STUDIES ON POLYACETYLENE PRODUCTION IN NORMAL AND
TRANSFORMED TISSUE CULTURES OF BIDENS ALBA

By

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ABSTRACT

The organs of *Bidens alba* each have a different composition of polyacetylenes (PAs). Factors affecting production of these compounds in tissue and organ cultures, and characteristics of cultures showing sustained synthesis of PAs were investigated. Additional studies evaluated transmission of tumour markers and leaf PAs in sexually produced offspring.

Polyacetylenes were separated and quantitated by high pressure liquid chromatography. Twenty-one compounds, representing six chromophores, were identified or indicated. The principal compounds were phenylheptatriyne (PHT), found in leaves and stems; phenyldiynene (PDE), found in stems; PDE-OAc, found in roots; and entetraynene acetate (ETE-OAc), found in roots.

Production of PAs past the third passage could not be achieved in callus from normal plants, despite variations in medium formulations and environmental parameters. Calluses from crown galls induced on *B. alba* and *B. pilosa* by two strains of *Agrobacterium tumefaciens*, however, continued to produce PAs after three years in culture. Transformed callus lines showed wide fluctuations in levels and a declining proportion of PDE-OAc over time. Leaf and stem PAs were not found in tumour callus several months old. Callus contained PAs not found in the plant.

Root cultures were used to determine the effect of environmental, hormonal, and nutritional parameters on growth, PA level and PA composition. Low temperatures, darkness, high kinetin levels, and increased sucrose/nitrate ratios increased production.
of PAs by roots. The proportion of PDE-OAc increased in light, high kinetin medium, and late log phase of growth. Leaf and stem PAs could not be induced in root cultures.

Plants regenerated from nopaline galls rooted and set fertile seed. Selfs and crosses with normal plants demonstrated inheritance of tumour characteristics to the fourth generation. However, $F_2$ and succeeding generations had sharply reduced levels of nopaline, and showed differential loss of transformation markers. Transformed $F_1$ plants could form octopine galls and double transformant tissue was obtained. Transformed plants were morphologically different from normal plants.

Crosses between B. alba and B. pilosa, which did not contain PAs in leaves or stems, yielded $F_2$ which segregated for PHT synthesis, but not in Mendelian ratios. Synthesis of PHT was dominant but showed depressed levels in the $F_2$ which was incompatible with a gene dosage effect.
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Polyacetylenes are widespread in two large dicot families (Umbelliferae and Compositae) as well as several smaller families and in certain groups of fungi. Over the past thirty years these compounds have been extensively investigated primarily by chemists and much is now known about the biosynthetic pathways leading to specific compounds. In recent years studies on the bioactivity of many of these compounds have shown that a number of them have powerful phototoxic antibiotic activities. These two aspects, however, scarcely exhaust the points of interest of this class of compounds. Many plants synthesize a different set of polyacetylenes in each organ or appendage type, sometimes with little overlap from one to the other. The stringent control exercised over the biosynthesis of a given plant's complement of polyacetylenes present a number of interesting questions; however, very little research in the area of either physiological or biochemical aspects of the control of polyacetylene synthesis had been reported when this thesis was begun. Over the past few years tissue cultures of plants have been used extensively in several cases for investigating not only the biosynthetic steps of secondary products but also control of the pathways; e.g., the work on the crucial involvement of phenylalanine ammonia lyase in phenylpropanoid metabolism. The studies reported in this thesis were undertaken with the purpose of developing a tissue culture system for investigating biosynthetic and regulatory aspects of poly-
acetylene synthesis in *Bidens alba*. However, initial results with callus cultures were not promising and other approaches were tried which led to tumour and organ cultures. Root cultures proved to be a sensitive system for studying physiological control of polyacetylene synthesis, and some characteristics of these cultures are presented here. In addition, preliminary studies were made of the inheritance of a major polyacetylene occurring in *B. alba*. Again, very little research has appeared on the genetics of polyacetylenes although the potential for involvement of this group of compounds in ecological adaptation would appear to be high.

Additional studies not directly related to polyacetylenes were carried out when results of work on crown gall cultures indicated that *B. alba* might behave differently from plants previously investigated for maintenance of transformation through meiosis.

This thesis is, therefore, not a completed and detailed examination of any one aspect of polyacetylenes but, rather, a survey of both characteristics of different tissue culture systems and also some genetic features of *B. alba* related to either polyacetylene synthesis or cultures of the plant.
CHAPTER I

INTRODUCTION
INTRODUCTION

An active area of current research in plant biochemistry is the production of secondary compounds in tissue cultures. One phase of the work reported in this thesis attempts to apply some of the techniques of tissue culture to the problem of producing a specific class of secondary compounds, polyacetylenes, in cultured material.

Polyacetylenes, also termed poly-yenes or acetylenes, are found in 19 higher plant families and in Basidiomycete fungi (9). They are most characteristic of members of the Umbelliferae, Compositae, Santalaceae, and related families (5). Higher plants account for 85% of the known compounds with the remainder isolated from fungi. Over 725 known acetylenes have been described (9). This group would seem, then, to be one of the most abundantly produced classes of secondary metabolites in plants, and thus warrant research into the factors affecting its synthesis. Polyacetylenes are considered to be a biogenetically uniform group having a common derivation from oleic acid and containing one or more acetylene bonds, usually conjugated (3,5). The common biosynthetic origin of polyacetylenes is shown in Fig. 1, which also shows the proposed relationships between precursor fatty acids and related epoxy and acetylenic compounds (4).

The plant chosen for investigating these compounds was Bidens alba var. radiata, a widely distributed member of the Compositae. This species has been segregated from the more commonly known B. pilosa by Ballard (1). Bidens alba contains
Fig. 1. Proposed pathways of biogenesis of the crepenynic family of acetylenic fatty acids. Asterisk denotes postulated enzyme-bound intermediates. Source: Hitchcock and Nichols; Plant Lipid Biochemistry, 1971.

three easily detected polyacetylenes: phenylheptatriyne (PHT), phenyldiynene (PDE), entetranyiene (ETE), and acetylated and alcohol forms of these. These compounds have characteristic distributions in different parts of the plant and thus offer a relatively simple but interesting system for investigating production in cultured material. Figure 2 shows the principal acetylenes found in B. alba and their distribution in the plant.

Much is now known about the biosynthetic pathways of polyacetylenes as a result of work in the labs of Bohlmann, Bu'Lock, Jones, the Sørensens, and many others. Bohlmann et al. summarized this research in 1973 in what is still the standard reference for work in polyacetylenes (3). Nevertheless, much remains to be done; as emphasized by Thaller (10) who noted that "Although the general principles of polyacetylene biogenesis are thought to be understood, many details still wait to be
Fig. 2. Principal polyacetylenes in organs of Bidens alba.
confirmed, not least the biogenesis of the triple bond itself."

A similar view was stated by Bohlmann in 1973 (7).

Tissue cultures have been used extensively for studying metabolic pathways in a variety of higher plants. Cultures that synthesize compounds of interest offer several advantages for the study of their biosynthesis and control. 1) Active cell-free systems and purified enzyme preparations can be prepared more easily due to the absence of compounds causing deactivation of enzymes, notably phenols and quinones (8). 2) Tissue cultures often show higher incorporation rates of labelled precursors than intact plants or tissue slices; allowing greater flexibility in identification techniques. As an example, suspension cultures of Catharanthus roseus had deuterium incorporation levels two orders of magnitude higher than intact plants, allowing the use of mass spectrometry for detection of labelled compounds (2). 3) Under suitable conditions a relatively homogeneous cell population can be obtained and environmental and nutritional parameters closely controlled, allowing rapid and precise evaluation of changed conditions.

An example of how cell cultures may contribute in a unique way to understanding the enzymology of secondary product formation is Zenk's summary of work on the synthesis of alkaloids of Catharanthus roseus (13). Excellent reviews of the use of tissue and organ cultures for the solution of problems in secondary plant metabolism have been published recently by Overton and Picken (8) and Kurz and Constabel (7).

Obtaining synthesis of secondary products, however, has presented a considerable challenge to tissue culturists.
Callus and suspension cultures are most often accompanied by loss or greatly reduced production of secondary compounds. The basic problem facing researchers attempting to obtain secondary products from tissue cultures has been stated succinctly by Krikorian and Steward (6):

Is it possible to reproduce at will the particular biochemistry of any particular organ, tissue or cell. Can one induce potentially totipotent cells to express the biochemistry that they normally achieve in a given morphological setting, without the necessity of reproducing that setting intact by the growth of the whole organism?

In recent years the efforts of many workers has resulted in achieving secondary product levels equal to or exceeding those of the intact plants for metabolites in several product categories (12), none of which were fatty acid derivatives. Thus, one phase of research may be said to be realizing part of the goal above; achieving levels of compounds comparable to those of the plant. The other part, selective activation of pathways, remains a challenging problem in nearly all systems.

In order to study the polyacetylenes in B. alba for this thesis, it was first necessary to develop analytical techniques for identifying and quantifying them and these results are presented in Chapter II. The primary objective of the research reported here was to try to develop a tissue culture system which could be used for biosynthetic, and possibly enzymological, studies. In attempting to find cultures which would produce acetylenes, variations in media composition and other factors were tried. However, no unambiguously positive results could be obtained by these methods, which are summarized in Chapter
IV. Fortuitous circumstances led to investigating transformed, crown gall, cultures for polyacetylenes and they were found to be consistent producers. These results are reported in Chapter V.

An alternative approach to the problem of obtaining cultures which would produce polyacetylenes was to use root cultures; they offered advantages for investigating factors affecting the specific compositional patterns in the plant. Chapter VI presents results showing characteristics of these cultures and the degree to which polyacetylene composition could be altered in them.

The work with crown gall cultures led to the finding that *B. alba* was unusually suitable for regeneration of plants from one type of tumour callus and these plants showed an unexpected ability to stably transmit the tumourous state from one generation to another. These results are presented in Chapter III.

A final area of this thesis concentrates on the inheritance of phenylheptatriyne (PHT) production. This is the main polyacetylene of leaves of *B. alba*. There has been only one report of work on the inheritance of polyacetylenes in higher plants (11). The availability of two interfertile species (*B. alba* and *B. pilosa*), which differed in their leaf compounds presented an opportunity to determine the number of loci involved in PHT inheritance and also to learn something about the dependence of compound level on gene dosage. These results are found in Chapter VII.
LITERATURE CITED


11. Van Fleet, D. S. 1970. Enzyme localization and the genetics of polyenes and polyacetylenes in the


CHAPTER II

IDENTIFICATION OF ACETYLENES

AND THEIR QUANTITATION
INTRODUCTION

Identification of the major acetylenic compounds in *B. alba* plants and cultures and their quantitation were central to the studies for this thesis. The procedures for compound identification and results are reported in this chapter.

Identification of the UV absorbing moiety, the chromophore, in acetyleneic compounds is often straightforward since conjugated poly-ynene systems typically exhibit sharp absorption bands which are diagnostic for the number and position of double and triple bonds in the chromophore. Published tables correlate UV maxima and intensity with structure (5,6). Conjugated triple bonds have a set of absorption peaks with very high molar extinction coefficients (ε values) in the UV region from 210-290 nm and quite small amounts of compounds can be detected (ε > 100,000 for three or more triple bonds). Conjugation of triple and double bonds results in a second set of longer wavelength peaks, with lower ε values, extending into the region 290-410 nm. The relative absorption shown by the two sets of peaks depends on the ratio of the number of double and triple bonds and thus can yield some information about the chromophore (5). Additional information on the general nature of the terminal groups or other substitutions can be inferred from the order in which compounds, especially different forms of the same chromophore, separate by chromatography (9).

Methods of separating acetylenes rely chiefly on liquid chromatography, both column and thin-layer. Solid phases used
include alumina (7), silica gel (9), and Sephadex LH-20 (1). Reverse phase silica gel was used by Bu'Lock and Smith (8) to separate acetylenic fatty acids. High pressure liquid chromatography (HPLC) was used for preparative separation of cis/trans isomers by Rose et al. (14). Use of liquid column (CC) and thin-layer chromatography (TLC) are undoubtedly the methods of choice for isolating minor components of complex mixtures at the levels required for characterization of new compounds, however recoveries are seldom quantitative according to Jones and Thaller (11). For a quantitative study a different method seemed advisable. In the present study separation was by HPLC. This method offers several advantages: 1) many acetylenes are unstable in purified form, especially in light, and HPLC uses a closed system which minimizes exposure to light during separation; 2) Quantitation of compounds is easily carried out after standard curves are obtained; 3) Analysis of samples is relatively fast—the turnaround time was 30 minutes for analytical separations in this study; 4) The availability of a variable-wavelength detector allows selection of a wavelength that minimizes detection of peaks from non-acetylenic compounds and increases the reliability of peak identification and quantitation.

In order to simplify discussion of the results which follow, the structures and names of all the compounds isolated or reported to occur in B. alba and B. pilosa are shown in Fig. 3.
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Fig. 3. Compounds discussed: structures, systematic names, and abbreviated names.
Extraction and Preparation of Samples for HPLC Analysis

All work was carried out with room light off and crude extracts were kept in petroleum ether (30-60°C, J. T. Baker Chem. Co., Phillipsburg, N. J. "Resi-Analyzed", or equivalent) until immediately prior to analysis to minimize degradation of compounds by light.

For analytical work 1-2 g samples of tissue were used. Before fresh weights were determined liquid cultured organs were patted dry with paper towels, suspension cultures were filtered onto Miracloth (Calbiochem), and agar cultures had adhering agar scraped off. Duplicate samples were taken and dried for 24 hours at 100°C, cooled to room temperature and dry weights determined. After weighing, samples were homogenized with 15 ml of redistilled methanol in 20 x 150 mm glass culture tubes by breaking and shearing the tissue with a glass pestle made in the laboratory which closely fitted the culture tubes. After homogenization, 5 ml of distilled water was added followed by 5 ml of petroleum ether (30-60°C). The contents were then shaken vigorously for 20 seconds and centrifuged to clarify the organic phase which was then drawn off by pipette. The brei was extracted three times with petroleum ether, aliquots combined, made up to volume, and a UV spectrum made of the crude extract. For liquid medium extractions 25 ml of medium was extracted four times with petroleum ether. Several series of preliminary extractions of leaf tissue and cultured
roots were extracted up to five times with petroleum ether and two times with methanol. The results showed that the average ratio of acetylenes for the n+1/n extraction was equal or less than 0.33. This indicated that three extractions would recover approximately 96% of the compounds extractable.

If analysis was to be immediate then the crude extract was evaporated to dryness with nitrogen gas in dim light and the remaining oil dissolved in HPLC grade methanol. If analysis was to be delayed then the sample was stored at -70°C in petroleum ether. The UV spectra of samples stored as long as six months were identical to initial spectra. In order to maximize the accuracy of the HPLC analysis, samples were made up in methanol at a level such that the average HPLC peak would have an optical density (OD) of about 0.2. Accordingly, the OD of HPLC peaks resulting from injection of different amounts of a crude extract of combined leaf and root material was correlated with absorbance at 287 nm. The following relation generally gave the volume required, given a 20 μl sample injection:

\[ V_s = OD \times V_p \times 0.035 \]

where \( V_s \) is the volume of methanol (ml), and \( V_p \) is the volume of petroleum ether (ml). For preparative work the constant is 0.002 instead of 0.035.

Spectra were run on a Pye-Unicam SP8-100 UV/VIS spectrophotometer set at bandwidth of 0.5 nm in petroleum ether (30-60°C) unless otherwise noted.

High Pressure Liquid Chromatography

Separation and quantitation of the polyacetylenes in the
oil resulting from extraction was accomplished by use of a Varian Aerograph model 5000 liquid chromatograph with a Series 634 UV/visible spectrophotometer as the detector unit. Detecting wavelength was 287 nm for all quantitative work. Varian Micropak MCH-10 reverse-phase analytical (30 cm x 4 mm) and preparative (50 cm x 8 mm) columns were used. The solvents were HPLC grade acetonitrile and distilled water. At the time this work was started no previous reports of HPLC use for acetylene analysis could be found so it was necessary to develop a program of changes in solvent composition and flow rate that would optimize the following considerations: 1) maximize resolution of components in a mixture containing all the polyacetylenes occurring in *E. alba* plants; 2) Minimize the amount of time required for the program to cycle; 3) Flush out non-polar compounds tending to be retained on the column and re-establish starting conditions. Two solvent systems were tried; acetonitrile/water and methanol/water; the former seemed to give the best results overall and was used throughout the work. Solvent composition and flow rate were programmed to the following cycle: (time in minutes: % acetonitrile) 0:61, 10:61, 20:100, 24:100, 27:61; (time: flow rate in ml/minute) 0:1, 19:1, 20:2, 24:2, 27:1. A similar program was developed for preparative work: (time: % acetonitrile) 0:55, 16:60, 23:65, 28:100, 32:55. Flow was 5 ml/minute at all times. Changes in flow and composition were linear over time.

In general the eluting peaks were homogeneous since the programs were developed using a complex mixture of compounds from both roots and leaves. Eluting peaks were collected and
a UV spectrum obtained for each peak when different types of samples were run. This was done for the following reasons: to insure that coelution of major compounds did not occur; to check that coeluting very minor compounds did not change in amount; and to detect new compounds which might elute at assigned retention times. To insure homogeneity, major peaks were split into two halves and the two spectra compared. As described below, in those instances in which two compounds with different chromophores eluted at a single peak, it was possible to determine the composition by collecting the peak and obtaining the composite spectrum. The advisability of following this type of procedure should be emphasized, especially when cultural conditions change significantly or the material being evaluated is suspected of being variable in composition. At the beginning and end of each set of analyses a standard mixture of leaf and root compounds was run to calibrate the system.

HPLC Quantitation of Major Compounds

The HPLC trace was quantitated using standard curves for the three principle compounds: phenylheptatriyne (PHT), phenlydiynene-OAc (PDE-OAc), and entetraynene-OAc (ETE-OAc). Compounds were isolated on a preparative column and made up to specific concentrations using published extinction coefficients. Known amounts were then repeatedly run on an analytical column and the resulting traces measured for peak height and area. Four to six levels were each run six or more times for each compound. The least squares linear regression for the data points was obtained with the linear regression function of a Texas Instruments SR-51 calculator. These relations of
quantity versus peak height and area were used to quantitate all peaks of the same chromophore. Compounds with the same chromophore but a different terminal group, e.g. ETE-ol instead of ETE-OAc, were converted as the ratio of the molecular weights of the variant and the standard. The standard curves of the three base compounds are shown in Fig. 4.

The detailed procedure for converting a peak trace to amount of compound per gram of sample follows. An output from the spectrophotometer activates a recorder pen assembly such that an output of 200 mv drives the pen full scale and corresponds to an OD of 2.00, and output of 50 mv to an OD of 0.5, etc. The output can be varied from 1 mv (OD 0.01) to 200 mv. If $R_v$ is the number of mv for full scale then $0.01 \times R_v = \text{OD}$. Full scale deflection covers a distance of 240 mm so that $\text{OD} = P_h \times K \times R_v$, where $P_h$ is peak height (mm) and $K = 1/240 \times 0.01 = 4.167 \times 10^{-5}$.

Area is calculated by multiplying OD by peak width, in minutes ($t$), at $\frac{1}{2}$ peak height ($\frac{1}{2}t$).Multiplying by the ratio of total sample ($V_s$) to injection amount ($V_i$), the volume of the injection loop, and dividing by sample weight ($w$) gives the total area ($A_t$) per gram sample:

$$A_t = \frac{P_h \times \frac{1}{2}t \times R_v \times K \times V_s}{w \times V_i}.$$  

Area is then converted to ug compound per gram sample with the inverse equations of the standard curves: ETE-OAc $\text{ug/g}=2.805m-0.0$; PDE-OAc $\text{ug/g}=24.15m-0.016$; PHT $\text{ug/g}=30.26m-0.121$.

In a few instances relatively minor compounds coelute together; in these cases the composite peak was collected, measured, and a UV spectrum run. Knowing the extinction coeffi-
Fig. 4. HPLC standard curves showing the relationship between amount of compound and peak area. A) PDE-OAc. B) ETE-OAc. C) PHT. Vertical bars are ± 1 SD.
cient and assuming noninteraction of the components it is possible to calculate the concentrations of each compound, since absorption (or optical density) is additive, by solving a pair of simultaneous equations of the form

\[ \text{OD}_{\lambda_1} = \varepsilon_{\lambda_1} c_1 + \varepsilon_{\lambda_2} c_2 \]
\[ \text{OD}_{\lambda_2} = \varepsilon_{\lambda_1} c_1 + \varepsilon_{\lambda_2} c_2 \]

where \( \lambda_1 \) and \( \lambda_2 \) are the two wavelengths of observation, \( \varepsilon \) is the extinction coefficient and \( c \) is the concentration (13, p. 240). Although the method, in principle, is applicable to any number of spectrally distinct components by adding an additional equation and wavelength for each addition, it was only very occasionally used in this study for peaks with three components. The results were checked in several instances against a known mixture and agreement was within 10%. A refinement of this approach using more points and a computer program to reconstruct the component spectra and calculate concentrations has recently been published (15). Workers have sometimes estimated the amounts and composition of mixtures directly from the crude lipid extract (8). In this study quantitation from the crude extract was only used in one study involving leaf extracts, in which one compound predominated, and as a check on results using standard curves.

**Quantitation of Minor Compounds**

In a few cases minor compounds were tentatively indentified and it was not practical to establish a standard curve directly. Quantitation of the HPLC peaks of these compounds was done by estimating the standard curve slope from the following consid-
erations. From a spectrum of the purified minor compound the extinction coefficient at the detecting wavelength ($\varepsilon_{\lambda_d}$) was determined by measuring the peak height at that wavelength ($H_d$) and the nearest peak ($H_p$), and taking the ratio times the known $\varepsilon$ of that peak. Thus $\varepsilon_{\lambda_d} = (H_d/H_p) \times \varepsilon_{\lambda_{\text{max}}}$. The ratio of $\varepsilon_{\lambda_d}$, the extinction coefficient of a compound with a known standard curve, to $\varepsilon_{\lambda_d}$ times the slope of the regression line of the known compound ($m'$) yields the estimated slope for the minor compound ($m$): $m = (\varepsilon_{\lambda_d}/\varepsilon_{\lambda_{\text{d'}}}) \times m' \times (mw/mw')$. The final term ($mw/mw'$) corrects for the difference in the molecular weights of the minor compound ($mw$) and the known ($mw'$) compound. The accuracy of the method was checked by calculating the slopes for PHT and PDE-OAc from the data for ETE-OAc. In the former case the calculation yields a slope of 29.1 vs. 30.3 derived from actual determination; for the latter compound the calculated slope is 23.5 vs. 24.1. Agreement was within about 3% for the calculated and determined slopes. All slopes determined in this way were assumed to intersect the origin. Thus, one carefully determined standard curve can form the basis for calculating the curves for related compounds if small amounts of the purified compounds are available for reference spectra and the $\varepsilon$-values are known. The following compounds were quantitated by this method:

EDE-OAc ($mw$ 230) slope=0.03 spectrum reference (1a)

DTE-ol ($mw$ 182) " 0.0031 " " " (5)
DTE-OAc ($mw$ 224) " 0.0038 " " " (5)
ETE-al ($mw$ 178) " 0.0034 " " " (3)
Gas Chromatography/Mass Spectrometry (GC/MS)

Preparative HPLC was used to obtain sufficient compound for GC/MS analysis: eluting peaks were collected, extracted with petroleum ether, dried with sodium sulfate, evaporated with nitrogen gas and dissolved in spectral grade n-hexane. Samples were then injected into a Finnegan model 1020 automated GC/MS with a 30 m x 0.25 mm, WE-554 fused silica capillary column. The initial oven temperature of 150°C was held one minute with a 10°C/min ramp to 250°C which was held for 10 minutes. Injection temperature was 250°C. Ion source temperature for EIMS was 95°C and the electron energy 70 eV.

