom stage showed 100% floral organogenesis in this research (as compared with 0% reported by Tran Thanh Van).

In a 1978 review, Tran Thanh Van herself somewhat disclaimed her original observation. In this review she stated that "direct flower formation is only expressed at a precise physiological stage i.e. after fertilization of the terminal flower on the donor plant. At other stages, vegetative-floral shoots and vegetative buds are formed instead of direct flower formation on the epidermal surface of the thin cell layers." (Tran Thanh Van and Trinh, 1978). However, there are no original publications in which this statement is substantiated or quantified.

In any case, the expression of direct flower formation was definitely observed at stages earlier than fertilization of the terminal flower (i.e. earlier than the green-fruit stage) in the research for this thesis. While at least 41% of the floral branch explants from green-fruit plants showed direct flower formation (compared with 100% in Tran Thanh Van's reports), at least 18% also did so from full bloom plants (compared with 0% in Tran Thanh Van's reports).

There are many possible explanations for the differences between the results reported in this thesis and those reported by Tran Thanh Van. A possible explanation is that the differences were caused by subtle genetic factors. As described in the Literature Review, genetic factors may influence the expression of in vitro organogenesis even in different genetic lines of the same cultivar. Certainly, different culture responses of different cultivars of Nicotiana tabacum have been well documented. Thus, there are definitely intraspecies genetic factors which regulate in vitro organogenesis in tobacco. Consequently, even though the same cultivar of tobacco was used in the research for this thesis as was used in the work by Tran Thanh Van, there could well be genetic differences associated with the seed source.

Other possible explanations for the differences between the results of Tran Thanh Van and of this thesis include the nutritional status of the donor plants (due to possible differences in the methods of growing tobacco plants) and intertissue and intercellular correlations (due to differences in the size of the epidermal explants). The latter is the most likely explanation for the greater complexity of organogenetic origins observed in the work for this thesis.

The research using the epidermal explant system posed many technical problems which are detailed in the Results section of this thesis. These problems were evidenced by unpredictably variable and low experimental yields, which in turn led to low

replication and occasional failure in repeating experiments due to lack of any organogenetic growth. These problems rendered the epidermal explant system inappropriate for further research and reduced the number of definitive conclusions which could be drawn from the data reported in this thesis. However, some conclusions and implications can be made, as discussed below.

The results reported in this thesis clearly showed that the gradient of *in vitro* floral expression was present not only in the green-fruit stage of tobacco, but also at two earlier stages of floral development. However, the gradient was most strongly expressed at the green-fruit stage, and less at the full bloom and floral bud stages. It is not felt that anything can be inferred from this observation due to insufficient replication of the data. However, more intensive studies quantifying the floral gradient at various developmental stages (*e.g.* pre-floral induction, during induction, and post-induction) would most likely give valuable information concerning the mechanism of floral induction and related physiological gradients in *Nicotiana*.

One implication which can be drawn from this research, which has already been discussed, is that the mechanisms involved in the regulation of floral and vegetative organogenesis are probably associated with two distinct but interrelating physiological gradients present in the stem of flowering *Nicotiana tabacum*.

The second tissue culture system studied, the leaf disk system, was developed for the purpose of this thesis. This

system employed 12.7 mm leaf disks cultured on modified Murashige and Skoog medium. The leaf disk system had many advantageous characteristics. On appropriate media, this system produced 100% yield and complete homogeneity in organogenetic response. The response was both rapid and vigorous. Organogenesis was well developed in 19 to 26 days. The system demonstrated a high propagation potential with complete retention of organogenetic capacity. Moreover, it was demonstrated that tobacco clones, involving large numbers of individuals, could be quickly and effectively produced from leaf disks, an obvious advantage to both plant breeding and physiological research programs.

The leaf disk system displayed a large degree of intrinsic homogeneity. The organogenetic response of the system to hormonal and environmental manipulation was usually uniformly expressed in 100% of the explants. Also, plant imposed factors were shown to have little effect on the organogenetic response. That is, excision locus on the leaf, leaf position on the plant, and developmental stage of the donor plant were all shown to be irrelevant to the organogenetic response of leaf disk cultures.

It was also demonstrated in this thesis that the emergence of organogenetic structures in the leaf disk system was a response to direct medium contact. Morphogenetic differences arising from different orientations of the leaf disk in culture were a consequence of differing responsiveness of the adaxial and abaxial surfaces of the leaf disk when in contact with the agar medium. On shoot-producing medium there was no difference

between the responsiveness of the abaxial and adaxial surfaces (i.e. the culture response was identical for explants with abaxial or adaxial contact). However, on nodule-producing medium there was a difference in responsiveness between the disk sides, in that the abaxial surface produced much more de novo growth than the adaxial side, when in contact with the medium. But for both surfaces and both media, the organogenetic response was an expression of the same organogenetic process; namely nodule formation followed by shoot formation.

Observations of the type, origin, and distribution of organogenetic structures in the leaf disk system, as reported in the Results section of this thesis supported the following conclusions:

1) Organogenesis, specifically the production of nodules and shoot apices, was induced by direct medium contact. This was true for both nodule- and shoot-producing media.

2) Organogenetic development, especially shoot development, was promoted by subsequent removal from direct medium contact. This was true only for the nodule-producing medium.

