PHENOLIC SYNTHESIS IN *PERILLA* SUSPENSION CULTURES

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ABSTRACT

PHENOLIC SYNTHESIS IN PERILLA SUSPENSION CULTURES

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Suspension cultures of Perilla ocymoides L., grown in a defined liquid medium, lose the biosynthetic potential for perillanin [cyanidin-3(6-p-coumarylglucoside)-5-glucoside], the characteristic pigment of the species. The culture, however, accumulates caffeic (3,4-dihydroxycinnamic) acid both in the free and esterified forms (in a 1:4 ratio) during the early-exponential phase and preceding maximum growth. The data on the biosynthesis of caffeoyl compounds, from trans-cinnamic acid-2-^{14}C, indicate active phenolic synthesis and reflect the pool size of both compounds during culture growth.

Increased levels of growth substances influenced the rate of accumulation of caffeoyl compound so that cytokinin stimulated, while auxin repressed, phenolic synthesis. Either of the hormones, however, caused an increase in the ratio of caffeoyl ester to free acid, especially during mid-exponential growth.

The regulatory role of caffeoyl compounds was discussed in relation to their accumulation during culture growth.

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TABLE OF CONTENTS

LIST OF FIGURES

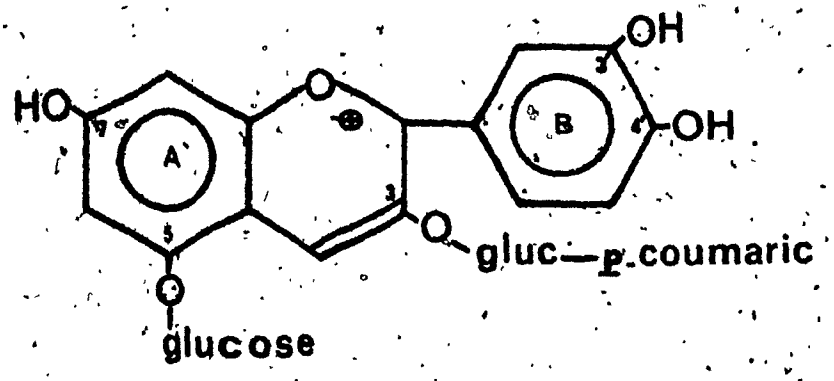
Page

LIST OF TABLES

I. INTRODUCTION

One of the major problems in plant biology is to know how cultured cells come to differ from one another with regard to their ability to reproduce their characteristic metabolites. Few higher plant tissues have been reported to retain their potential for secondary metabolite synthesis when grown *in vitro* (Staba, 1963, 1969; Carew and Staba, 1965; Street *et al.*, 1965; Krikorian and Steward, 1969 and references cited therein). However, the majority of cultured plant tissues apparently lose this property due to modification of their metabolism which may be caused by changes in the composition of the nutrient culture medium, growth regulators or the environmental conditions surrounding the cultured tissue (Steward, 1963; Street *et al.*, 1965). Experiments which have been carried out with intact plants demonstrated that both environmental variables and growth regulators affect plant growth and the biochemical events leading to the accumulation of its specific metabolites.

Perilla ocymoides L. (Labiateae) is a perennial herb with redish-purple stems and leaves. Its colouration is mainly due to the presence of an anthocyanin pigment, perillanin [cyanidin-3-(6-p-coumarylglucoside)-5-glucoside] (Fig. 1), which prevails in the young seedling and the mature plant (Watanabe *et al.*, 1966). Therefore, this plant



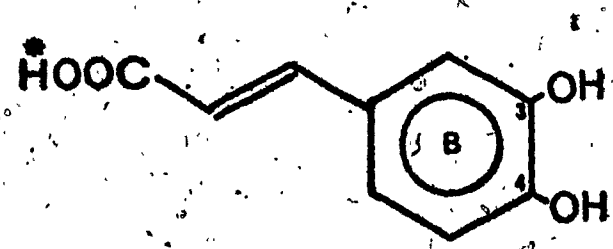


Figure 1. Structures of perillanin (top) and caffeic acid (bottom). * denotes site of ester formation.

material was conceived suitable to establish a suspension culture in order to investigate the biosynthetic potential for its characteristic anthocyanin pigment and other related phenolic compounds.

While several attempts failed to induce pigment formation in vitro, however, the suspension culture produced one major phenolic metabolite, caffeic (3,4-dihydroxycinnamic) acid, both in the free and ester forms. This remarkable property was considered by itself a valid reason to study the biosynthesis and accumulation of such ubiquitous constituent in relation to culture growth.

This investigation will concern itself with (a) the initiation of a cell suspension culture from Perilla cotyledons, (b) the determination of its growth characteristics under defined culture conditions, (c) the isolation and identification of its major phenolic constituents, (d) studying their biosynthesis from ^{14}C-labelled cinnamic acid, and (e) examining the effect of added auxin or cytokinin on their accumulation during the different stages of culture growth.

The fact that caffeoyl compounds constitute the major secondary metabolites of Perilla cultures makes this study particularly interesting in view of the widely accepted role of phenolic compounds in plant growth and metabolism (Galston, 1969; Kalefi and Kodyrov, 1971; Stenlid, 1962, 1970).

II. LITERATURE REVIEW

1. Callus Tissue and Cell Suspension Culture

The idea of culturing cells and tissues *in vitro*, with the aim of utilizing their totipotency, was first put forth by Haberlandt (1902), but it was not until 1934 that the late P.R. White succeeded in establishing an actively growing organ culture from tomato roots (White, 1934). At about the same time, Gautheret successfully initiated tissue cultures from wound callus of some woody plants (Gautheret, 1935). These were grown on an agar medium containing a sugar, as a carbon source, and a mixture of inorganic salts.

In 1954, Muir *et al.*, developed the cell suspension culture technique which consisted of free cells and cell clumps growing in an agitated liquid medium (Muir *et al.*, 1954). The advantage of suspension culture lies in the fact that it, ideally, allows the cells to be free from restraints or any contact with neighbouring cells which might induce morphogenesis. Thus, the cell is potentially able to express its 'biochemical totipotency' (Krikorian and Steward, 1969).

Few tissues or cells would be able to grow on such simple media as that of White (White, 1943). Most tissues, however, require the addition of some undefined natural

extracts such as coconut milk, yeast extract or casein hydrolysate for their continued growth in culture. Nevertheless, some cultures were established on completely defined media (Street and Henshaw, 1966 and references cited therein). These media are usually more complex than the original basal medium of White, and incorporate such supplements as vitamins, growth substances and sometimes mixtures of amino acids. A source of carbon such as sucrose, or some other sugar, is invariably necessary, since chlorophyll-rich tissues grown under high light intensity may not be autotrophic with respect to carbon (Hildebrandt et al., 1963).

Many tissues also require the addition of one or more growth substances for their continued growth in culture. The most important growth substance is auxin. The main synthetic auxins used are napthaleneacetic acid (NAA), and 2,4-dichlorophenoxyacetic acid (2,4-D) as well as the naturally occurring auxin indoleacetic acid (IAA). Gautheret (1954) published a tabulated review of the tissues which require auxins for their in vitro growth. On the other hand, some tissue cultures are apparently auxin-autotrophic (Bitancourt, 1955) or become adapted after repeated subculture on auxin-free media (Gautheret, 1955).

The other important group of growth substances, the cytokinins, is known to promote cell division. The most

commonly used are the synthetic cytokinins, kinetin (6-furfurylaminopurine) and benzyladenine, as well as the naturally occurring cytokinin, zeatin 6(4-hydroxy-3-methylbut-2-enylamino)purine. Cytokinins and auxins have been reported to interact in the control of growth and morphogenesis of some tissue cultures (Skoog and Miller, 1957), and regulate the levels of certain phenolic metabolites (Skoog and Montaldi, 1961).

