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THE EFFECTS OF INDOLEACETYLAMINO ACIDS
ON CALLUS GROWTH AND SHOOT REGENERATION
IN TOBACCO AND TOMATO

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Michael Douglas Peterson

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THE EFFECTS OF INDOLEACETYLAMINO ACIDS ON CALLUS GROWTH AND SHOOT REGENERATION IN TOBACCO AND TOMATO

By

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ABSTRACT

THE EFFECTS OF INDOLEACETYLAMINO ACIDS
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Plant tissue cultures can continue to grow long after the exogenous indoleacetic acid is gone from the culture medium. Since it is known that plants conjugate exogenous indoleacetic acid with L-aspartic acid and store the complex in their tissues, it was thought that the continued growth of the cultured tissues might be dependent on some stored complex as a source of auxin. Accordingly, a number of indoleacetylamino acids were prepared and their effects on tissue growth determined.

Indoleacetyl-L-alanine supported growth almost as well as free indoleacetic acid in tobacco callus tissues and was very much more active than free indoleacetic acid in supporting the growth of Marglobe tomato callus tissue. It was also very much more effective than free indoleacetic acid in initiating undifferentiated callus development from Marglobe tomato hypocotyl sections. Consistent with this great auxin activity was the fact that it inhibited shoot formation in all tissues.

In contrast, indoleacetyl-D-alanine supported very little growth of any tissue in any medium. It did, however, ensure the survival of tissues where the absence of auxin caused the tissues to die. This persistent survival in the presence of a severely limited auxin activity favored the growth of many shoots in tissues already known to be capable of producing shoots.

Indoleacetyl-β-alanine and indoleacetylglycine had effects which were intermediate between those of indoleacetyl-L-alanine and indoleacetyl-D-alanine.

There is evidence that indoleacetic acid and the indoleacetylamino acids have qualitatively different effects. The suppression of organogenesis in favor of callus formation, which occurs in the presence of most of the complexes cannot be entirely due to a greater availability of free indoleacetic acid since the addition of a small amount of free indoleacetic acid itself along with the complex restores organogenesis.

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ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
IAA	indoleacetic acid
Iβala	indoleacetyl-β-alanine
IDala	indoleacetyl-D-alanine
IGly	indoleacetylglycine
ILala	indoleacetyl-L-alanine
ILasp	indoleacetyl-L-aspartic acid
uM	micromolar

Introduction

A plant is composed of a number of cell types, each type fulfilling a particular physical or biochemical role. Certain cells, called parenchymatous cells, are relatively unspecialized, which is to say they usually are not irreversibly differentiated into cell types with a single special function. Occupying internal regions of the plant, such as the pith and cortex of stems, such cells constitute storage compartments for substances the plant needs to conserve and sometimes perhaps as storage compartments for substances the plant needs to be rid of (9).

Parenchymatous cells, in contrast to their more differentiated neighbors, can often be induced to undergo differentiation as in the restoration of severed vascular connections and in the formation of adventitious roots or adventitious shoots. Restoration of vascular connections occurs in two ways. When a woody plant is girdled, calluses arising from parenchymatous cells of the bark (phloem) grow from the edges of the wound. When the two calluses from the upper and lower sides meet, differentiation of some of the cells into new cambium and conducting elements takes place. On the other hand, in herbaceous species, severing of a vascular bundle can cause the "vascularization" of adjacent parenchymatous cells to bridge the gap and thus reestablish unbroken conduits (24).

When entirely new roots are required, parenchymatous cells are induced to multiply, but in this case they differentiate into the growing points of roots and therefore ultimately into all of the many cell types characteristic of roots. The ease with which new roots are initiated is very different from plant species to plant species. In general, however, induction is assisted by the presence of exogenous auxin, such as indolebutyric acid (13).

The production of adventitious shoots from parenchymatous cells is often more difficult than the production of roots and has never been accomplished in some plants. On the other hand, shoot production from the parenchymatous cells of leaves, stems or roots is an important method of reproduction in some plants (27). In general, the application of cytokinins favors shoot formation if shoot formation can be induced at all. Whether roots or shoots are formed from a given explant is often determined by the ratio of exogenous auxin to exogenous cytokinin (22). However, if any cell divisions at all are to occur and therefore if there is to be any potential at all to produce either roots or shoots, a minimum level of auxin is usually required. Therefore if the fragment of plant does not contain any auxin-producting region, the fragment will fail to grow and ultimately die unless there is an exogenous auxin source. See Table I.

Table I. Biological Effects of Different Auxin and Cytokinin Levels on Tobacco Stem Pith Tissue (15)

		Cytokinin Level		
		none	low	high
Auxin Level	none	death	death	death of shoots
	low	death	slow callus growth	shoots
	high	death	vigorous callus growth, perhaps some roots	tight callus growth

The Culturing of Isolated Plant Cells and Plant Tissues

In the presence of exogenous auxins, especially in the presence of stable auxins such as 2,4-D, there is a tendency for parenchymatous cells to proliferate indefinitely as a mass of undifferentiated, friable tissue which is similar to wound callus and can be cultured in a sterile nutrient medium (33). Such masses of undifferentiated tissue have many uses and methods for initiating and maintaining them have been widely explored. In the case of tobacco, tomato, potato, carrot, soybean, etc., an explant of parenchymatous tissue (often obtained from hypocotyls or from the pith of the stem of a vegetative, i.e., not-flowering plant) is placed on a solid agar medium containing inorganic salts, vitamins, an auxin, a cytokinin and a carbon source.

Cultured plant tissues can be classified into two groups, primary and secondary. Primary callus is that which has been always grown as a block of tissue, usually on agar. In the case of such primary cultures, it is difficult to be sure that the tissue organization which was present in the parental plant has been completely lost. Secondary callus is that which has, at some point in its history experienced long-term growth as a suspension culture in liquid medium. In this case most of the tissue has probably been derived from a single cell at some stage in the culturing process and, therefore, it is probably safe to say that all parental tissue organization has indeed vanished.

The Regeneration of Plant Organs from Cultured Callus

Just as parenchymatous cells can be induced to differentiate into vascular tissue, roots and shoots *in vivo*, so too can cultured callus sometimes be induced to produce vascular tissue, roots and shoots *in vitro*. Not surprisingly, the high concentrations of stable auxins which initiate callus growth from plant fragments also favor the persistence of callus and strongly inhibit shoot formation (17). Moreover, with callus as with stem explants, high cytokinin levels tend to inhibit root formation and to stimulate shoot formation (15). On the other hand, a low cytokinin to auxin ratio tends to favor root formation from either callus or stem explants, provided the auxin is not so active that only callus growth results.

It has often been possible to generate roots and shoots and thus whole plants from primary callus tissues derived from a number of different plant species. However one cannot be sure that new plants do not owe some of their different cell types to residual organization carried through the primary callus. In contrast, secondary callus has rarely been induced to regenerate new plants and, indeed, retrieval of plants from secondary callus has been achieved with regularity only with carrot and tobacco (4,11). No one has yet been able to regenerate plants from either primary or secondary callus of most plants (29). Nevertheless, the fact that any species at all can regenerate entire plants from single cells means that, at least in these species, undifferentiated parenchymatous cells (and callus cells derived therefrom) retain all of the information required for a normal, mature individual. Whether this "totipotency" is a general characteristic of undifferentiated plant cells remains to be seen. The fact that many diverse plant tissues seem to revert to one common callus cell type implies that there have been no irreversible changes associated with the development of these diverse tissues. The situation is quite different with many mammalian tissues which retain characteristic differences when they are cultured. In the mammalian case, differentiation does seem to have caused heritable changes which may well be irreversible. On the other hand, we cannot be sure on the basis of superficial appearances that there have not also been some irreversible changes in the majority of cultured plant tissues. The fact that it still remains impossible to regenerate plants from the cultured callus of most species may indicate that the cells of such calluses, like animal tissue cultures, have undergone irreversible heritable changes which are less apparent than in the case of the animal tissues. At the moment, we cannot decide between this pessimistic view and the more optimistic view that many cultured plant cells are really "totipotent" but that we have not yet discovered the right signals to induce organogenesis.