The first conclusion was drawn from the disk orientation results, as discussed above. The second conclusion was drawn from general observation in the disk orientation and other experiments. On nodule-producing medium, the greatest shoot development always occurred on those parts of the explant lifted away from the agar due to convolution (typical of abaxial contact) or rolling up (typical of adaxial contact) of the leaf

disk during culture. This promotion of development could be a consequence of exposure to air or removal of a factor(s) in the medium. The fact that this promotion of development only held true for nodule-producing medium, but not for shoot-producing medium, suggested an involvement of kinetin. The two media differed only in their respective concentrations of kinetin (10^{-4} M vs. 10^{-5} M). The promotion of development by subsequent removal of the growth regulator has certainly been demonstrated for auxin involvement in embryogenesis (Kohlenbach, 1977; also see Literature Review). The hypothesis that high kinetin concentrations inhibit development (which is equivalent to removal of high kinetin concentrations stimulating development) was also supported by the hormone manipulation studies which will be discussed below.

The results from the experiment testing leaf disk response to different IAA and kinetin concentrations elucidated several modes of organogenetic regulation (Section IV-2-ii-a). The conclusions drawn from the data are as follows:

1) The absolute concentration of kinetin is the most important factor regulating vegetative shoot, nodule, and callus production (Figure 15). Vegetative shoot organogenesis only occurred at kinetin concentrations greater than or equal to 10^{-6} M. Callus production on the other hand, basically only occurred at kinetin concentrations less than or equal to 10^{-6} M. Propagation nodules only occurred at kinetin concentrations greater than or equal to 10^{-5} M. (In each case, the auxin:

cytokinin ratio was irrelevant, with one exception as discussed below.) The absolute dependence on cytokinin for vegetative bud formation on tobacco leaf explants has also been demonstrated by Gupta et al. (1966) and Mullins et al. (1976), as described in the Literature Review (Section II-4).

2) At 10^{-6} M kinetin, which supports both callus and vegetative shoot production, the IAA concentration also regulates organogenesis. This could be concluded from the observation that the number of explants forming shoots decreased with increasing IAA concentration at 10^{-6} M kinetin. However, several possible modes of regulation could explain this phenomenon: a) At 10^{-6} M kinetin, the mode of organogenetic regulation may be the auxin: cytokinin ratio (e.g. Figure 16a), as established for many other tissue culture systems (e.g. Skoog, 1970; also see Literature Review). b) At 10^{-6} M kinetin, endogenous hormone concentrations in the leaf disk explants are possibly exerting some regulatory effect on organogenesis in association with the exogenous IAA concentration. c) At 10^{-6} M kinetin, IAA is counteracting the effect of kinetin to some extent, thereby reducing the effective concentration of kinetin. In this case, the mode or organogenetic regulation is still a threshold mechanism governed by the absolute concentration of kinetin, as concluded in No. 1 above. This could fully explain the decrease in vegetative organogenesis with increasing IAA concentration in the 10^{-6} M kinetin cultures. This explanation also best fits other conclusions and observations from the data, as discussed below.

3) IAA counteracts the effect of kinetin to some extent. The evidence for this conclusion comes from several observations. i) There was low tissue viability and little de novo growth in the 10^{-3} kinetin cultures, except at the highest concentration of IAA (Table 14). ii) There was an increase in shoot development with increasing IAA concentration in the 10^{-4} kinetin cultures (Figure 13). iii) Only 10^{-6} M kinetin or less supported callus growth, except at the highest IAA concentrations in the 10^{-5} M kinetin cultures (Table 14).

4) Kinetin induces vegetative shoot organogenesis, but inhibits its development (or perhaps certain aspects of development, such as leaf development). The evidence for this conclusion comes in part from the leaf disk orientation results, as previously discussed. Further evidence was derived from the hormone manipulation studies: i) The degree of shoot development was a function of the kinetin concentration. The 10^{-3} M kinetin cultures only developed shoot apices, the 10^{-4} M cultures developed microshoots, and the 10^{-5} M cultures developed leafy vegetative shoots several centimeters long (Figures 12, 13, 14). In other words, the greater the kinetin concentration, the less the degree of shoot development. ii) Assuming the conclusion that IAA counteracts kinetin is valid, then further evidence that higher concentrations of kinetin inhibit shoot development is given by the observation that in the 10^{-4} M kinetin cultures, the degree of shoot development increased with increasing IAA concentration (Figure 13). This conclusion is

also supported by the work of Chih-hung et al. (1978).

5) The mode of regulation for root organogenesis combines an IAA threshold effect with an auxin:cytokinin ratio effect. Evidence for this conclusion is derived from two observations. i) In the 10^{-3} and 10^{-4} M IAA cultures, the frequency of root organogenesis was proportional to the auxin:cytokinin ratio (Figure 16b). ii) Only IAA concentrations greater than or equal to 10^{-4} M permitted root growth, in spite of appropriately high auxin:cytokinin ratios at lower IAA concentrations (Figure 15). This conclusion is supported by the work of Coleman and Greyson (1977) who demonstrated the auxin requirement for root organogenesis on tomato leaf disks cultured on a cytokinin-free medium.

These five conclusions and the modes of organogenetic regulation which are implied by these conclusions provide significant insight into the study of organogenetic regulation. Due to the inherent homogeneity demonstrated in the leaf disk system, it seems quite possible that this system could be used for investigations even at the molecular level of hormonal regulation, and certainly for many further studies at the physiological level of hormonal action.