2. Biochemical Behavior of Cells and Tissues in Culture

Until the 1960's most of the work with tissue cultures was concerned with such morphogenetic aspects as tissue differentiation (Gautheret, 1966), control of phloem and xylem formation (Wetmore and Rier, 1963) and the control of shoot and root differentiation (Gautheret, 1966). However, increasing attention has been focused on the biochemical totipotency of cultured tissues in an attempt to control the production of their desirable metabolites. It has always been envisaged that the biosynthetic potential of tissue cultures might be controlled by modifying the composition of the culture media in which the cells grow. However, one of the main problems encountered with this technique is that the metabolism of the cultured cells is usually a function of a number of environmental conditions in which they grow, such as the nutrient medium composition, light quality, temperature and growth regulators (Staba, 1969;

et al.,
Street, 1966; Steward et al., 1964; Steward, 1963). Therefore, it has often been found that the particular metabolism of a certain plant species is not usually reproduced when it is cultured in vitro (Krikorian and Steward, 1969 and references cited therein). Furthermore, the cultured tissue may produce compounds that are not characteristic of the parent plant (Forrest, 1969; Liau and Ibrahim, 1973). There have been unsuccessful attempts to produce certain commercially valuable compounds in in vitro cultures, such as the volatile oils from peppermint (Krikorian, 1965) or latex from rubber (Mahlberg, 1962). On the other hand, the formation of anthocyanin pigments has been demonstrated in tissue and cell cultures (Ball et al., 1972), and has been induced in cultures derived from acyanic plants, such as Haplopappus gracilis (Reinert et al., 1964; Ardenne, 1965; Constabel et al., 1971), poplar (Matsumoto et al., 1970) and in carrot, flax and Jerusalem artichoke (Ibrahim et al., 1971). The formation of other phenolic compounds has also been reported in a number of suspension cultures (Hahlbrock and Wellmann, 1970; Steck et al., 1971; Davies, 1972). The following tables illustrate examples of tissues which reproduce (Table 1), or fail to reproduce (Table 2) their characteristic metabolites when cultured in vitro.

Table 1: Tissue cultures which reproduce their characteristic metabolites in vitro.

Tissue Culture	Metabolite	Reference
Atropa belladona	Atropine	West & Mika, 1957
Nicotiana tabacum	Nicotine	Speake et al, 1964
Camillia sinensis	Caffeine	Ogutuga & Northcote, 1970
Trichocereus spachianus	Candicine	Steinhart, 1962
Solanum xanthocarpum	Solasonine	Heble et al., 1968
Peganum harmala	Harmine	Reinhard et al., 1968
Nicotiana tabacum	Scopolin	Sargent & Skoog, 1961
Dioscorea deltoidea	Diosgenin	Kaul & Staba, 1968
Citrus aurantium	Methoxyflavones	Brunet & Ibrahim, 1973
Petroselinum hortense	O-Glycoflavones	Hahlbrock & Wellmann, 1970

Table 2: Tissue cultures which fail to reproduce their characteristic metabolites in vitro.

Tissue Culture	Metabolite	Reference
Mentha piperata	Volatile oils	Krikorian, 1965
Carica papaya	Papain	Krikorian, 1965
Ricinus communis	Lipids	Brown et al., 1970
Digitalis purpurea	Cardiac glycosides	Stohs & Staba, 1965
Iberis sempervirens	Mustard oil	Krikorian, 1965
Linum usitatissimum	C-Glycoflavones	Liau & Ibrahim, 1973
Euphorbia marginata	Latex	Mahlberg, 1962

These are but a few examples which have been investigated. Evidently,,more tissue cultures will have to be examined in order to investigate their biosynthetic potential for secondary metabolite formation.

3. Phenolic Compounds in Tissue Cultures

The formation of phenolic compounds in vitro has been reported in a limited number of cultured tissues. Nevertheless, the metabolites produced may be of such variety as that found in the intact plants. The subject has been reviewed by Staba (1963; 1969), Carew and Staba (1965),

Street et al., (1965), and Krikorian and Steward (1969). Table 3 contains a listing of the phenolic compounds which have been reported to occur in callus tissue or suspension cultures.

4. Biosynthesis of Phenolic Compounds

The biosynthesis of the phenolic acids, phenylalanine, and tyrosine, has been demonstrated first with microorganisms (Davis, 1955). It has been shown that phenolic compounds in higher plants are mostly derived from the aromatic amino acids, phenylalanine and tyrosine, which in turn are formed via the shikimic acid pathway (Neish, 1960; Gamborg, 1966; Yoshida, 1969). The phenylpropanoid, or cinnamic, acids are formed by the enzymic deamination of the aromatic amino acids: phenylalanine, by phenylalanine ammonia-lyase (Koukol and Conn, 1961) or tyrosine, by tyrosine ammonia-lyase (Neish, 1961) to form cinnamic and p-coumaric acids, respectively. These phenolic acids are then successively hydroxylated and methylated to form a variety of substituted cinnamic acids (McCalla and Neish, 1959) as shown in Figure 2.

Substituted cinnamic acids are rarely found in the free state but are usually bound as the ester or glycoside forms (Ibrahim, 1961; Neish, 1964). The substituted benzoic acids are apparently formed from their corresponding