Purification of PHT

Several hundred mg of PHT was isolated using the following procedure: 1 kg of fresh B. alba leaves was extracted four times with 4 l of cold methanol, extracts combined and evaporated under reduced pressure to 750 ml and an equal volume of water added and followed by extraction seven times with 500 ml of heptane. Heptane extracts were combined, evaporated to 250 ml, dried with sodium sulfate and applied to 4 silica gel 60 columns (70-230 mesh ASTM, E. Merck, Darmstadt) 70 mm x 80 mm made up in heptane. Elution with heptane and collecting 100 ml fractions gave PHT in fractions 7-10. These fractions were combined and evaporated under reduced pressure. Formation of light yellow crystals occurred after most of the solvent was lost. These were redissolved in heptane and recrystallized twice at 4°C to yield white, elongated crystals which were 95% pure by CG/MS. The major impurity was PDE-OAc (3.5%).
Plants

Bidens alba seedlings were harvested 55 days after sowing. Plants were grown in an incubator at 25°C in soil. Illumination was 12 hours at 6,000 lux. Flower parts were from mature plants grown under similar conditions.
RESULTS

Separation

An HPLC trace of the mixture of leaf and root extracts used to calibrate runs is shown in Fig. 5. Not all the components which were eventually identified are shown: some compounds only occurred under certain conditions or in cell cultures. A list, in elution order, of compounds that were positively or tentatively identified in plants and cultures of B. alba is shown in Table 1. Compounds identified as "a" or "b" in the compound number column are identical in molecular weight and nearly identical in UV and mass spectra. They are presumed to be cis/trans isomers. The normal order of elution from silica gel columns, i.e., in order of increasing polarity, is reversed for reversed phase columns. Although different polarity groups mainly separate together there is some overlap; some hydrocarbons elute before acetates, e.g. (V) before (IVa,b). The correspondence in elution between analytical and preparative columns differed in only one instance: (II) eluted after (XVI) and PHT on the former and before these compounds from the latter column.

Identification: Enetetraynene Compounds

Three sets of compounds were isolated which had very similar UV spectra (Fig. 6) differing by only ½ to 1 nm in the detecting region. The spectra agreed with published data for the ETE chromophore (2,3,17) and the order of HPLC elution suggested quite different polarities for the compounds. The
Fig. 5. HPLC trace of calibration mixture of leaf and root polyacetylenes of *Bidens alba*. (1a,b) PDE-ol; (2a,b) DTE; (3) PDE-OAc; (4) ETE-ol; (5) PDE; (6) PHT; (7a,b) ETE-OAc; (8a,b) ETE.
Table 1. HPLC retention times for polyacetylenes isolated from plants and cultures of *B. alba*.

<table>
<thead>
<tr>
<th>Compound no.</th>
<th>Compound</th>
<th>Column retention time (min.)</th>
<th>analytic</th>
<th>preparative</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIa</td>
<td>PDE-ol</td>
<td>5.1</td>
<td>9.0</td>
<td></td>
</tr>
<tr>
<td>VIb</td>
<td>PDE-ol</td>
<td>5.6</td>
<td>9.6</td>
<td></td>
</tr>
<tr>
<td>IX</td>
<td>PHT-ol*</td>
<td>6.0</td>
<td>10.5</td>
<td></td>
</tr>
<tr>
<td>XIV</td>
<td>EDE-ol*</td>
<td>6.4</td>
<td>11.3</td>
<td></td>
</tr>
<tr>
<td>XIIa</td>
<td>DTE-ol*</td>
<td>7.3</td>
<td>13.1</td>
<td></td>
</tr>
<tr>
<td>IIIa</td>
<td>ETE-ol</td>
<td>7.8</td>
<td>14.0</td>
<td></td>
</tr>
<tr>
<td>XIIb</td>
<td>DTE-ol*</td>
<td>8.0</td>
<td>14.3</td>
<td></td>
</tr>
<tr>
<td>VII</td>
<td>PDE-OAc</td>
<td>8.7</td>
<td>16.6</td>
<td></td>
</tr>
<tr>
<td>XV</td>
<td>ETE-OAc*</td>
<td>9.1</td>
<td>18.4</td>
<td></td>
</tr>
<tr>
<td>X</td>
<td>PHT-OAc</td>
<td>9.5</td>
<td>17.1</td>
<td></td>
</tr>
<tr>
<td>IIIb</td>
<td>ETE-ol</td>
<td>10.2</td>
<td>17.7</td>
<td></td>
</tr>
<tr>
<td>XIIIa</td>
<td>DTE-OAc*</td>
<td>11.2</td>
<td>20.0</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>PDE</td>
<td>11.8</td>
<td>20.5</td>
<td></td>
</tr>
<tr>
<td>XIIIb</td>
<td>DTE-OAc*</td>
<td>12.6</td>
<td>22.1</td>
<td></td>
</tr>
<tr>
<td>XVI</td>
<td>TDE*</td>
<td>12.8</td>
<td>23.3</td>
<td></td>
</tr>
<tr>
<td>VIII</td>
<td>PHT</td>
<td>12.8</td>
<td>23.3</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>ETE-al</td>
<td>13.7</td>
<td>22.6</td>
<td></td>
</tr>
<tr>
<td>IVa</td>
<td>ETE-OAc</td>
<td>14.1</td>
<td>24.8</td>
<td></td>
</tr>
<tr>
<td>IVb</td>
<td>ETE-OAc</td>
<td>16.7</td>
<td>27.6</td>
<td></td>
</tr>
<tr>
<td>Ia</td>
<td>ETE</td>
<td>19.7</td>
<td>29.2</td>
<td></td>
</tr>
<tr>
<td>Ib</td>
<td>ETE</td>
<td>20.1</td>
<td>29.9</td>
<td></td>
</tr>
</tbody>
</table>

*Tentative identification--based on spectrum and retention time only.*
Fig. 6. UV spectra of entetraynene compounds. A) entetraynene hydrocarbon. B) entetratyene acetate.
Fig. 7. Mass spectra of entetraynene hydrocarbons. A) isomer "a". B) isomer "b".
Fig. 8. Mass spectra of entetraynene alcohols. A) isomer "a". B) isomer "b".
Fig. 9. Mass spectra of entetraynene acetates. A) isomer "a". B) isomer "b".
Fig. 10. UV spectra of entetraynene aldehyde in different solvents. A) spectrum in petroleum ether (30-60 C). B) partial spectrum in petroleum ether (solid line) and in methanol (dotted line). Peak at 390 nm due to contaminating ETE-OAc.
Fig. 11. UV and mass spectra of phenyldiyne hydrocarbon. A) UV spectrum. B) mass spectrum.
Fig. 12. Mass spectra of phenyldiynene alcohols. A) isomer "a". B) isomer "b".
Fig. 13. UV and mass spectra of phenyldiyynene acetate. 
A) UV spectrum. B) mass spectrum.
Fig. 14. UV and mass spectra of phenylheptatriyne hydrocarbon, A) UV spectrum. B) mass spectrum.
Fig. 15. Mass spectrum of phenylheptatriyne acetate.
Fig. 16 (top). UV spectrum of endiynene acetate.
Fig. 17 (btm). UV spectrum of dientrynene acetate.
ETE chromophore is distinguishable from the tetrayndiene isomer by UV spectrum differences (10,17). The mass spectrum of the hydrocarbons shows a parent ion of 164 (Fig. 7), corresponding to the formula $C_{13}H_8$; the alcohols a parent ion of 180 (Fig. 8) which corresponds to $C_{13}H_{10}O$; the acetates have a parent ion of 222 (Fig. 9) which corresponds to $C_{15}H_{10}O_2$ and have characteristic base peaks at m/e 43 ($CH_3CO$). The identifications of the compounds in this group are based on the UV spectra, elution orders, mass spectra, and reported occurrences of these compounds in _B. pilosa_. As shown in Table 1, two forms of each compound are found, almost certainly cis/trans isomers.

The conjugated aldehyde of the ETE chromophore was also isolated and tentatively identified on the basis of its UV spectrum (Fig. 10a) as reported (2,3) and retention time. In addition the aldehyde function was indicated by the loss of the long wavelength peaks when the compound was dissolved in methanol (Fig. 10b) (12). The additional peaks in this figure are due to contaminating ETE-OAc. Repeated attempts to obtain a mass spectrum with purified compound using the GC/MS were unsuccessful—apparently because of instability of the compound under GC conditions.

**Phenyldiynene Compounds**

The presence of the alcohol and acetate of phenyldiynene in roots of _B. pilosa_ has been reported (4) but not the hydrocarbon. Three isolates with a UV spectrum in agreement with this chromophore (Figs. 11,13) (3,7) were found and HPLC retention times suggested the above compounds. The mass spectrum of the hydrocarbon (Fig. 11) shows the likely parent ion to be 166 with a base peak at 165 (-H); the alcohols (Fig. 12)
have the expected parent ion of 182 and the acetate (Fig. 13) a parent ion of 224 which corresponds to the formulas $C_{13}H_{10}$, $C_{13}H_{10}O$, and $C_{15}H_{12}O_2$ respectively. The mass spectra of known standards of all three compounds were identical to those of the isolates. Only the alcohol occurred as two isomers.

**Phenylheptatriyne**

This aromatic polyacetylene occurs primarily as the hydrocarbon in leaves and stems of *B. alba* and is easily identified by its characteristic UV spectrum (Fig. 14a) which has been reported (4). Minute amounts of what appeared to be the alcohol were isolated but in insufficient quantity for further analysis. The mass spectrum of PHT has the expected parent ion at 164 for $C_{13}H_8$ (Fig. 14b) and was identical with the spectrum of known compounds. The acetate has a parent ion of 222 (fig. 15) corresponding to $C_{15}H_{10}O_2$ and a prominent peak at m/e 43 ($CH_3CO$).

**Other Polyacetylenes**

A final group of compounds occurred at levels too low to allow further characterization, or loss occurred under GC conditions. The only data obtainable were UV spectra and HPLC retention times. Compounds indicated on this basis were an endiynene alcohol and acetate (EDE-ol, EDE-OAc) (Fig. 16); two dienatriynene alcohols and acetates (DTE-ol, DTE-OAc) (Fig. 17) and an endiyntriene hydrocarbon (EDT). The absorption maxima and relative intensities agree with published values (5,10,16).
Polyacetylenes in B. alba

Table 2 shows the composition of B. alba seedlings and flowers of mature plants. B. pilosus L. (=B. pilosa L.) was examined by Bohlmann et al. (4). Roots of their material contained PDE-OAc (VII), ETE (I), ETE-al (II), ETE-ol (III), and ETE-OAc (IV); leaves contained PHT (VIII), PHT-ol (IX), PHT-OAc (X), and trace pentaynene (XVII). It is not known whether the material investigated here is the same taxon as the one that Bohlmann et al. examined, however the composition was similar. The two reports differ in three respects: B. alba lacked the ETE-al in roots and the alcohol and acetate of PHT in leaves, although the compounds occurred in flowers and seeds. B. alba contained EDE-OAc which was not reported for “B. pilosus”. The absence of the aldehyde was interesting because this compound was found later in crown gall cultures. No trace of the pentaynene was ever observed in any B. alba material. UV spectra for several very minor compounds not reported by Bohlmann et al. (4) were obtained from tumour cultures (XI-XV). Although only three chromophores occur at significant levels in B. alba each may have up to four different terminal groups (as in I-IV) and each may occur in cis or trans forms.
Table 2. Polyacetylene composition of *Bidens alba* var. *radiata* seedling organs and flowers.

<table>
<thead>
<tr>
<th>Organ</th>
<th>PHT-ol</th>
<th>PHT-OAc</th>
<th>PHT</th>
<th>PDE-OAc</th>
<th>PDE</th>
<th>ETE-ol</th>
<th>ETE-OAc</th>
<th>ETE</th>
<th>EDE-OAc</th>
<th>Total product (mg/g d.w.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root</td>
<td>-</td>
<td>-</td>
<td>5.7</td>
<td>58.3</td>
<td>2.9</td>
<td>0.4</td>
<td>4.9</td>
<td>19.3</td>
<td>0.8</td>
<td>7.7</td>
</tr>
<tr>
<td>Stem</td>
<td>-</td>
<td>-</td>
<td>54.2</td>
<td>2.2</td>
<td>43.5</td>
<td>-</td>
<td>t</td>
<td>t</td>
<td>-</td>
<td>2.3</td>
</tr>
<tr>
<td>Petiole</td>
<td>-</td>
<td>-</td>
<td>94.1</td>
<td>-</td>
<td>5.9</td>
<td>-</td>
<td>t</td>
<td>t</td>
<td>-</td>
<td>1.5</td>
</tr>
<tr>
<td>Leaf</td>
<td>-</td>
<td>-</td>
<td>98.3</td>
<td>-</td>
<td>1.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4.6</td>
</tr>
<tr>
<td>Flower head</td>
<td>-</td>
<td>-</td>
<td>97.3</td>
<td>-</td>
<td>2.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>11.9</td>
</tr>
<tr>
<td>Petal</td>
<td>-</td>
<td>6.0</td>
<td>92.7</td>
<td>-</td>
<td>1.2</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4.2</td>
</tr>
<tr>
<td>Seed</td>
<td>0.8</td>
<td>19.8</td>
<td>78.2</td>
<td>-</td>
<td>1.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>6.9</td>
</tr>
<tr>
<td>Pollen</td>
<td>-</td>
<td>-</td>
<td>100.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.1</td>
</tr>
</tbody>
</table>

*a* Minus ligulate petals

*b* Ligulate petals.

*c* Small amounts of material made it impossible to detect minor compounds.

Symbols: -, not detectable; t, trace; d.w., dry weight.
DISCUSSION

There are advantages and disadvantages of using HPLC for the studies reported later. Obtaining enough material for further analysis was relatively fast and efficient; a few or, sometimes, only one run of sample on a preparative column usually yields enough material for GC/MS analysis (approximately 10 ug). The loading capacity, however, is rather low and minor compounds required a number of runs and sometimes re-extraction and purification with a second run. Cis/trans isomers are easily separated as shown in this study and others (14). Quantitation is simple once the requisite standard curves have been determined. This can be coordinated with the part above and the compounds isolated used to establish the curves. The standard curves can also be calculated with reasonable precision. For extensive work with the same group of compounds standard curves are efficient and if a programmable integrator is brought into the system then routine peak identification and quantitation can be made automatic. However in other situations TLC or CC may be faster and in some cases estimation directly from a crude extract may be possible. Detection of the eluting peaks is controlled by the set wavelength and selecting this value is necessarily a compromise for complex mixtures. If the wavelength is too short peaks of no interest may be picked up and obscure minor peaks of interest. If the value is too long then sensitivity of detection of some compounds may be quite low. The detecting wavelength may coin-
cide with a valley in the region of the absorption spectrum with relatively low $\varepsilon$ values. The set value should be in a region in which as many as possible of the compounds expected would have high absorbance, even for valleys. For this study 287 nm was chosen since it was at a maximum for one of the principal chromophores, entetraynene, and was still fairly sensitive for phenyldiynene. Phenylheptatriyne had the poorest sensitivity (Fig. 4). If a particular chromophore present in only small amounts is to be monitored it may be possible to select a wavelength nearly unique to it, e.g., pentaynene (XVII) was reported to be present in trace amount in *B. pilosa* by Bohlmann et al. (4). There was no indication of it in the regular runs but it could easily be missed if it was at low concentration. It has a peak at 410 nm which is not overlapped by any of the other expected acetylenes. Samples were run at this wavelength at high sensitivity but there was still no indication of the compound, suggesting that it was absent or present at very low levels.


12. Lam, Jörgen. Personal communication.


CHAPTER III

INHERITANCE OF TUMOUR CHARACTERISTICS IN BIDENS ALBA
INTRODUCTION

The studies reported in this chapter were an offshoot of the research on tissue cultures for polyacetylene production. They are presented here in order to keep the sections dealing with tissue cultures together and to introduce the background information on crown gall.

Crown gall is a neoplastic disease of plants induced by strains of Agrobacterium tumefaciens carrying one of a group of plasmids (Ti plasmids) essential for tumour induction (49, 50). A portion of the plasmid (T-DNA) becomes covalently linked to host plant DNA (46) in the nucleus (12). In vivo transcription of T-DNA has been demonstrated (15) and a minimum of seven polyadenylated RNAs have been identified from octopine tumours (33) and nine from nopaline tumours (40). Three specific mRNAs from T-DNA have been shown to be translatable in a wheat germ system (42). One of the products appeared to be the enzyme lygopine dehydrogenase, which produces octopine in tumour cells. The presence of T-DNA is essential for maintenance of transformation (56, 57) which is generally considered to be confirmed by three criteria (19): 1) production of opines by the tissue, 2) growth of tissue explants on a simple medium devoid of phytohormones, and 3) demonstration of the presence of T-DNA in the tissue. A fourth trait is also used: insusceptibility of tumour-derived plants to infection by virulent A. tumefaciens strains (52). Opines fall into three established groups (34): 1) octopine and
related amino acid derivatives, 2) nopaline and related compounds, 3) agropine. A fourth type, agrocinopines, has recently been partially characterized and described (17). The first two groups appear to be widely accepted as being specific to crown gall tissue (22,32) and there are no reports of the last two being found in normal tissue.

A characteristic feature of nopaline tumours is their tendency to differentiate into relatively normal organs and tissues (teratomata) when the tumour either grows near the apex of the plant (2,47) or when it is cultured on a synthetic medium (7,8,28). However under appropriate conditions agropine and octopine tumours may also be induced to differentiate (54). Regeneration, from single cells, of flowering plants which set seed when grafted to normal plants has been demonstrated (9,48).

Regeneration of complete, rooted tobacco plants from tumours has been shown by Einset and Cheng (16) and from single cells by Sacristan and Melchers (39). However, tissue from the regenerated plants showed a loss of tumour markers in both studies. Loss of markers has also been reported for regenerants of Arabidopsis thaliana (1). In another case (using in vitro transformation) regenerants showed a variety of combinations of markers (23). In general, then, teratomas usually differentiate to form shoots but not roots (9,26,48,54) and regenerated plants may show a loss of tumour markers.

Braun, using tobacco, was the first to show complete recovery in plants grown from seed set by transformed plants (9). Later, studies of Braun's line (RT37) using DNA solution hybridization (56) and Southern blots (25) confirmed the loss
of T-DNA; from F\textsubscript{1} plants in the former study and from S\textsubscript{1} and anther-derived haploid plants in the latter study. Yang et al. (58) have demonstrated retention of the ends of T-DNA (from pTi-T37) in F\textsubscript{1} plants of the same line although all other markers were lost. Turgeon et al. (48) identified the stage at which recovery occurred by checking flower parts and haploids derived from anthers. Their results indicated that the loss of T-DNA occurred at meiosis. This finding was confirmed in later studies (6, 25, 56).

The two problems in these reports (nonrooting of regenerants and loss of T-DNA at meiosis) are of considerable interest to molecular biologists who anticipate use of the Ti plasmid as a vector, with inserts in the T-DNA, for incorporating new genetic material into host plant genomes (10, 30, 35, 52). The feasibility of insertion of exogenous DNA into T-DNA, and stable incorporation into host DNA, has been shown (18, 20, 36). Thus it is significant that two groups recently reported evidence for the transmission of T-DNA into the F\textsubscript{1} and S\textsubscript{2} generations of tobacco. Wullems et al. (52) used \textit{in vitro} transformation of tobacco protoplasts to obtain a nopaline-type clone which was regenerated into a rootless shoot and, as a graft, set seed when crossed with normal tobacco yielding F\textsubscript{1} plants which retained transformation markers. Otten et al. (37) obtained a tobacco mutant (GV2100) with lysopine dehydrogenase activity which was able to root, set seed, and produce S\textsubscript{1} and F\textsubscript{1} progeny retaining markers of the inciting plasmid. They demonstrated Mendelian inheritance of T-DNA markers and obtained both homo- and hemizygous S\textsubscript{2} plants. The plasmid
used was pTiB6S3 containing a Tn7 insertion which, however, was deleted in plant nuclear DNA along with adjacent T-DNA sequences (13,37). Recently T-DNA with a defined gene inserted into (and inactivating) a locus affecting root formation has been shown to be stably transmitted to the \( R_1 \) generation (4a). These results demonstrate the practicality of obtaining complete plants which can reproduce sexually and transmit foreign T-DNA from generation to generation in a predictable pattern. There is some evidence that expression of inserted genes may require additional genetic manipulations (10,27).

Reported here are the results of work with *Bidens alba* showing the production of transformed whole plants and transmission of tumourous traits through four generations using bacteria containing an unmodified nopaline-type plasmid and *in vivo* transformation. These results agree with the work of others but show some interesting differences.
MATERIAL AND METHODS

Bacterial and Plant Cell Lines

Bidens alba var. radiata (Schultz-Bip.) Ballard as delimited by Ballard (4, 29) was obtained from southern Florida, USA. Voucher specimens are on file at UBC. Agrobacterium tumefaciens strain A208 (containing plasmid pTi-T37) and A277 (pTi-B6-806) were both obtained from Dr. M. P. Gordon, U. of Washington, Seattle. The origins of the strains are described in Sciaky et al. (43) and Chilton et al. (11). Galls induced by A208 produce nopaline and those of A277, octopine.