One reason for the pessimistic view is the fact that plant tissues often undergo changes in their chromosome number while they are being cultured. Indeed, there is considerable evidence that cultured tissues may lose their regenerative capacity through changes in chromosome number and other aberrations (30).

Potential Advantages of Plant Regeneration

Several promising areas of research depend on our ability to harness plant cell totipotency. They are: 1) Cloning plants from single cells; 2) Genetic transfer between species using protoplast fusion and; 3) Mutation formation in single plant cells and selection for desirable characteristics at the single cell level (4).

Being able to clone plants is of great importance to the commercial grower who wishes to produce a great number of genetically identical individuals. Until the advent of plant tissue culture techniques, cloning was accomplished using highly organized tissues such as stem sections and leaves. However, these methods might allow genetic changes in the clone to occur and accumulate in the form of chimeras. In fact, a number of the clones represented by our older cultivars may be chimeral in nature. For example, the cells of the apical meristem that give rise to the epidermis (tunica) may be different from the cells that give rise to the internal portions of the plant (corpus) (9). Stable chimeras normally only occur in species where buds arise from places these two regions of the meristem remain quite distinct and cells of the two almost never intermingle. It is known that such chimeras can be initiated as a result of a bud arising at the graft union between two genetically different individuals (28).

Culturing plant tissue fragments has in several cases, such as the orchid, made it possible to reproduce vegetatively plants that could not be easily propagated using other methods (17). Such cloning usually does not involve the establishment of an undifferentiated callus culture but rather the proliferation of an organized tissue, often the apical meristem. Virus-free stocks of plants have been established in this manner. An explanation of this might be that apical meristems sometimes are able to grow faster than the viral infection can spread (22).

There is currently much interest in the possibility of obtaining hybrids between plant species that cannot hybridize in nature. To date, protoplast fusion with subsequent plant regeneration has been achieved only in the case of pairs of species that do hybridize in nature (4,16,21). Protoplast fusion and heterokaryon formation have been accomplished between many other more distantly related species, but no plants have been regenerated from such fused cells (6). Experience with somatic animal cell hybrids indicates that, at least in fusions between cells of very distantly related species such heterokaryons are likely to be unstable. Usually part or all of one of the genomes is excluded. However there is reason to believe that, in cases where there seems to have been a complete loss of one genome by cytological criteria, there may be in fact a retention of some of the genetic information from that genome. For instance, in one example, an enzyme coded for by the apparently missing chromosome complement continued to be produced (7). Thus there is reason to hope that it may some day be possible to transfer genes between widely different species which certainly could never cross using the regular sexual process and probably could not make successful heterokaryons.

The considerable advances of mutagenesis and screening techniques in microorganisms in recent years are beginning to be applied to single plant cells (4). Single cells are better suited than are whole plants for the recovery of some desired mutant types for several reasons. Whole plants are large organisms with long generation times; the large nutrient pools make difficulties in assessing biochemical mutations since failure to synthesize substances may become apparent only after some time; whole plants are composed of cells in many differentiated states; they are diploid, or often, polyploid, and again this presents grave problems in the analysis of mutations. In contrast, single haploid cells are small with short generation times and small nutrient pools; they are usually composed of uniform cells in an undifferentiated state; genetically there is one copy for each trait, so that recessive traits can be expressed.

Cell lines with disease resistance (12), cell lines that have herbicide tolerance (19), cell lines that over or underproduce certain amino acids (20), and cell lines that are tolerant of high levels of different elements (8) have already been produced. However only in tobacco has a trait selected at the single cell level been expressed on the whole plant level (3). Unfortunately, a major drawback to the application of all single cell techniques in the selection of desirable plant characteristics is that we are usually unable to distinguish these desirable characteristics in their single cell manifestations.

Auxins in Plant Tissue Culture

Currently 2,4-dichlorophenoxyacetic acid (2,4-D) and indoleacetic acid (IAA) are the most widely used forms of auxin for the growth of tissue cultures. Of the two, 2,4-D is the more persistent in tissue culture media and it is probably for this reason that it seems to be about 100 times as active as indoleacetic acid in the tobacco callus growth bioassay. Since it is often necessary to incubate plant fragments on a culture medium containing a high level of auxin activity to induce friable callus formation, 2,4-D has sometimes been used routinely as a constituent of callus induction media, especially when the plant species being investigated does not respond well to the more labile IAA (29). As expected from the fact that it is more active in supporting callus growth than IAA, it is easy to show that 2,4-D is much more inhibitory to shoot formation than is indoleacetic acid. Furthermore, friable callus initiated using 2,4-D frequently cannot be induced to produce shoots, even after it has been transferred for some time to IAA. Perhaps in providing enough auxin activity to induce callus we have been providing too much auxin for retention of an organogenic potential.

In many cultured tissues that do form shoots, a very low level of indoleacetic acid is either required for or greatly stimulatory to shoot production (26). Usually a certain auxin to cytokinin ratio, rather than the auxin level *per se* promotes the most bud formation. But might not the concentration of indoleacetic acid that is optimal for shoot induction be somewhat less than the concentration of IAA initially present in the culture medium. This would be true if the initial concentration needs to be high in order to allow for the rapid disappearance of IAA. That is to say, the rapid disappearance of auxin may make it necessary to apply an ititial level which produces a long-term inhibition of shoot production. Thus the situation with indoleacetic acid in tissue cultures may be analogous to the situation which we suspect may exist in 2,4-D-initiated callus, that is, in order to provide enough auxin activity for the growth of parenchymatous cells, it may be necessary to supply too much auxin activity for shoot induction. Therefore it seemed possible that the presence of a large reservoir of a stable but relatively inactive auxin or auxin precursor might permit cell multiplication without inhibiting bud formation.

Whole plants may be regulating internal levels of free indoleacetic acid in this way, by the reversible formation of auxin complexes which in turn may or may not have some auxin activity in their own right. Free IAA does not seem to occur in plants except in extremely low concentrations. Most of the IAA is bound, presumably either by rather stable amide linkages or by more labile ester linkages. The best characterized of these IAA complexes are various naturally occurring isomers of indoleacetyl-meso-inositol (2). When plant tissues are supplied with generous amounts of indoleacetic acid, the IAA is taken up and converted into indoleacetyl-L-aspartic acid, indoleacetyl-1-glucose and into much smaller smounts of other complexes (1,25,31). The laboratories of Hamilton (10) and Rekoslavskaya (23) have shown that these stored forms of auxin may be used to support the growth of tissue cultures.

The fact that auxin-dependent tissues will often undergo several mitotic divisions in the absence of exogenous IAA before ceasing to grow suggests that the indoleacetic acid initially presented to the cultures may have been taken up and stored as IAA complexes. We say "as complexes" rather than "as free IAA" because it has been shown that the uptake of exogenous indoleacetic acid rarely results in any accumulation of free IAA in the tissues (1). If an appreciable amount of the exogenous IAA is stored as complexes in the tissues, it would indeed seem to be serving as a reservoir for the slow release of an auxin, hence the continued growth.