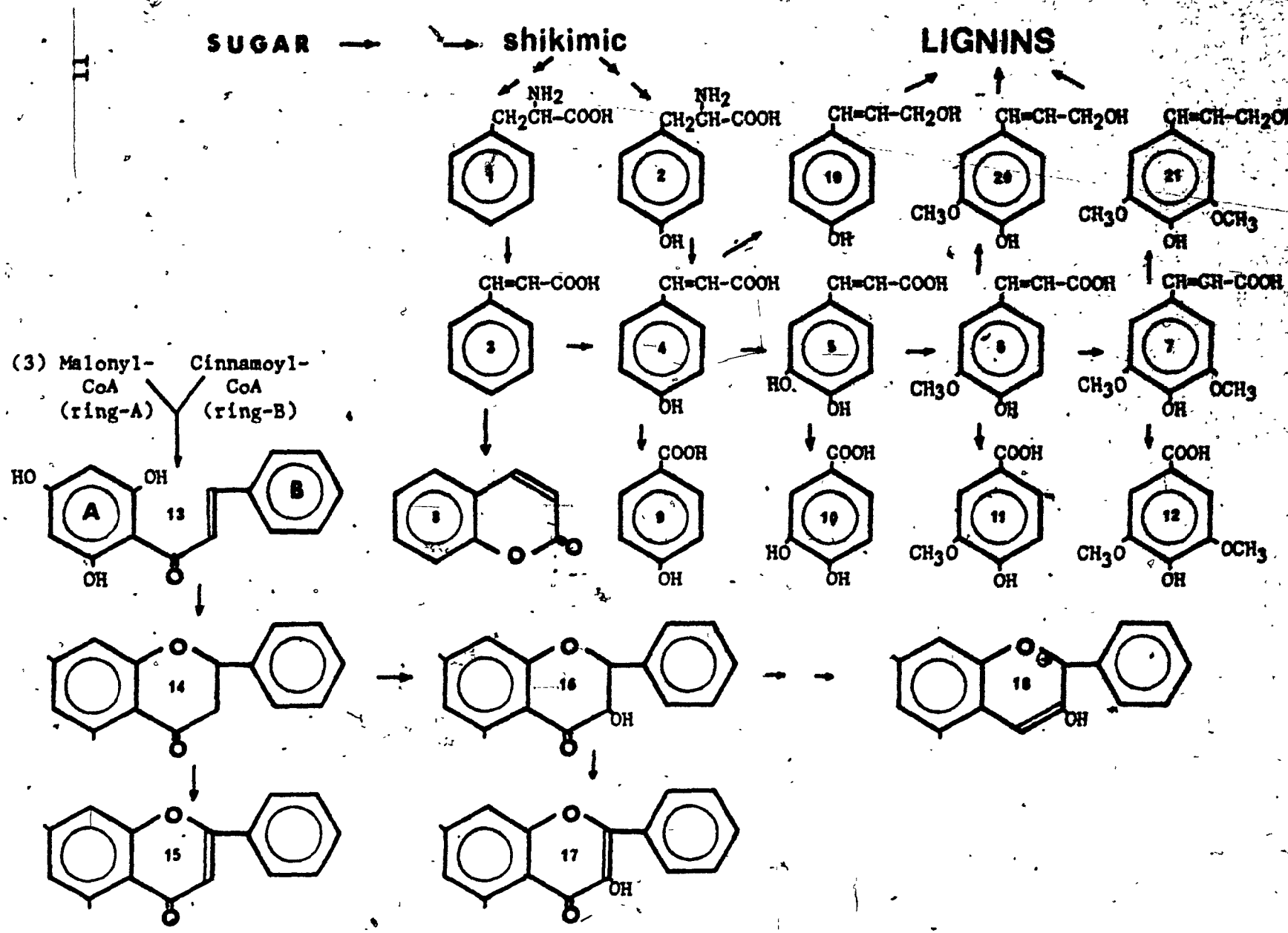


Figure 2. Summary of the pathways for the biosynthesis of phenolic compounds in plants (see following page for compounds 1-21).

Figure 2 (continued)

1, L-Phenylalanine

2, L-Tyrosine

3, trans-Cinnamic acid

4, p-Coumaric acid (p-hydroxycinnamic)

5, Caffeic acid (3,4-dihydroxycinnamic)

6, Ferulic acid (3-methoxy-4-hydroxycinnamic)

7, Sinapic acid (3,5-dimethoxy-4-hydroxycinnamic)

8, Coumarin (o-hydroxy-cis-cinnamic)

9, p-Hydroxybenzoic acid

10, Protocatechuic acid (3,4-dihydroxybenzoic)

11, Vanillic acid (3-methoxy-4-hydroxybenzoic)

12, Syringic acid (3,5-dimethoxy-4-hydroxybenzoic)

13, Chalcone

14, Flavanone

15, Flavone

16, Flavanonol

17, Flavonol

18, Anthocyanin

19, p-Hydroxycinnamyl alcohol

20, Coniferyl alcohol (3-methoxy-4-hydroxycinnamyl)

21, Sinapyl alcohol (3,5-dimethoxy-4-hydroxycinnamyl)

Table 3: Phenolic compounds isolated from callus or suspension cultures.

Phenolic Compound	Source	Reference
Coumarins	Tobacco	Sargent & Skoog, 1960
	Ruta graveolens	Reinhard et al., 1968; Steck et al., 1971
Stilbenes	*Pinus resinosa*	Jorgensen & Balsillie, 1969
Lignins	*Pinus strobus*	Hasegawa et al., 1960
	Daucus carota	Koblitz, 1962
	Lactuca sativa	Ganapathy & Hildebrandt, 1967
Catechins, Leucoanthocyanins and Tannins	*Sequoia sempervirens*	Ball, 1960
	Acer pseudoplatanus	Goldstein et al., 1962
	Juniperus communis	Constabel, 1963
	Camillia sinensis	Forrest, 1969
	Paul's Scarlet rose	Davies, 1972
Flavonols	Cotton	Smith, 1961
	Parthenocissus tricuspidata	Bleichert & Ibrahim 1974

Table 3 (continued):

Flavones	*Petroselinum hortense*	Halhlbrock & Wellmann, 1970
	Citrus peel -	Brunet & Ibrahim, 1973
Iso-Flavone	Soybean	Miller, 1969
Anthocyanins	*Haplopappus gracilus*	Constabel et al., 1971
	Impatiens balsamina	Klein & Hagen, 1961
	Daucus carota	Sugano & Hayashi, 1967
	Corn	Strauss, 1960
	Parthenocissus tricuspidata	Stanko & Bradinskaya, 1962
	Dimorphotheca auriculata	Ball et al., 1972
	Helianthus tuberosus	Ibrahim et al., 1971
	Linum usitatissimum	Ibrahim et al., 1971
	Daucus carota	Ibrahim et al., 1971
	Rosa multiflora	Ibrahim et al., 1971
	Malus pumula	Ibrahim et al., 1971

cinnamic acids (Fig. 2.) through the loss of two carbons from the three-carbon side chain (Basyouni et al., 1964). Coumarins are believed to be formed by cyclization of the side chain of an orthohydroxy cinnamic acid to form the

lactone ring (Figure 2). This step appears to take place after hydrolysis of the cis-form of the cinnamic acid glycoside (Brown, 1965).

Flavonoid compounds are formed through the condensation of three units of acetate or malonate with one molecule of substituted cinnamic acid, presumably as its coenzyme-A ester. It has been demonstrated, in higher plants, that Ring-A of cyanidin, an anthocyanidin (Grisebach, 1957); quercetin, a flavonol (Watkin et al., 1957) and phloridzin, a dihydrochalcone (Hutchison et al., 1959) are indeed formed from acetate. In all cases, ring-B was shown to be formed from shikimic acid, phenylalanine or cinnamic acid (Figure 2). It has also been suggested that other phenolic compounds may be formed exclusively from acetate (Birch et al., 1955).

The complex phenolic polymer, lignin, is derived from the phenylpropane monomers, coniferyl and sinapoyl alcohols (Freudenberg, 1959) as well as p-hydroxycinnamoyl alcohol (Nord and De Stevens, 1953). These alcohols are apparently derived from their corresponding cinnamic acids (Brown et al., 1959; McCalla and Neish, 1959). The enzymatic reduction of cinnamic acids to cinnamyl alcohols (Ebel and Grisebach, 1973) and ferulic acid to coniferyl alcohol (Gross et al., 1973) has been recently demonstrated in suspension cultures of soybean and the cambial tissues of Forsythia sp. respectively.

5. Role of Phenolic Compounds in Plants

Phenolic constituents have often been assigned the role of protecting plants against insect and fungal attacks. Many examples of this are available from the literature and are summarized in the reviews by Farkas and Kiraly (1962), Kuc (1964), and Cruikshank and Perrin (1964).

More recently, phenolic compounds are becoming more implicated in various metabolic roles. Gortner and Kent (1958) have shown that the enzyme IAA-oxidase complex was activated by p-coumaric acid, a monohydroxyphenol and inhibited by the diphenols caffeic, ferulic and chlorogenic acids in pineapple. Sondheimer and Griffin (1960) have demonstrated that p-coumaric acid can overcome the inhibition, by chlorogenic acid, of IAA-oxidase in peas. Similar results have been reported with the strawberry plant (Runkova et al., 1972) and sweet-potato roots (Imbert and Wilson, 1972). The general pattern, therefore, seems to be that monohydroxy phenols activate, while dihydroxy phenols inhibit the enzyme. This finding may suggest that phenols play an indirect role in the regulation of growth and metabolism by protecting the native auxin (Galston, 1969; Stenlid, 1962). In support of this view, Bottomley et al., (1965) have found that the increase in weight of etiolated pea seedlings, irradiated with red light, was accompanied by an increase in the amount of a quercetin (dihydroxyflavonol) triglucoside esterified with

p-coumaric acid, while the amount of kaempferol (monohydroxyflavonol) triglucoside and its p-coumaroyl ester remained constant. It is interesting to note that monohydroxy phenolic acids, while inhibited tobacco callus growth, were found to be more active in promoting budding than the dihydroxy acids, though the latter enhanced callus growth (Lee and Skoog, 1965). The authors concluded that while kinetin was primarily responsible for induction of bud formation in tobacco cultures, however, the phenolic compounds influenced the effectiveness of kinetin either by raising or lowering the level of IAA through the IAA-oxidase system or by functioning as auxins. Phloridzin, a dihydrochalcone glucoside (with a monohydroxy B-ring), has been reported to inhibit growth of Avena coleoptile (Thimann and Marre, 1954), promote root growth in peas (Lavollary and Laborey, 1948) and inhibit phosphorylation in chloroplasts (Izawa et al., 1966). Koves and Varga (1959) have shown that monohydroxy phenolic acids, especially salicylic acid, act as germination inhibitors in dry fruits. More recently, a number of phenolic compounds have been demonstrated to play an important role as growth inhibitors (Kalefi and Kodyrov, 1971). It has also been reported that some phenolics may participate in oxidation-reduction systems and inhibit ATP formation in plant mitochondria (Nagornaya, 1968; Stenlid, 1970).