Culture Methods

All tissue cultures were grown at 25°C in 100 x 10 mm² plastic culture plates on Schenk and Hildebrandt (SH) medium (41) with 7 g/l agar, pH 5.7. Hormones were omitted except as noted. Medium was sterilized by autoclaving. For tumour induction and culture the following procedure was used: the bacterium was grown at 25°C on medium containing 0.8% nutrient broth, 0.1% yeast extract, and 0.5% sucrose (17a). A drop of the 48 hour culture was placed on a plate of SH medium and spread and pieces of B. alba callus (approx. 1 cm² x 3 mm) were added to the plate. The callus had previously been isolated from stem tissue and maintained as callus on SH medium plus 4 mg/l NAA for 2½ years. The viscous suspension of bacteria resulting after ten days growth was taken up in a syringe and inoculated into a stem internode punctured through with the needle. This method of growing the inoculum was tested.
vis-a-vis liquid nutrient medium by inoculating *B. alba* and *Helianthus annus* 'Mammoth Russian', suggested as an assay plant by Gordon (19), with both strains. Results (not shown) showed slightly larger galls resulting from the liquid medium but the difference was not considered significant. Total cell counts for liquid medium yielded $2 \times 10^9$ bacteria/ml and $8.5 \times 10^9$ for agar cultures. Tumours were harvested after three months of growth, sterilized according to the method of Johnson et al. (21), and incubated in darkness for 3 weeks on SH medium to which filter-sterilized solutions of carbenicillin disodium (Ayerst Labs) and kanamycin sulfate (Sigma), 0.5 g/l and 0.13 g/l respectively, had been added. Seeds were sterilized by immersion for one minute in 70% ethanol followed by thirty minutes in 10% commercial bleach with 1% detergent (Sparkleen) and then rinsed four times in sterile distilled water.

Gall explants were transferred to fresh medium every three weeks. After the appearance of organized shoots from A208 tissue (approximately six weeks after harvest) cultures were transferred to a 25°C illuminated incubator (Lifeline fluorescent bulbs, Sylvania) with an illuminance of 6000 lux and a photoperiod of 16 hours. Plants were transferred to medium in flasks as necessary. Rooting in the callus surrounding the base of the shoots began about 3½ months after harvest. After the rooted plants were well established they were potted in sterile potting soil and grown to maturity in a growth chamber as above except that illuminance was 8000 lux and the relative humidity was initially 70%.
Opine Assay

Leaf tissue, minus petiole, was used except as noted, for nopaline determination. Preliminary work showed that the nopaline content increased from the top leaves to the 3rd or 4th leaf down (in young plants). Samples were taken from the 4th leaf or lower. For gall tissue only the outer, nonwoody layer of tissue, which was peeled off, was used. About 0.25 gram of fresh tissue was weighed and crushed thoroughly with an equal amount of 95% ethanol and centrifuged. The supernatant was taken to dryness on a rotary evaporator and redissolved in distilled water. An amount of extract equivalent to 0.05 gram of tissue was applied to a sheet of Whatman no. 1 chromatography paper. On each sheet three sets of standards were run, namely, arginine (Sigma), octopine (Sigma), and nopaline (Calbiochem), so that one set contained 2 ug of each component, the second 4, and the third 6 ug. Samples were electrophoresed for two hours by the procedure of Otten et al. (38), the paper dried and sprayed thoroughly with a solution of phenanthrene-quinone according to the method of Yamada and Itano (55). After drying the intensity of sample spots was estimated under long wave UV by comparison to the standards. If a sample was outside the standard range it was rerun at twice or half the amount above. In this way the amount of opine in a sample spot was graded into the following categories: absent, present in trace amount, or present at 2, 3, 4, 5, or 6 ug/spot. Multiplying by the appropriate factor yields the estimated opine/g fresh weight. A series of replicate runs were made with sample order randomized from run to run and the grade assignments
compared; the assessments on average varied less than one grade (data not presented). In Table 3 when only hemizygous plants resulted the homozygote level is assumed to be twice the hemizygote.

**Crosses**

*Bidens alba* var. *radiata* has small flowers organized into heads of 40-60 florets. The anthers surround the style in a manner typical for the Compositae making it technically impractical to remove them in crosses. However, the particular line of plants used in this study is largely self-incompatible; when plants are isolated from pollinators and allowed to self, seed-set is only 4-8% of the level obtained when the plants are out-crossed (data not presented). To minimize selfs in crosses the heads to be used in the cross were chosen at a stage in flowering such that most of the styles had emerged from the anthers and were covered with pollen on the non-receptive portion of the style tip. The recipient heads were then blown vigorously to disperse as much of the pollen as possible. Remaining unopened florets were plucked out and discarded. The cross was then made by rubbing the heads together. Under these conditions an estimated background level of selfs of about 5% was expected, assuming equal viability of pollen from the two plants. Crosses of transformed to wild type plants were to normal untransformed plants grown from the same seed pool as the parental plants.
RESULTS AND DISCUSSION

Inheritance of Nopaline Synthesis

One objective of this study was to see if Mendelian inheritance of a T-DNA marker could be demonstrated for B. alba in the absence of single-cell clones. Explanted gall tissue formed shoots and then roots after approximately six weeks and 3½ months respectively. Two different sets of galls (inoculated at different times) showed the same capacity for regenerating rooting plants. These regenerants were the parental generation (R). The second generation (R₁) was produced by selfing parental plants and selecting the seed from one of these with highest nopaline content as the line for further study. Outcrosses and further selves were made as shown in Table 3. Individual R₁ plants should, therefore, be equivalent to single-cell regenerants but the different plants would not necessarily be clones of the same line since the parent plant was derived from a multicellular tumour fragment.

If it is assumed that the parent (R) for the R₁ generation was homozygous then the crosses which follow can only fit the data if losses of the trait occur; 6% for the R₁, about 43% for the F₁, and 24% for the R₂. If it is assumed that the parent was hemizygous then the R₁ can only be correct if low and high levels are in the same category (i.e., nopaline⁺) and the departure from a 1:3 ratio is not significant. This implies that gene dose is not resolved by nopaline level. The F₁ crosses and the R₂ do not differ significantly (by chi-square test).
from the expected ratios in this case. However, the final generation shown, R₂F₁-C, does differ significantly from an expected 1:3 ratio. These plants were the progeny of a selfed plant which must have been hemizygous, since the parent was a wild-type plant which had received transformed pollen. However all of these plants were nopaline negative. Thus the results appear to be incompatible with either assumption unless the trait is lost at a variable rate. Data presented in the next section suggests that the first assumption is more likely to be correct.

An additional self, not shown in Table 3, was also made; an F₁-A plant was selfed but did not yield enough plants for statistical analysis (six nopaline⁺, one nopaline⁻) but the result did demonstrate inheritance of nopaline synthesis to the fourth generation.

Along with the apparent loss of nopaline synthesis as a trait, nopaline levels also declined in the successive generations of F₁, R₂, and R₂F₁-C; declining from 300 ug/g f.w. (R₁) to 20 ug/g f.w. (R₂) and to 0.0 (R₂F₁-C); to 100 ug/g f.w. (F₁-A) and 60 ug/g f.w. (F₁-C). Although levels consistently declined after the R₁, the amount varied considerably from generation to generation. This is not easily explained but, considering the centrality of arginine and α-ketoglutarate (substrates for nopaline synthase) in metabolism, it seems unlikely that these results are due to physiological factors. Comparisons of the nopaline levels in tissues grown under different conditions (light/dark, with/without auxin) and different tissue types (leaf and root, callus and leaf) showed that levels differed by no more than a factor of two at most (data not presented).
Table 3. Nopaline synthesis in successive generations of transformed *Bidens alba*.

<table>
<thead>
<tr>
<th>Generation</th>
<th>Origin</th>
<th>Nopaline level (% plants analyzed)</th>
<th>Nopaline amount (\mu g/g f.w.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R</td>
<td>Parental regenerants</td>
<td>N.D. 54 46</td>
<td>13 260</td>
</tr>
<tr>
<td>R&lt;sub&gt;1&lt;/sub&gt;</td>
<td>R (high) self</td>
<td>6 31 63</td>
<td>16 300</td>
</tr>
<tr>
<td>F&lt;sub&gt;1&lt;/sub&gt;-A</td>
<td>W.t. &lt;sup&gt;b&lt;/sup&gt; &lt;female&gt; X R&lt;sub&gt;1&lt;/sub&gt; &lt;male&gt; (low)</td>
<td>46 54 0</td>
<td>39 100</td>
</tr>
<tr>
<td>F&lt;sub&gt;1&lt;/sub&gt;-C</td>
<td>R&lt;sub&gt;1&lt;/sub&gt; &lt;female&gt; (low) X W.t. &lt;male&gt;</td>
<td>40 60 0</td>
<td>43 60</td>
</tr>
<tr>
<td>R&lt;sub&gt;2&lt;/sub&gt;</td>
<td>R&lt;sub&gt;1&lt;/sub&gt; (low) self</td>
<td>24 45 31</td>
<td>84 20</td>
</tr>
<tr>
<td>R&lt;sub&gt;2&lt;/sub&gt;F&lt;sub&gt;1&lt;/sub&gt;-C</td>
<td>F&lt;sub&gt;1&lt;/sub&gt;-C (low) self</td>
<td>100 0 0</td>
<td>21 n.d.</td>
</tr>
</tbody>
</table>

<sup>a</sup>Estimated nopaline for homozygote.

<sup>b</sup>Untransformed, wild-type, plants.

Abbreviations: n.d., not detectable; N, total plants assayed.
I conclude that nopaline synthesis is transmitted through meiosis but that variable rates of loss of expression of the trait prevent determination of the number of loci involved in the nopaline trait.

**Differential Trait Expression**

To check for loss of the tumour marker auxin autotrophy (aut$^+$), approximately equal numbers of nop$^+$ and nop$^-$ plants were randomly selected from the $R_2$ and $F_1$ lines and stem internode sections (5 mm slices) were explanted to SH medium. Explants were transferred as required and growth evaluated at the end of ten weeks. Seven of the nop$^-$/aut$^+$ cultures were grown on medium supplemented with 5 mM arginine (1) for an additional four weeks. Six remained negative and one culture was positive. The results of growth on regular medium are shown in Table 5. Note that the ratio of nop$^+$ to nop$^-$ has no significance. Results for the two lines, $F_1$ and $R_2$, were similar and combined. The data suggests that there was not only a loss of nopaline synthesis, while auxin autotrophy was retained (10 out of 28), but that there appeared to be a bias to the direction of loss; the retention of nopaline synthesis and loss of autotrophy (5 out of 26) occurring less frequently. This is also indicated in the generation $R_2F_1-C$ (Table 4) which had 4 out of 21 plants showing hypertrophy of the lower stem but all 21 lacked nopaline. I assume that auxin autotrophy is a reliable indicator of transformation since I have not been successful in getting habituated tissue after repeated attempts. However, the possibility that passage through tissue culture affects this trait is not ruled out.
Several studies have shown that differential loss of traits may occur in cells transformed in vitro by bacteria (52,53) or plasmids (14,23). Loss may occur after differentiation (44,51) or be lost in roots and retained in the parent callus (36). The latter has also been observed in this study in octopine negative roots from an oct^+ A277 callus of B. alba.

The data from these two sections suggest a progressive loss of T-DNA or non-expression from the R_1 generation onward. Known features of the organization of the T37 T-DNA are also consistent with this view; the presence of multiple copies of T-DNA was suggested by the work of Yang et al. (57) and work by Lemmers et al. (25) indicated that nopaline tumours can carry from one to at least four T-DNA copies in tandem; evidence by Zamбрьski et al. (59) indicated that T-DNA was organized as two to five tandem repeats inserted into repetitive DNA. Chilton et al. (12) concluded that at least some T-DNA copies were tandem and that there were two or more inserts (including inserts in T-DNA). Decreases of T-DNA in differentiated tissue, relative to parent callus, has been observed (56) as well as alter-
ation in the pattern of border fragments produced by restriction analysis in callus and differentiated tissues (25). These studies show that T-DNA may undergo alterations as the host cells change state and these alterations are consistent with the type of changes seen in *B. alba*. However, Barton et al. (4a) have reported that levels of nopaline varied greatly among R₁ seedlings of a cloned nopaline parent and the copy number for nopaline synthase T-DNA showed little correlation with product level. Perhaps inactivation and loss can both occur as separate alterations. The data above does not allow a choice to be made between these two alternatives.

Superinfection

There is conflicting data on whether transformed plants are susceptible to superinfection with the same or other strains of *A. tumefaciens*. In some cases (48, 52, 58) it was found that transformants were resistant to reinfection. However, Otten et al. (37) reported that both homo- and hemizygous S₁ plants formed galls upon inoculation with three different strains of bacteria. Double transformants (producing both octopine and nopaline) were obtained. I inoculated forty nopaline positive R₂ plants with strain A277 and obtained gall formation on 92.5% of plants (each plant was inoculated in three places); 77.5% of the plants formed galls comparable in size to those formed on four control plants; 15% showed a weaker response and 7.5% showed no indication of gall formation. There were no consistent differences in the galls induced on low and high nopaline plants. Some nopaline positive plants were inoculated with strain A208. Galls formed, but extremely slowly; it took several months for
tumour growth to become evident.

Tissue from an octopine gall on a low nopaline plant was grown on either SH medium or SH medium plus arginine (SH-A). The levels of both opines showed marked changes with time, however on SH-A medium octopine was consistently present at higher levels than nopaline. A representative assay of callus after two months culture is shown in Fig. 18. Callus from internode tissue of the parent plant shows only nopaline on both media (lanes 1 & 3) whereas A277 gall callus from the same plant shows quite low levels of both compounds on regular medium (lane 2) and a higher relative level of octopine on SH-A medium (lane 4). Normal control callus shows no trace of either compound (lane 5).

Morphological variation

Plants from the R1, F1 and later generations, which were transformed by at least one of the two criteria showed several consistent differences in morphology from control plants: 1) transformed plants were invariably smaller than controls (Fig. 19A). 2) Transformed plants had well developed basal lateral branches resulting in a "pitchfork" habit (Fig. 19A, B). 3) Nearly all older transformed plants showed irregular and abnormal thickening of the stem cortex, increasing both towards the base of the plant and with age. In young plants this swelling was most pronounced in the hypocotyl and upper root, giving them a radish-like appearance (Fig. 19B). With age the lower nodes especially became swollen and bulbous (Fig. 19C). Frequently the petiole bases of lower leaves would also show extreme hypertrophy (Fig. 19D). This was especially evident if the leaves were broken at the petiole base. 4) Ray flowers frequently showed growth of a
Fig. 18. Paper electropherogram of single and double transformant callus. Lanes 1, 3: single transformant on normal and arginine medium respectively. Lanes 2, 4: double transformant on normal and arginine medium. Lane 5: normal callus on arginine medium. Amount of tissue per sample: 1 and 2, 0.05 g; 3 and 4, 0.02 g; 5, 0.03 g. Abbreviations: s, standards; arg, arginine; ?, unknown; oct, octopine; nop, nopaline.
Fig. 19. Morphological differences in transformed and normal B. alba plants. A) Left, normal plant; right, R₂ transformant. B) Left, F₁ transformant showing basal hypertrophy; right, control. C) Old R₁ plant showing enlargement of nodes. D) Old R₁ plant showing enlargement of petiole base. E) Left, normal flower; right, R₂ flower, note spur development on rays.
spur of petal tissue from the inner (upper) side (Fig. 19E). Similar aberrations have been described for transformed tobacco plants (4,22). In contrast, Otten et al. (15) described $F_1$ and $S_1$ plants as appearing normal, apparently as a result of a deletion in the T-DNA of that line. Other differences appeared to be due to passage through tissue culture since they occurred in both transformed plants and regenerants from stem segments but at much lower frequency in normal, seed-grown, plants. Similar effects of in vitro culture have been shown for a number of other plants (24,45).

Hypertrophic growth on some of the plants grown from seed resembled gall growth, suggesting soil-borne contamination of the plants by bacteria. A number of tests were performed to exclude this possibility. I obtained the following results:

1) normal seeds germinated and grown in soil from hypertrophic plants were normal. 2) Seeds from transformed plants which were germinated on sterile medium and transferred to sterile soil gave plants that showed the same combinations of markers as in Table 4 as well as hypertrophy. 3) Normal seedlings wounded and inoculated with soil from hypertrophic plants healed normally. 4) Gall-like growths from plants which were nopaline negative in the leaves were negative in the galls. 5) Normal internode and "gall" tissue from hypertrophic plants surface-sterilized and explanted to hormone-free medium showed autotrophic growth with no sign of bacterial contamination. These results indicated that there was no contamination of the soil by bacteria carrying a normal Ti plasmid.

Some of the morphological correlates of transformation in offspring of B. alba shown in this study have been seen in
tobacco. Binns et al. (6) described regenerant plants with thickened nodes and abnormal shoots arising from them. In the plants here the shoots from swollen nodes were normal. Wullems et al. (52,54) described plants regenerated from somatic fusion hybrids of crown gall and normal cells as being shorter, thicker and having decreased apical dominance as primary grafts. After two or more graft transfers nearly normal shoots resulted. These authors also showed that spraying normal plants with kinetin resulted in plants similar to primary graft plants. Wullems et al. (52) also noted the small size and slower development of an $F_1$ nopaline-producing plant. These results contrast with those of Otten et al. (37) who described their $F_1$ and $R_1$ plants as being completely normal in appearance.

Three types of cultures have been isolated from A208 tumours: a compact, slow-growing callus tissue with no tendency to form teratomata; a loose, faster growing callus which shows no organ formation; and shoot-forming callus which grows only as relatively normal, though diminutive, clusters of shoots. From the latter, lines have been isolated in which root formation is suppressed in about 90% of the explants from a given clump. The tendency to form roots can be almost eliminated and lines can be isolated in which both shoot and root formation is suppressed. Amasino and Miller (3) described three tissue types similar to these and each was characterized by a specific balance of hormones. They showed that normal callus grown on medium with hormone levels corresponding to those of the transformed cultures assumed
the same growth form. Whether each of the growth forms in _B. alba_ has its own particular hormone balance is not known although the work of Amasino and Miller (3) suggests that this is the case, indicating that isolates from tumour explants of _B. alba_ are capable of assuming several stable states.
SUMMARY

Bidens alba differs from plants commonly used in crown gall research in its capacity for regeneration of whole plants from galls induced by an unmodified T-37 plasmid and its retention of T-DNA traits through several sexual generations. However, it shows a marked decrease in expression of nopaline synthesis from generation to generation and loss of either auxin autotrophy or nopaline synthesis can occur suggesting either progressive loss of T-DNA or changes in its expression. Nopaline positive plants are capable of having octopine galls induced on them to yield double transformants, suggesting that insusceptibility to transformation is not a reliable indicator that tissue is transformed. Transformed plants were distinguished by several morphological differences some of which were probably due to an altered hormone balance in such plants, others possibly due to passage through tissue culture.
LITERATURE CITED


CHAPTER IV

STUDIES OF NORMAL CALLUS OF BIDENS ALBA
INTRODUCTION

This chapter describes attempts to obtain production of polyacetylenes in normal callus culture of B. alba. In an effort to uncover clues to the possible requirements of such cultures a survey of reports of other plants which produced unusual fatty acid products was made (for recent reviews see 22, 27). The most useful of such cultures appear to have been those of two species of Malva, which produced levels of cyclopropane and cyclopropene fatty acids (sterculic and malvalic acids and their dihydro analogs) comparable to or exceeding those of the seeds (43). Callus cultures were used to establish the biosynthetic pathway for these compounds (42). The biosynthesis of fatty acids having a terminal cyclopentenyl ring was studied with suspension cultures of Idesia polycarpa, which were found to elongate a precursor into the final product (23, 28). However, cultures of a related plant, Hydnocarpus anthelminica, were unsuitable for studying these compounds (33). Specialized fatty acids have been found in callus of Pinus elliottii (19) and Petroselinum crispum (6). In the latter high levels of petroselenic acid (18:1,6c) found in the plants were absent from cultures, but an isomer, vaccenic acid (18:1,11c), was present. There seem to be no reports of fatty acids restricted to seed tissue being produced in significant amount in callus, although Jones (17) obtained formation of erucic acid (22:1,13c) in embryo-like structures which differentiated from Crambe callus.
The reports summarized above represent instances of tissue cultures producing more than trace quantities of unusual fatty acids. However, no special modifications to cultural conditions were made to enhance or induce the production of these compounds. These plants would appear to be constitutive producers under the standard conditions used. Many negative reports, however, demonstrate that production of specialized fatty acid derivatives, as with other groups of secondary compounds, is exceptional in tissue cultures. (11,17,20,29,35,36,40). No reports could be found of attempts to influence expression of unusual fatty acids through nutritional changes, although it has been reported that plant membrane phospholipids could be experimentally altered (24).

Reports of polyacetylenes in normal tissue cultures of Compositae have uniformly been restricted to short-term cultures (less than three months). Jente (16) reported the earliest instance of establishing callus cultures and determining the polyacetylenes present. She found extremely low levels of 0.5 ug/g fresh wt. in callus of Centaurea ruthenica versus 5.0 mg/g f.w. in roots of the plant. Polyacetylenes were found only in cultures which had been grown in light or alternating light and dark. The compounds identified in callus were different from those occurring in the intact plant, although biogenetically related. There was no difference in acetylenes between cultures established from roots or leaf material (16). Ichihara and Noda (14) investigated Cardamum tinctorius seedlings and callus cultures established from roots, hypocotyls and cotyledons of seedlings. Tissues were
incubated with illumination for 14 days before harvesting. Under these conditions most acetylenic compounds increased in amount, however no data on cultures over two weeks old were reported. These authors also demonstrated synthesis of polyacetylenes in protoplasts and chopped seed preparations. *Tagetes erecta* was investigated by Setia (31) for biosynthesis of both polyacetylenes and thiophenes. Callus cultures failed to produce significant amounts of either type of compound when the medium (B₅) contained 1 ppm 2,4-D. However, cultures grown on medium either lacking hormones or with 2,3,5-trichlorobenzoic or 2,3-dichlorobenzoic acids (1-4 ppm) were able to synthesize thiophenes but not polyacetylenes in low but isolatable amounts. Spectral evidence suggested the presence of a polar triyndiene chromophore but only in minute amounts. Cultures began to die within 6 to 7 days and no growth occurred after 14 days. The highest product yield was found after 13 to 16 days (31).

The following points summarize the main findings of these studies: 1) light may be necessary for product formation. 2) Types of hormones used in the medium could be a factor in sustaining biosynthesis. 3) Compounds normally not found in significant amount may accumulate in cultured tissue.

The work summarized in this chapter attempted to improve on these studies and establish cultures which would synthesize polyacetylenes as a normal part of callus growth. Several reviews of factors affecting a wide range of secondary products synthesized in tissue cultures served as guides for selecting parameters to evaluate for polyacetylene production (1,5,38).
MATERIALS AND METHODS

Explants

Seeds were washed in 70% ethanol for one minute and then soaked ten minutes in a 10% commercial bleach (Javex) solution with 0.5% detergent as a wetting agent, gently agitated, rinsed three or more times in sterile distilled water and germinated in dark at 25°C on moistened and sterilized filter paper discs. After germination seedlings were transferred to the light and grown to approximately four cm in length and then divided into roots, hypocotyl, and cotyledons and explanted to separate plates of medium.

Media

The following media formulations were tested: White (41), Nitsch (26), Heller (12a), ER (7), MS (25), Hildebrandt (13), B₅ (9), and SH (30); MS medium was made up with equimolar concentrations of ferrous sulphate and disodium EDTA (32). All formulations, except as noted, were made up with 0.7% agar (Difco, Bacto-agar), 3% sucrose (Fisher Scientific), 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma) and pH 5.7, adjusted with potassium hydroxide or hydrochloric acid. Media were autoclaved 20 minutes at 15 lb in⁻² and then poured into 15 x 90 mm plastic petri plates. The standard incubation for cultures was 25°C with no illumination.