My research dealt with how well various indoleacetic acid-amino acid complexes could serve as exogenous sources of auxin for plant tissue cultures. This work was especially concerned with the development of improved regeneration of whole plants from callus.

Summary of the Rationale for this Research

Indoleacetylamino acid complexes might have the double advantage of constituting a long lasting source of a stable but low auxin activity, thus perhaps preserving organogenic potential while permitting continued cell division.

Methods

I. Cell Culture Medium.

A. Basal medium: slightly revised version of the medium in Murashige and Skoog, 1962:

Constituent	Concentration (mg/l)	(Molar)
KNO_3	1900	1.88×10^{-2}
NH_4NO_3	1650	2.06×10^{-2}
$MgSO_4 \cdot 7H_2O$	370	1.50×10^{-3}
$CaCl_2 \cdot 2H_2O$	440	2.99×10^{-3}
KH_2PO_4	170	1.25×10^{-3}
$MnSO_4 \cdot H_2O$	16.8	9.94×10^{-5}
H_3BO_3	6.2	1.00×10^{-4}
$ZnSO_4 \cdot 7H_2O$	10.6	3.69×10^{-5}
$NaMoO_4 \cdot 2H_2O$	0.25	1.14×10^{-6}
$CuSO_4 \cdot 5H_2O$	0.025	1.57×10^{-7}
$CoCl_2 \cdot 6H_2O$	0.025	1.05×10^{-7}
KI	0.83	5.00×10^{-6}
$FeSO_4 \cdot 7H_2O$	27.8	1.00×10^{-4}
$Na_2EDTA \cdot 2H_2O$	37.3	1.00×10^{-4}
Thiamine HCl	1.0	2.96×10^{-6}
Myo-inositol	100	5.55×10^{-4}
Sucrose	30,000	8.76×10^{-2}
Agar	9000	

B. Preparation of media:

1. Before autoclaving, all media were adjusted to pH 6.0 with KOH.
2. When agar was used, sucrose was autoclaved separately from the other constituents of the medium.
3. Volumes of media greater than or equal to 250 mls were autoclaved for 15 minutes at a steam pressure of 15 lbs. Volumes less than 250 mls were autoclaved for 12 minutes.
4. No constituents were filter sterilized. Auxins and cytokinins were added to the media before autoclaving.
5. Culture media were stored at room temperature in the dark.

II. Tissues.

A. Tobacco cultivar "Wisconsin 38":

1. Callus which had always been maintained on solid medium: a one year old pith culture initiated and grown in the dark at 27 ± 1°C on solid basal medium to which were added 17 uM indoleacetic acid and 1.4 uM kinetin.

2. Callus which had been through a liquid suspension phase: a one year old pith culture, initiated and grown in the dark at 27 ± 1°C on solid basal medium to which were added 17 uM indoleacetic acid and 1.4 uM kinetin; then transferred, after the establishment of rapidly growing callus, to liquid basal medium also containing 17 uM indoleacetic acid and 1.4 uM kinetin and grown in the dark.

B. Tomato cultivar "Marglobe":

1. Hypocotyl sections from seedlings, grown sterilely in the dark at 27 ± 1°C for six days on solid basal medium plus 1 mg per liter each of nicotinic acid (8.12×10^{-6} M) and pyridoxine HCl (4.86×10^{-6} M).

2. Callus which had been through a liquid suspension phase: a two year old culture derived from anthers; callus initiated on solid basal medium to which were added 1 mg per liter each of nicotinic acid and pyridoxine HCl, 29 uM indoleacetic acid, 2.3 uM 2,4-D and 1.4 uM kinetin. After initiation of vigorous growth, callus was transferred to a liquid medium with the same constituents. After about one year as a suspension culture, growth was continued for another year on a liquid medium from which

the 2,4-D had been omitted but which contained all the other constituents.

III. Growth conditions during experiments.

A. Suspension cultures were grown in the dark at 27 ± 1°C in 50 mls of culture medium in 125 ml Erlenmeyer flasks with foam stoppers. Flasks were shaken at 125 revolutions per minute on a rotary shaker.

B. Callus cultures:

1. Calluses were grown in the light at 22 ± 1°C on a 16 hr light- 8 hr dark cycle under GE Delux cool-white fluorescent tubes at a distance to give 550-750 lux of illumination.

2. Calluses were grown in the dark in controlled-temperature incubators at 27 ± 1°C.

IV. Biological variables studies.

A. Growth:

1. Growth of callus was recorded as (final fresh weight-initial fresh weight)/initial weight or as increase in volume/initial volume.

2. Growth of cell suspensions was recorded as the settled volume of cells after settling for 30 minutes at one times gravity. Settled volumes were measured using graduated conical-bottom 50 ml test tubes with screw caps.

B. Organogenesis:

1. "Shoots" were defined as centers from which at least one green, leaf-like structure emanated, distinguishable at 6x magnification.
2. The roots counted were those arising directly from the callus, as descernable at 6x magnification. In this procedure, lateral roots were not counted.

V. Syntheses of IAA-amino acid complexes.

The complexes of indoleacetic acid with glycine, D-alanine, L-alanine and β-alanine were prepared in the following manner which is a modification of the method of Weiland and Hörlein (32). The reaction sequence is shown below. Ten millimoles of IAA were dissolved at 0°C with stirring in 30 ml of tetrahydrofuran plus 1.4 ml of triethylamine (10 millimoles). Over a period of 30 seconds 0.96 ml of ethylchloroformate (10 millimoles) were added dropwise with rapid stirring (I). After another 5 minutes of stirring at 0°C, 10 millimoles of the sodium salt of the amino acid were added in 8 ml of H_2O. The mixture was allowed to warm to room temperature with continuous stirring (II).

1. $$R-C(=O)OH + N(C_2H_5)_3 \longrightarrow R-C(=O)O^- + H\overset{+}{N}(C_2H_5)_3$$

2. $$R-C(=O)O^- + H\overset{+}{N}(C_2H_5)_3 + Cl-C(=O)-OC_2H_5 \longrightarrow$$

$$(C_2H_5)_3\ NHCl + \underset{\mathrm{I}}{R-\overset{\displaystyle O}{\overset{\|}{C}}-O-\overset{\displaystyle O}{\overset{\|}{C}}-O-C_2H_5}$$

3. $$R-\overset{\displaystyle O}{\overset{\|}{C}}-O-\overset{\displaystyle O}{\overset{\|}{C}}-O-C_2H_5 + H_2N-CH(R')-CO_2^-\ Na^+ \longrightarrow$$

$$\underset{\mathrm{II}}{R-\overset{\displaystyle O}{\overset{\|}{C}}-NH-CH(R')\ CO_2^-\ Na^+} + CO_2 + C_2H_5OH$$

R = indole

R' = side chain of the amino acid

The aqueous lower phase was then adjusted to pH 3.6 with HCl and extracted 3 times with 25 ml of chloroform in order to extract any unreacted indoleacetic acid. The pooled chloroform phases were extracted twice with 25 ml water to remove any product carried over into the chloroform and these washings were back-extracted once with 20 ml chloroform. The chloroform washed original aqueous phase and the aqueous washings of the chloroform washings were combined and acidified to pH 2.9 with HCl in order to convert the product into its non-polar protonated form. The acidified combined aqueous phases were then extracted 3 times with 25 ml of 1-butanol. The pooled butanol extracts were washed once with 50 ml of water. The water wash was back-extracted twice with 5 ml of 1-butanol. The butanol phases were pooled and the butanol was removed by distillation at reduced pressure (about 15 mm Hg). The viscous residue was dissolved in approximately 15 ml of 50% 2-propanol in water (v/v). The crude product was then passed over a Sephadex LH-20 column, 4 cm x 30 cm, using more 50% aqueous 2-propanol as the solvent. The products were eluted after about 450 to 500 ml of solvent had passed over the column. The residual unreacted IAA was not eluted until 630 to 670 ml had passed. The three 20 ml fractions containing most of the product were pooled and concentrated by distillation at reduced pressure (about 15 mm Hg). The products were then crystallized from ethylacetate by adding hexane. Note, however, that the indoleacetylglycine crystallized from the water as the 2-propanol was removed by distillation.