6. Regulation of Phenolic Synthesis in Plant Tissue Cultures

The regulation of phenolic synthesis in tissue cultures is, at the moment, a controversial subject. Not only is it found that most tissue cultures lose the biosynthetic potential for their characteristic metabolites, but also that some cultures may produce compounds that are not found in the parent plant. Examples of this are the anthocyanins which have been found in cultures derived from acyanic plants (Ardenne, 1965; Constabel et al., 1971; Ibrahim et al., 1971). These are excellent examples of the induction of secondary metabolite formation which may be explained in terms of derepression of enzyme systems leading to phenolic synthesis by the components of the culture medium (Bonner, 1965).

The requirement for growth substances for secondary metabolite synthesis by a number of tissue and cell cultures has been fairly well documented. Cytokinin requirement has been reported for the formation of anthocyanins by carrot and Jerusalem artichoke (Ibrahim et al., 1971), 2,4-D (Ardenne, 1965) or NAA (Blakely and Steward, 1961) by apparently different strains of Haplopappus gracilis and o-chlorophenoxyacetic acid by Cape marigold (Ball et al., 1972) tissue cultures.

Blakely and Steward (1961) found that high levels of coconut milk or NAA resulted in green cultures of H. gracilus whereas low levels of coconut milk and NAA resulted

in the formation of anthocyanins. On the other hand, Constabel et al., (1971) using another strain of H. gracilus reported that increasing the level of cytokinin resulted in the formation of anthocyanins.

Arnold and Alston (1961) working with Impatiens balsamina found that the amount of anthocyanin produced by cultured hypocotyl segments depended upon the origin of the segment along the length of the hypocotyl. It was later discovered that the distribution of anthocyanin among the hypocotyl segments could be altered by NAA, and that the action of auxin could be reversed by the inhibitor, triiodobenzoic acid (Arnold and Albert, 1964).

Klein and Hagen (1961) found that anthocyanin production could be accelerated in cultures of I. balsamina by the addition of kinetin, or after injury, and could be suppressed with mannitol or ethionine, and delayed with triiodobenzoic acid.

Strauss (1960) showed that anthocyanin production by corn culture could be promoted by the addition of aspartate and cystine, and inhibited by riboflavin, methionine, asparagine, glutamine, and valine.

Sugano and Hayashi (1967) reported a good example of the nature of auxin regulation of phenolic synthesis in carrot tissue culture. They found that anthocyanin formation was induced by IAA, whereas 2,4-D stimulated the formation of hydroxycinnamic acids.

Lignification in tobacco tissue cultures has been found to be increased by increasing the levels of kinetin (Koblitz, 1962; Bergmann, 1964; Kumar, 1971) or 2,4-D (Ganapathy and Hildebrandt, 1967) and has been reported to be inhibited with high concentrations of calcium ions (Lipetz, 1962). Bergmann (1964) also found that the increase in lignin content of tobacco culture by high kinetin was accompanied by a hundred-fold increase in the number of tracheids such that, in this case, the kinetin appeared to have an indirect effect on the accumulation of lignin.

The formation of scopolin (scopoletin glucoside) in tobacco tissue cultures is apparently dependent on the presence of kinetin in the culture medium; and that the conversion of scopolin to scopoletin is accelerated in the presence of high concentrations of IAA (Sargent and Skoog, 1960). Furthermore, the release of free scopoletin into the culture medium is augmented in the presence of high levels of IAA, and that a high level of kinetin prevents the auxin-triggered release of scopoletin into the medium (Skoog and Montaldi, 1961). These authors suggested that the internal levels of scopolin and scopoletin, as well as the incorporation of these phenols into cell wall materials, are regulated by the balance between auxin and kinetin.

Callus cultures of red pine do not normally produce the phenols, pinosylvin and pinosylvin monomethylether, but

will do so upon [illegible], which may suggest the stress induction of phenolic formation (Jorgensen and Balsillie, 1969).

It can be seen from this short review that the information available on the regulation of phenolic synthesis in in vitro cultures is still too fragmentary to allow a complete picture of the mechanisms involved.

III. MATERIALS AND METHODS

1. Plant Material

Seeds of Perilla ocymoides c.v. Crispa were kindly supplied by Dr. J.H. Miller, Department of Bacteriology and Botany, Syracuse University, New York. They were germinated in the greenhouse with 18-hour photoperiods. The cotyledons of one-week-old seedlings were used to establish both the callus tissue and cell suspension cultures.

2. Callus Tissue and Suspension Culture

a. Induction of callus:

The cotyledons of seven-day-old seedlings of Perilla were excised and soaked in 50% chlorinated solution (Javex bleach, 5.25% available Cl_2) for ten minutes. The surface-sterile cotyledons were then rinsed several times with sterile distilled water. The cotyledons, after being halved lengthwise, were then placed, cut edge down, onto the nutrient culture medium which was solidified with agar. All operations were performed aseptically under a transfer hood (Lab-Con Co., Corp., Kansas City, Mo.).

The cultured explants were maintained in the dark, at room temperature (ca 22°C), in glass prescription bottles (15 X 5 X 5 cm) each containing about 30 ml of the culture medium. The callus tissue formed was subcultured at four to five-week intervals by transferring small pieces

(ca 50-100 mg) onto the fresh medium.

b. Initiation of suspension cultures:

Forty-day-old callus tissue which was highly friable was used. Approximately two-gram lumps of callus tissue were transferred aseptically to 500-ml Erlenmeyer flasks each containing 125 ml of sterile culture medium. The flasks were stoppered with cotton plugs which were covered with aluminum foil and agitated on a gyrotary shaker (Model V-S, New Brunswick Scientific, New Brunswick, N.Y.) at a rate of 120 r.p.m. and a room temperature of about 24 ± 1°C in diffuse light.

The callus masses were transformed into a loose suspension within fourteen days. The establishment of the cell culture was confirmed by examining samples from the suspension under the light microscope. Single cells, as well as small and large cell aggregates were evident by microscopic examination.

The cell suspension was subcultured by transferring, under aseptic conditions, an aliquot of the old culture to a flask containing fresh medium. The inoculum was obtained by withdrawing the culture through a large plastic syringe (30 ml) fitted with a long piece (20 cm) of glass tubing. The ratio of inoculum to fresh medium was established at 1:10 and the inoculum being taken at the stationary phase of growth (approx. four weeks old). Normally, an inoculum

of about 1.5 g fresh weight of cells was transferred to a 500-ml flask containing 125 ml of fresh medium.

c. Culture media:

The nutrient culture medium used for suspension cultures of Perilla consisted of the macro-elements, micro-elements, vitamins, iron, and inositol of Murashige and Skoog (1962) which was diluted to 50%. It was supplemented with 3% (88mM) sucrose, 1.0 mg/l (5.4μM) NAA and 0.1 mg/l (0.47μM) kinetin.