Supplements

Inorganic salts, organic acids (except fatty acids),
glucose, inositol, and kinetin were added to the medium prior to autoclaving. All other compounds were filter-sterilized with 0.2 micron Millipore filters and added after autoclaving. Fatty acid supplements were added to cooled medium after filter-sterilizing, shaken vigorously to form an emulsion and then poured. A number of different emulsifiers for lipids were tried including ethylene glycol, glycerin, Triton X-100, Tween-20, -40, and -80, ethanol, lecithin, sodium acetate and tragacanth; these in various combinations. All are listed in standard references on emulsions (2,37) as suitable for lipids of the type added. However, most were unsatisfactory due to the low ratio of lipid to aqueous phase in medium. In addition, heated medium enhanced emulsion breakdown and creaming. The best combination obtained, which formed a stable emulsion, was fatty acid: Tween-80: ethanol in the ratio 35:8:7 (v/v/v). Controls showed little effect on callus growth from the Tween-80 and ethanol at the highest levels used. Oleic, linoleic, and linolenic acids were Purified grade, containing at least 90% of the free acid (Fisher Scientific).

Callus Testing

Callus for use in tests of medium supplements were lines which had been subcultured two or more times and showed the presence of small amounts of acetylenes. Four explants were made to each of three plates and a total of six lines tested. After growth on the medium for 2-3 weeks the callus from each plate was combined, extracted, and a UV spectrum taken and checked for any indication of polyacetylene synthesis. If an effect was to occur it might only be found with certain lines
so as many lines as possible were tested. Approximately three-quarters of the callus from a petri plate was taken for extraction; the remainder was left for subculturing. The petroleum ether extract was evaporated with a stream of nitrogen, made up to 5 ml, and a UV spectrum made of it. This would usually represent the extract from at least 5 g of callus and possibly more. Under these conditions the UV spectrum from approximately 0.02 g of roots or 0.01 g of leaf gave spectra in which the main chromophores could be recognized. Hence, the polyacetylenes in callus containing 0.5% of the level found in the plant should have been readily recognizable. The spectrum for a line was classified as negative or positive for product and compared to later spectra of the same line to determine the effect of further subculturing. Figure 20 shows the overall structure of the evaluation procedure followed in attempting to find some defined condition which would result in product synthesis in callus.

**Mercuric Chloride Treatment**

Conditions for mercuric chloride elicitation were adapted from data of Hargreaves (12). A concentration of 3 mM mercuric chloride and exposure times of 5, 10, and 15 minutes were used. Treated and control calluses were rinsed five times in sterile distilled water and incubated for four days on SH medium with 4.0 mg/l NAA. Duplicate plates of five calluses for each of four lines were tested at each treatment combination. Two lines had been cultured for two years and two for four years at the time of the experiment.
Seedling explants to 8 different media.

Selection of 3 media with best growth → Harvest and evaluate all callus for composition.

Evaluation of growth vs. hormone levels.

Standard auxin level.

Establishment of 220 lines from seedling.

Growth for three months.

Harvest and evaluate extract of all lines. → Discard line if spectrum featureless.

Cultures showing some indication of product.

Subculture. → Evaluation of medium variations.

Re-examine. → Evaluation of each variation with at least 6 lines per treatment.

Fig. 20. Diagram of evaluation procedure for polyacetylene production in callus cultures of B. alba.
RESULTS AND DISCUSSION

Growth on Different Media

The initial step in culturing B. alba was to find how growth was affected by different media. Initial work showed that callus formation occurred on B₅ medium with 1.0 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid) and this level of auxin was used in all media.

Eight formulations were tried with five explants of each seedling organ explanted per plate and triplicates for each organ and medium. Growth was evaluated at the end of three weeks by visually ranking the amount of new tissue formed. Results are shown in Table 5.

There was little difference in growth response among the three organs. Media having low salt levels (Hildebrandt, White, Nitsch, and Heller) produced distinctly less growth than those with higher salt levels. The low salt group was eliminated from further work after additional subcultures showed no product formation, and ER medium was eliminated due to its similarity to MS and its lack of product. The remaining media (MS, SH, B₅) provided the best growth and were used in subsequent experiments.

Table 5. Relative growth of B. alba explants on different media.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Medium type</th>
<th>Hildebrandt</th>
<th>White</th>
<th>Nitsch</th>
<th>Heller</th>
<th>SH</th>
<th>MS</th>
<th>ER</th>
<th>B₅</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Hypocotyl</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Cotyledon</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+++</td>
</tr>
</tbody>
</table>

Symbols: +, least growth; ++++, most growth.
Callus and Product Formation

Explants callused readily on 2,4-D supplemented SH, B$_5$, and MS media, although some root formation occurred with leaf tissue, and by the end of the first month most of the original explant had been replaced by callus. Extraction of callus tissue at the end of the second month of culture showed no PHT was present in leaf or stem callus. Leaf callus had low levels of ETE-R (the particular form could not be determined from the spectrum, although it was probably the acetate) due to root formation. There were only trace amounts of acetylenes in stem and root callus. By the end of the third month polyacetylenes were not detectable in most of the cultures. If traces were still present it was always either ETE-R or PDE-R (presumably the acetate).

Mercuric Chloride Treatment

Mercuric chloride is an abiotic elicitor which has been found to cause production of phytoalexins in some plants (10). After other methods had been tried and found not to result in product synthesis, this approach was tried. Callus treated with mercuric chloride turned brown and showed extensive, but not complete, cell death. There was no indication of elicitation of polyacetylenes. It is possible that the age of the lines was a factor in the negative results; Fett and Zacharius (8) found that phytoalexin production could be induced in a recently established pea cell suspension culture by bacteria but a similar line in culture for 15 years showed no response. At the time this experiment was done all the lines used were at least two years old.
Miscellaneous Treatments

A few factors were checked in a series of cursory experiments for obvious effects on product formation. The effect of temperature shock was checked by exposing cultures to either low (4°C) or high (40°C) temperatures overnight, returning them to standard conditions, and harvesting two days later. Calluses were also incubated under a germicidal lamp for times ranging from two hours to overnight and similarly harvested. Cultures were harvested at all phases of growth, including very late in the passage when most of the medium was gone and the texture of the callus became quite loose. Calluses contaminated with a variety of unknown fungi and bacteria were harvested. None of the material checked under these conditions showed any indication of product synthesis.

Summary of Results from Medium Modifications

As shown in Fig. 20, the next step in evaluation was to determine if there was any effect from the source of the explant on product in the subcultured callus. At the time callus from the three organs was harvested (fourth passage) none of the cultures were producing appreciable levels of product. It was concluded that under these conditions the source of the explant was unimportant. The explanted organs originally had characteristic patterns and levels of polyacetylenes similar to those in Table 2.

Opinions on the importance of the source of the explant for subsequent biosynthetic capacity vary widely; Böhm (3) considers it irrelevant, while Staba (34) cites evidence supporting the importance of explant source. Its importance for subse-
quent morphogenesis has been reviewed by Thorpe (38a) but effects on secondary product synthesis seem to be variable. Loh et al. (21) recently reported that no difference in product could be demonstrated for callus from different parts of Cannabis sativa; cannabinoids were not synthesized in any of the calluses from different parts of the plant. However, the amount and type of hormones required for growth was specific for the origin of the explant. Callus from the different organs of Bidens seedlings seemed to show about the same amount of growth, as shown in Table 5.

In the next stage of evaluation the level of auxin for best growth was determined for both NAA (α-napthalene acetic acid) and 2,4-D. Best growth for 2,4-D was at 1.0 mg/l and for NAA, 4.0 mg/l. There was no detectable difference between NAA and 2,4-D in product formation, however, so 2,4-D was used for the balance of the experiments, except as noted.

The next step was to culture as many separate seedlings as practical and determine if there were significant differences in the callus derived from these, i.e. would a small percent of the seedlings be constitutive for polyacetylene synthesis in callus. Altogether callus from 220 seedlings was checked for product; some were grown on MS medium, some on SH, and some on B₅. Callus was subcultured three times and evaluated during the fourth passage. Most of the lines showed no indication of any polyacetylenes at this stage and were, therefore, discarded. A few showed low levels and these were continued and used for the experiments with various media and environmental factors.
Table 6. Medium ingredients evaluated for ability to induce polyacetylene synthesis.

<table>
<thead>
<tr>
<th>Hormones</th>
<th>2,4-D</th>
<th>NAA</th>
<th>NAA x kinetin</th>
<th>GA₃ x kinetin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugars and vitamins</td>
<td>Inositol</td>
<td>Sucrose</td>
<td>Sucrose (+,- light)</td>
<td>Glucose</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ascorbic acid</td>
</tr>
<tr>
<td>Primary metabolites</td>
<td>Acetate (A)</td>
<td>Malate (M)</td>
<td>Citrate (C)</td>
<td>A + M, A + C, M + C, A + M + C</td>
</tr>
<tr>
<td>Macronutrients</td>
<td>KNO₃, MgSO₄, NH₄H₂PO₄, CaCl₂, KH₂PO₄</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Product precursors or related products</td>
<td>Oleic acid</td>
<td>Linoleic acid</td>
<td>Linolenic acid</td>
<td>Combinations of all three</td>
</tr>
</tbody>
</table>
The objective of the next step was to test as many as possible of the lines that still showed traces of product against variations in medium and determine if product level continued the sharp decline shown by most lines or if the decline in product could be stopped or reversed. The only evaluation made was whether or not the UV spectrum of a line showed constancy or improvement in product level. There was no exact measurement of growth or other characteristics except in one or two cases. Table 6 lists the factors examined. The Appendix contains detailed tables showing the medium components which were varied, range of levels tested, number of levels tested, and some general comments on how growth was affected (see Appendix Tables 1 to 5).

The final result was that none of the treatments induced measurable levels of polyacetylenes. The product level in all of the lines which had been used for these experiments fell below the limit of detection within two further subcultures or less. Although changes in texture and growth rate varied considerably with the treatments, none induced product synthesis. Fatty acid supplements resulted in hard, dense callus somewhat like the transformed callus described later, but there was no trace of product in any of the extracts. Greening of callus was induced by lowering sucrose level to less than 1% and illuminating the callus, but again the results were negative.

It is conceivable that loss of biosynthetic capacity might be irreversible and that attempting to regain synthesis once lost, would not be practical. However, many non-producing
cultures have been shown to be capable of synthesizing secondary compounds when exposed to elicitors of various types, including heavy metals (10). Safynol, a polyacetylenic phytoalexin, is produced by *Carthamus tinctorius* in response to infection (15). More recently safynol has been elicited in suspension cultures of *C. tinctorius* by cell wall preparations from two different fungal pathogens (39). One of the culture lines of *B. alba* established for the work in this chapter has been shown to synthesize low levels of PHT in response to a fungal broth preparation (4). These reports show that callus and suspension cultures which have stopped making polyacetylenes, nevertheless, retain the capacity to make them if appropriately treated. The approach used in this chapter, however, did not result in finding defined conditions for continuous synthesis.
SUMMARY

1) Media containing high levels of salts (MS, SH, ER, \( B_5 \)) gave the best callus growth for *Bidens alba*. Maximum growth was obtained with either 1.0 mg/l 2,4-D or 4.0 mg/l NAA. Choice of medium did not affect the loss of polyacetylene synthesis in successive cultures.

2) The part of seedlings cultured (root, hypocotyl, or cotyledon) had little effect on the growth of callus and no effect on polyacetylene synthesis.

3) If *B. alba* seedlings differ in the capacity to continue polyacetylene synthesis in callus, then the fraction of plants which show continued synthesis of product is less than could be detected in a sample of 220 seedlings.

4) Production of polyacetylenes could not be achieved by varying selected nutritional and environmental parameters.


41. White, P. R. 1954. The cultivation of animal and plant cells. Ronald Press, N.Y.


CHAPTER V

STUDIES ON TRANSFORMED CALLUS OF BIDENS ALBA
INTRODUCTION

In attempts to obtain cultures of *Bidens alba* which would produce polyacetylenes, two further approaches were tried: the establishment of cultures of crown gall tumours and root cultures. In this chapter the results of studies on tumours, and tumour cultures of *B. alba*, *B. pilosa*, and *B. cynapiifolia* are presented.

No systematic survey of the secondary products of crown gall tumours or of cultures prepared from them seems to have been reported in the more than seventy years that crown gall has been intensively studied by plant physiologists, biochemists, pathologists, and molecular biologists. The following paragraphs summarize the main reports that deal primarily with secondary products in tumours or cultures.

Kado (31) has reviewed and tabulated recent literature on primary and secondary metabolites of crown gall tumour cells. Older references may be found in Krikorian and Steward (38) and Klein (34). Kado concluded that of the many primary metabolites and enzymes investigated, most appear to differ quantitatively rather than qualitatively. Often it appeared that differences were due to higher growth rates for tumour cells than for normal cells (31).

Of secondary metabolites, alkaloids have been most investigated. Klein (33), referring to unpublished data of Mika, states that *Datura stramonium* tumours contain almost 500% more hyoscine than roots and up to 300% more tropane alkaloid than
the whole plant. Tomatine, a glycosylated steroid alkaloid, is reportedly found in tomato stem galls (15,35) and sporadically in normal callus cultures over two years old, but not in suspension cultures (55). Catharanthus roseus tumour cultures produce alkaloids in callus and liquid cultures similar to those found in the plant but at lower levels. Suspension cultures were able to effect conversion of vindoline to other alkaloids (4). Non-protein amino acids are found in some intact galls; \( \gamma \)-hydroxyvaline occurs in stems, leaves, and tumours of Kalanchoe daigremontiana (50) at levels higher than in non-tumourous tissue (39). Bean leaves inoculated with Agrobacterium tumefaciens strain 13333 produce \( \gamma \)-amino butyric acid which promotes tumour growth at levels as low as 1 \( \mu \)g per leaf (48).

Ubiquinone is present in tumours of Parthenocissus sp. at levels similar to those of normal tissues (46). However, suspension cultures of tobacco tumour cells contained less than half as much ubiquinone as normal cells and one-third as much as much as a normal line selected for high yield (28). Brown and Tenniswood (12) reported that normal tobacco callus three weeks after subculture, differed in composition from crown gall tumour callus. The latter contained fewer of the coumarin compounds found in normal cells but at higher levels. The authors emphasize that transformation leads to marked qualitative changes in the metabolism of phenolic compounds.

In recent years Japanese investigators have shown interest in the production of secondary products from crown gall. Misawa (43) has reviewed several patents for production of compounds
from tumour cultures, including berberine from *Coptis japonica*, saponins and sapogenins from ginseng, betanin from several plants, and stevioside from *Stevia rebaudiana*. Ginseng tumour callus contained about the same level of crude saponin as normal callus.

*Matricaria chamomilla* appears to be the only Composite for which data on secondary products in normal and transformed tissues is available. Reichling et al. (53) determined essential oils, flavonoids, coumarins, and phenolic acids in flowers, herb, root, and tumours of this plant. They found that stem tumours contain approximately 1/10 the level of essential oils of flowers and about the same amount as herb and root tissue. Among the compounds identified were two acetylenic compounds, cis- and trans-en-yne-dicycloethers, which were present in all parts of the plant and in both nopaline and octopine type tumours. Flavonoids and two coumarins (herinarin and umbelliferone) were present in shoots and flowers but not in roots or tumours. The occurrence of inulin in Jerusalem artichoke was investigated by Kaneko (32) and found not to be present in either normal or tumour callus.

In summary, there is only one report in which a polyacetylene has been detected in transformed cells and a comparatively few reports of other secondary products. The literature on the physiology and biochemistry of crown gall is extensive but it is clear that there is no way to predict what effect transformation will have on most aspects of cell metabolism, including secondary products. Teuscher, however, has suggested (60) that since crown gall tumours contain secon-
tary products, they might retain this characteristic in culture; paralleling tumourous endocrine cells of animals, which can produce hormones while cultured on artificial substrates. Evidence to date neither confirms nor disconfirms this conjecture although the evidence presented here shows that it applies to polyacetylenes in two species of *Bidens*.

The objective of the work described in this chapter was to determine if tumour cultures might be useful in polyacetylene research, and whether polyacetylene expression was different in transformed cells. Initial work attempted to increase levels of product in transformed cultures.
MATERIALS AND METHODS

Bacterial and Plant Lines

Origins and culture methods for Bidens alba var. radiata and Agrobacterium tumefaciens strains are given in Chapter 3. Also found there are the methods for transforming plant tissue and culturing both tissues and bacteria. Seeds of Bidens pilosa var. minor (Blume) Sherff were obtained from Belize City, Belize through Dr. Thor Arnason. Seeds of Bidens cynapiifolia H. B. K. were obtained from Jamaica, W. I. through the Chemistry Department of the University of West Indies.

Culture of Teratomas

All cultures were grown in 15 mm x 90 mm plastic petri plates on SH medium with 7 g/l agar, 3% sucrose, pH 5.7, and no hormones except as noted. Illumination was from Lifeline fluorescent bulbs (Sylvania) with illuminance of 6,000 lux. Teratogenic callus from tumours of A. tumefaciens strain A208 spontaneously differentiated shoots and then formed roots in callus at the base of the shoots. If organized tissue was repeatedly excised from callus and the callus cultured, then differentiation eventually stopped occurring. This happened whether the tissue was grown in light or dark. If, however, differentiated shoot tissues were left attached to the light-grown callus then more shoots formed. The cluster of shoots could be divided and grown separately. This process was repeated for at least eighteen subcultures. Cultures were subcultured every three weeks or harvested. The whole process
is similar to the technique of propagation by means of pre-
cocious axillary branches as described by Krikorian (37). Some
divisions did not root and these non-rooting divisions were
subdivided repeatedly to obtain a line which had a continuous
history of suppressed roots. Some teratomas were grown in liquid culture in 125 ml Erlenmeyer
flasks with 75 ml of medium and incubated in dark or light.
These cultures were subcultured once before harvesting.

Transformation of Tissue Slices

In an attempt to increase the yield of explanted tumours,
an alternative method of inoculation to that described in Chap­
ter III was tried. Stem internode segments of B. alba plants
approximately 4 cm long were surface sterilized with 70% ethanol
for one minute followed by ten minutes in a 10% commercial
bleach solution (Javex) and rinsed 4X with sterile distilled
water. The end 5 mm were trimmed off and the remaining stem
cut into 5 mm slices and placed on solidified 1% agar in petri
plates. Slices were incubated overnight in dark at 25°C and
inoculated the next day with enough 48-hour bacterial suspen­sion to cover the surface of each segment. After two weeks,
small outgrowths 1-2 mm in diameter were explanted to antibiotic
medium. Only one of these explants survived to yield callus,
the line designated F277 IV. This line produced octopine and
grew rapidly as loose, friable callus. A similar method of
transformation has been described by Braun (9) and more recently
by Barton and Chilton (2).
RESULTS AND DISCUSSION

Physical Characteristics of Callus Tissue

Callus derived from both types of plant tumours differed from normal callus in several characteristics: 1) transformed callus was harder and denser than normal callus, appearing to have more lignified tissue. Clumps of transformed callus had to be cut with a scalpel for subculturing while normal callus could be sliced easily with thin forceps. 2) The ratio of wet to dry weight for tumour cultures was always lower than for normal tissue, the former was typically about 12 and the latter about 18. 3) Growth of normal tissue was generally faster than transformed tissue. Doubling time for dense transformed tissue was typically 30 days but only 11 days for normal tissue. 4) Tumour callus contained scattered cavities containing an amber oil resembling the material found in resin canals of intact plant organs. These were almost certainly accumulations of acetylenic compounds. Similar structures were not found in normal tissue. An exception to this pattern was tumour line F277 IV, which was derived from a tumour induced on a stem section; this line was similar to normal tissue in all the points above except that it produced polyacetylenes. The basis for these differences is not clear, although it may reflect differences in levels and ratios of hormones which become established in callus (see later discussion).
Pigmentation and Product Level

The polyacetylenes investigated in this study are colourless compounds, but their detection is relatively easy, involving a simple extraction and UV spectrophotometry of the extract. However, this method requires from 10 mg to 1 g of material and the number of such tests that could be run set a severe limit on the detection of lines with high yield. A group-specific TLC spray reagent for most acetylenes is known (44) but it is not sensitive enough for small groups of cells squashed directly onto a TLC plate and developed, as has been successful with nicotine selection in tobacco (45). Therefore, some other method was necessary if selection of infrequent high yielding cultures was to be practical.

Soon after a number of transformed (oct+) callus lines had been established variation in colour of the calluses was apparent. Some had dark brown spots in varying amounts while others were nearly white. Preliminary work suggested a correlation between amount of colour and acetylene content. An experiment was set up to test this idea. Fifteen different calluses from one line were graded into five groups on the basis of the amount of pigment present. The average product levels for the groups are shown in Fig. 21. The correlation coefficient for this data was 0.72 and the critical value (P=0.01) was 0.68. The null hypothesis was rejected (P<0.01). Composition (not shown) changed in a regular way also; class 1 showed a higher proportion of PDE-OAc (88%) than class 5 (75%) and the other classes had intermediate percentages. The bulk of the remaining product was ETE-OAc.
Fig. 21. Total polyacetylenes for transformed calluses graded by amount of pigmentation. Class 1 white, class 5 heavily mottled. Bars show ±1 SD.
The correlation of pigmentation with product level suggested that continued selection of lines might lead to increased levels over time. Accordingly, the lines from the previous experiment were separated into a high group and a low group based on their classification by colour (not on their product level), with a total of fourteen in each category. At each subculture the most highly pigmented callus was transferred in the high lines and the whitest callus in the low lines. At eight months only the highest of the pigmented lines and the lowest of the white lines were retained (four each) and colour selection continued. Fig. 22 shows the results after nearly 18 months of selection. The initial line was derived from an explant originally started in culture 10 months earlier. There was little difference over the first period of selection between the two groups, indeed at one point the white line exceeded the dark line by a small margin. It appeared that selection on the basis of pigmentation had little effect in differentiating the lines over time, or even maintaining an initial difference. The total level, however, increased appreciably for both series during this interval. After the series were selected on the basis of product level at eight months a significant difference in the two groups began to appear. The points at 10½ months represent the levels after selection; in effect the starting points for the next selection series. Over the next 10 months the low line declined nearly 50% while the high line increased by 43%. The sharp change in relative levels is shown in the decreasing ratio of low to high lines in Fig. 22.
Fig. 22. Total polyacetylenes in high and low pigmentation callus groups over time. Ratio of low lines to high lines is in parentheses. Arrowhead indicates reselection of groups by level of product.
The later divergent trends may indicate an effect of selection on the reduced set of lines, but the absence of a control series without selection makes this uncertain. The pigmentation, once selected, was stable for the three years the lines were maintained.