Thus, in view of what has just been discussed, the work described in this thesis has extended the knowledge of organogenetic regulation on tobacco leaf explants in several ways. While the studies by Gupta et al. (1966) and Mullins et al. (1976) both confirmed that vegetative buds on tobacco leaf explants were induced by appropriate kinetin concentrations, their

observations had certain limitations. Gupta et al. used a limited range of hormone concentrations, a relatively more complex medium (White's supplemented with coconut milk), and only seedling leaves for explanting. Furthermore, their explants included portions of the leaf midrib. Consequently, the tissue composition of their explants were not truly comparable either to the explants used in the work for this thesis or those of other published studies. The results reported by Gupta et al. were not as quantitatively definitive as those reported in this thesis, probably due to the greater complexity and lesser homogeneity of their system (for reasons mentioned above).

Mullins et al. (1976) used tobacco leaf disks comparable to those of the leaf disk system reported in this thesis, but used an auxin-free medium containing 5×10^{-6} M kinetin. Since they did not test other concentrations of kinetin, nor use any auxin in their medium, their work did not elucidate how growth regulators effected regulation of organogenesis, as reported here. Their results were also not as homogenous and definitive as in this thesis. The maximum organogenetic response they reported for any experimental condition was 85% of the disks showing an average of 10 buds per disk. In the work for this thesis, the usual response was 100% of the disks showing hundreds of buds per disk. The leaf disk system of Mullins et al. also showed a great deal of variation due to plant imposed factors (e.g. physiological stage, excision position, and disk orientation, as described in the Literature Review, Section II-4)

that was not apparent in the leaf disk system reported in this thesis. Since the system reported by Mullins et al. was evidently more complex and subject to multiple sources of variation, their work was also not as definitive as that reported here. Moreover, as in the work of Gupta et al., Mullins et al. did not elucidate the mode of growth regulator action on the regulation of organogenesis.

The work by Chih-hung et al. (1978) was the most analogous to that reported here, though the report was not available until after the research for this thesis had been completed. Though they did not test as great a range of auxin and cytokinin concentrations, they did report that cytokinin stimulated bud formation whereas auxin promoted root formation, thus confirming part of the results reported here. Moreover, they experimentally established what was simply implied from observations reported here, namely that cytokinin induces vegetative organogenesis but retards its development. Specifically, Chih-hung et al. reported that cytokinin was only required for the first 5 to 6 days of culture, and that subsequent removal accelerated shoot and root growth. However, neither Chih-hung et al., nor any previous group working with tobacco leaf culture, reported the phenomenon of nodule formation on tobacco leaf explants, or the definitive effects of auxin and cytokinin in regulating organogenesis.

An interesting, though somewhat speculative, point for discussion is the phenomenon of propagation nodules in the leaf disk system.

Kohlenbach's 1977 review of somatic embryogenesis describes how the presence of reduced nitrogen and high auxin concentration (approximately 10^{-6} M or greater) in the medium causes inhibition of embryoids in Daucus cultures and production of nodule-like structures. The nodules in the Daucus cultures are described as being composed of small superficial cells dense in cytoplasm and large vacuolated central cells. These nodule-like structures develop numerous well-formed embryos if the auxin is reduced or omitted. These embryoids can be cultivated into plants. Furthermore, these nodule-like structures (called embryogenic clumps) can be propagated in the presence of high auxin concentrations.

Kohlenbach's description of embryogenic clumps in Daucus cultures bears striking similarity to the description of propagation nodules produced on the tobacco leaf disk cultures. The medium producing the propagation nodules has the same characteristics, i.e. reduced nitrogen and high auxin concentration. The propagation nodules also often show the type of histology described by Kohlenbach (preliminary histological studies not reported in this thesis). Moreover, as reported in the Results section, the propagation nodules can readily be propagated (i.e. subcultured) on medium containing high auxin and cytokinin concentrations.

However, preliminary histological studies (not reported in this thesis) showed that the de novo shoots formed on the nodules were definitely adventitious and were not embryoids. The shoots

were unipolar structures openly linked to the tissue in which they were formed. Embryoids, on the other hand, are bipolar structures with a closed radicular pole and no continuity with surrounding tissue, according to the commonly accepted definition of embryoid as established by Haccius and Lakshmanan in 1969. Nonetheless, it is still possible that the propagation nodules of the tobacco leaf disk system are analogous to the embryogenic clumps in systems such as that of Daucus. Possibly, with appropriate medium manipulation (e.g. removal of auxin) the propagation nodules could be induced to produce embryoids. This would certainly be an interesting avenue for future studies.

Another interesting avenue of investigation to pursue with the leaf disk system would be to more carefully test the system for potential expression of floral organogenesis. The standard concentrations of IAA and kinetin used in this work (10^{-4} and 10^{-5} M kinetin and 10^{-5} M IAA) are usually inappropriate for floral organogenesis in tobacco tissue culture. For example, Hillson and LaMotte (1977) reported inhibition of floral organogenesis (and stimulation of vegetative organogenesis) on stem segments of tobacco at IAA concentrations greater than or equal to 10^{-6} M and kinetin concentrations greater than or equal to 10^{-5} M. Tran Thanh Van, Thi Dien and Chlyah (1974) reported that 10^{-5} M kinetin inhibited floral organogenesis and induced vegetative organogenesis on the floral branch epidermal explants of tobacco. In other words, even if the leaf disk system does have potential for in vitro floral organogenesis, it probably

cannot be expressed under the conditions standardly used in this research; namely, high auxin and cytokinin concentrations. However, it was shown in this thesis that lower IAA and kinetin concentrations alone do not induce floral organogenesis on the leaf disks (Section IV-2-ii-a). This does not imply that leaf tissue does not have the potential for *in vitro* expression of floral organogenesis.