The callus cultures were established and subcultured on the same medium without dilution. The latter contained 3% sucrose, 2.0 mg/l NAA and 0.5 mg/l kinetin. The medium was solidified with 0.8% agar and its pH was adjusted to 5.6-5.8. The composition of the medium is given in Appendix A. All culture media were autocalved for twenty minutes at 15 p.s.i. before being used.

3. Growth Measurements

The parameters used to determine the growth of suspension cultures were (a) packed cell volume, (b) oven-dry weight, and (c) soluble protein content as determined by the Lowry method. The packed cell volume was obtained by centrifuging the culture in graduated centrifuge tubes at 6,000 r.p.m. (I.E.C., 8 X 50 ml head) for ten minutes. It was found that the fresh weight in grams of filtered cells was more or less numerically equal to the packed cell volume

in millilitres (± 5%). However, it was difficult to determine the fresh weight of the cells accurately due to the presence of adhering water, especially in samples with low fresh weights.

The dry weight was determined after drying the centrifuged cells in a vacuum oven at 40°C for five days. Dry weight determinations were performed on a standard analytical balance of 0.1 mg accuracy.

The protein content was determined by the method of Lowry (Lowry et al., 1951) using freshly prepared acetone powders of cells. Acetone powders were prepared by homogenizing the centrifuged cells with five volumes of cold acetone (-20°C) for one minute using a Virtis homogenizer, then filtering with suction. The residue was washed several times with cold acetone, dried in air for 30 minutes and stored in the freezer.

For protein determination, 100-mg samples of the acetone powder were mixed with 5 ml of Tris-HCl buffer, pH 8 and the mixture was left in the refrigerator for 30 min with periodic shaking. It was then centrifuged at 15,000 r.p.m. (I.E.C., 8 X 50 ml head) for 20 min and the supernatant was collected. The reagents used in the protein determinations consisted of:

Solution A: 2% Na_2CO_3 in 0.1N NaOH

Solution B: 0.5% $CuSO_4 \cdot 5H_2O$ in 1% sodium-potassium tartrate

Solution C: 50 ml solution A + 1 ml solution B

Solution D: 1N Folin reagent (Fisher Scientific Co).

One 100µl of the protein solution was diluted to 1.0 ml with Tris-HCl buffer, mixed with 5 ml of solution C and allowed to stand for ten minutes, after which 0.5 ml of solution D was added and mixed rapidly. The optical density of the solution was determined after 30 min, at room temperature, at 600mµ in a Unicam SP-800 spectrophotometer. Results were compared with a standard curve which was prepared with bovine serum albumin (J.T. Baker Chemical Co.).

4. Microscopic Examination of Cell Cultures

The cell cultures were examined microscopically at each sampling in order to determine the shape of the cells and to estimate the proportion of free cells to cell clumps. Photomicrographs of the cells were also taken at the various stages of growth.

5. Preparation of Cell Culture Extracts

The cells were harvested from the culture medium by centrifugation and the wet cells were crushed with hot alcohol (95%) using a Potter-Elvehjem homogenizer (Bellco Glass Inc., Vineland, N.J.). The crushed cells were then transferred to a glass thimble of a soxhlet extractor and were extracted with 80% ethanol for twelve hours. The

alcoholic extracts were flash evaporated under reduced pressure and the residue was dissolved in a minimum amount of 80% alcohol (ca 0.5 ml) and used for chromatography. This extract contained the free phenolic acids and the soluble phenolic esters.

The alcohol-insoluble residue was dried in a vacuum oven at 40°C and stored in the freezer until needed for analysis. This residue contained the phenolic compounds chemically bound to the cell wall material.

6. Hydrolysis of the Alcohol-Soluble Extracts and Cell Wall Residues

Alcoholic extracts were flash evaporated and the aqueous residue was suspended in a suitable volume of 2N NaOH. Dry powders were suspended directly in the alkali. About 2 ml of alkali were used per 100 mg of the dry powder. The vials containing the reaction mixture were flushed with nitrogen gas and allowed to stand for two hours at room temperature in the dark. This treatment cleaved the ester linkages associated with phenolic compounds. The alkaline hydrolysis was then acidified to pH 2, using 5N HCl, and heated on a boiling water bath for one hour in order to break any glycosidic bonds present. The acid hydrolysate was liquid-liquid extracted with ether and the latter was flash evaporated to dryness. The residue was then taken up

in 80% ethanol for chromatography.

7. Chromatographic Analysis

The extracts were chromatographed on thin-layer plates coated with cellulose. The plates were prepared by mixing 27 g of cellulose powder (Avicel SF, F.M.C. Corp., Penn.) with 120 ml of distilled water in a blendor for one minute and spreading the mixture, 0.3 cm thick, on five 20 X 20 cm glass plates using a spreader (Desaga, Heidelberg, Germany). The plates were air-dried overnight before use. The solvent system used for the separation of the phenolic compounds consisted of benzene-acetic acid-water (10:15:1), which formed a single phase.

Previous assays with different solvent mixtures showed that this ratio offered optimum separation of the phenolics in the extract. When two-dimensional chromatography was performed 15% aqueous acetic acid was used for the second direction.

8. Nitrobenzene Oxidation of the Alcohol-Insoluble Residue

The cell wall residue before being analysed for lignin monomers was thoroughly extracted in a soxhlet with 95% ethanol, ethanol-benzene mixture (1:1) aand pure benzene, in sequence, in order to remove all extractives. The method used for the degradation of cell wall constituents was a modification of that of Stone and Blundell (1951). Fifty mg

of the dry residue was transferred to a steel bomb together with 2 ml of 2N NaOH and 20µl of nitrobenzene. The mixture was heated in a sand bath at 150°C for various periods of time ranging from30 min to 4 hours. The mixture was allowed to cool, diluted with water, and then extracted with ether to remove the excess nitrobenzene. The aqueous layer was further acidified, flash evaporated to dryness, and the residue was dissolved in alcohol for chromatography.

9. Quantitative Determination of Phenolic Compounds

The phenolic compounds were assayed by a direct in situ fluorometric method (Ibrahim, 1969) using a Turner Filter Fluorometer model 111 (Palo Alto, Calif.). This instrument allows fluorometry of fluorescent compounds directly on TLC plates. The plate holder is moved at a fixed scanning speed (2 cm/min) past the scanning slit which has a fixed height (15 mm) and a variable width. The 110-810 UV-lamp was combined with the 7-54 and 34A filters for primary activation while the 2A filter was used for secondary screening. This combination gave an exitation peak at 325mµ and an emission peak at 415mµ. The output of the fluorometer was connected with a recorder (Servo Riter II, Texas Instruments, Inc.) with variable chart speed which transformed the fluorometric readings into two-dimensional peaks. The areas under the peaks were determined with the aid of a planimeter, and the corresponding values were

determined from a standard curve which was prepared by running authentic samples of the known compound in the same manner.

The chromatograms were prepared by banding the extracts as narrow bands, 4 cm long, along one end of the TLC plate. After development, the extract was resolved into bands of the constituent phenolics which were visualized in UV-light. The thickness of the bands did not exceed the slit height of the instrument (15 mm). These bands were then assayed by scanning along the length of each band, which ensured that the entire band was quantitatively measured. This method was found to be superior to that of measuring single spots (Ibrahim, 1969) since a circular spot has a tendency to spread much wider than a band during chromatography.

10. Isotope Feeding and Determination of Radioactivity

Duplicate suspension cultures were administered, under aseptic conditions, 2μCi of cinnamic acid-2-^{14}C (sp. act. 1.4 mCi/mmole, I.C.N. Tracerlab, Irwine Calif.) per flask. The initial inoculum consisted of approximately 0.5 g fresh weight cells obtained at the early stationary phase. The administration of label was carried out at weekly intervals in order to study the incorporation of the phenolic precursor into the alcohol-soluble fraction and cell wall residue throughout the entire period of culture growth.