Segregation of tumour callus into visually different types has been reported by Scott (56), Amasino and Miller (1), and Meins (42). The last two studies were able to find nutrient and hormone conditions which could induce formation of the different tumour types. Differences in secondary products were not examined. Other studies have reported correlations between callus colour and the presence of colourless products (8,26). In some cases related compounds were coloured and provided a selectable character. It is possible that the pigment in *B. alba* callus represented polymerized polyacetylenes, a common reaction of these compounds when exposed to light or to drying out in air, but a separate analysis of the pigment was not attempted. The connection between pigment and product level remains unclear.

Figure 23 shows the combined polyacetylene composition data for the two selected lines, including gall tissue and the callus prior to selection. Composition showed a distinct downward trend in PDE-OAc percentage over time. Long-term changes in product ratios have been reported for the bebane and protopine type alkaloids in *Papaver bracteatum* cultures (67) but the reason for such slow changes is not clear. On the basis of the results from work with roots reported later, the indication would be that levels of cytokinins were declining.
Fig. 23. Percentage change in phenyldiynene acetate over long-term culture of transformed callus.

Changes in composition and product level for callus of octopine line F277 IV, which grew rapidly, were followed at three-day intervals over the course of a 27 day culture period. No consistent pattern was found for composition; the PDE-OAc percentage fluctuated between 45% and 60% with a mean at 52%. Polyacetylene level varied little from 0.04 mg/g dry wt. and this line showed no tendency to accumulate product during any phase of the culture cycle.
Stability of Polyacetylene Production over Time

Several different lines of transformed callus were cultured and harvested for analysis at various times after explanting. Figure 24 shows these results. All of the lines except that derived from *E. pilosa* showed instability of level over long-term culture. Levels of total product ranged up to 20% of the level found in cultured roots but the level to which the cultures seemed to be stabilizing was less than 10% of that value. (Table 10 shows the values for cultured roots).

Wide fluctuations in secondary compound production from tissue cultures are well known, although stable lines producing various product have been reported (e.g. 3,41,66). The determinants of stability are not known. Tumour cells might be expected to be at least initially unstable for reasons related to their origin; T-DNA, which maintains the transformed state, can undergo alterations resulting in deletions of nearly all or portions of the insert, leading to changes in expression of those T-DNA genes which affect cell functions. Evidence that this occurs in *E. alba* has been presented in Chapter III. Extended culture of the transformed lines might result in more stable production but more than twenty-five subcultures have not effected such a change in the lines studied here. However, there is no evidence that T-DNA alterations account for the long-term instability. It seems more likely that changes in the T-DNA would produce a variety of different callus types early in culture and these would be relatively stable to further changes.
Fig. 24. Total polyacetylenes over time of different callus lines of transformed B. alba and B. pilosa. F208 lines are A208 (nop+) type B. alba, F277 an A277 (oct+) line, and B208 an A208 line of B. pilosa. Each point is the value obtained for the combined callus from two or more plates.
Detailed Analysis of Polyacetylenes in Transformed and Normal Tissue

Shown in Table 7 is a breakdown of the composition and product levels for the two species of *Bidens* cultured. Results for both normal as well as singly and doubly transformed callus are shown. The data show the unique pattern of polyacetylene biosynthesis found in tumour cells. There is a consistent pattern of ratios and of the production of compounds found only at detectable levels in tumour cells.

*In situ* tumours of *B. alba* differ from the surrounding tissue in having low levels of PHT and high levels of PDE-OAc instead of PDE. Octopine and nopaline galls were qualitatively similar but differed markedly with respect to the proportion of PDE. Growth of tumours as callus, away from the influence of the host tissue, resulted in a different pattern of compounds. The most interesting change was the occurrence of significant amounts of the ETE aldehyde (ETE-al) in all tumour calluses. This compound was not detected in normal tissue or in galls. It is interesting that this compound was found by Bohlmann (as noted in Chapter II) in the material he worked with but was not present in *B. alba* except under these conditions. Small percentages of the putative dientriynene alcohol (DTE-ol) were found consistently in all three types of cultured tumour callus. The triendiynene alcohol (TDE-ol) was also detected only in tumour callus but at levels too low to estimate. All the callus types showed convergence to a similar range of compounds and proportions suggesting that the differences between gall types were a function of the plant environment. The
Table 7. Comparison of polyacetylene level and composition of normal plant organs, transformed cultures, and galls of *B. alba* and *B. pilosa*. Values are the average of at least five different samples. Callus values were averaged over one year.

<table>
<thead>
<tr>
<th>Tissue/Species</th>
<th>Total product (mg/g d.w.)</th>
<th>Polyacetylene Composition (wt % of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PHT</td>
<td>PDE-OAc</td>
</tr>
<tr>
<td><strong>E. alba</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaf</td>
<td>4.6</td>
<td>98.3</td>
</tr>
<tr>
<td>Stem</td>
<td>2.3</td>
<td>54.2</td>
</tr>
<tr>
<td>Root</td>
<td>8.7</td>
<td>5.7</td>
</tr>
<tr>
<td>Oct⁺ stem gall</td>
<td>0.24</td>
<td>7.4</td>
</tr>
<tr>
<td>Nop⁺ stem gall</td>
<td>0.41</td>
<td>7.1</td>
</tr>
<tr>
<td>Oct⁺ callus</td>
<td>0.30</td>
<td>-</td>
</tr>
<tr>
<td>Nop⁺ callus</td>
<td>0.25</td>
<td>-</td>
</tr>
<tr>
<td>Oct⁺-nop⁺ callus</td>
<td>0.26</td>
<td>-</td>
</tr>
<tr>
<td>Normal callus</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
### B. pilosa

<table>
<thead>
<tr>
<th></th>
<th>Leaf</th>
<th>Stem</th>
<th>Root</th>
<th>Nop(^+) callus</th>
<th>Normal callus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>0.1</td>
<td>3.5</td>
<td>0.08</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- 43.2</td>
<td>- 50.5</td>
<td>- 77.8</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- 56.7</td>
<td>- 0.4</td>
<td>- 4.3</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- t</td>
<td>- 40.1</td>
<td>11.8</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- 0.4</td>
<td>- 6.1</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\(a\) Includes ETE-OAc (A) and (B).  
\(b\) Double transformant.

Symbols, abbreviations: -, not detectable; t, trace; d.w., dry weight.
absence of PDE in cultures of nopaline galls was the most striking change although PHT was also absent from subcultured callus. Callus cultures characteristically had higher percentages of PDE-OAc, compared to ETE-OAc, than any of the plant organs. No acetylenes were detected in normal callus of either species.

*Bidens pilosa* had a simpler, more restricted range of compounds than *B. alba*; PHT and PDE were both absent from plants and cultures. Nopaline tissue was generally similar to roots, however EDE-OAc was absent from transformed callus and ETE-al was found only in callus. As in the other tumour cultures, the ratio of PDE-OAc to ETE-OAc was larger than in plant organs.

Two features of Table 7 are notable: 1) the appearance in tumour cultures of compounds not found in either normal plant material or in galls. 2) The absence of compounds from cultured tumour tissue which were found in the galls. The accumulation of metabolic intermediates or the occurrence of new compounds in plant tissue cultures is not unusual according to Staba (58) and there are many reports describing such results (e.g. 7, 20, 21, 59), including one for polyacetylenes in *Centaurea ruthenica* (29) by Jente. Although the compounds found by Jente were present in very low levels (approximately 0.1% of the levels found in her plants) there seemed to be a completely different set of compounds in callus as compared to the plants. The compounds were shown to be biogenetically related, however. It is interesting that Jente's cultures only produced acetylenes under light, or alternating light and dark,
but not in dark. *B. alba* cultures showed a similar pattern to that of roots described later; illumination decreased the level of acetylenes by at least 60% (results not shown).

The question of what factors might account for novel compounds has been discussed by Böhm (7) who suggests that the changed localization of substrate and enzyme is important and also the formation of new compounds from accumulated intermediates. Bu'Lock (13) states that secondary product enzymes generally have low specificities and modify a variety of substrates; the final product produced depending on the type of substrate available. Other important factors, according to Bu'Lock, are the physiological history and conditions of culture. Which of these factors is relevant to tumour callus is not clear; certainly the physiological and nutritional status of the callus must be different. One factor would be the changed hormonal level of tumour tissue as compared to normal tissue. Work described later with exogenously supplied hormones suggests that at least part of the difference, namely the relative proportions of PDE-OAc and ETE-OAc, can be accounted for by increased cytokinin levels in callus. There is, however, no data on the actual hormone levels in *Bidens* callus to confirm this suggestion.

The possibility that intermediates might accumulate can be evaluated if the biosynthetic pathways for the compounds are known. A biosynthetic scheme for the polyacetylenes which occur in *B. alba* is shown in Fig. 25. This information was compiled from Bohlmann et al. (5). Jente and Richter (30) have isolated labelled PHT (VIII) from *Coreopsis lanceolata* fed with
Fig. 25. Biosynthetic pathways leading to polyacetylenes occurring in Bidens alba. Source: Bohlmann, Burkhardt, and Zdero: Naturally occurring acetylenes, 1973.
labelled crepenynic acid (XX) and oleic acid (XVIII), confirming this part of the pathway. Assuming the essential correctness of Fig. 25, it is clear that nearly all of the compounds found in the studies with *B. alba* were terminal products which only undergo modification at the terminal methyl group; oxidation to the alcohol or aldehyde or acetylation. An alternative biosynthetic route to the aromatic acetylenes has been suggested by Sorensen (ref. 9, Chap. I) in which compound (XI) is the precursor for cyclization. Other than this suggestion, there is no known or postulated interconversions of any of these compounds except the compound which was tentatively designated EDE-0Ac. All of the acetylenes positively identified were C\textsubscript{13} compounds or derivatives, thus when a compound was isolated which had the endiynene chromophore spectrum, it was assumed that it would have a C\textsubscript{13} chain length, before any terminal modifications. In fact, however, although a C\textsubscript{10} acetylene with this chromophore is well known as matricaria ester, I have been unable to find a record of a C\textsubscript{13} acetylene with this structure. A possibility is that the compound is an intermediate between compounds XXIV and XXV. If this scheme is correct then EDE-0Ac could be the C\textsubscript{14} precursor for (I), (XVI), and (XI). The low levels found in tissues and the apparent instability to GC/MS analysis did not allow further characterization of the compound.

The presence of PHT and PDE in stems and stem galls, including the basal callus of plantlets, suggests their synthesis is not tightly linked to a structure or signal that only occurs in one type of differentiated organ; as, for example,
seems to be the case with many seed storage lipids. The report by Bohlmann et al. (5) that PHT can be found in the roots of several species of Coreopsis (a genus closely related to Bidens) and one other species of Bidens, Bidens cernua, supports this view. However, extracts of roots of Coreopsis verticillata and B. cernua growing locally showed no indication of PHT, although both are reported to contain it (5). Variability in expression of aromatic acetylenes synthesized by a different scheme from that shown in Fig. 25 has been reported for Artemisia capillaris. Plants growing by the seaside in Japan contain capillen and cappillin only in the roots, but plants growing along riverbanks contain both compounds in roots and leaves (65). The lack of PHT and PDE in the cultured tumour tissue suggests that the factors common to stems and leaves are not maintained in culture, although they apparently are transmitted from stems to the basal callus of unrooted plantlets, as shown in the experiment described on p. 118. Thus the problem of expression of these compounds seems not to be strongly linked to morphological differentiation. However, it is difficult to imagine that related plants would have such different hormone distributions that the root of one would be similar to the leaf and stems of another. The implication, then, seems to be that a combination of factors, or a balance between hormones, environmental, and nutritional conditions is responsible.

Effect of transformation on Bidens cynapiifolia

To test for the possibility that infection by A. tume- faciens might induce polyacetylene synthesis in normally
non-producing tissues, *Bidens cynapiifolia* plants were inoculated with A208 (nop\(^+\)) bacteria. Large galls formed on the stems three months after infection and these were devoid of UV-detectable acetylenes, as were the stems and leaves of the plant. Although it is very unlikely that genes carried by plasmid T-DNA would specifically cause synthesis of polyacetylenes, as for opines, nevertheless this simple experiment could test for this possibility. The negative result supports the indirect involvement of transformation in acetylene synthesis in tumour tissue.

**Transformed Plantlets**

The availability of rooting and non-rooting plantlets on the same medium made it possible to obtain information on whether roots were a factor in polyacetylene synthesis in leaves, especially PHT formation.

Plantlets were diminutive but well-formed with small leaves 5-10 mm long and 2-3 mm wide. Several shoots were easily grown in a standard petri plate. Table 8 shows the results of a number of sample analyses. Each sample was composed of the leaves from several plantlets. Formation of PHT continued in unrooted plants, although the composition was somewhat different from that of normal leaves; there was a characteristic increase in level of the PHT alcohol. Unrooted plants were perhaps most similar to seed in composition, except that the proportions of PHT-ol and PHT-OAc were reversed (cf. Table 2). Leaves of rooted plants were most like normal leaves. Rooted plants had appreciably more PHT than unrooted plants suggesting that roots may have pro-
Table 8. Comparison of polyacetylenes in leaves of rooted and unrooted transformed plantlets. Coefficient of variation in parentheses.

<table>
<thead>
<tr>
<th>Polyacetylene composition (wt %)</th>
<th>Total product (mg/g d.w.)</th>
<th>( a_n )</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHT</td>
<td>PHT-OAc</td>
<td>PHT-ol</td>
</tr>
<tr>
<td>Rooting</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\( a_n \) Number of samples analyzed.

vided some factor which either promoted an increase in synthesis or decreased destruction of PHT. A shielding effect from increased chloroplast pigments may have reduced destruction by light; rooted shoots were markedly darker green than non-rooted shoots. Chlorophyll, however, is unlikely to be essential for protection of acetylenes; the white petals of ray flowers contain levels of PHT about 40% of those of leaves (Table 2).

Rootless shoots were grown in liquid culture in dark and in light to determine if light was necessary for PHT formation. These results are shown in Table 9. Due to the lack of expansion of leaf lamina grown in dark, it was necessary to harvest stems as well as leaves and petioles. This explains the significant differences in composition between these cultures and the agar-grown shoots. Light-grown cultures contained less than 20% of the amount of product of those grown in dark. The main difference in composition was the high proportion of PDE in illuminated cultures, which was nearly twice as high as in dark cultures. Dark cultures were
Table 9. Comparison of polyacetylenes in light- or dark-grown transformed plantlets. Coefficient of variation in parentheses. Light-grown plants received 6,000 lux continuous light.

<table>
<thead>
<tr>
<th>Culture type</th>
<th>Product composition (wt %)</th>
<th>Total product (mg/g d.w.)</th>
<th>b&lt;sub&gt;N&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PHT</td>
<td>PDE</td>
<td>ETE-OAc</td>
</tr>
<tr>
<td>Dark-grown</td>
<td>51.8</td>
<td>40.8</td>
<td>7.4</td>
</tr>
<tr>
<td>Light-grown</td>
<td>19.8</td>
<td>79.0</td>
<td>0.6</td>
</tr>
</tbody>
</table>

aIncludes PHT, PHT-OAc, and PHT-ol. bNumber of samples analyzed.

similar in level and composition to seedling stems (cf. Table 2). Since these were subcultures of dark-grown shoots, the amount of carry-over of compounds from the green cultures used as a source should have been small. The presence of PHT and PDE in etiolated shoots indicated, then, that chloroplasts and light are not required for their synthesis. In a general way this agrees with results for *Crepis rubra*; chopped seeds did not require light for formation of crepenynic acid (25). In contrast, chloroplast involvement is reported for epoxide acetylenes in safflower (27) and in a cell-free homogenate of *Chrysanthemum flosculosum* for production of a bicyclic acetylene (6).

Hagimori et al. (24), in a similar type of study, used differentiated shoots and callus, either of which could be grown to be green or colourless, and found that chloroplasts were not essential for the synthesis of digitoxin in *Digitalis purpurea*. 
Effect of Kinetin on Plantlets

The effect of medium supplemented with kinetin on the acetylene content of both leaves and the basal callus of illuminated transformed plantlets is shown in Fig. 26. A surprising result was that leaves were much more sensitive to the effect of kinetin, especially in level of product, than was callus. The two tissues seemed to be almost completely independent of each other in respect to their acetylenes; composition differed sharply and was consistent for each part across the range of kinetin tested. The results suggested that polyacetylenes are not transported from leaf to callus. In situ synthesis of acetylenic acids has been shown in sweet quandong (14) and in other plants (27).

Increasing kinetin generally resulted in an increasing proportion of PDE-OAc in leaves, although the proportion was less than one-half that of the callus. Significant amounts of ETE-OAc were only present in leaves when auxin was provided with kinetin. The levels of product in the callus may have been reduced by light destruction; callus contained only small amounts (visual estimate) of chlorophyll. The effect of kinetin on total product in leaves is difficult to rationalize; as shown later, root cultures showed increased product over the same range of kinetin tested in shoots. When 0.1 mg/l NAA was included along with kinetin, leaf product dropped sharply but in callus the level was stable. The joint effect of auxin and kinetin was not further investigated but it appeared there may have been interaction between the two hormones, affecting product level as well as composition in
**Fig. 26.** Effect of kinetin on polyacetylenes in shoots and callus of transformed plantlets. PDE-OAc, PHT, and ETE-OAc include all forms of these chromophores. C is callus, L is leaf material. Bars represent ± 1 standard deviation.
leaves.

There is evidence that factors transferred from roots to leaves in the transpiration stream regulate the rate of growth and photosynthetic activity in leaves (17). Other studies indicate that cytokinins are produced in the roots and provide a source for leaves with the stems possibly serving as a reservoir (18,57). Exogenous application of benzyladenine can compensate for chlorophyll reduction resulting from partial root excision (16). The stimulatory effect of rooting on greening and product seemed to agree with a cytokinin effect; however, kinetin in the medium did not produce the same effect. The form or type of cytokinin supplied may be critical.

Aspects of Crown Gall Physiology

Production of secondary compounds in crown gall cultures (other than the characteristic opines) at levels significantly higher than for normal tissue cultures is unlikely to be general (e.g., coloured products would certainly not have gone unnoticed), but the results in this chapter suggest that polyacetylene synthesis may be peculiarly affected by transformation in at least two closely related species of Bidens. Although the net effects of transformation on cells are by no means entirely clear some of the reported changes may bear on secondary product synthesis.

Increased ion-transport capabilities or altered membrane permeability, especially to $K^+$, has been suggested to explain results showing ion-activation by high salt medium of biosynthetic pathways (11,63). Later work on the uptake of quini-
crine showed that normal cell membrane permeability was unchanged by raising the level of salts (64). Lentz et al. (40) confirmed the difference in internal levels of $K^+$ but found that regulation of uptake was different in habituated normal cells and tumour cells. Normal cells increased the rate of $K^+$ uptake when internal levels were low but tumour cells did not. The levels of $K^+$, $Mg^{++}$, and $Ca^{++}$ were all found to be higher in tumour cells of potato discs than in normal cells. Levels of $K^+$ and $Mg^{++}$ were unaffected by 2,4-DNP, indicating that active transport was not involved. Radosevich and Galsky concluded that increased concentrations of certain cations may be a specific property of crown gall tumours (51).

There is evidence that membrane permeability may control the quantity of anthocyanins in rose and red cabbage, and possibly the qualitative composition of lignins in poplar stems (52). Krauspe (36) has pointed out that changes in permeability also occur temporarily after wounding and Braun has suggested that transformation results in a persistent activation of the wound response (10).

Studies of membrane lipids were reported by Phillips and Butcher (49) who found that the overall pattern of phospholipids was similar for normal and tumour callus cultures but tumour cells contained more phosphatidylcholine and less phosphatidic acid and phosphatidylinositol than normal cells. Cockerham et al. (19) evaluated the possibility that membrane changes were due to secondary differences associated with the rapid growth of transformed cells. Working with *Vinca rosea* they found that fully transformed cells had less than one-half
the amount of phospholipids of normal cells. Partially transformed cells were similar to normal cells and when stimulated to grow at a rate comparable to fully transformed cells the level of phospholipid did not change appreciably. The authors concluded that membrane alteration is a direct result of transformation and not a consequence of growth rate. Para-finic hydrocarbons of tobacco were compared in cultured teratoma tumour tissue, habituated callus and intact plants (61). Tumour cells were found to be qualitatively identical to seedlings in composition.

A third characteristic feature of most types of crown gall tissue is the capacity for growth on medium lacking auxins and cytokinins. Evidence exists that, for at least some plants, transformation results in higher than normal auxin levels in tumour tissue (1,10,22). However, other studies show that this is not generally true (47,62). The mechanism for alteration of hormone level, whether by directly coded T-DNA synthetic genes or by modulation of plant hormone biosynthesis, is still not known (54).

The connection between these three areas is not clear at present and more information is needed on changes in permeability, membrane composition of transformed cells, and hormone levels in normal and transformed cells at similar growth stages. Resolving the ways in which tumour cells differ from normal cells would certainly help to understand why transformed B. alba cells produce polyacetylenes and normal callus cells do not.
SUMMARY

Crown galls induced on _B. alba_ by two different strains of _Agrobacterium tumefaciens_ were cultured and investigated for polyacetylene production and composition. An effort to use a visual difference in some calluses as guide for selecting those with higher product levels resulted in a line with a higher content but an uncertain relation to the selection criterion. Callus grown over several years showed a continuing ability to synthesize polyacetylenes at levels up to 20% of that in cultured roots. However, levels fluctuated widely over time, and composition showed a decrease in the proportion of phenyldiynene acetate (PDE-OAc). Octopine, nopaline, and double transformant callus, all showed similarities in composition characterized by high levels of PDE-OAc and the presence of minor compounds not found in plant tissues. Work with transformed plantlets showed that roots and light were unnecessary for synthesis of leaf and stem acetylene compounds. Leaves of plantlets generally showed inhibition of phenylheptatriyne synthesis by kinetin in the medium, but callus associated with the plantlets showed an increase. Crown gall callus of both _B. alba_ and _B. pilosa_ produced appreciable levels of polyacetylenes over time and accumulated biogenetically related acetylenes.
LITERATURE CITED


CHAPTER VI

STUDIES ON ROOT CULTURES OF BIDENS ALBA
INTRODUCTION

The variations in medium components, additives and environmental conditions that were tried in unsuccessful attempts to obtain cultures of *Bidens alba* that produced appreciable levels of polyacetylenes have been described in Chapter III. Rather than continue to experiment with aging callus which produced no acetylenes it seemed more promising to work with a system which already produced acetylenes and to determine how the level and composition was affected by factors known to enhance levels of secondary products or lipids in other systems. If substantially increased product could be achieved then these conditions should serve as a better starting point for obtaining polyacetylenes in callus and suspension cultures. In addition, information from root cultures could help in understanding the factors controlling biosynthetic patterns in the plant. The relevance of work with excised roots to the physiology of the intact plant has been affirmed by Street (5).