For instance, it is known that when the apex of a tobacco plant becomes florally induced, the axillary buds below are progressively induced to form floral branches. These same axillary buds form vegetative branches if the tobacco plant is detopped before floral induction. Thus, the axillary buds are vegetative until they are induced to the flowering state by some mechanism apparently transmitted progressively down the stem. This mechanism is probably the basis for the gradient of *in vitro* floral expression seen in stem cultures of *Nicotiana tabacum* L. This mechanism, possibly a biochemical factor, is probably not transmitted to the tobacco leaves, since floral organogenesis has never been observed on leaf disk explants under the conditions standard for floral organogenesis on stem explants. However, this this does not imply that the leaf tissue would not be receptive to this floral mechanism. For example, some very suggestive and promising work has been reported by Wardell (1977) in which a certain fraction of purified DNA from stems of flowering tobacco plants induced flowering in vegetative tobacco apices when applied topically to those apices. Perhaps this type of work

could be applied to the leaf disk system. Since the leaf disk culture described here offers a system of vigorous, almost synchronous, induction of vegetative apices (i.e. the system produces hundreds of shoot apices per explant within a day or two), the possible floral induction of these apices might provide a large population of apices simultaneously induced to the flowering state in a strictly controlled, almost synchronous fashion. Such a system might be extremely useful in studies on the molecular basis of floral induction.

The last point for discussion in this thesis is a comparison of the epidermal and leaf disk systems, as studied here. Both systems offer several advantages not available in many other tissue culture systems. To quote Thorpe (1978):

> Our knowledge of the physiology and biochemistry of the organ-forming process is very limited. A major problem in this area has been the lack of a truly suitable experimental system for such studies; a common drawback of all callus organogenetic systems being the fact that only a few cells in the callus mass are directly involved in the organ-initiation process. Furthermore, this process tends not to be synchronous. Thus the presence of the dilution effect and asynchrony significantly reduces the usefulness of callus for studies on the physiology and biochemistry of organogenesis.

In this respect, both the leaf disk and epidermal explant systems offer the advantages of rapid, reasonably synchronous, homogenous (at least in Tran Thanh Van's reports of the epidermal system) systems of organogenesis. However, even ignoring the difficulties encountered in this research when attempting to reproduce Tran Thanh Van's work, the leaf disk system has certain

inherent advantages over the epidermal explant system. For instance, the latter obtains explants from an extremely small portion of a mature tobacco plant (e.g. in Tran Thanh Van's work only the bases of floral branches of green-fruit stage tobacco plants are used), as compared with the leaf disk system which obtains explants from a major portion of a mature tobacco plant, namely the leaves. Thus, hundreds of tobacco plants would be required in the epidermal explant system to supply the same number of explants that a single tobacco plant could supply for the leaf disk system. This aspect might be very important in biochemical studies involving analysis for endogenous substances involved in organ formation. Such endogenous substances may be present in small amounts thus requiring large numbers of explants at each stage of organ initiation and development. The extremely high productivity of the leaf disk system would be a further advantage in such a situation.

Furthermore, the difficulties encountered in this research in the attempts to reproduce Tran Thanh Van's work are, in this author's opinion, indicative of the instability of the epidermal explant system. Homogenous explants are difficult to obtain since the manual excision techniques cannot ensure the same number of cell layers per explant and the variation observed in the organogenetic expression indicates a very delicate balance of factors involved in the regulation of organogenesis. The leaf disk system on the other hand, was extremely stable, in that homogenous results were nearly always obtained. Therefore, it is

most likely a better system for plant physiologists and biochemists to reproduce for their specific needs. The apparent lack of floral organogenesis in the system need not be a deterrent, since, as already discussed, there are many promising avenues of research which could be explored.

Although one must agree with Everett et al. (1978) who stated that, "It can be concluded that there is no one ideal experimental system for hormone research", it does seem evident that the leaf disk system described here offers unique advantages for future study.

References

Aghion-Prat, D; Néoformation de fleurs in vitro chez Nicotiana tabacum L; Physiol. vég. 3: 229-303 (1965) (Biological Abstracts Vol. 47, No. 103978, 1966).

Bajaj, V.P.S. and P. Nitsch; In vitro propagation of red cabbage (Brassica oleracea L. var. Capitata); J. Exp. Bot. Vol. 26 No. 95: 883-890 (1975).

Banerjee, S. and S. Gupta; Morphogenesis in tissue cultures of different organs of Nigella sativa; Physiol Plant. 33: 185-187 (1975a).

Banerjee, S. and S. Gupta; Embryoid and plantlet formation from stock cultures of Nigella tissues; Physiol. Plant. 34: 243-245 (1975b).

Brossard, D.; Root organogenesis from foliar discs of Crepis capillaris L. Wallr. cultured in vitro: cytochemical and microspectrophotometric analysis; New Phytol. 79: 423-429 (1977).

Burk, L.G.; Clonal and selective propagation of tobacco from leaves; Plant Sci. Letters, Vol. 4: 149-154 (1975).