At all stages in these operations the distributions of the product and the unreacted IAA were followed by thin layer chromatography of the solvent phases and location of the indoles on the chromatogram using the Ehrlich indole reagent. Yields in most cases were somewhat under 40%.

Indoleacetic acid was obtained from Aldrich Chemical Company, 940 West Saint Paul Avenue, Milwaukee, Wisconsin 53223. Ethylchloroformate and β-alanine were obtained from Eastman Organic Chemicals, 1187 Ridge Road West, Rochester, New York 14650. Glycine, D-alanine and L-alanine were obtained from Sigma Chemical Company, P.O. Box 14508, Saint Louis, Missouri 63178. Already prepared indoleacetyl-L-aspartic acid was obtained as the dicyclohexylammonium salt from Calbiochem, P.O. Box 12087, San Diego, California 92112.

Results

The D-alanine, L-alanine, β-alanine, glycine and L-aspartic acid complexes of indoleacetic acid were used as auxin sources in growth and regeneration experiments with tissue cultures from tobacco and tomato. Some experiments in which there was no organogenesis provided data on growth of callus. In those experiments where "shoots" and roots appeared in addition to callus, measurements of growth included contributions of mass made by organized tissues. Probably in some cases the organized tissues themselves contributed endogenous hormones which permitted further growth of callus and therefore the factors contributing to organogenesis cannot always be separated clearly from the factors contributing to callus growth.

I. Effects of Indoleacetylamino Acids on Growth of Tobacco and Tomato Cells in Culture

A. Effects of indoleacetylglycine and indoleacetic acid on the growth of suspensions of tobacco cells.

Indoleacetylglycine persists for much longer in the suspension medium than does indoleacetic acid itself. One week after the beginning of the experiment, when indoleacetic acid had long since disappeared from the medium, about 30% of the indoleacetylglycine remained, as detected by the

Ehrlich indole reagent on thin layer chromatograms of chloroform extracts of the media. Nevertheless, the total amount of growth supported by IAA is about the same as that supported by indoleacetylglycine at all concentrations tested, down to less than 1 micromolar.

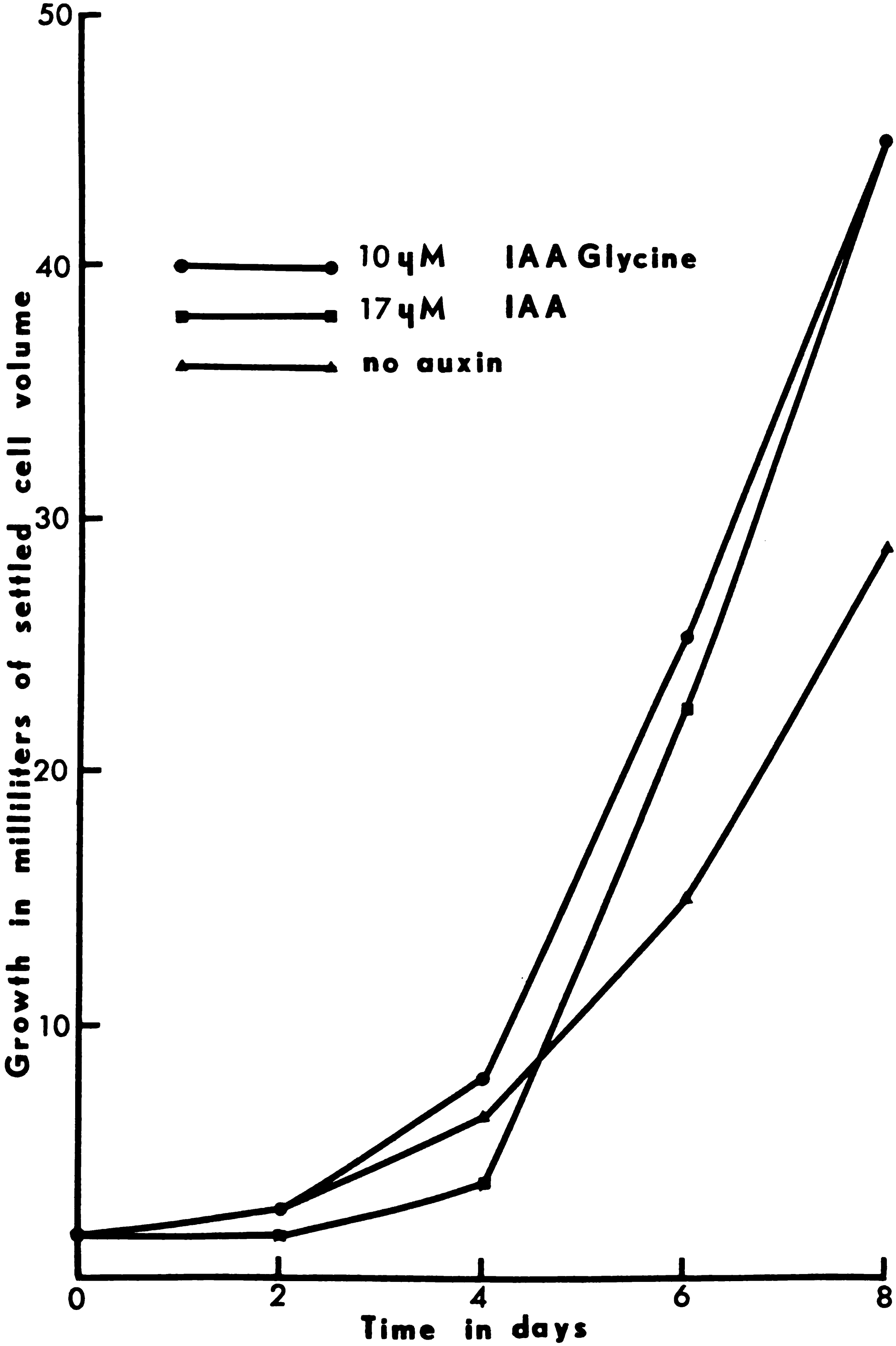
As shown in Figure 1, there seems to be a considerable carry-over of auxin from the inoculum since growth persisted in these auxin-dependent cells for 8 days even in the absence of exogenous auxin. Indoleacetylglycine at the concentration used supported the same amount of growth as did the slightly lower concentration of IAA. Moreover, indoleacetylglycine did not inhibit growth for the first few days as IAA always did at almost any concentration (down to 0.6 uM). In fact, indoleacetylglycine did not have this inhibitory effect at any concentration tested (up to 10 uM).

The persistence of growth in the absence of any exogenous auxin should be noted. As was pointed out in the introduction, this phenomenon suggests that auxin is somehow stored in the cells, and that the stored auxin is capable of supplying all of the auxin needs of the cells for many days.

B. Effects of indoleacetyl-L-alanine, indoleacetyl-D-alanine and indoleacetic acid on the growth of tobacco pith callus tissue on solid media.