Excised root cultures have been used in secondary product studies to determine the site of biosynthesis of compounds, examples being work demonstrating the production of atropine in roots of *Atropa belladonna* (45), the biosynthesis of nicotine in root tips of tobacco, and anabasine in a separate region of the root (17,35). Other studies have used root cultures to show an increase in hyoscyamine when protein synthesis was inhibited by puromycin (11), to determine the effect of precursors and inhibitors on levels of tomatine in excised
tomato roots (32), and to investigate the biosynthesis of tropane alkaloids in *Datura metel* (26). Root cultures of *Dioscorea deltoidea* produced only trace amounts of diosgenin while callus derived from roots produced appreciable amounts (21). There is a report of a patent for production of saponins from ginseng root cultures (20). In general, however, there seems to have been surprisingly little use made of root cultures for either biosynthetic studies or as sources for the large number of useful compounds which occur in roots.

Excised root cultures of *B. alba* provided suitable experimental material for the following reasons. 1) The roots could be subcultured repeatedly on a defined medium, thus providing genetic continuity. 2) The physical and chemical environment could be easily controlled and growth under both light and dark conditions could be studied. 3) Growth, with an appropriate hormone level, was rapid, allowing experiments to be conducted on material which had doubled several times, so that only a small percentage of the final culture was from material grown under the standard conditions of the inoculum source. 4) Cultured roots lacked secondary thickening, allowing much easier homogenization for extraction and eliminating a variable which could affect product synthesis. 5) Cultured roots produced a relatively stable pattern of polyacetylenes at levels and composition comparable to intact roots.

The studies reported here outline the characteristics of *B. alba* root cultures and the effect of various treatments on polyacetylene level and composition. The overall objective was to develop a culture system which would facilitate work
on factors which determine the pattern of compounds found in
the intact plant. The results show the value of this currently
rather little used technique of tissue culture (defined in
the broad sense) for investigating the control of polyacetylene
synthesis.
MATERIALS AND METHODS

Culture Methods

Root cultures were established from single roots arising from either sterile leaf or stem explants of *B. alba* grown on SH agar medium which contained 4.0 mg/l NAA. Stock cultures were stored on SH agar plates without hormones and were kept in the dark at 25°C. Roots were stored in this way for four to six months between transfers. Under these conditions growth was quite slow and when a tip segment was placed in liquid shake culture a lag phase of approximately seven days occurred before vigorous growth resumed. Roots were also stored in liquid medium without hormones. A single segment was used to start a liquid culture, which was then used to start both experimental and continuing stock cultures. The latter served as the source for the next experimental and stock culture, and so on. Transfers were made by pulling out, with forceps, a tuft of young roots from the parent clump and inoculating the experimental or stock flask. Equality of explants was determined visually. Although this method was not precise, typical sets of explants had a fresh weight of 0.2 g and a coefficient of variation of 15%. The variation in weights at time of harvest compared favourably with studies using single roots (17,33a). If environmental conditions were being evaluated, and the medium was identical for all conditions, then inoculated flasks were randomized before being assigned to an experimental group. Roots were then grown for
11 days in 125 ml Erlenmeyer flasks containing 75 ml of liquid SH medium with 0.5 mg/l NAA. Medium pH was 5.7, adjusted with KOH or HCl, and flasks were stoppered with foam plugs (Identiplug, Western Scientific) covered with two layers of paper towel and sealed at the neck with a rubber band. Incubation was at 25°C in dark on a rotary shaker at 125 rpm. Medium was autoclaved for 20 minutes at 15 lb/sq. in. These are the standard conditions and any variations are noted.

Transformed Roots

Transformed roots were cultured from both A208 and A277 callus lines, which rooted as described in Chapter III. Both lines were tested for the presence of opines and found to contain the expected types. However, the A277 line after a few months in culture was negative for octopine. The A208 line remained positive for nopaline. Cultures were grown as for normal roots.

Harvesting

Cultures were harvested by pouring off the medium and gently removing the clump of roots with forceps. The clump was patted dry between paper towels, weighed and a representative one gram sample used for extraction and analysis. The remaining tissue was dried between paper towels for 24 hours at 100°C, allowed to cool to room temperature and weighed. Sample and total dry weight (per flask) were calculated from the ratio of fresh to dry weight. "Growth" as used here means the cumulative dry weight per flask versus time.
Analysis

Roots were extracted and prepared for HPLC analysis and quantitation as described in Chapter 2.

Average doubling time ($t_d$) was calculated with the following equation: $t_d = t / \left( \log(w_1/w_0) / \log(2) \right)$, where $t$ is the time interval for growth and $w_0$ and $w_1$ are the starting and ending weights respectively.
RESULTS AND DISCUSSION

Comparison of Polyacetylene Composition of Cultured and Intact Roots

Table 10 shows a comparison of polyacetylene level and composition for *E. alba* seedling roots and dark-grown cultured roots. Intact roots contained small amounts of PHT and PDE which were not found in cultured roots. The small amounts of both compounds may have been contributed by the crown of the root; plants were harvested by cutting off the roots at ground level and the upper portion of the root clump included some stem material. The principal difference in composition between the two types of roots was the higher percentage of PDE-OAc in intact roots and a corresponding lower percentage of ETE-OAc. Higher proportions of PDE-OAc were found in cultured roots when either NAA or kinetin levels were higher than the standard 0.5 mg/l NAA (see later), suggesting that intact roots may contain levels of hormones higher than those used for the cultured roots. The differing environment and the presence of the shoot must prevent the excessive callusing found in cultured roots at higher hormone levels.

The overall similarity of the two types of roots seemed to justify use of cultured roots as a representative system for the study of polyacetylenes. The next step was to see how various factors affected the level and composition of acetylenes in this material.
Table 10. Polyacetylene composition of seedling and cultured roots of *B. alba* grown under standard conditions.

<table>
<thead>
<tr>
<th>Root type</th>
<th>Polyacetylene composition (wt % of total)</th>
<th>Total product (mg/g d.w.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PHT</td>
<td>PDE-0Ac</td>
</tr>
<tr>
<td>Seedling</td>
<td>5.7</td>
<td>58.3</td>
</tr>
<tr>
<td>Cultured</td>
<td>-</td>
<td>38.7</td>
</tr>
</tbody>
</table>

Symbol: -, not detectable.
**Effect of Temperature on Roots**

Figure 27A shows the dependence of polyacetylene level and final dry weight per flask on incubation temperature. With increasing temperature over the range 18-33°C product level decreased at very nearly a constant rate. Growth also showed a nearly linear response to temperature; increasing up to 30°C but decreasing sharply at 33°C. Cultures grown at 15°C and 35°C showed no appreciable growth at either temperature over the culture period of 11 days and are not shown. Tomato roots are also reported to grow best at 30°C with a sharp decline in growth at higher temperatures, and other species show a similar sensitivity (40).

Figure 27B shows the effect of temperature on composition. Only the major component, PDE-OAc is shown, since most of the remaining product was ETE-OAc, which changed reciprocally to PDE-OAc. Low temperatures did not significantly affect PDE-OAc and high temperatures (30-33°C) resulted in a maximum decrease of about 10% in this component. It might be supposed that, at higher than normal temperatures, the composition of cultures would tend to resemble those grown longer at lower temperatures; however, as Fig. 29B shows, this was not the case. In older cultures grown at 25°C the proportion of PDE-OAc increases. Thus the small decline appears to be caused by other factors. The overall composition was relatively stable over changes in temperature while product levels changed sharply.

A decline in alkaloid formation with increasing temperature has been reported by Courtois and Guern (8) for Catharanthus roseus suspension cultures. Their explanation for the effect
Fig. 27. Effect of growth temperature on cultured B. alba roots. A) Effect on polyacetylene level and growth. B) Effect on polyacetylene composition. Each point is the average for three flasks. Bars show ± 1 SD.
was that the ratio between biosynthesis and degradation was modified by temperature. Given the small change in composition over the temperature range tested, and the large changes in product level, this explanation seems unlikely for *B. alba* roots, unless degradation was either nonselective or nearly equally balanced for the different polyacetylenes.

One possible explanation of the inverse relation between product level and temperature could be that substrate availability is increased at lower temperatures. Many plants show increases in fatty acid levels (30) and unsaturation as temperature decreases (10,18,31). However, there are exceptions to this general rule (12,42) and in some plants genetic control can override physical factors (19). Nevertheless, if cultured *B. alba* roots are typical, then higher levels of linoleic acid resulting from cold treatment, might be diverted to product synthesis.

The effect of temperature on lipid composition is thought to act either directly on the desaturase system or through increased levels of dissolved oxygen at lower temperatures (15, 16). Since oxygen is required for fatty acid desaturation (9), the same argument might also apply to the formation of the triple bond of polyacetylenes; there is evidence that oxygen is required for this reaction as well (4,14). It would be interesting to know the effect of different oxygen levels on polyacetylene synthesis in cultured roots.

Alternatively, the enzymes for triple bond formation may be inhibited or inactivated at temperatures not inhibitory to growth. Haigh et al. (14) found that the conversion of oleate
to crepenynate in chopped *Crepis rubra* seeds was completely inhibited at 32 °C. However, the effect of a range of temperatures on oleate conversion was not reported.

Martin (28) has reported that temperatures below 25 °C reduced nitrogen utilization in *Ipomoea* to a greater extent than sucrose utilization. If a similar effect occurs in *Bidens* then it could account, at least partly, for the levels of polyacetylene. A later experiment (see below) shows that increased sugar to nitrate ratios favoured increased product formation.

Martin (28) notes that "little if anything seems to have been reported on temperature optima for growth versus those for metabolite production." The data reported here are the first for polyacetylenes. Further work needs to be done to elucidate the mechanism causing product level and growth to show opposite trends.

**Effect of pH on Roots**

Results from growing roots in unbuffered medium are shown in Fig. 28. The pH of the medium after 10 days growth of all except the roots grown at pH 3.3 and 9.0 was 5.5 ± 0.1. Roots cultured at the lowest and highest pH showed very little growth or polyacetylene content. Composition (not shown) was similar for all pH values except the two extremes, which showed increased PDE-OAc. Both maximum growth and product coincided with the standard medium pH of 5.7. The range for growth of *B. alba* roots, as well as the capacity for adjusting pH, was similar to reports for cultures of other plants (6, p.46). Growth and product level generally declined as the starting
Fig. 28. Effect of pH on growth and polyacetylene content of cultured B. alba roots. Cultures were grown for ten days under standard conditions. Results are average values for three flasks for each point. Bars show ±1 SD; dry weight SD values were similar to product values.
pH was lower or higher than 5.7. Differences in the amount of growth may be due to the time required by the roots to change the pH of the medium to an equilibrium level permitting rapid growth.

Effect of Culture Age on Roots

Figure 29A shows the relationship between age of culture and the amount of tissue formed, the polyacetylene content per gram of tissue and per flask, and average doubling time. Growth followed a pattern typical of many types of cultures and showed the following phases: lag phase (days 0-2), exponential or log phase (days 2-14) and stationary phase (from day 14). The polyacetylene level of root cultures, per gram of tissue, decreased during the lag phase and reached a minimum during the period of most rapid growth (days 4-6), increased during mid-to-late log phase (days 8-14) and declined again as growth ceased. Total acetylenes per flask changed little during early log phase growth but rapidly increased as growth began to slow and decreased as growth began to stop, suggesting that the compounds were being metabolized or excreted into the medium. Extraction of the medium in several experiments, however, failed to show significant amounts of acetylenes; levels were typically less than 1 ug per ml of medium.

Figure 29B shows the change in composition as root cultures age; only the PDE-OAc component is shown. Low levels of PDE-OAc were correlated with the period of most rapid growth and nearly doubled as growth ceased. A much more pronounced change in composition of tobacco roots has been reported by Solt et al. (35). In the early part of the culture period anabasine and
Fig. 29. Effect of culture age on growth and polyacetylenes of B. alba root cultures. A) Total product per gram root and per flask, doubling time, and growth. B) Composition of polyacetylenes. Each point is the average for three flasks. Bars show ± 1 SD. Dry weight scale is logarithmic.
nicotine were present at a ratio of 2:1, but toward the end of the passage the ratio changed to 4:1. The authors concluded that the two products were synthesized in separate portions of the root. Analysis of separate regions of Bidens roots, however, showed little difference in composition (see below), but large differences in amount of product.

Level of secondary product formation in cell cultures is sometimes strongly dependent on the growth stage of the culture (7) or the rate at which the culture grows (34). Product synthesis is frequently associated with late log and stationary phase, especially in microorganisms (25,27). However, studies of alkaloid production in roots of tobacco (17) and tomato (32) show a strong correlation between growth and amount of product, characteristic of compounds synthesized in young tissue. Other evidence would make the late synthesis of acetylenes unlikely in B. alba root cultures: resin canals, the structures in which acetylenes accumulate in roots (36, 43), form in B. alba root tips ahead of the earliest visible xylem tissue (data not presented) and the polyacetylene level in the youngest growth of a culture is higher than in the older core portion (see later). The minimum level of polyacetylenes occurred during the period of most rapid growth, suggesting either that synthesis did not keep pace with growth or that primary metabolites were unavailable for secondary product synthesis. Work with fungi has indicated that carbon is shunted to lipid production as protein and nucleic acid syntheses diminish (22) and that a rapid increase in lipids following nitrogen limitation in fungi reflects a constant rate of lipid
synthesis as cell proliferation slows. The rapid rise in polyacetylenes that follows the period of most rapid growth suggests that a similar effect occurs in root cultures. Evidence supporting this effect was provided in the next experiment.

The late drop in average tissue product level was somewhat unexpected and when combined with the decline in total product per flask indicated that breakdown of the polyacetylenes was occurring. Sorensen (37) notes that in those cases where uptake of labelled polyacetylenes by plants has been studied, they are rapidly metabolized with a half-life of one to two days. Evidently root polyacetylenes are not inert end products, passively accumulated; rather, they seem to be quite sensitive to the continual changes which occur under batch culture conditions.

Effect of Sucrose/Nitrate Ratios on Roots

The effect of changes in the sucrose and potassium nitrate levels on polyacetylene production and composition is shown in Table 11. The normal level of sucrose and nitrate in SH medium is 3% and 0.25% respectively with nitrate accounting for two-thirds of medium nitrogen. Growth and product increased as sucrose increased from 1.2% to 6% but both declined sharply at higher concentrations of sucrose. Composition showed a more complex pattern: PDE-OAc decreased as sucrose increased and increased as nitrate level decreased. Doubling the sucrose level from 3% to 6% resulted in a 25% increase in product; decreasing nitrate level by ½ and ¾ increased it by 37% and 25%. Similar effects on fat accumulation in fungi have been
Table 11. Effect of changes in carbohydrate/nitrate ratios on polyacetylene levels of B. alba root cultures. Results are averages for three flasks of each medium type grown under standard conditions.

<table>
<thead>
<tr>
<th>Medium type</th>
<th>Root dry wt/ flask (g)</th>
<th>PDE-OAc&lt;sup&gt;b&lt;/sup&gt; wt. % of total</th>
<th>Total product&lt;sup&gt;b&lt;/sup&gt; (mg/g d.w.) (CV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25 1.5</td>
<td>0.19</td>
<td>30.9&lt;sub&gt;ab&lt;/sub&gt;</td>
<td>2.85&lt;sub&gt;b&lt;/sub&gt; (26)</td>
</tr>
<tr>
<td>0.25 3.0</td>
<td>0.31</td>
<td>36.1&lt;sub&gt;bc&lt;/sub&gt;</td>
<td>3.12&lt;sub&gt;b&lt;/sub&gt; (10)</td>
</tr>
<tr>
<td>0.25 6.0</td>
<td>0.33</td>
<td>30.6&lt;sub&gt;ab&lt;/sub&gt;</td>
<td>3.91&lt;sub&gt;a&lt;/sub&gt; (12)</td>
</tr>
<tr>
<td>0.25 12.0</td>
<td>0.26</td>
<td>28.3&lt;sub&gt;a&lt;/sub&gt;</td>
<td>0.56&lt;sub&gt;c&lt;/sub&gt; (27)</td>
</tr>
<tr>
<td>0.25 16.0</td>
<td>0.12</td>
<td>24.2&lt;sub&gt;a&lt;/sub&gt;</td>
<td>0.70&lt;sub&gt;c&lt;/sub&gt; (21)</td>
</tr>
<tr>
<td>0.125 3.0</td>
<td>0.34</td>
<td>41.2&lt;sub&gt;c&lt;/sub&gt;</td>
<td>3.89&lt;sub&gt;a&lt;/sub&gt; (14)</td>
</tr>
<tr>
<td>0.063 3.0</td>
<td>0.30</td>
<td>37.9&lt;sub&gt;c&lt;/sub&gt;</td>
<td>4.28&lt;sub&gt;a&lt;/sub&gt; (4)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Standard SH medium.

<sup>b</sup> Means within a column followed by the same letter are not significantly different at the 5% level by Duncan's multiple range test.
found. The C:N ratio is reported to be the most important nutritional parameter for lipid production by fungi (44). Although it also appears to be important in root cultures, the effect of other components should be investigated, e.g., the effect of different levels of myo-inositol and phosphate and ammonium versus nitrate. Hagimori et al. (13) found that reduction of basic nitrogen by one-third increased the yield of digitoxin from Digitalis purpurea without suppressing growth. The levels of sucrose optimal for polyacetylene synthesis in this experiment (6%) were considerably lower than the 40% level of glucose reported to be optimal for many fungi (44).

Zenk (46) has noted that in many cases increasing sucrose level above the normal 2-3% improves secondary product yield. The concentration of sucrose was a key factor in obtaining very high yields of rosmarinic acid from Coleus cultures, 7% sucrose being optimal (47). Similar results have been reported for shikonin production from ginseng (41), anthraquinones from Cinchona pubescens (29), phenolics from Paul's scarlet rose (24), and alkaloids and polyphenols from Catharanthus roseus (25). Although not all secondary products are increased by higher levels of sucrose (41), the number of such cases suggests that sugar level is one of the factors that should be evaluated early in any attempt to enhance product yield.
Effect of Kinetin on Roots

The results of growing *B. alba* roots in medium supplemented with kinetin are shown in Fig. 30 and Table 12. No auxin was added to these cultures. Figure 30 shows that low levels of kinetin increased growth moderately, but at 0.135 mg/l growth increased sharply and additional kinetin had little effect on growth; however, total product increased over the expected level (from Fig. 29) of 2.5 mg/g for 11 day cultures, to a maximum of over 5.0 mg/l at the highest level of kinetin (1.2 mg/l). There was also a regular effect on composition as shown in Table 12. The weight percent of ETE-ol, ETE and the putative EDE-OAc were changed relatively little by the level of kinetin. The percentage of PDE-OAc and ETE-OAc, however, showed regular and reciprocal changes with kinetin level; the former increased by 14.4% from the lowest to the highest kinetin level while the ETE-OAc declined by 11.5%. The expected percentage of PDE-OAc for 11 day cultures grown with 0.5 mg/l NAA would be 36-40% (Fig. 29) which agrees with the level of total polyacetylenes in cultures grown at kinetin levels of 0.045 and 0.135 mg/l, i.e., at the levels marking the transition to increased growth and product. Roots grown at the highest level of kinetin were thicker and shorter than those grown at lower levels. In this respect they resembled roots grown in light, transformed roots, and older roots; all of which had higher levels of PDE-OAc. These points suggest that the level of cytokinins may be one of the factors responsible for both the thickened growth and the increased PDE-OAc level. Other, unknown factors seem to be involved in determining the level
Fig. 30. Effect of varying kinetin concentration on total polyacetylenes and growth of cultured *B. alba* roots. Each point is the average for three flasks. Bars show ± 1 SD.
Table 12. Effect of varying kinetin concentration of medium on polyacetylene composition in \textit{B. alba} roots. Roots were grown under standard conditions except for omission of NAA. Figures are the average of three flasks for each concentration. Coefficient of variation in parentheses.

<table>
<thead>
<tr>
<th>Kinetin (mg/l)</th>
<th>Polyacetylene composition (wt % of total)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PDE-OAc&lt;sub&gt;b&lt;/sub&gt;</td>
<td>EDE-OAc</td>
</tr>
<tr>
<td>0.0</td>
<td>29.9</td>
<td>14.4</td>
</tr>
<tr>
<td></td>
<td>(0.4)</td>
<td>(0.5)</td>
</tr>
<tr>
<td>0.005</td>
<td>33.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.5</td>
</tr>
<tr>
<td></td>
<td>(0.1)</td>
<td>(19.1)</td>
</tr>
<tr>
<td>0.015</td>
<td>32.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.3</td>
</tr>
<tr>
<td></td>
<td>(8.5)</td>
<td>(20.6)</td>
</tr>
<tr>
<td>0.045</td>
<td>36.3</td>
<td>12.0</td>
</tr>
<tr>
<td></td>
<td>(1.9)</td>
<td>(0.8)</td>
</tr>
<tr>
<td>0.135</td>
<td>41.3</td>
<td>11.4</td>
</tr>
<tr>
<td></td>
<td>(2.3)</td>
<td>(6.6)</td>
</tr>
<tr>
<td>0.405</td>
<td>44.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.6</td>
</tr>
<tr>
<td></td>
<td>(1.4)</td>
<td>(4.1)</td>
</tr>
<tr>
<td>1.22</td>
<td>44.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.2</td>
</tr>
<tr>
<td></td>
<td>(0.8)</td>
<td>(2.2)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Total for ETE-OAc<sub>ab</sub> (A)+(B).

<sup>b</sup>Means within a column followed by the same letter are not significantly different at the 5\% level by Duncan's multiple range test.
of product.

Butcher and Street (5) have discussed studies showing an increased percentage of dry weight for roots grown with auxin and provided evidence that this was due to increased sucrose uptake. In all the studies reported here with *B. alba*, however, increases in kinetin resulted in a decreased fresh weight to dry weight ratio and auxin in increased ratios. Whether kinetin stimulated sucrose uptake is not known but the effects were similar to those from increasing the carbon/nitrogen ratio.

**Effect of NAA on Roots**

The relationship between NAA concentration and polyacetylene level and growth is shown in Fig. 31A. Growth was nearly a linear function of NAA level except at the highest level. Product level showed a more complex relationship with higher levels of NAA clearly inhibiting product accumulation. Due to the amount of tissue formed at the higher levels of NAA, nutrient limitation could have affected the amount of product, however Fig. 29 shows that a decline in product did not take place until tissue dry weight in excess of 0.64 g/flask was formed and this level was only reached at the highest NAA level. The standard level of 0.5 g/l was near the point of maximum product.

Changes in composition are shown in Fig. 31B. Changes in the proportion of PDE-OAc were less pronounced than with kinetin but showed the same trend. In general, roots showed an increase in PDE-OAc formation as either NAA or kinetin levels increased.

It is interesting that both kinetin and NAA increased
Fig. 31. Effect of NAA concentration on cultured roots of B. alba. A) Effect on growth and product level. B) Effect on polyacetylene composition. Each point is the average for three flasks. Bars show ± 1 SD.
growth but only kinetin showed an increase in product over the level of roots grown in medium lacking hormones.