Chailakhyan, M. Kh., T.V. Bavrina and T.N. Konstantinova; Morphogenetic potentials of tobacco calluses of stem and leaf origin; Doklady Bot. Sc. Vol. 232-234: 16-19 (1977).

Chang, W. and Y. Hsing; In vitro flowering of embryoids derived from mature root callus of ginseng (Panax ginseng); Nature Vol. 284: 341-342 (1980).

Chih-hung, Hsu; Wang Siung and Liu Kwei-yun; Studies on organogenesis in the culture of the leaf explant of Nicotiana tabacum L.; in: Proceedings of the China-Australia Symposium on Plant Tissue Culture, Peking, China, May 26-30, 1978; ed: Hu Han; Academia Sinica, Peking; pp. 501-507 (1978).

Chlyah, H.; Etude morphogénétique, histologique et carylogique de l'organogenèse au niveau des tissus superficiels d'un segment de tige de Torenia fournieri (Lind.) cultivé in vitro. Approches quantitatives; Thèse doctorat d'Etat; Université de Paris (1974).

Chouard, P. and D. Aghion; Modalities de la formation de bourgeon florale sur des cultures de segments de tige de tabac; C.R. Acad. Sci. D. 252: 3864-3867 (1961).

Coleman, W.K. and R.I. Greyson; Analysis of root formation in leaf disks of Lycopersicon esculentum Mill. cultured in vitro; Ann. Bot. 41: 307-320 (1977).

Cousson, A. and K. Tran Thanh Van; In vitro control of de novo flower differentiation from tobacco thin cell layers cultured on a liquid medium; Physiol. Plant. 51: 77-84 (1981).

Everett, N.P., T.L. Wang, and H.E. Street; Hormone regulation of cell growth and development in vitro; in: Frontiers of Plant Tissue Culture 1978, Proceedings of the 4th Int. Cong. of Plant Tissue and Cell Culture held at the University of Calgary, Alberta, Canada, Aug. 20-25, 1978; ed: T.A. Thorpe; Int. Ass. for Plant Tissue Culture; pp. 307-316 (1978).

Gupta, G.R.P., S. Guha and S.C. Maheshwari; Differentiation of buds from leaves of Nicotiana tabacum Linn. in sterile culture; Phytomorphology 16: 175-182 (1966).

Haccius, B. and K.K. Lakshmanan; Adventiv-embryonen--embryoide--adventiv-knopsen. Ein beitrag zur klarung der begriffe; Österr. Botan. Z. 116: 145-158 (1969).

Hillson, T.D. and C.E. LaMotte; In vitro formation and development of floral buds on tobacco stem explants; Plant Physiol. 60: 881-884 (1977).

Holdgate, D.P.; Propagation of ornamentals by tissue culture; in: Applied and Fundamental Aspects of Plant Cell, Tissue, and Organ Culture; ed: J. Reinert and Y.P.S. Bajaj; Springer-Verlag, Berlin-Heidelberg-New York; pp. 18-43 (1977).

Kamate, K., A. Coussin, T.H. Trinh and K. Tran Thanh Van; Influence des facteurs génétique et physiologique chez le Nicotiana sur la néoformation in vitro de fleurs à partir d'assises cellulaires épidermiques et sous-épidermiques; Can. J. Bot. 59: 775-781 (1981).

Kasperbauer, M.J. and G.B. Collins; Reconstitution of diploids from leaf tissue of anther derived haploids in tobacco; Crop. Sci. 12(1): 98-101 (1972).

Kohlenbach, H.W.; Basic aspects of differentiation and plant regeneration from cell and tissue cultures; in: Plant Tissue Culture and Its Bio-technological Application; Proceedings of the First Int. Cong. on Medicinal Plant Research, Section B, held at the University of Munich, Germany, Sept. 6-10, 1976; ed: W. Barz, E. Reinhard, and M.H. Zenk; Springer-Verlag; Berlin-Heidelberg-New York; pp. 355-366 (1977).

Kohlenbach, H.W.; Comparative Somatic Embryogenesis; in: Frontiers of Plant Tissue Culture 1978, Proceedings of the 4th Int. Cong. on Plant Tissue and Cell Culture held at the University of Calgary, Alb. Canada, Aug. 20-25, 1978; ed: T.A. Thorpe; Int. Ass. of Plant Tissue Culture; pp. 59-66 (1978).

Konstantinova, T.N., L.V. Gofshtein, O.I. Molodyuk, T.V. Bavrina and N.P. Aksenova; The histones of vegetative and reproductive calluses of trapezoid tobacco; Doklady Botanical Sciences {Dokl. Akademii Nauk. SSSR} 214-216: 43-45 (1974).

Letham, D.S. and C.O. Miller; Identity of kinetin-like factors from Zea mays; Plant and Cell Physiology 6: 355-359 (1965).

Mauseth, J.D. and W. Halperin; Hormonal control of organogenesis in Opuntia polyacantha (Cactaceae); Am. J. Bot. 62(8): 869-877 (1975).

Mullins, M.G., S. Harsono and D.J. Batten; Sources of variation in cytokinin induced bud formation in leaf disks of Nicotiana tabacum; Phytomorphology 26(2): 176-182 (1976).

Murashige, T.; Plant propagation through tissue cultures; Ann. Rev. Plant Physiol. 25: 135-66 (1974).