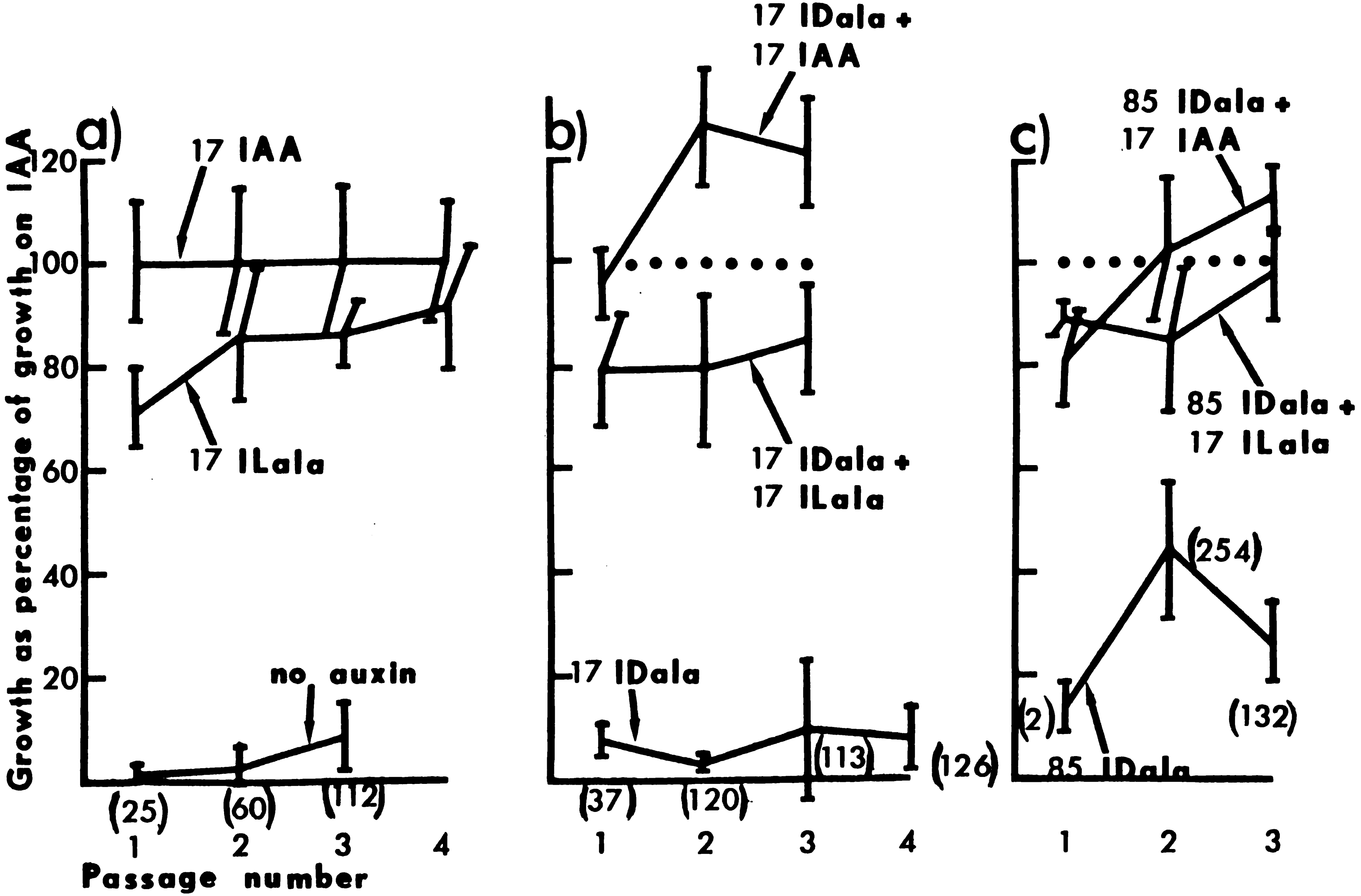
Figure 2 shows some of the effects of maintaining tobacco tissue cultures on indoleacetylamino acids through several passages. Indoleacetyl-D-alanine had very little effect on either callus growth or "shoot" initiation except at the

Figure 1. The effects of exogenous indoleacetic acid and indoleacetylglycine on the growth of suspensions of tobacco cells. Growth was measured at two day intervals as milliliters of settled cells after 30 minutes at 1 x g. The cells were taken from basal liquid medium contain 17 uM IAA and 1.4 uM kinetin and grown for two days on a similar medium containing no indoleacetic acid. The cells were then transferred to the test media, all of which contained 1.4 uM kinetin. Although these cells had an absolute requirement for an auxin and would have ultimately died in the medium lacking indoleacetic acid, they continued to grow well for a total of 10 days after their removal from an external source of auxin. This implies that the tissues were able to store indoleacetic acid or a product of indoleacetic acid which served for a time as an internal auxin source.



50
40
30
20
10
0
2
4
6
8
Time in days
Growth in milliliters of settled cell volume
10 μM IAA Glycine
17 μM IAA
no auxin

Figure 2. The effects of indoleacetyl-L-alanine, indoleacetyl-D-alanine and indoleacetic acid on the growth of tobacco pith callus tissue on solid media. Five callus inocula, each weighing about 60 mg, were placed in a 10 cm petri plate containing basal medium plus 1.4 uM kinetin and the indicated auxin. Incubation was in the dark for 4 weeks, whereupon one piece of carefully selected undifferentiated callus from each block of tissue was sub-cultured for another 4 weeks on the same medium as the parent block. The initial callus was sub-cultured in this manner 2 or 3 times giving a total of 3 or 4 "passages". At the end of each passage the callus block the small piece used for transfer, was weighed. Growth data represent increase in fresh weight/initial weight. Between 4 and 10 plates were used per treatment. Numbers before the names of the various auxins are concentrations (micromolar). The numbers in brackets indicate the number of "shoots" per plate arising as a result of each passage. Standard deviations among the weights of the blocks grown on 17 uM IAA are shown in the upper part of Figure 2a. Means of growth on 17 uM IAA for the 4 passages were 35.2, 38.6, 36.9 and 30.3.


a)
b)
c)
Growth as percentage of growth on IAA
120
100
80
60
40
20
17 IAA
17 ILala
no auxin
(25)
(60)
(112)
17 IDala +
17 IAA
17 IDala +
17 ILala
17 IDala
(37)
(120)
(113)
(126)
85 IDala +
17 IAA
85 IDala +
17 ILala
(254)
(2)
(132)
85 IDala
1
2
3
4
Passage number

highest concentration where there was a slight stimulation of callus growth without any decrease in the number of "shoots" formed. Both indoleacetyl-L-alanine and IAA itself supported vigorous callus growth and suppressed all "shoot" formation. The D-alanine complex had very little effect on the growth without organogenesis when it was combined with either the L-alanine complex or with IAA. The high auxin activity of the L-isomer and the very low auxin activity of the D-isomer is consistent with the idea that these complexes are serving as sources of IAA after their hydrolysis by the cell's peptidases which are likely to be almost entirely specific for the L-isomer.

On the other hand, as we shall see, there is some reason to believe that these complexes may be auxins in their own right. If so, hormone receptor sites must also be specific for L-isomers.

C. Growth of Marglobe tomato callus on solid media.

Tomato tissue, unlike tobacco tissue, seems to lose the capacity to produce roots and shoots in culture soon after callus growth is initiated. We wondered whether the responses of tomato tissues to indoleacetic acid and its amino acid complexes might differ depending on the level of organization of the tissues used. For this reason tomato cell suspensions and tomato stem hypocotyls, representing a very wide range of organization, were grown on these various auxins.