Twenty four hours after introduction of the label, the cells of a duplicate sample (two culture flasks) were filtered off and thoroughly washed with water and cold alcohol, while applying suction. The cells were then successively extracted with hot 95% and 70% ethanol and aliquots from the combined extracts were counted for radioactivity determination. The chromatographed extracts were autoradiographed using Medical X-Ray films (Kodak Royal Blue, R.B. 2) and the latter were developed in order to locate the radioactive compounds.

The amounts of caffeic acid and its ester were determined by fluorometry of triplicate samples. The spots were then carefully scraped off the TLC plates and transferred to scintillation vials containing 2g of powdered silica gel, in order to hold the cellulose powder into suspension, and 15 ml of scintillation liquid. The latter consisted of 5 g/l 2,5-diphenyloxazol (P.P.O. Spectra Fluor T.M., Nuclear Chicago, Illinois) in spectral grade toluene.

Aliquots (ca. 5mg) of the alcohol insoluble (cell wall) residue were combusted in duplicates by means of the Schöniger flask method. Six ml of phenylethylamine-methanol-toluene (1:2:1) mixture was used to absorb the $^{14}CO_2$, and triplicate-one ml samples of the radioactive mixture were assayed for activity. All radioactive samples were counted

using a three-channel Unilux II liquid scintillation spectrometer (Nuclear Chicago, Illinois) at an efficiency of approximately 80%. Counts per minute were converted to disintegrations per minute (D.P.M.). Quench correction was made by the channels ratio method using an efficiency calibration curve.

IV. RESULTS

1. Growth of Perilla Suspension Culture

Growth of Perilla suspension cultures was studied in order to determine the duration of the growth cycle and attempt to define the different phases of cell growth. Duplicate samples were taken at weekly intervals and were assayed for fresh weight, dry weight and protein content. The culture was initiated with an inoculum obtained from a four-week-old culture which was grown in the control medium. The latter contained 1.0 mg/l (5.4 μM) NAA and 0.1 mg/l (0.47 μM) kinetin. The growth curves are presented on a semi-logarithmic plot in order to define the growth phases and accentuate the early stages of protein synthesis and phenolic formation.

The growth curves for fresh and dry weights (Fig. 3) show a lag phase of approximately one week, followed by an exponential phase which continues for approximately two weeks, at the end of which it appears to level off reaching the stationary phase. Growth analysis shows that there was approximately a 10-fold increase in both fresh and dry weights at the early exponential phase, as compared with a three-fold increase for the remaining growth period. Although cells reach the stationary phase of growth after three to four weeks in culture, they, nevertheless, remain viable and can be subcultured in a fresh medium over a

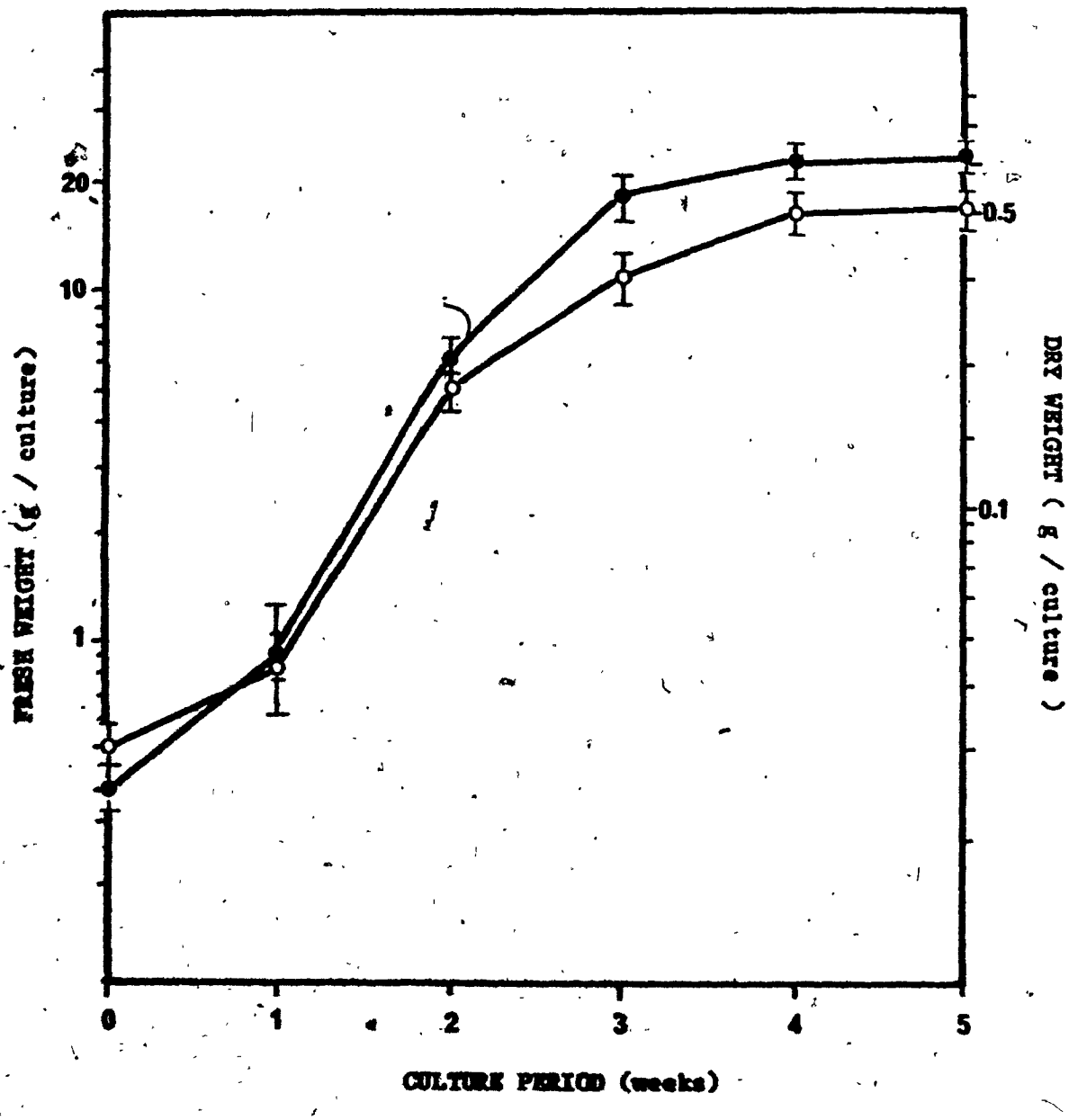


Figure 3. Fresh weights (-○-) and dry weights (-●-) of Perilla cells cultured in control medium.

further period of two weeks.

As expected the growth of Perilla cells was characterized by a rapid increase (approximately 3 to 5-fold) in the level of soluble protein during early exponential growth (Fig. 4). This is evident whether the protein content is calculated per culture (Curve A), or per gram fresh weight of cells (curve B). This was followed by a sharp decrease in protein content during the rest of growth cycle (curve B). Evidently, when the results are calculated per culture, the protein content appears to increase proportionately with growth. This representation certainly may seem unrealistic since late exponential and stationary cells usually grow by expansion, rather than by cell division and increased dry weight.