Murashige, T.; Clonal crops through tissue culture; in: Plant Tissue Culture and its Bio-technological Application, Proceedings of the First Int. Cong. on Medicinal Plant Research, Section B, held at the University of Munich, Germany, Sept 6-10, 1976; ed: W. Barz, E. Reinhard and M.H. Zenk; Springer-Verlag, Berlin-Heidelberg-New York; pp. 392-403 (1977).

Murashige, T.; The impact of plant tissue culture on agriculture; in: Frontiers of Plant Tissue Culture 1978; Proceedings of the 4th Int. Cong. of Plant Tissue and Cell Culture held at the University of Calgary, Alberta, Canada, Aug 20-25, 1978; ed: T.A. Thorpe; The Int. Ass. of Plant Tissue Culture; pp. 15-26 and 518-524 (1978).

Murashige, T. and F. Skoog; Revised medium for rapid growth and bioassays with tobacco tissue cultures; Physiol. Plantarum 15: 473-497 (1962).

Narayanaswamy, S.; Regeneration of plants from tissue cultures; in: Applied and Fundamental Aspects of Plant Cell, Tissue, and Organ Culture; ed: J. Reinert and Y.P.S. Bajaj; Springer-Verlag, Berlin-Heidelberg-New York; pp. 179-250 (1977).

Nitsch, C.; The role of growth regulators in flowering as demonstrated by in vitro techniques; in: Hormonal Regulation in Plant Growth and Development; Proc. Adv. Study, Inst. Izmir, 1971; ed: H. Kaldeway and Y. Vardar; Verlag Chemie, Weinheim; pp. 413-421 (1972).

Oka, S. and K. Ohyama; In vitro initiation of adventitious buds and its modification by high concentration of benzyladenine in leaf tissues of mulberry (Morus alba); Can. J. Bot. 59: 68-74 (1981).

Pierik, R.L.M.; Vegetative propagation of horticultural crops in vitro with special attention to shrubs and trees; Acta Horticulturae 54: 71-82 (1975).

Pierik, R.L.M. and H.H.M. Steegmans; Freesia plantlets from flower buds cultivated in vitro; Neth. J. Agric. Sci. 23: 334-337 (1975).

Prabhudesai, V.R. and S. Narayanaswamy; Differentiation of cytokinin-induced shoot buds and embryoids on excised petioles of Nicotiana tabacum; Phytomorphology Vol. 23 (3-4): 133-137 (1973).

Ringe, F. and J.P. Nitsch; Conditions leading to flower formation on excised Begonia fragments cultured in vitro; Plant and Cell Physiol. 9: 639-652 (1968).

Sangwan, R.S. and H. Harada; Chemical regulation of callus growth, organogenesis, plant regeneration, and somatic embryogenesis in Antirrhinum majus tissue and cell cultures; J. Exp. Bot. Vol. 26, No. 95: 868-881 (1975).

Sattler, R.; A technique for the study of floral development; Can. J. Bot. 46: 720-724 (1968)

Skoog, F.; Aspects of growth interactions in morphogenesis of tobacco tissue cultures; in: Les Cultures de Tissus de Plantes; Coll. Internat. C.N.R.S., No. 193: 115-135 (1970).

Skoog, F. and C.O. Miller; Chemical regulation of growth and organ formation in plant tissue cultured in vitro; Symp. Soc. Exp. Biol. 11: 118-131 (1957).

Speigel-Roy, P. and J. Kochba; Application of tissue culture for plant improvement; in: Plant Tissue Culture and Its Bio-technological Application, Proceedings of the First Int. Cong. on Medicinal Plant Research, Section B, held at the University of Munich, Germany, Sept. 6-10, 1976; ed: W. Barz, E. Reinhard, and M.H. Zenk; Springer-Verlag, Berlin-Heidelberg-New York; pp. 404-414 (1977).

Thi Dien, Nguyen and M. Tran Thanh Van; Differentiation in vitro et de novo d'organes floraux directement à partir des couches minces des cellules de type epidermique de Nicotiana tabacum. Etude au niveau cellulaire; Can. J. Bot. 52: 2319-2322 (1974).

Thorpe, T.A.; Physiological and biochemical aspects of organogenesis in vitro; in: Frontiers of Plant Tissue Culture 1978, Proceedings of the 4th Int. Cong. of Plant Tissue and Cell Culture held at the University of Calgary, Alberta, Canada, Aug. 20-25, 1978; ed: T.A. Thorpe; Int. Ass. for Plant Tissue Culture 1978, pp. 49-58 (1978).

Thorpe, T.A., K. Tran Thanh Van and T. Gaspar; Isoperoxidases in epidermal layers of tobacco and changes during organ formation in vitro; Physiol. Plant. 44: 388-394 (1978).

Tran Thanh Van, M.; In vitro control of de novo flower, bud, root, and callus differentiation from excised epidermal tissues; Nature 246: 44-45 (1973a).

Tran Thanh Van, M.; Direct flower neoformation from superficial tissue of small explants of Nicotiana tabacum L.; Planta (Berl.) 115: 87-92 (1973b).

Tran Thanh Van, K.*; Regulation of morphogenesis; in: Plant Tissue Culture and Its Bio-technological Application, Proceedings of the First Int. Cong. on Medicinal Plant Research, Section B, held at the University of Munich, Germany, September 6-10, 1976; ed: W. Barz, E. Reinhard and M.H. Zenk; Springer-Verlag, Berlin-Heidelberg-New York; pp. 367-385 (1977).