The effect of hormones on production of secondary products in tissue cultures can be critical in some cases, e.g. (29), but there seem to be no general rule for deciding which to use for any given product. Inhibition by high levels of auxin, however, is frequently reported (3,25) and this was found for polyacetylene production at levels of NAA which promoted the most rapid growth in *Bidens*.

**Effect of Light on Roots**

The effect of light on root cultures grown with kinetin or NAA is shown in Table 13. Two features were immediately apparent: 1) the product ratios of illuminated cultures were quite different from dark-grown roots; the proportion of PDE-OAc was over twice that of dark cultures. 2) Kinetin resulted in 2 to 3 times higher total product levels than NAA. Total product level was highest at the higher kinetin level, confirming the effect seen with dark-grown cultures in Fig. 30. Comparison of light-grown kinetin cultures with similar dark-grown cultures (Fig. 30) showed that light reduced the product level to about 1/3 of dark conditions. However, an even sharper decrease was seen in NAA and hormone-free cultures; total product level was less than 1/6 and 1/16 of the dark levels. Kinetin markedly enhanced product level in light-grown cultures relative to NAA and hormone-free media. This result was contrary to the effect shown on transformed shoots; kinetin up to 3.3 mg/l caused decreases in leaf product. Evidently leaf tissue responds to the same hormonal environment very differ-
Table 13. Effect of light and hormones on root growth and polyacetylene composition. Coefficient of variation in parentheses. Each combination is the average of five flasks. Illuminated roots received 1400 lux continuous light (+).

<table>
<thead>
<tr>
<th>Hormone (mg/l)</th>
<th>Light</th>
<th>Dry wt/flask (g)</th>
<th>Product composition (wt %)</th>
<th>Total product (mg/g d.w.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>PDE-OAc</td>
<td>EDE-OAc</td>
</tr>
<tr>
<td>NAA (0.5)</td>
<td>-</td>
<td>0.67 (12)</td>
<td>30.1</td>
<td>9.0</td>
</tr>
<tr>
<td>NAA (0.5)</td>
<td>+</td>
<td>0.36 (16)</td>
<td>74.3</td>
<td>7.0</td>
</tr>
<tr>
<td>None</td>
<td>+</td>
<td>0.04 (13)</td>
<td>83.5</td>
<td>3.2</td>
</tr>
<tr>
<td>Kinetin (0.1)</td>
<td>+</td>
<td>0.16 (27)</td>
<td>82.8</td>
<td>4.9</td>
</tr>
<tr>
<td>Kinetin (1.0)</td>
<td>+</td>
<td>0.21 (26)</td>
<td>70.7</td>
<td>8.0</td>
</tr>
</tbody>
</table>

aIncludes ETE and ETE-ol, the total of which amounted to less than 1% for illuminated cultures.
ently from roots; the proportion of PDE-OAc in illuminated leaves increased slightly with increasing kinetin. Light-grown roots, however, showed decreasing PDE-OAc with increasing kinetin. Changes in composition between light and dark-grown *Ruta graveolens* cultures producing $C_8$ and $C_9$ volatile oil components have also been reported (7); light-grown cultures resembled leaves in composition and dark-grown cultures were similar to roots. Changes in nutrient conditions had little effect in this system; composition of the volatile oil in cultures was chiefly determined by light. *Bidens* roots seem to be constrained by morphology to produce a particular set of acetylenes, and although the relative proportions of these may be altered, sharp discontinuities in the type of product or the form of the product were not observed.

**Effect of Dedifferentiation on Polyacetylenes of Transformed Roots**

In this experiment transformed roots (nop$^+$) were used to examine the effect of dedifferentiation on polyacetylene production. Transformed roots were similar to normal roots in types of polyacetylenes present and in the enhancement of growth by NAA; although 0.5 mg/l, as used here, was probably higher than necessary for good growth, it was used for consistency. The primary differences between the two types of roots was the level of PDE-OAc, which was about 10% higher than in normal roots. Transformed roots were used because they seemed to be more sensitive to kinetin than normal roots, showing a greater tendency to form callus in the older portion of the roots and to shed cells into the medium. Kinetin was preferred
as the hormone since earlier experiments had shown it to increase product level at the concentration necessary for dedifferentiation. The details on separation of the roots into different regions and promotion of dedifferentiation are shown in the legend of Table 14.

Two main trends were evident from the data: 1) product level decreased sharply as the organization of the culture decreased; the youngest portion of the root had the highest polyacetylene level and the oldest portion, the lowest level. Suspension culture levels were the lowest of the series, indicating that organization played a crucial role in accumulation of polyacetylenes. 2) The second trend was the change in composition of the cultures. Within the organized root EDE-OAc was similar in proportion to normal roots, however as organization was lost the proportion of EDE-OAc increased between three and six times to become the dominant acetylene compound present. Both PDE-OAc and ETE-OAc decreased in percentage. Another feature of composition in the disorganized tissue was the appearance of small amounts of PHT and the putative dientriynene acetate (DTE-OAc) which were otherwise not found in cultured roots. The change in relative percentages of the two forms of ETE-OAc was also striking; ETE-OAc(A) showed a steady increase relative to the (B) isomer. No other culture condition studied resulted in the near equality of these two isomers.

None of the effects from the parameters studied in this chapter could reasonably be extrapolated to give the type of composition found in the callused roots and suspension cultures.
Table 14. Effect of dedifferentiation on cultures of transformed roots. Callusing was promoted by growing roots in liquid medium with 1.5 mg/l kinetin for one passage and transferring the most heavily callused roots to flasks for the next passage. The free cells from the same passage were used as the inoculum for suspension cultures. Root tip was the first 2 cm of root from the tip, middle root was the next 3-4 cm, and the remainder of the root was the oldest root. Roots and suspension cultures grown under standard conditions except as noted.

<table>
<thead>
<tr>
<th>Tissue stage</th>
<th>Relative callus</th>
<th>Polyacetylene composition (wt % of total)</th>
<th>Total product (mg/g d.w.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root tip</td>
<td>0</td>
<td>PDE-OAc: 47.1, EDE-OAc: 9.2, DTE-OAc: -</td>
<td>A: 0.4, B: 4.3, 39.0, 2.72</td>
</tr>
<tr>
<td>Middle root</td>
<td>0</td>
<td>PDE-OAc: 43.7, EDE-OAc: 9.0, DTE-OAc: -</td>
<td>A: 0.6, B: 5.1, 41.6, 2.36</td>
</tr>
<tr>
<td>Oldest root</td>
<td>+</td>
<td>PDE-OAc: 45.8, EDE-OAc: 9.5, DTE-OAc: t</td>
<td>A: 0.9, B: 6.2, 37.7, 0.64</td>
</tr>
<tr>
<td>Callused root</td>
<td>++</td>
<td>PDE-OAc: 28.8, EDE-OAc: 30.2, DTE-OAc: 1.9</td>
<td>A: 3.2, B: 1.7, 13.8, 20.4, 0.49</td>
</tr>
<tr>
<td>Callused root</td>
<td>+++</td>
<td>PDE-OAc: 19.9, EDE-OAc: 55.4, DTE-OAc: 1.1</td>
<td>A: 2.8, B: 1.4, 8.2, 11.2, 0.06</td>
</tr>
<tr>
<td>Suspension culture</td>
<td></td>
<td>PDE-OAc: 31.2, EDE-OAc: 41.4, DTE-OAc: 1.3</td>
<td>A: 3.0, B: 5.4, 9.0, 8.7, 0.02</td>
</tr>
</tbody>
</table>

\(^a\) Grown with 1.5 mg/l kinetin. Symbols: 0, no visible callusing; +, ++, +++ increasing callus formation; -, no detectable compound.
The appearance of PHT especially was quite unexpected. The implication is that the organization in roots determines both level and composition and loss of organization not only changes the relative proportions of typical compounds but also allows the synthesis of compounds either normally not present or present at much lower levels.

Staba (38) has suggested that shoot or root cultures could be modified for growth in fermentors by using high levels of growth regulators to reduce their size. An example he cited was degenerated Chrysanthemum shoot cultures which were grown with 20 ppm benzyladenine. Although it was simple to derive less organized cultures from roots it did not look promising to use these for further studies, due to the low levels of product.

One problem is that if polyacetylenes are secreted into the medium in cell suspensions then they are much more liable to degradation, especially if light is present. A possible method for increasing the production of polyacetylenes under these conditions is suggested by the work of Beiderbeck (1) and others (2) who show that a suspension culture of Matricaria chamomilla tumour cells yield a marked increase in the recovery of lipophillic compounds when the cells are cultivated in a two phase system consisting of nutrient medium and synthetic triglyceride, which accumulates the product. Production of coniferyl aldehyde, in the same culture, increased 60-fold by adding activated charcoal to the medium and recovering adsorbed product from the charcoal (23). A similar system could work with suspension cultures of B. alba tumour cells; a lipophillic
phase could efficiently accumulate small amounts of non-polar polyacetylenes released into the medium. Charcoal might work in recovering acetylenic alcohols and, perhaps, some of the postulated and known acidic intermediates. Use of the method with root cultures or suspension cultures might stabilize excreted product and yield a better estimate of the amount of polyacetylenes actually produced by cultured roots.

**Stability of Root Cultures**

The final table of this chapter is a compilation of the data for control cultures which were run for each experiment, including controls for some experiments not reported here. The total time that this particular isolate was actively subcultured was five months, however the line had been maintained on agar plates as described in Materials and Methods for nearly three years prior to being actively cultured.

The results, shown in Table 15, generally showed reproducibility from run to run, however an overall drift in the data over time was clear, especially between weeks 8 and 13. When the data was grouped into two classes, weeks 3-8 and 13-20, and tested for significance using a two-tailed t-test, the means for PDE-OAc, ETE-OAc (A+B), total product, and dry weight were all significantly different ($P<0.01$) using either group as the standard. The significance of the changes in the minor components was not calculated. *Bidens* root cultures, then, showed statistically significant changes over time in all the parameters measured: composition, total product, and growth.

The cultures appeared to change between weeks 8 and 13.
Table 15. Stability of composition, polyacetylene level, and growth over time of B. alba root cultures. Figures are the average of N flasks for each age. Cultures grown under standard conditions. Origin of reisolate described in text.

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>N</th>
<th>PDE-OAc</th>
<th>EDE-OAc</th>
<th>ETE-ol</th>
<th>ETE-OAc A</th>
<th>ETE-OAc B</th>
<th>ETE</th>
<th>Total product (mg/g d.w.)</th>
<th>Dry wt/flask (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>3</td>
<td>36.1</td>
<td>8.6</td>
<td>0.7</td>
<td>5.5</td>
<td>47.3</td>
<td>1.8</td>
<td>3.4</td>
<td>0.43</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>34.3</td>
<td>9.1</td>
<td>0.6</td>
<td>2.1</td>
<td>52.1</td>
<td>1.8</td>
<td>3.4</td>
<td>0.27</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>38.8</td>
<td>6.9</td>
<td>0.3</td>
<td>4.7</td>
<td>47.6</td>
<td>1.7</td>
<td>4.9</td>
<td>0.40</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>35.7</td>
<td>8.9</td>
<td>0.7</td>
<td>3.5</td>
<td>49.5</td>
<td>1.7</td>
<td>3.2</td>
<td>0.35</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>36.1</td>
<td>9.0</td>
<td>0.8</td>
<td>6.6</td>
<td>45.3</td>
<td>2.2</td>
<td>4.5</td>
<td>0.31</td>
</tr>
<tr>
<td>13</td>
<td>3</td>
<td>41.4</td>
<td>6.7</td>
<td>0.8</td>
<td>3.3</td>
<td>45.5</td>
<td>2.3</td>
<td>1.9</td>
<td>0.63</td>
</tr>
<tr>
<td>14</td>
<td>3</td>
<td>43.4</td>
<td>3.7</td>
<td>0.9</td>
<td>2.0</td>
<td>47.9</td>
<td>2.1</td>
<td>2.9</td>
<td>0.34</td>
</tr>
<tr>
<td>16</td>
<td>2</td>
<td>44.6</td>
<td>7.6</td>
<td>0.6</td>
<td>5.7</td>
<td>39.8</td>
<td>1.7</td>
<td>2.1</td>
<td>0.57</td>
</tr>
<tr>
<td>17</td>
<td>2</td>
<td>37.6</td>
<td>8.3</td>
<td>0.9</td>
<td>2.1</td>
<td>48.7</td>
<td>2.4</td>
<td>3.6</td>
<td>0.29</td>
</tr>
<tr>
<td>19</td>
<td>3</td>
<td>44.2</td>
<td>7.7</td>
<td>0.8</td>
<td>2.3</td>
<td>42.6</td>
<td>2.4</td>
<td>3.2</td>
<td>0.60</td>
</tr>
<tr>
<td>20</td>
<td>2</td>
<td>44.8</td>
<td>8.4</td>
<td>1.1</td>
<td>2.1</td>
<td>41.8</td>
<td>1.7</td>
<td>3.4</td>
<td>0.66</td>
</tr>
<tr>
<td>^a 3</td>
<td>2</td>
<td>44.9</td>
<td>7.6</td>
<td>0.8</td>
<td>2.5</td>
<td>42.5</td>
<td>1.7</td>
<td>2.3</td>
<td>0.60</td>
</tr>
</tbody>
</table>

Statistical Analysis

\[(A+B)\]

\[
\begin{array}{cccc}
3-8 & \bar{x} & 36.7^{**} & 52.3^{**} & 3.9^{**} & 0.34^{**} \\
N=15 & SE= & 0.82 & 0.76 & 0.243 & 0.021 \\
13- & \bar{x} & 42.7 & 47.6 & 2.8 & 0.51 \\
20 & N=15 & SE= & 0.72 & 0.76 & 0.224 & 0.039 \\
\end{array}
\]

^a Reisolate. **(P<0.01).
During this time they were being maintained in liquid medium without hormones and without agitation. Active growth was started by transferring to the standard growth medium with shaking. As a check a new culture was started from the same line which had been stored on agar medium. After growing up and subculturating, the material was harvested as usual. This reisolate is shown in Table 15. Inexplicably, it showed almost identical characteristics to the second group of active roots; differing only in total product level. A possibility is that an unnoticed change occurred in either the environmental conditions or in the medium, resulting in a similar change in both cultures. Note though that transformed callus showed a decline in PDE-OAc over some 42 months in culture (Fig. 23). Considering the more rapid growth of roots relative to callus, the physiological ages of the cultures which resulted in similar changes in composition were probably comparable. Whether the change in the roots occurred as a result of a chance selection of roots with slightly different characteristics from the main stock culture, from some change due to storage or medium, or from a developmental change of physiology is unclear.

Rücker et al. (33) have reported that root cultures of *Digitalis purpurea* produced cardiac glycosides for two passages (two months) but were not detected on the third passage. Growth of roots also increased substantially with each subculture.

Steward and Krikorian (39) have stated that "... root-tip cultures of many plants, (both dicots and monocots) even-
ual 'peter out'. This does not appear to be the case for *B. alba* root cultures. However, if a very stable line was required for experimental work, then more information on the long-term stability of *Bidens* root cultures would be required. The ease with which a clone can be maintained on agar allows a line to be effectively 'stored' while in active use. However, the stability of slow-growing agar cultures must still be demonstrated. I do not think that the small, but significant, change in the root cultures shown over the course of these experiments invalidates the results or conclusions drawn from them.
SUMMARY

Excised root cultures of *Bidens alba* grown in darkness with 0.5 g/l NAA in liquid SH medium were evaluated for growth, polyacetylene level and polyacetylene composition as nutritional or environmental conditions were altered. Factors which increased polyacetylenes in roots above the standard level were: late log phase growth, lowered temperature, high levels of kinetin, and high sucrose to nitrate ratios. Factors which decreased polyacetylene levels were: log and stationary phase growth, high temperature, low sucrose to nitrate ratios, high levels of NAA, and illumination. Composition was shifted to increased synthesis of phenyldiynene acetate by high levels of kinetin and illumination.

Transformed roots were evaluated for the effect of organization on product level and composition. Young root tips had the highest level of product and the oldest, basal, region of roots, the lowest. Disorganization of roots, induced by high kinetin levels, resulted in low levels of product with a quite atypical composition. As cultures, roots showed a small but statistically significant change in characteristics over time. Nevertheless, *B. alba* roots are a sensitive system for investigating factors affecting polyacetylene synthesis.
LITERATURE CITED


13. Hagimori, M., T. Matsumoto, and Y. Obi. 1982. Studies on the production of Digitalis cardenolides by plant tissue culture. III. Effects of nutrients on digi-


CHAPTER VII

CHARACTERISTICS OF HYBRIDS BETWEEN

BIDENS ALBA AND BIDENS PILOSA
INTRODUCTION

The genetic control of polyacetylene synthesis has received little study. However, if cell cultures are to be used for research into the control of the pathway it would be of interest to know if polyploidy affects synthesis, and whether the genes for synthesis of individual compounds are inherited in a simple Mendelian pattern. In the course of the studies presented earlier, *Bidens pilosa* (sensu lato) plants from several parts of the world were examined for PHT in leaves. One of these accessions, *B. pilosa* var. *minor* (Blume) Sherff, from Belize City, Belize was chosen for crossing studies with *B. alba*. Characteristics of this accession which made it suitable for this study were the following: 1) it does not synthesize detectable levels of PHT (or any other polyacetylenes) in its leaves. 2) It crosses readily with *B. alba* as the female parent. 3) Several characters appear to distinguish the two taxa sufficiently to allow ready identification of *F₁* hybrid plants and selves. The following points were to be investigated: 1) evidence for the hybridization of *B. alba* and *B. pilosa*; 2) levels of leaf PHT in *F₁* and *S₁* plants; 3) inheritance pattern and levels for leaf polyacetylenes in *F₂* plants.

The only studies in which higher plant crosses have been made and the offspring evaluated for acetylenes are those of Van Fleet (17) who worked with *Coreopsis saxicola* and *C. grandiflora*. His results are difficult to evaluate since no quantitative data were given for either polyacetylene levels or for
numbers of plants used in crosses, and all the data are apparently for $F_1$ plants. However, the following points summarize the relevant aspects of Van Fleet's results: 1) root polyacetylenes of parents were similar and hybrids showed no qualitative changes. 2) Most of the parents contained PHT as the principle acetylene in stems and leaves but some $C. \ grandiflora$ and most of the hybrids had a mixture of $C_{13}$ triene-triyne and phenyldiynene. 3) Hybrids appeared to be additive for polyacetylenes from both parents but the proportions of compounds differed widely from the parental levels (17). Wild and horticultural forms of $Dahlia\ scapigera$ (2) and $D.\ coccinea$ (8) have been compared but the genetic relatedness of the taxa was not given if known. Studies on the heritability of acetylene levels in fungi have been reported by Bistis and Anchel (4) and Carey et al. (7). Levels in dikaryons appear to depend on the mating types involved as well as on the component homokaryon strains. However, there was no evidence of a gene dosage effect.

Hybridizing a PHT producing taxon with a compatible nonproducing taxon could indicate if gene dose affects PHT level or if PHT synthesis is dominant or recessive. In my view crossing a producer and a non-producer may be considered to be the inverse of polyploidization with respect to the trait PHT synthesis. Levin (11) has recently reviewed the literature on polyploidy effects on cell metabolism and it is clear that enzyme activities of primary metabolism vary widely with changes in ploidy, sometimes showing dosage effects, sometimes compensation, and sometimes decreasing below compensation.
level—depending on the enzyme and the plant examined. The effect of ploidy changes on secondary products, other than flavonoids, seems to be less investigated but Levin (11) cites a number of examples of drug alkaloids and terpenes showing large to moderate increases in quantities with increases in ploidy level. Qualitative changes were also found in one study. Grant (9) discusses gene dosage effects observed in vitamin A content of corn endosperm and flower colour in *Dahlia* and *Antirrhinum*. There seems to be no regular correlation between ploidy and product level even in autopolyploids. The situation with allopolyploids, as in the present study, might be expected to be even more variable.
MATERIALS AND METHODS

Crosses

Seeds for the parental strains of Bidens alba L. var. radiata (Schz. Bip.) Ballard ex Melchert and B. pilosa var. minor (Blume) Sherff were sown in flats of sterilized black soil, sphagnum, and leaf mulch (2:1:1) and grown under Lifeline fluorescent bulbs (Sylvania) with an illuminance of 6,000 lux and photoperiod of 18 hours at a temperature of 25°C and 70% relative humidity.

Parental flower heads were selfed by rubbing together flowering heads of the same plant and crossed by rubbing the other species. Crossed heads were tagged and recrossed as new flowers opened. Heads setting seed were collected at maturity and divided into the resulting four groups (i.e. selfs of each type and the two reciprocal crosses). These seeds were then sown in separate flats and grown under glasshouse conditions with the natural light for Vancouver, B.C. from August to October. \( F_1 \) and \( S_1 \) were sown in 1981 and the \( F_2 \) in 1982. To check for year to year variation B. alba plants were grown from the same seed source as the parental generation and sown at the same time as the \( F_2 \).

After the first flowers in a cluster of head began to open, a plastic bag, perforated with approximately 50 needle holes, was placed over the cluster and closed at the base by a strip of plastic. Head diameter of unbagged heads was measured from
the tip of one ray flower to the tip of the opposite flower in heads in which all flowers had opened. The ray flowers were counted and plucked from the head and the length of the corolla measured from the point of attachment to the ovary to the most distant extension of the petal. Pilosity was estimated visually with the parental plants serving as + and ++ standards for B. alba and B. pilosa respectively. Seed awns of F₁ and F₂ having a comparable length were counted, but setate less than approximately 1/5th the length of the two main awns were not included. Voucher specimens of the two parents and the F₁ hybrids are deposited at UBC.

Extractions

Preliminary results showed that PHT concentration in leaves varied with position on a given plant. Since it was not practical to evaluate whole plants, the 3rd leaf from the base, not including the cotyledons, was used for quantitation of the S₁ and F₁. For the F₂, the 3rd and 6th fully opened leaves from the top of the plant were used. In a preliminary set of extractions of 12 plants, the PHT content of both leaves at the third node was compared in order to determine if there was appreciable variation in leaf pairs. The correlation coefficient was 0.98 and the null hypothesis rejected (P < 0.01). Therefore only one leaf was sampled per node. Only the lamina of the leaf was used for extraction. Extractions were performed as described in Chapter 2.

PHT accounts for over 95% of the acetylenes in leaves of B. alba (see Table 2), and any additional acetylene chromophores are readily detected in the spectrum, either by the
presence of additional absorption peaks or by changes in the ratios between PHT peaks. Therefore the total PHT content was estimated from the UV spectrum of the combined petroleum ether extracts. The amount was determined from the absorbance at 310 nanometers using the published extinction coefficient of 33,700 (10). A tangent to the valleys on either side of the peak at 310 nm was drawn and peak height measured from this baseline. A similar baseline was drawn for the peak at 290 nm and the ratio checked for agreement with the ratio for pure compound. This procedure was adapted from Morton (14, p.60).