2. Induction of Secondary Metabolite Synthesis

A number of attempts were made to induce anthocyanin synthesis by Perilla suspension cultures. These attempts included exposure of the cultures to different light intensities and photoperiods as well as exposure to low temperature (ca. 12°C) during the dark periods. Other cultures were established in order to determine the optimum requirements for both auxin and cytokinin in relation to growth of the culture and its potential for secondary metabolite synthesis. The same nutrient medium was supplemented with different levels of growth substances as shown in the

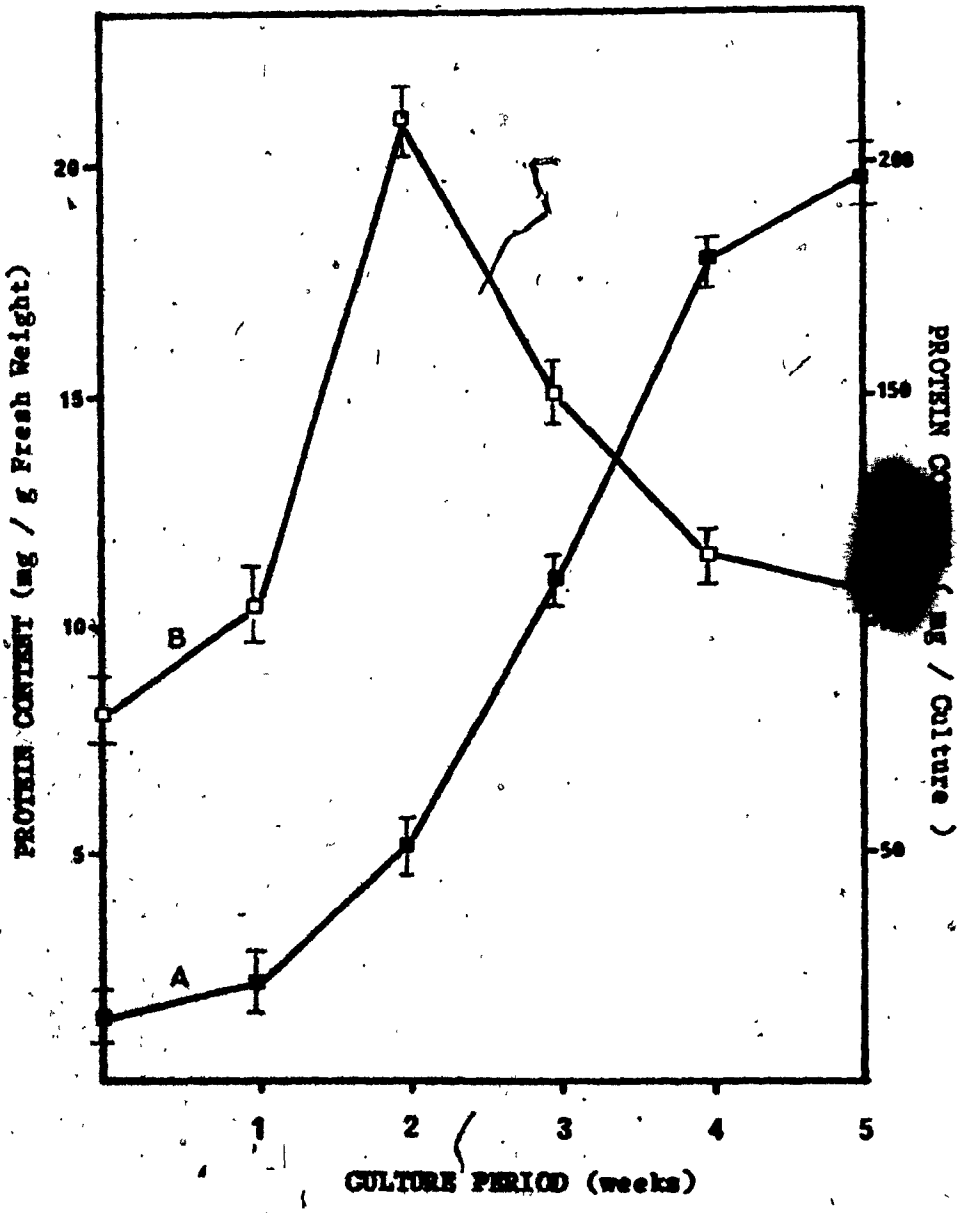


Figure 4. Protein content of Perilla cells cultured in control medium and represented as mg/g fresh weight (—□—) or mg/culture (—■—).

following table and the fresh and dry weights of duplicate samples were determined at weekly intervals.

Treatment	NAA		Kinetin	
	mg/l	Molar	mg/l	Molar
Control (C)	1.0	5.4×10^{-6}	0.1	4.7×10^{-7}
High auxin (HA)	5.0	2.7×10^{-5}	0.1	4.7×10^{-7}
High kinetin (HK)	1.0	5.4×10^{-6}	2.0	9.4×10^{-6}

The growth curves of both high-auxin (HA) and high-kinetin (HK) treatments are shown in Figure 5. These curves show that the overall growth of the HA treatment compares fairly well with that of the control culture (Fig. 3), whereas the HK treatment resulted in a decrease in both fresh and dry weights. Analysis of these curves (Fig. 5) shows that the HA treatment produced approximately 9-fold and 14-fold increases in fresh and dry weights compared with 3- and 8-fold increases for the HK treatment, respectively at the exponential phase. However, there was a rapid increase in growth rate of the HA culture during the remainder of growth which resulted in tissue yields comparable with those of the control culture. The HK treatment, on the other hand, maintained a slow but steady growth rate throughout the entire period.

It should be noted that while the kinetin concentration used with the HK cultures was 20 times higher than that of the control medium, however, it resulted in increased