The tomato cell suspension, totally lacking the ability to form any organized structures, was tested for growth on agar. See Table II. Interestingly, in this case, the L-alanine, glycine and β-alanine complexes all supported more growth at every concentration than did IAA. In fact, the L-alanine complex supported more growth at the lowest concentration than IAA did at the highest. There were no marked synergisms and no marked inhibitions by excess auxin even in the case of the L-alanine complex where the highest concentration (57 uM) gave almost the same growth as the lowest concentration (0.57 uM). The D-alanine complex supported very little growth at low concentrations but at 57 uM it was almost as good as IAA.

The media used in Table II were also used to study the responses of hypocotyl sections. See Table III. All of the complexes except indoleacetyl-D-alanine were responsible for initiation of unorganized, fast-growing callus. Roots and "shoots" were produced in many of the treatments and these data are reported in Table VI. The D-alanine complex and IAA itself did not support the development of much callus but instead supported the growth of "shoots" or roots. In general there was a good correspondense between callus initiation in the hypocotyl sections and growth of callus from the suspended cells.

Table II. Growth of Marglobe Tomato Callus on Solid Media

Suspended cells (about 0.5 g fresh weight) were removed from liquid medium by means of a mesh scoop and inoculated onto 6 cm petri plates containing basal medium to which were added 1 mg per liter each of nicotinic acid and pyridoxine HCl, benzyladenine (8.9 uM) and the indicated concentrations of indoleacetic acid or indoleacetic acid-amino acid complexes. Data represent increase in fresh weight/initial weight after 37 days growth in the light. Weight after 37 days in the absence of any auxin was arbitrarily chosen to represent the initial weight.

	Callus Growth (fresh weight gain/initial weight)		
Kind of Auxin	57 uM auxin	5.7 uM auxin	0.57 uM auxin
IAA	13.1	6.4	1.6
IDala	11.2	0.74	0.49
IDala + IAA 9 : 1	11.1	3.7	0.23
ILala	15.2	17.1	14.1
ILala + IAA 9 : 1	14.0	15.4	12.3
IGly	15.3	13.8	6.6
IGly + IAA 9 : 1	15.0	15.4	7.7
Iβala	13.5	17.8	2.5
Iβala + IAA 9 : 1	15.2	12.8	2.2

Table III. Induction of Callus from Marglobe Tomato Hypocotyl Segments

Data taken from the experiment also reported in Table VI. Sterile hypocotyls from seedlings grown 6 days in the dark were cut into sections 0.5 cm long and placed on a solid basal medium to which were added 1 mg per liter each of nicotinic acid and pyridoxine HCl, benzyladenine (8.9 uM) and the indicated concentrations of indoleacetic acid or indoleacetic acid-amino acid complexes. There were 6 replicates, 3 on each of two 6 cm plates. Callus formation was assessed after 53 days growth in the light. At this time, 3 calluses from each treatment were photographed and paper tracings of the photographs were cut out and weighed. The weights were taken to the 3/2 power as estimates of tissue volume, and the average volumes were calculated. Data represent increase in volume/initial volume. Volume after 53 days in the absence of any source of auxin was arbitrarily chosen to represent the initial volume, since the hypocotyl sections denied auxin failed to grow at all and died.

	Callus Initiation (volume gain/initial volume)		
Kind of Auxin	57 uM auxin	5.7 uM auxin	0.57 uM auxin
IAA	29.3	26.1	3.6
IDala	3.8	3.8	6.2
IDala + IAA 9 : 1	10.0	4.5	4.4
ILala	69.9	67.5	15.2
ILala + IAA 9 : 1	67.0	75.6	18.8
IGly	75.1	69.4	0.86
IGly + IAA 9 : 1	96.0	75.5	1.8
Iβala	85.6	9.3	0.37
Iβala + IAA 9 : 1	82.7	50.8	2.5

II. Effects of Indoleacetylamino Acids on Regeneration of "Shoots" and Roots from Tobacco and Tomato Cells in Culture

The use of auxins in tissue culture is usually thought of in terms of the support of growth of undifferentiated cells. Nonetheless, auxins are often necessary components of media designed to induce "shoot" formation from callus. Because these tissues are not able to make their own auxin until the appearance of differentiated structures which can serve as sources of hormones, a low level of auxin is required to keep the cells alive. One of the difficulties in achieving regeneration of plants from cultured plant tissues may be the problem of supplying the tissue with auxin over the long periods of growth which are often necessary to induce "shoot" formation. IAA would not seem to be a satisfactory auxin source for regeneration experiments because of its rapid disappearance from the culture medium. The persistence of the indoleacetylamino acids and the long-term auxin activity associated with this persistence would seem to make them more useful than IAA in attempts to provide the stable very low levels of auxin which might be expected to help in the initiation of "shoots".

In order for an IAA complex to be useful in regeneration experiments, IAA must not be released from the complex at a rate that is inhibitory to "shoot" formation. One might expect the organogenic capacity of cultured tissues to be more suppressed by those IAA complexes that have high activity in supporting growth, than by those IAA complexes that have a limited auxin activity in this regard. One might also expect that the activity of the complexes in supporting growth would be related to the ease with which they can be metabolically converted into free IAA. For instance, the L-isomer of the alanine complex should be much more active in supporting growth than the D-isomer and as we have seen, such is the case. This argument implies, of course, that the complex serves as an auxin only by breakdown to IAA. However, it is difficult to understand the behavior of IAA itself in either growth or regeneration experiments because the auxin activity of IAA relative to the IAA complexes seems to vary from one plant species to another and, as we shall see later on, from one kind of medium to another.

A. Effects of indoleacetylamino acids on the regeneration of "shoots" from tobacco callus

When tobacco tissues were grown on a kinetin-containing medium lacking nicotinic acid and pyridoxine HCl, "shoot" formation was inhibited most by IAA itself. See Table IV. All IAA complexes inhibited "shoot" formation less than did IAA. The glycine and L-alanine complexes, which supported good cell growth (Fig. 1 and 2), inhibited "shoot" initiation more than did the D-alanine complex, which only weakly supported callus growth. Indoleacetyl-D-alanine strongly promoted "shoot" formation even at the highest levels. It was better for this purpose than the other complexes, or than indoleacetic acid itself, at any concentration tested. It was also better than no auxin at all.

The effects of the various auxin complexes on the regeneration of "shoots" from tobacco callus were investigated in the presence of a different cytokinin, benzyladenine, nicotinic acid and pyridoxine HCl. Results are shown in Table V. In this experiment, the inhibitions of "shoot" formation by indoleacetyl-L-alanine, indoleacetylglycine and indoleacetyl-D-alanine were similar to the inhibitions of "shoot" formation by these complexes in the previously used media. In addition, the effects of two other complexes, indoleacetyl-L-aspartic acid and indoleacetyl-β-alanine were studied. Indoleacetyl-L-aspartic acid caused about the same degree of "shoot" initiation as did indoleacetyl-D-alanine, while indoleacetyl-β-alanine inhibited "shoot" formation about as much as did indoleacetylglycine.