**Chromosome Counts**

Flower buds were fixed in modified Carnoy's solution consisting of chloroform: absolute ethanol: glacial acetic acid (4:3:1, v/v/v) (1) and anthers stained in aceto-orcein or aceto-carmine, mounted in Hoyer's medium (3) and squashed. Counts were made on meiotic pollen mother cells.

**Statistics**

Correlation coefficients, chi-square, and student's t-tests were calculated according to Strickberger (16). For t-tests when more than two populations were involved, means were evaluated in pairs.
RESULTS AND DISCUSSION

\( F_1 \) Vegetative Characters

Typical leaves from parental and \( F_1 \) plants are shown in Fig. 32. Leaves are from the 6th node or higher. *Bidens alba* typically develops simple leaves at the first few nodes, changing to trifoliate at about the 5th node. A few upper leaves have 5 leaflets. *Bidens pilosa* leaves are typically trifoliate at lower nodes and have 5-7 leaflets at the upper nodes. Leaves of \( F_1 \) hybrids with *B. alba* as the pistillate parent had 5-7 leaflets in the upper nodes and 3 leaflets at the lower nodes. Putative \( F_1 \) plants with *B. pilosa* as the pistillate parent were indistinguishable from *B. pilosa* in all characters scored (Table 16), apparently the result of selfing.

Pilosity of the \( F_1 \) was intermediate to similar to the *B. alba* parents. The trait is not easily evaluated precisely, but it does appear to be more variable in the hybrids than in the parents. About half the \( F_1 \) were like *B. alba* and half intermediate between the two parents.

\( F_1 \) Floral Characters

The number of ray flowers per head were similar in the two parents (Table 16) and the difference was not statistically significant. Both ray flower length and flower diameter were closely similar, as would be expected, and only the former (which had somewhat less variance) is listed in Table 16. The average length of ray flower corollas in the \( F_1 \) was nearly intermediate between the parental types, differing by less than
Fig. 32. Typical leaves from parental and F₁ hybrid Bidens plants. Leaves were taken from the sixth node, or higher, from the base. Column A) B. alba; B) B. pilosa; C) B. alba♀ X B. pilosa♂.
Table 16. Comparison of characters of *B. alba*, *B. pilosa*, and *F₁* hybrids.

<table>
<thead>
<tr>
<th></th>
<th><em>B. alba</em>, <em>S₁</em></th>
<th><em>F₁</em>, <em>B. alba♀</em></th>
<th><em>B. pilosa</em>, <em>S₁</em></th>
<th><em>F₁</em>, <em>B. pilosa</em>&lt;sup&gt;a&lt;/sup&gt;♀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ray flowers</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no./head (SE)</td>
<td>5.61 (0.048)</td>
<td>5.47 (0.062)</td>
<td>5.09 (0.045)</td>
<td>5.24 (0.076)</td>
</tr>
<tr>
<td>corolla length (mm)</td>
<td>16.58&lt;sup&gt;*&lt;/sup&gt; (0.091)</td>
<td>11.78 (0.13)</td>
<td>5.91&lt;sup&gt;**&lt;/sup&gt; (0.033)</td>
<td>5.97&lt;sup&gt;**&lt;/sup&gt; (0.058)</td>
</tr>
<tr>
<td>style present</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>N</td>
<td>254</td>
<td>151</td>
<td>153</td>
<td>50</td>
</tr>
<tr>
<td>No. awns/achene (SE)</td>
<td>2.12 (0.016)</td>
<td>2.19 (0.025)</td>
<td>3.11 (0.025)</td>
<td>3.06 (0.022)</td>
</tr>
<tr>
<td>Phtosity</td>
<td>+</td>
<td>+,+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>PHT (mg/g d.w.) (SE)</td>
<td>4.8 (0.29)</td>
<td>2.8 (0.35)</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>N</td>
<td>22</td>
<td>15</td>
<td>27</td>
<td>15</td>
</tr>
</tbody>
</table>

<sup>*</sup>Differs significantly from *F₁*, *B. alba* P<0.05; <sup>**</sup>P<0.01.

Abbreviations: *N*, number of units scored; *SE*, standard error; *nd*, not detectable; *d.w.*, dry weight.

<sup>a</sup>Putative hybrid.
Fig. 33. Typical flower heads of *B. alba*, F₁, and *B. pilosa*. X 0.7

Fig. 34. Ray flowers from (left to right) *B. pilosa*, F₁, and *B. alba*. X 6.7
5% from the arithmetic mean. Parents and F₁ differed significantly for this character. Representative flower heads from the parental and F₁ plants are shown in Fig. 33.

In Table 16 "style present" means that the ray flowers were scored "+" if they had an evident style. According to Ballard (1) these flowers are fertile in B. pilosa var. minor. The trait is suppressed in the F₁ as in the B. alba parent, suggesting that absence of a style is dominant. Figure 34 shows typical ray flowers for parents and F₁; note the lack of a style in B. alba and the F₁ and the intermediate corolla length in the F₁.

Differences in number of achene awns does not quite reach significance at the 0.05 level; the average probability for the mean of the F₁ and B. alba compared to the mean of the B. pilosa S₁ and putative F₁ is 0.08. The difference between B. alba and the F₁ is not significant.

Time of flowering in the F₁ is intermediate between the parents (Table 16). All seeds were planted on the same day in identical soil and grown together in the same glasshouse.

**Chromosome Counts**

Ballard (1) showed that B. alba is a tetraploid (x=24) and B. pilosa a hexaploid (x=36). Since these counts would imply that the hybrid would be pentaploid (x=30), and probably infertile, counts were made on selected plants used in this study. Figure 35 shows a tracing of representative preparations of telophase pollen mother cells and confirms Ballard's results. Meiosis in the hybrids seemed to be regular.

The characteristics of the F₁ plants may be summarized as
Fig. 35. Pollen mother cell squashes of telophase I nuclei. A) *B. alba* (x=24), B) *F₁* hybrid (x=30), C) *B. pilosa* (x=36). X 500
follows: 1) $F_1$ are intermediate between the two parents in flowering time, ray flower corolla length, and pilosity. 2) $F_1$ are similar to the $B. \text{ pilosa}$ parent in leaf morphology and similar to the $B. \text{ alba}$ parent in achene awn number and in lack of fertile ray flowers. 3) Hybrids have the expected chromosome number.

**PHT Content of Hybrids**

The mean PHT level in hybrids was close to one-half the level in the $B. \text{ alba}$ parent (Table 16) suggesting a gene dosage effect on product level. However, because of the limited number of plants which were recovered from the crosses, and the high variance in $B. \text{ alba}$ and the $F_1$ hybrids, the values obtained were not statistically significant. Nevertheless the data were not incompatible with a dosage effect and this possibility could be evaluated further in the $F_2$.

**PHT Levels in $F_2$ Plants**

Progeny from selfed $F_1$ segregated into plants with or without PHT in leaves. Figure 36 shows representative spectra for each type. The sharp peak at 250 nm (ε148,000) for PHT makes possible detection of levels much lower than those found in $B. \text{ alba}$, hence there was little ambiguity in classifying the plants for this trait. None of the plants showed any significant increase in other acetylene chromophores. The frequency distribution of PHT levels for 65 $F_2$ plants is shown in Fig. 37. Scores are the average of levels in leaves at the 3rd and 6th nodes from the top. The distribution in both series was similar, with a correlation coefficient of $r=0.88$
(N=58), the null hypothesis (random correlation) rejected (P<0.01).

Analysis of the F₂ data is considerably complicated by the difference in ploidy level in the two parents, as shown earlier. The fertility and apparently regular meiosis of the F₁ are difficult to account for in view of the presumed pentaploid level. As Fig. 37 shows, seven of the 65 plants were PHT negative, indicating that B. alba chromosomes likely pair with pilosa homologues. A possibility is that the base chromosome number is six instead of twelve. This would allow regular division. However, Stuessy has recently reviewed the systematics of the Heliantheae (15) and there is no indication that a base number of six occurs in the subtribe containing Bidens, Coreopsidinae, and only four genera are reported to have a base of six. Calculation of gamete ratios and F₂ ratios on the basis of a decaploid F₁ gives a very low frequency for the occurrence of the recessive (PHT negative): 1 in 1190. Even if doses of one and two B.alba chromosomes (versus a maximum of eight in the F₂) is grouped with the null class the expected frequency is less than half of that found and the probability (by chi-square test) is much less than 0.01. The results are clearly not likely to be explained by positing a 10xF₁. A second alternative is to calculate the expected ratios assuming the pentaploid F₁ produces 2n and 3n gametes with all combinations equally probable. Letting "A" be the alba allele and "a" the pilosa, the following combinations and ratios are expected: 1 AA: 3 AAa: 6 Aaa: 6 Aa: 3 aa: 1 aaa. In the F₂ 16 out of 400 plants should be recessive. The chi-square value of
Fig. 36. Representative UV spectra of petroleum ether extracts of B. alba X B. pilosa F$_2$ plants. A) Typical spectrum of PHT positive leaves; B) typical spectrum of PHT negative leaves.
Fig. 37. Frequency distribution of PHT levels in 65 F₂ progeny of B. alba X B. pilosa. Mean PHT level for seven B. alba control plants was 4.9 mg/g dry wt.

30.4 again has a probability far less than 0.01 and this hypothesis is rejected. If only 2n gametes are assumed to be formed then the following gametic ratios are expected: 1 AA: 6 Aa: 3aa and the corresponding zygote ratios: 1 AAAA: 12 AAAa: 42 AAaa: 36 Aaaa: 9 aaaa. Under this assumption the chi-square value is 0.845 which has a probability greater than 0.1. This would imply that PHT synthesis segregates as a single-factor Mendelian trait. However, this explanation is counter to the chromosome number found in pollen mother cells.

A second aspect of the data in Fig. 37 is the asymmetry of the distribution. Only one plant had a level comparable to the control plants (4.2 mg/g vs. 4.9 mg/g respectively) with the bulk of the scores clustered around 0.6 mg/g. A seasonal difference seems not to be involved since control plants grown from seed from the same pool as the parental plants in Table 16, and sown at the same time, gave a level very close
to that of the $S_1$ (4.9 mg/g vs. 4.8 mg/g). The results clearly show that PHT level is not proportional to gene dosage. All of the alternatives discussed result in roughly symmetrical distributions with maxima at combinations having from two-fifths to one-half of the $F_2$ chromosomes from B. alba. If a simple dosage effect was present then the distribution in Fig. 37 would peak at about 2.4 mg/g f.w. Apparently additional loci which affect level but not presence or absence of the trait are segregating in the $F_2$.

**Future Work**

Evidence presented by Ballard (1) suggests that the two taxa examined here are derived from a common parent, B. odo-rata. Given the dominant character of PHT synthesis this would suggest that B. pilosa var. minor has either lost the capacity to activate a functional gene set or to have acquired the ability to convert PHT to a derivative. There is no suggestion of the latter either in the literature (6, Table VIII) or in the present results; PHT appears to be a terminal product. It would be interesting, then, to test a range of non-producers like pilosa var. minor (especially diploid lines) in crosses with producers to see if trans complementation is possible. An $F_1$ with enhanced synthesis of PHT could serve as an initial indicator for lines in which complementation might be occurring. Levy, in related studies, (12) has interpreted the gain of flavonoid compounds in autoploid cultivars of Phlox drummondii as functional derepression of previously silent structural genes, a fact Mears (13) has taken to imply that the genes were active in wild plants but became inactive.
in the cultivars and were reactivated by chromosome doubling with colchicine.

The existence, according to Bohlmann (5,6) of another species of *Bidens* and several species of *Coreopsis* in which PHT is synthesized in roots and not leaves raises a question about differentiation: is the PHT pathway a biosynthetic module the expression of which depends on a signal(s) tightly linked to organ differentiation as a morphological state or does expression reflect less specific conditions such as hormone balance, with different sites of expression normally due to dissimilarity in hormonal (or other) conditions in the different organs to which expression is confined? If the latter is true then synthesis of root compounds in leaves could be triggered by altering inducer levels either artificially or genetically. Some evidence that this may be the case with flavonoids is discussed by Mears (13): many changes in flavonoid tissue specificity occurred during induction of autotetraploids from diploid cultivars of *Phlox drummondii*. It would be extremely interesting to see what pattern of expression occurs in crosses between taxa producing PHT in roots only with taxa producing PHT in stems and leaves only, especially if tissue hormone levels were determined as well.
Hybrids between tetraploid (4x=48) *B. alba* and hexaploid (6x=72) *B. pilosa* yield fertile F₁ (5x=60) exhibiting a mixture of morphological traits and a roughly intermediate level of PHT in the leaves. Selfed F₁ plants gave an F₂ which segregated for PHT synthesis. Synthesis is dominant but showed depressed levels incompatible with a single gene dosage effect. The ratios of segregants do not appear to agree with expected values for the pentaploid.


AFTERWORD

The studies presented in this thesis have shown some of the possibilities for using Bidens alba tissue and organ cultures for studying the physiological factors controlling polyacetylene level and composition. Callus cultures from normal plants could not be induced to produce detectable levels of polyacetylenes; however, transformed callus did produce a characteristic set of compounds, some of which were novel, at fractional levels of those found in the plant. Further work needs to be done to determine what factors account for the difference between normal and transformed cells; e.g., is the composition and level of hormones the critical difference or are other physiological factors involved?

Root cultures proved to be a simple, efficient, and consistent system for investigating polyacetylene synthesis. Factors having a significant effect on composition or level included the following: temperature, growth phase, carbohydrate/nitrate ratio, hormone type and level, and presence of absence of light. Whether these determinants are integrated into fewer, or perhaps one, basic system controlling the level and composition of product remains to be determined. It is possible that all the factors above result in changes in the ratios and levels of hormones which, in turn, act on biosynthesis. The studies presented here have indicated some factors which can modify the polyacetylene ratios in roots but not how non-root compounds can be induced. Other signals are apparently necessary for
production of leaf and stem compounds in roots. Dedifferentiation of roots resulted in production of some of these compounds at low levels, indicating that the specific type of tissue organization is important for specificity. The central problem of how the expression of organ specific compounds are regulated, however, remains to be solved. *Bidens alba*, however, has several characteristics which make it useful for studies of this type.

Results from crosses of *B. alba* and *B. pilosa* indicated that at least one of the principal compounds of *B. alba*, PHT, was inherited as a unit, but other loci apparently affect levels of product. The availability of an interfertile species lacking the biosynthetic pathway for PHT offer additional possibilities for studying the control of polyacetylene expression, although the combination used in this study resulted in problems of interpretation due to the different ploidy levels involved.

Transmission of crown gall tumour characteristics through sexual reproduction presents an opportunity to see how addition of foreign DNA to a plant genome is integrated into the total life cycle of the plant. In addition, it suggests that insertions of other genes would be retained, and *B. alba* may be particularly suitable for genetic engineering studies.

In summary, this thesis is a contribution to understanding the way in which a fascinating and important set of compounds, polyacetylenes, are integrated into the complex web of pathways which function so precisely in this, and many other, rather ordinary weeds.
Appendix Table 1. Variations in medium hormones used for culturing Bidens alba callus. All media were made with 3% sucrose.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Range tested (mg/l)</th>
<th>No. levels tested</th>
<th>Medium</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-D</td>
<td>0-2.0</td>
<td>5</td>
<td>B₅</td>
<td>Maximum growth at 1.0 mg/l.</td>
</tr>
<tr>
<td>NAA</td>
<td>0.02-10.0</td>
<td>9</td>
<td>SH</td>
<td>Maximum growth at 4.0 mg/l.</td>
</tr>
<tr>
<td>NAA x Kinetin</td>
<td>0.01-1.2 x</td>
<td>11</td>
<td>SH</td>
<td></td>
</tr>
<tr>
<td>GA₃ x Kinetin</td>
<td>0.04-0.4 x</td>
<td>9</td>
<td>SH</td>
<td>Maximum growth at 0.125 mg/l each.</td>
</tr>
</tbody>
</table>

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; NAA, α-napthalene acetic acid; GA₃, gibberellic acid.

Appendix Table 2. Variations in sugars and vitamins used for culturing B. alba callus.

<table>
<thead>
<tr>
<th>Supplement</th>
<th>Range tested (g/l)</th>
<th>No. levels tested</th>
<th>Medium</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inositol</td>
<td>0-1.6</td>
<td>7</td>
<td>a₁B₅</td>
<td>Little difference in growth.</td>
</tr>
<tr>
<td>Sucrose</td>
<td>2.5-80</td>
<td>6</td>
<td>1SH</td>
<td>Maximum growth at 50 g/l.</td>
</tr>
<tr>
<td>Sucroseᵇ</td>
<td>2.5-80 (+light)</td>
<td>6</td>
<td>1SH</td>
<td>Maximum growth at 50 g/l.</td>
</tr>
<tr>
<td>Glucose</td>
<td>15-30</td>
<td>2</td>
<td>1SH</td>
<td>Poor growth.</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>0.01-0.4</td>
<td>5</td>
<td>1B₅</td>
<td>Best growth on 0.05 g/l, 0.2 and above killed cells.</td>
</tr>
</tbody>
</table>

ᵃConcentration of 2,4-D (mg/l) precedes medium abbreviation. ᵇIllumination 6000 lux, continuous.
Appendix Table 3. Variations in SH medium supplemented with primary metabolites. All media made with 1 mg/l 2,4-D and 2% sucrose.

<table>
<thead>
<tr>
<th>Supplement</th>
<th>Range tested (molar)</th>
<th>No. of levels tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate (A)</td>
<td>$10^{-1}-10^{-4}$</td>
<td>4</td>
</tr>
<tr>
<td>Malate (M)</td>
<td>$10^{-2}-10^{-5}$</td>
<td>4</td>
</tr>
<tr>
<td>Citrate (C)</td>
<td>$10^{-2}-10^{-5}$</td>
<td>4</td>
</tr>
<tr>
<td>A + M</td>
<td>$3 \times 10^{-4} (A) + 3 \times 10^{-4} (M)$</td>
<td>1</td>
</tr>
<tr>
<td>A + C</td>
<td>&quot; &quot;</td>
<td>1</td>
</tr>
<tr>
<td>M + C</td>
<td>$2.2 \times 10^{-4} (M) + 7.4 \times 10^{-4} (C)$</td>
<td>1</td>
</tr>
<tr>
<td>A + M + C</td>
<td>$2.2 + 2.2 + 7.4 \times 10^{-4}$</td>
<td>1</td>
</tr>
</tbody>
</table>
Appendix Table 4. Variations in SH medium macronutrients used for culturing B. alba callus. Composition in mg/l except sucrose (g/l). "C" is the control level, the standard level for SH medium. All media made with 1 mg/l 2,4-D.

<table>
<thead>
<tr>
<th>Sucrose</th>
<th>KNO₃</th>
<th>MgSO₄</th>
<th>NH₄H₂PO₄</th>
<th>CaCl₂</th>
<th>KH₂PO₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>2500</td>
<td>400</td>
<td>300</td>
<td>200</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>1250</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>625</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>313</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>C</td>
<td>C</td>
<td>600</td>
<td>C</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>C</td>
<td>C</td>
<td>1200</td>
<td>C</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>C</td>
<td>C</td>
<td>2400</td>
<td>C</td>
<td>0</td>
</tr>
<tr>
<td>100</td>
<td>625</td>
<td>C</td>
<td>1200</td>
<td>C</td>
<td>0</td>
</tr>
<tr>
<td>50</td>
<td>C</td>
<td>C</td>
<td>0</td>
<td>C</td>
<td>680</td>
</tr>
<tr>
<td>50</td>
<td>1250</td>
<td>C</td>
<td>0</td>
<td>C</td>
<td>680</td>
</tr>
<tr>
<td>50</td>
<td>625</td>
<td>C</td>
<td>0</td>
<td>C</td>
<td>680</td>
</tr>
<tr>
<td>50</td>
<td>1250</td>
<td>C</td>
<td>0</td>
<td>C</td>
<td>1360</td>
</tr>
<tr>
<td>C</td>
<td>C</td>
<td>800</td>
<td>C</td>
<td>C</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>C</td>
<td>1600</td>
<td>C</td>
<td>C</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>400</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>800</td>
<td>0</td>
</tr>
<tr>
<td>50</td>
<td>1250</td>
<td>C</td>
<td>600</td>
<td>C</td>
<td>0</td>
</tr>
<tr>
<td>50</td>
<td>1250</td>
<td>C</td>
<td>600</td>
<td>C</td>
<td>0</td>
</tr>
</tbody>
</table>

a Control level, standard level for SH medium.
Appendix Table 5. Variations in SH medium supplemented with mixed fatty acids. Lipids solubilized in ethanol and Tween-80. Figures are the average of six calluses. All media made with 3% sucrose and 1.0 mg/l 2,4-D.

<table>
<thead>
<tr>
<th>Supplement</th>
<th>Oleic acid (ml/l)</th>
<th>Linoleic acid (ml/l)</th>
<th>Linolenic acid (ml/l)</th>
<th>Fresh wt. (g/callus)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2.0</td>
</tr>
<tr>
<td>0.31</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2.2</td>
</tr>
<tr>
<td>0.31</td>
<td>0.05</td>
<td>0</td>
<td>0</td>
<td>1.4</td>
</tr>
<tr>
<td>0.31</td>
<td>0</td>
<td>0.05</td>
<td>0.05</td>
<td>1.8</td>
</tr>
<tr>
<td>0.31</td>
<td>0.05</td>
<td>0.05</td>
<td></td>
<td>1.6</td>
</tr>
<tr>
<td>0</td>
<td>0.05</td>
<td>0.05</td>
<td></td>
<td>1.3</td>
</tr>
<tr>
<td>1.0</td>
<td>0</td>
<td>0</td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>0</td>
<td>1.0</td>
<td>0</td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>1.0</td>
<td></td>
<td>2.0</td>
</tr>
<tr>
<td>0.95</td>
<td>0.05</td>
<td>0</td>
<td></td>
<td>1.5</td>
</tr>
<tr>
<td>0.95</td>
<td>0</td>
<td>0.05</td>
<td></td>
<td>0.8</td>
</tr>
<tr>
<td>0</td>
<td>0.1</td>
<td>0.1</td>
<td></td>
<td>1.2</td>
</tr>
<tr>
<td>0.95</td>
<td>0.05</td>
<td>0.05</td>
<td></td>
<td>1.4</td>
</tr>
<tr>
<td>1.0</td>
<td>0.1</td>
<td>0</td>
<td></td>
<td>1.5</td>
</tr>
<tr>
<td>1.0</td>
<td>0</td>
<td>0.1</td>
<td></td>
<td>1.2</td>
</tr>
<tr>
<td>b</td>
<td>0</td>
<td>0</td>
<td></td>
<td>1.9</td>
</tr>
</tbody>
</table>

a Ethanol, Tween-80 control.
b Control, no additives.