Tran Thanh Van, M., H. Chlyah, and A. Chlyah; Regulation of organogenesis in thin layers of epidermal and sub-epidermal cells; in: Tissue Culture and Plant Science, Proceedings of the 3rd Int. Cong.; ed: H.E. Street; Academic Press, London and New York; pp. 101-139 (1974).

*Dr. Tran Thanh Van changed her initial from M. to K. after 1975.

Tran Thanh Van, M. and A. Drira; Definition of a simple experimental system of directed organogenesis de novo: organ neoformation from epidermal tissue of Nautilocalyx lynchei; in: Les Cultures de Tissus de Plantes; Colloq. Int. Cent. Natl. Rech. Sci. 193: 169-176 (1970).

Tran Thanh Van, M. and N. Thi Dien; Etude au niveau cellulaire de la différenciation in vitro et de novo de bourgeons végétatifs de racines, ou de cal, à partir de couches minces de cellules de type épidermique de Nicotiana tabacum Wisc. 38; Can. J. Bot. 53: 553-559 (1975).

Tran Thanh Van, M., Nguyen Thi Dien and Averil Chlyah; Regulation of organogenesis in small explants of superficial tissue of Nicotiana tabacum L.; Planta (Berl.) 119: 149-159 (1974).

Tran Thanh Van, K. and H. Trinh; Morphogenesis in thin cell layers: concept, methodology, and results; in: Frontiers of Plant Tissue Culture 1978, Proceedings of the 4th Int. Cong. of Plant Tissue and Cell Culture held at the University of Calgary, Alberta, Canada, Aug. 20-25, 1978; ed: T.A. Thorpe; Int. Ass. for Plant Tissue Culture; pp. 37-48 (1978).

Vasil, I.R. and V. Vasil; Totipotency and embryogenesis in plant cell and tissue cultures; In Vitro 8: 117-127 (1972).

Wardell, William L.; Floral induction of vegetative plants supplied a purified fraction of deoxyribonucleic acid from stems of flowering plants; Plant Physiol. 60: 885-891 (1977).

Wareing, P.F. and I.D.J. Phillips; The Control of Growth and Differentiation in Plants, 2nd Edition; Pergamon Press; Oxford; (1978).

Yusufov, A.G. and S.S. Khachumova; Ability of isolated leaves to regenerate in sterile culture; Bot. Zh. (Leningr.) 60(1): 95-103 (1975) (in Russian; English abstract in Biological Abstracts Vol. 61, No. 10423, 1976).

Zenk, M.H.; The impact of plant cell culture on industry; in: Frontiers of Plant Tissue Culture 1978, Proceedings of the 4th Int. Cong. of Plant Tissue and Cell Culture held at the University of Calgary, Alberta, Canada, Aug. 20-25, 1978; ed: T.A. Thorpe; Int. Ass. of Plant Tissue Culture; pp. 1-13 (1978).

APPENDIX

Complete Yield Analyses of Epidermal Explant Experiments

Experiment	Explant type *	No. plated	% Infection	% excluding infected				Replicate/Total Yield % of plated explants	Comments*
				Necrosis	No Growth	Insufficient Development	Organogenesis		
a	b	c	d	e	f	g	h	i	j
2	GF-SF	98	21	27	23	18	31	24	o-p
3	Bd-SF	54	91	20		80		7	s-p
	Bd-M	54	100	-		-		0	
	Bd-B	54	100	-		-		0	
	Fl-FB	54	57	43		57		24	
	Fl-F	54	61	29		71		28	
	Fl-SF	54	74	43		57		15	
	Fl-M	54	98	0		(100)		2	
	Fl-B	54	100	-		-		0	
	GF-FB	54	26	50		50		37	
	GF-F	54	20	44		56		44	
	GF-SF	54	54	4		96		44	
	GF-M	54	67	83		17		6	
	GF-B	54	96	100		0		0	
	Total	918	79	42		58		12	
5	GF-FB	17	35	0	9	45	45	29	o-p
	GF-F	18	28	0	38	38	23	17	
	GF-SF	18	39	0	18	9	73	44	
	GF-M	18	33	0	8	25	67	44	
	GF-B	18	50	0	44	33	22	11	
	Total	89	37	0	23	30	46	29	
7	GF-FB	90	2	3	2	74	20	20	o-p
	GF-F	90	9	2	9	84	5	4	
	GF-SF	90	4	1	10	83	6	6	
	GF-M	90	14	4	25	62	9	8	
	GF-B	90	50	4	20	58	18	9	

Appendix continued.