Note that indoleacetyl-D-alanine and indoleacetyl-L-aspartic acid caused the initiation of more "shoots" than did any of the other complexes, and more than occured in the absence of any exogenous auxin, but not more than did indoleacetic acid itself. In contrast to the earlier results with a different medium (see Table IV), indoleacetic acid promoted "shoot" formation at concentrations of up to 57 uM. Perhaps this inability of IAA to inhibit "shoot" formation is causally related to the inability of IAA to initiate and

Table IV. "Shoot" Production from Tobacco Callus with two Different Kinetin Levels and Various Auxins

Twenty-five callus fragments, each about 60 mg, of undifferentiated tissue, which had been grown on basal medium containing 1.4 uM kinetin and 17 uM indoleacetic acid were transferred to each condition. Growth period was 4 weeks. The basal agar media contained the two indicated concentrations of kinetin and the indicated concentrations of auxins. Unbracketed numbers are totals of "shoots" from all 25 pieces. Numbers in brackets are pieces that produced at least one "shoot" under each condition.

"Shoots" Formed by 25 Pieces

Level of	IAA		ILala		IGly		IDala	
Auxin (uM)	Light	Dark	Light	Dark	Light	Dark	Light	Dark
			a) Kinetin 14 uM					
17	0	0	0	0	10(4)	10(2)	235(11)	76(12)
1.7	1(1)	0	2(2)	0	137(10)	177(11)	205(14)	187(10)
0.17	43(4)	10(2)	93(8)	160(10)	104(5)	43(6)	43(6)	2(1)
0	60(5)	30(5)	-	-	-	-	-	-
			b) Kinetin 1.4 uM					
17	0	0	0	0	14(4)	1(1)	146(19)	67(12)
0	12(3)	3(1)	-	-	-	-	-	-

Table V. "Shoot" Production from Tobacco Callus with Benzyladenine and Different Concentrations of Various Auxins

Six callus fragments, each about 50 mg, of undifferentiated tissue, which had been grown on basal medium containing 1.4 uM kinetin and 17 uM indoleacetic acid were transferred to a basal medium containing 8.9 uM benzyladenine, 1 mg per liter each of nicotinic acid and pyridoxine HCl and the auxin or auxins at the indicated concentrations. "Shoots" were counted after 29 days in the light. Unbracketed numbers are total "shoots" from the six pieces. Numbers in brackets are pieces of tissue that gave rise to at least one "shoot".

	"Shoots" Formed by 6 Pieces						
Total Auxin Concentration (uM)	57	5.7	0.57	0.057	0.0057	0.00057	0
IAA	43(4)	0	9(2)	55(6)	34(3)	5(2)	6(2)
IDala	13(1)	52(4)	18(1)	-	-	-	"
IDala + IAA 9 : 1	9(2)	33(6)	25(2)	-	-	-	"
ILala	0	0	5(3)	20(1)	-	-	"
ILala + IAA 9 : 1	0	0	0	43(3)	-	-	"
IGly	0	12(2)	16(3)	7(2)	-	-	"
IGly + IAA 9 : 1	0	13(2)	52(4)	118(4)	-	-	"
I ala	0	1(1)	9(3)	0	-	-	"
I ala + IAA 9 : 1	0	1(1)	21(2)	0	-	-	"
ILasp	56(4)	7(2)	9(2)	4(1)	-	-	"
ILasp + IAA 9 : 1	54(5)	12(5)	88(6)	5(2)	-	-	"

- indicates not tested

support growth of unorganized callus at low concentrations in tomato (See Table III), since in the tomato experiments, the same benzyladenine-nicotinic acid-pyridoxine HCl medium was used.

B. Effects of indoleacetylamino acids on the regeneration of "shoots" from tomato stem hypocotyl tissue and in the callus derived therefrom

One of the prime aims of this research was to explore the possible uses of the amino acid complexes of indoleacetic acid in helping to bring about the regeneration of plants from undifferentiated callus. Since we had no way of knowing the organogenic potentials of already established homogeneous callus cultures, we decided to measure the effects of the various auxin sources on organogenesis at various stages in the transformation of differentiated hypocotyl tissues into relatively undifferentiated callus. And, since we had already investigated the effects on established tobacco cultures, we decided to study organgenesis in another species where it is more difficult to achieve, namely in tomato. We looked at the effects of the auxin complexes both on the maintainance of organogenic potential and on the expression of this potential in the form of "shoots" and roots.

As shown in Table VI, those IAA complexes which favored undifferentiated callus formation from the hypocotyl sections also tended to suppress "shoot" and root formation. The better the callus growth, the stronger was the inhibition

Table VI. Production of "Shoots" and Roots from Marglobe Tomato Hypocotyl Segments

Data from the experiment reported also in Table III, quod vide. Numbers of "shoots" and roots were recorded after 53 days. Numbers to the left of the slash refer to "shoots" and numbers to the right refer to roots. Unbracketed numbers record the individual "shoots" or roots, while bracketed numbers record hypocotyl segments giving rise to at least one "shoot" or one root.

"Shoots" or Roots Formed by 6 Hypocotyl Sections

Total Auxin Concentration (micromolar)	57	5.7	0.57	0
Kind of Auxin				
IAA	0/50(6)	7(3)/31(5)	10(3)/0	no growth of any kind
IDala	7(1)/0	1(1)/0	23(2)/0	"
IDala + IAA 9 : 1	10(3)/0	8(3)/5(1)	8(2)/0	"
ILala	0/0	0/0	0/1(1)	"
ILala + IAA 9 : 1	0/2(1)	0/3(3)	0/0	"
IGly	0/3(2)	0/0	1(1)/0	"
IGly + IAA 9 : 1	0/0	0/5(2)	6(2)/0	"
Iβala	0/0	0/0	0/0	"
Iβala + IAA	0/2(1)	6(2)/11(2)	1(1)/0	"

of "shoots" and roots whereas the better the growth of "shoots" and roots, the worse the growth of callus. In the absence of any source of auxin, there was no growth at all and the hypocotyl segments eventually died. As expected (18), low concentrations of IAA caused "shoot" formation and high concentrations of IAA caused root formation; there was also considerable growth of a not very friable kind of callus at high levels of IAA. At all concentrations tested, indoleacetyl-D-alanine maintained the sections in a healthy condition and allowed the segments to produce "shoots", but no roots and no callus until "shoots" were initiated; many sections failed to produce either organs or callus and failed to grow although they seemed to remain healthy. In contrast, indoleacetyl-L-alanine supported good callus growth at all concentrations and organogenesis was almost entirely abolished. Indoleacetyl-β-alanine suppressed all organogenesis and also allowed the sections to produce large amounts of callus at higher concentrations. Interestingly, a small amount of IAA in the presence of larger amounts of indoleacetyl-β-alanine restored the organogenic potential which the β-alanine complex alone seems to have abolished. Indoleacetyglycine, like the β-alanine complex, supported massive callus growth at the higher concentrations but did not abolish organogenesis quite so effectively.

Callus tissues which had been initiated from the hypocotyl sections on media containing IAA, indoleacetyl-L-alanine or a combination of these were subcultured on media which had promoted "shoot" formation from the original hypocotyl sections (See Table VII). In general, tissues taken from hypocotyls which had produced no organs at all in the first passage, seemed to have lost organogenic potential in that they produced no organs even when they were transferred to media which had allowed organogenesis in the first passage. On the other hand, whenever the medium used in the first passage supported any organogenesis at all, the "callus" derived therefrom always retained organogenic potential. In other words, the degree of organization in the first passage played a large role in organogenesis in the second passage. Thus, there was a good deal of cryptic organization remaining in the first generation callus grown on indoleacetyl-L-alanine, but only if the complex was augmented with a low concentration of indoleacetic acid. This residual organization was very marked when IAA was the sole auxin. Indoleacetyl-L-alanine seemed to abolish organization in the first generation and in so doing to make organogenesis very difficult in the second generation. This observation has two profound implications. First, it would seem that indoleacetyl-L-alanine and indoleacetic acid are somehow doing different things since the observed results could not have been observed if the

Table VII. Formation of "Shoots" from Marglobe Tomato "Callus" as a Function of the Auxin Used to Initiate the "Callus"

"First generation" refers to tissues growing from the original hypocotyl sections (see Table III). "Second generation" refers to tissues growing from selected relatively undifferentiated regions of the first generation tissue. For the second generation studies, pieces of tissue about 80 mg in size, which had been initiated from hypocotyl sections on media containing the indicated first generation auxin source, were transferred to a second generation medium containing the indicated second auxin source. Five pieces were used per second generation treatment. Both the first and second generation media contained 8.9 uM benzyladenine and 1 mg per liter each of nicotinic acid and pyridoxine HCl. "Shoots" in the second generation were recorded after 53 days growth in the light. Unbracketed numbers refer to total "shoots", while bracketed numbers refer to pieces producing at least one "shoot".