a	b	c	d	e	f	g	h	i	j
7	Fl-F	63	5	2	15	83	0	0	o-p
	Fl-SF	72	6	3	10	87	0	0	
	Fl-M	63	0	11	29	60	0	0	
	Fl-B	72	6	9	22	69	0	0	
	Bd-SF	63	0	0	5	87	8	8	
	Bd-M	72	4	10	25	65	0	0	
	Bd-B	45	4	2	21	77	0	0	
	Total	900	10	4	15	75	6	5	
8	GF-FB	20	0-10	0	0	callus:	100	90+	o-p/alc
	GF-F	20	0-10	0	0		"	90+	
	Total	40	0-10	0	0		"	90+	
9	GF-FB	18	0	17	22	11	50	50	o-p/alc/ep-up
	GF-F	18	6	0	12	6	82	78	
	GF-SF	18	0	0	72	0	28	28	
	Total	54	2	6	36	6	53	52	
10	GF-FB	36	3	0	0	9	91	89	o-p/alc/ep-up
	GF-F	36	11	0	3	6	91	81	
	GF-SF	72	1	0	3	15	82	81	
	GF-M	72	7	0	9	40	51	47	
	GF-B	54	41	0	12	50	38	22	
	Fl-FB	36	0	0	11	11	78	78	
	Fl-F	36	6	0	3	18	79	75	
	Fl-SF	54	7	0	12	20	68	63	
	Fl-M	54	24	0	37	24	39	30	
	Fl-B	36	53	0	53	0	47	22	
	Bd-SF	36	11	0	0	28	72	64	
	Bd-M	36	0	0	6	58	36	36	
	Bd-B	36	22	0	0	25	75	58	
	Total	594	14	0	10	25	66	56	
12	GF-FB	18	0	6	22	11	61	61	o-p/alc/ep-up
	GF-F	18	6	0	35	12	53	50	
	GF-SF	18	6	0	12	18	71	67	
	Total	54	4	2	23	13	62	59	
13	GF-FB	18	0	17	0	83		83	o-p/alc/ep-up
	GF-F	18	0	0	6	94		94	
	GF-SF	18	0	0	6	94		94	
	GF-M	18	0	0	17	83		83	
	GF-B	18	6	0	24	76		72	
	Fl-FB	18	0	0	0	100		100	
	Fl-F	18	6	6	0	94		89	
	Fl-SF	18	0	0	0	100		100	
	Fl-M	18	0	0	17	83		83	
	Fl-B	18	0	11	33	56		56	

Appendix continued.

a	b	c	d	e	f	g	h	i	j
13	Bd-SF	18	0	0	17		83	83	o-p/alc/ep-up
	Bd-M	18	0	6	56		39	39	
	Bd-B	18	0	0	61		39	39	
	Total	234	1	3	18		79	78	
14	GF-FB	36	0	19	0	17	64	64	o-p/alc/ep-up/ thin
	GF-F	36	0	17	25	36	22	22	
	GF-SF	36	8	39	36	12	12	11	
	GF-M	36	0	64	14	19	3	3	
	GF-B	36	25	37	15	33	15	11	
	Fl-FB	36	0	0	3	19	78	78	
	Fl-F	36	0	6	31	25	39	39	
	Fl-SF	36	0	3	19	44	33	33	
	Fl-M	36	0	39	44	8	8	8	
	Fl-B	36	3	49	23	23	6	6	
	Bd-SF	36	0	3	47	28	22	22	
	Bd-M	36	0	53	42	6	0	0	
	Bd-B	36	3	49	17	26	9	8	
	Total	468	3	29	24	23	24	24	
15a	GF-FB	36	0	17	36	17	31	31	o-p/alc/ep-up
	GF-F	36	3	3	69	23	6	6	
	GF-SF	36	0	6	42	33	14	14	
	GF-M	36	0	0	86	11	3	3	
	GF-B	36	0	44	36	11	8	8	
	Fl-FB	36	6	0	44	26	32	31	
	Fl-F	36	0	0	53	14	33	33	
	Fl-SF	36	0	3	50	25	22	22	
	Fl-M	36	0	0	89	8	3	3	
	Fl-B	36	0	33	47	14	0	0	
	Bd-SF	36	17	0	0	33	67	56	
	Bd-M	36	6	0	38	41	21	19	
	Bd-B	36	17	3	43	33	20	17	
	Total	468	4	9	49	22	20	19	
15b	GF-FB	18	6	18	82	0	0	0	o-p/ep-up/thin/ alc
	GF-F	18	0	6	72	22	0	0	
	GF-SF	18	0	0	89	11	0	0	
	GF-M	18	0	6	96	0	0	0	
	GF-B	18	0	17	72	11	0	0	
	Total	90	1	9	82	9	0	0	

Appendix continued.

a	b	c	d	e	f	g	h	i	j
15b	GF-FB	18	11	6	94	0	0	0	o-p/ep-up/thin
	GF-F	18	6	35	65	0	0	0	
	GF-SF	18	6	0	88	12	0	0	
	GF-M	18	11	6	94	0	0	0	
	GF-B	18	33	67	33	0	0	0	
	Total	90	13	21	77	3	0	0	
	Bd-SF	18	6	0	0	24	76	72	o-p/ep-up/thin/
	Bd-M	18	0	0	56	33	11	11	alc
	Bd-B	18	6	24	18	41	18	17	
	Total	54	4	8	25	33	35	33	
	Bd-SF	18	0	0	11	50	39	39	o-p/ep-up/thin
	Bd-M	18	6	0	59	35	6	6	
	Bd-B	18	22	14	21	50	14	11	
	Total	54	9	4	31	45	20	19	

*Key to Abbreviations: GF: explants excised from green-fruit stage tobacco plants; Fl: explants excised from flowering tobacco plants; Bd: explants excised from floral bud stage tobacco plants; FB: explants excised from the base of floral branches; F: explants excised from the floral region of the tobacco stem; SF: explants excised from the sub-floral region of the tobacco stem; M: explants excised from the middle region of the tobacco stem; B: explants excised from the basal region of the tobacco stem; o-p: explants excised from open-pollenated tobacco plants; s-p: explants from self-pollenated tobacco plants; ep-up: explants plated epidermis up; alc: use of alcohol treatment during the sterilization procedure; thin:explants cut thinner than usual