Auxin in Medium for Second Generation	Auxin in Callus-initiating Medium					
	IAA		ILala + IAA		ILala	
	57 uM	5.7 uM	57 uM	5.7 uM	57 uM	5.7 uM
IAA:						
0.00057 uM	0	17(3)	3(1)	1(1)	0	0
No Auxin	2(1)	2(1)	1(1)	0	0	0
IDala:						
57 uM	0	12(1)	3(1)	0	0	0
5.7 uM	0	4(2)	0	0	0	0
0.57 uM	2(2)	16(2)	0	4(1)	0	0
IDala + IAA: (9 : 1)						
57 uM	0	0	12(1)	0	0	0
5.7 uM	3(1)	8(1)	0	0	0	0
0.57 uM	0	20(2)	0	0	0	0
Totals	7(4)	78(12)	19(4)	5(2)	0	0

complex was simply serving as a source of IAA. Second, the observation points up the fact that "callus" tissues can have very different regenerative capacities depending on the hormones used in their initiation.

DISCUSSION

The purpose of the research in this thesis was to explore whether indoleacetylamino acids could act as auxin sources in growth and regeneration experiments with cultured plant tissues. The specific aims were two. The first was to find an auxin source that did not suppress organogenic potential as much as did 2,4-D. The second was to find an auxin that would persist in tissue culture media so as to provide a continuous low level of indoleacetic acid to tissues when the production of shoots was desired. Both these aims were based on the hypothesis that the indoleacetylamino acids would act as biologically inert storage forms of indoleacetic acid. An early experiment showed that free indoleacetic acid does not remain in the medium in the presence of tissues, while indoleacetylglycine does remain. It was our thought that indoleacetic acid in complexed form might be safe from oxidative enzymes secreted by the tissues, since it had already been shown that all of the complexes used in our experiments were insensitive to horseradish peroxidase, under conditions where indoleacetic acid is extremely labile (5).

The auxin activity of indoleacetic acids may be due to two things: the ease with which they can enter the cell, and the susceptibility of the peptide bond to cleavage by peptidases. Indoleacetyl-L-alanine, indoleacetylglycine and indoleacetyl-β-alanine appear to be available sources of auxin, that is to say, they support, to varying degrees, unorganized callus growth in both tobacco and tomato which usually depends on a good supply of an active auxin. The other auxins which were tested, indoleacetyl-D-alanine and indoleacetyl-L-aspartic acid do not seem to be very good sources of available indoleacetic acid. However the fact that they often promoted "shoot" production better than no auxin shows that they do have some auxin activity even at concentrations less than micromolar.

It may be useful to introduce here the concepts of a complex's auxin activity relative to indoleacetic acid. Relative auxin effect is defined as: the concentration of indoleacetic acid that is optimal in a particular bioassay/ the concentration of indoleacetylamino acid that is optimal in the same bioassay. A relative auxin effect of less than one means that the auxin complex has a lower auxin activity than indoleacetic acid. A relative auxin effect of greater than one means that the auxin has a greater auxin activity than indoleacetic acid.

In tobacco tissues, when kinetin was the cytokinin, all complexes had relative auxin effects of less than one in the callus growth and "shoot" production bioassays. (It should be remembered that, the more stimulatory an auxin is to callus growth, the more inhibitory it becomes to "shoot" formation.)

Tobacco callus grew best on indoleacetic acid of all the auxins tested. As expected, "shoot" production was inhibited most by indoleacetic acid. The order of auxin activities of indoleacetic acid and the complexes was the same whether "shoot" induction or callus growth was measured. The order is: indoleacetic acid, indoleacetyl-L-alanine, indole-acetylglycine and indoleacetyl-β-alanine. However, there is some evidence that this order may be affected by the nature of the culture medium. See Table V.

Tobacco was chosen as an initial test species because of the ease with which plants can be regenerated from undifferentiated callus. Much work has been devoted to the discovery of a culture medium that would support such callus growth. A systematic search of this kind has not been carried out with many other species. The lack of an ideal culture medium for callus growth may be one of the problems confronting attempts to regenerate plants from callus.

Several experiments were conducted with the tomato cultivar Marglobe, in order to extend the application of indoleacetylamino acids to a species in which it is more difficult to regenerate organs from callus than in tobacco. Tobacco and tomato are both members of the family, Solanaceae. They both form adventitious roots easily from cuttings. Both will also regenerate plants from plant fragments. However, tomato tissue loses its capacity to produce "shoots" or roots as soon as, or soon after it is transformed into a friable callus culture. As mentioned earlier, it was suspected that perhaps the medium used to initiate callus formation might be at fault. The work described here investigated the roles of auxin complexes in initiating and maintaining callus growth and in the unfortunate abolishing of organogenic potential of the recently initiated callus. Any serious attempt to optimize either the basal medium or the cytokinin level for callus growth was outside the scope of my research. Thus, a slightly amended tobacco growth medium was used as the growth medium for tomato.

In studies of the induction of callus growth and support of undifferentiated callus growth of tomato, benzyladenine was the only cytokinin tried, thus precluding any cross-cytokinin comparisons.

In stimulating the growth of primary callus (immediately derived from hypocotyl sections), indoleacetyl-L-alanine, indoleacetylglycine and indolacetyl-β-alanine all had relative auxin effects greater than one, while the relative auxin effect of indoleacetyl-D-alanine seemed to be less than one. The same trend was observed with secondary callus tissue of Marglobe, which did not regenerate plants. These data suggest that the responses to auxins involved in tissue growth did not change appreciably with successive subcultures, even though organogenic potential was lost during this period.

The data discussed thus far can be explained on the basis of a slow release of indoleacetic acid from the complexes. However not all of the data can be so explained. As has been noted, indoleacetyl-L-alanine is particularly active in initiating callus and in maintaining callus growth in tomato hypocotyl sections. It also abolishes "shoot" initiation completely. By these criteria, one would suppose that indoleacetyl-L-alanine is an excellent source of available indoleacetic acid. This supposition is quite consistent with the fact that it is the L-isomer and presumably susceptible to hydrolysis by peptidases. However, the addition of a small amount of free indoleacetic acid to the very "available" complex restored organogenesis--a result which is in no way consistent with the argument that indoleacetyl-L-alanine is already providing the tissues with abundant indoleacetic acid. It would seem that indoleacetic acid and indoleacetyl-L-alanine must somehow be doing different things.

This possibility opens the door to entirely new areas of investigation. Thus, by varying the kinds of auxins used in generating "callus" we may be able to preserve organogenic potentials that are now being lost. And in attempting to regenerate shoots, we may find that different complexes elicit quite different responses. Certainly the number of possible auxin complexes is almost limitless and some of the complexes may have ideal properties for use in regeneration experiments.

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