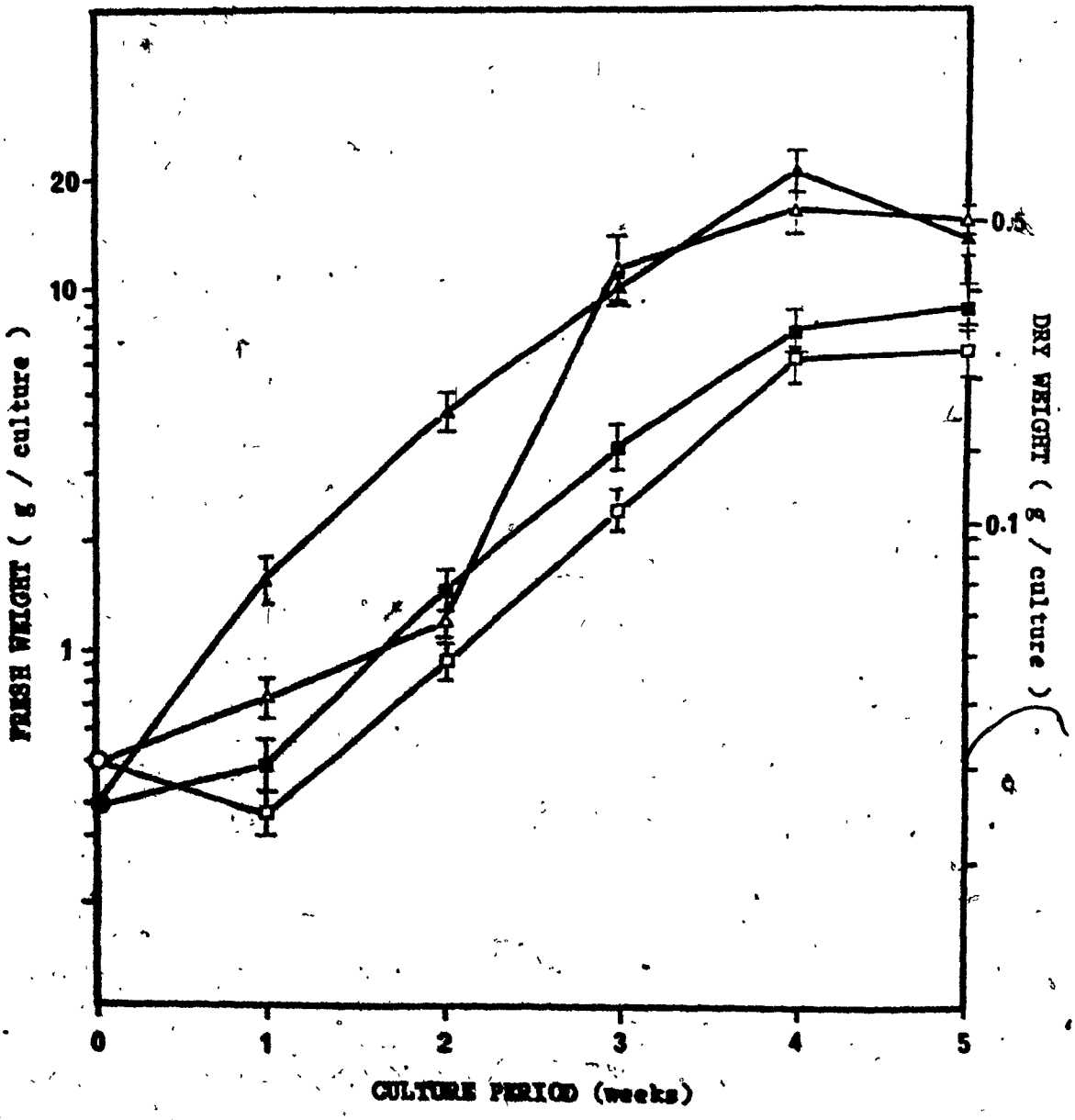


Figure 5. Fresh weights (open symbols) and dry weights (solid symbols) of Perilla cells cultured in high-auxin (-△-, -▲-) and high-cytokinin (-□-, -■-) media.

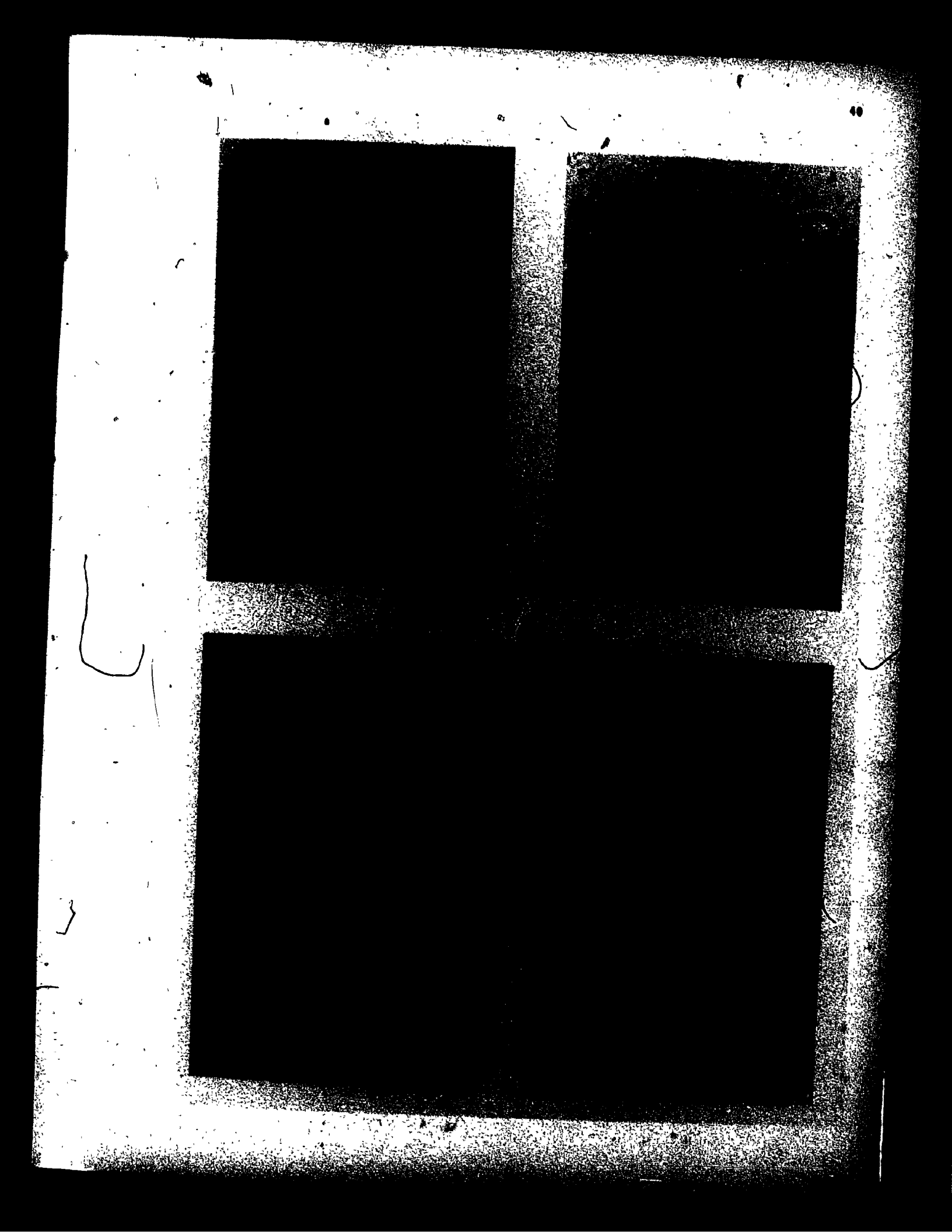
production of caffeoyl compounds as compared with the control cultures. HA treatment, on the other hand, caused a significant reduction in phenolic production (see section IV, 7). There was no formation of anthocyanin pigments in these experiments.

3. Growth Habit of Perilla Cultures

A suspension culture of Perilla, grown in the control medium consists of typically round cells, though some cells may be oval or elongated in shape, with well defined nuclei and cytoplasm (Fig. 6 a-d). The cells are surrounded by thin walls characteristic of cultured cells. The culture usually consists of a mixture of free cells and cell clumps of various sizes, depending on culture conditions and the stage of culture growth. The colour of culture is light brown though chloroplasts may be seen in young cells. This colour becomes increasingly more brown as the culture approaches senescence.

Stationary-phase cultures usually consist of about 80% free cells and cell pairs with the remaining 20% consisting of cell clumps of various sizes ranging from few to approximately hundred cells. When an inoculum of cells in the stationary phase is transferred to a fresh medium the proportion of free cells to cell clumps is apparently reversed so that by the end of the lag phase of growth the culture consists of approximately 90% clumps and 10% free

Figure 6. *Perilla* cells cultured in the control medium showing typical exponential-phase cells of various shapes, a,- d (420X).



cells (Fig. 7a). During the exponential phase of growth (Fig. 7b) this proportion gradually shifts to yield the ratio found at the stationary phase.

If a filtered inoculum consisting of a large proportion of free cells is cultured in a fresh medium then growth of the culture is characterized by an extended lag period. This and the above observations may indicate that initiation of cell growth in Perilla cultures takes place among the large clumps and that the high proportion of free cells observed at the stationary phase may be the result of their sloughing off from the large clumps. Cell division may be noticed in single free cells (Fig. 6d) or in small clumps (Fig. 7a) consisting of few cells. It may be that these cells, however, have been sloughed off from the large clumps during cell division.

It is interesting to note that in HA cultures there appears to be, at all times, a higher proportion of cell clumps (Fig. 7c) than in the control culture, which is maintained until the stationary phase of growth. The HK cultures, on the other hand, maintain a population of approximately 75% large clumps and 25% of small clumps and free cells throughout the culture period (Fig. 7c).

4. Identification of the Phenolic Constituents of Perrilla Cultures

The alcohol-soluble fraction of Perilla cells was subjected to two-dimensional chromatography in order to

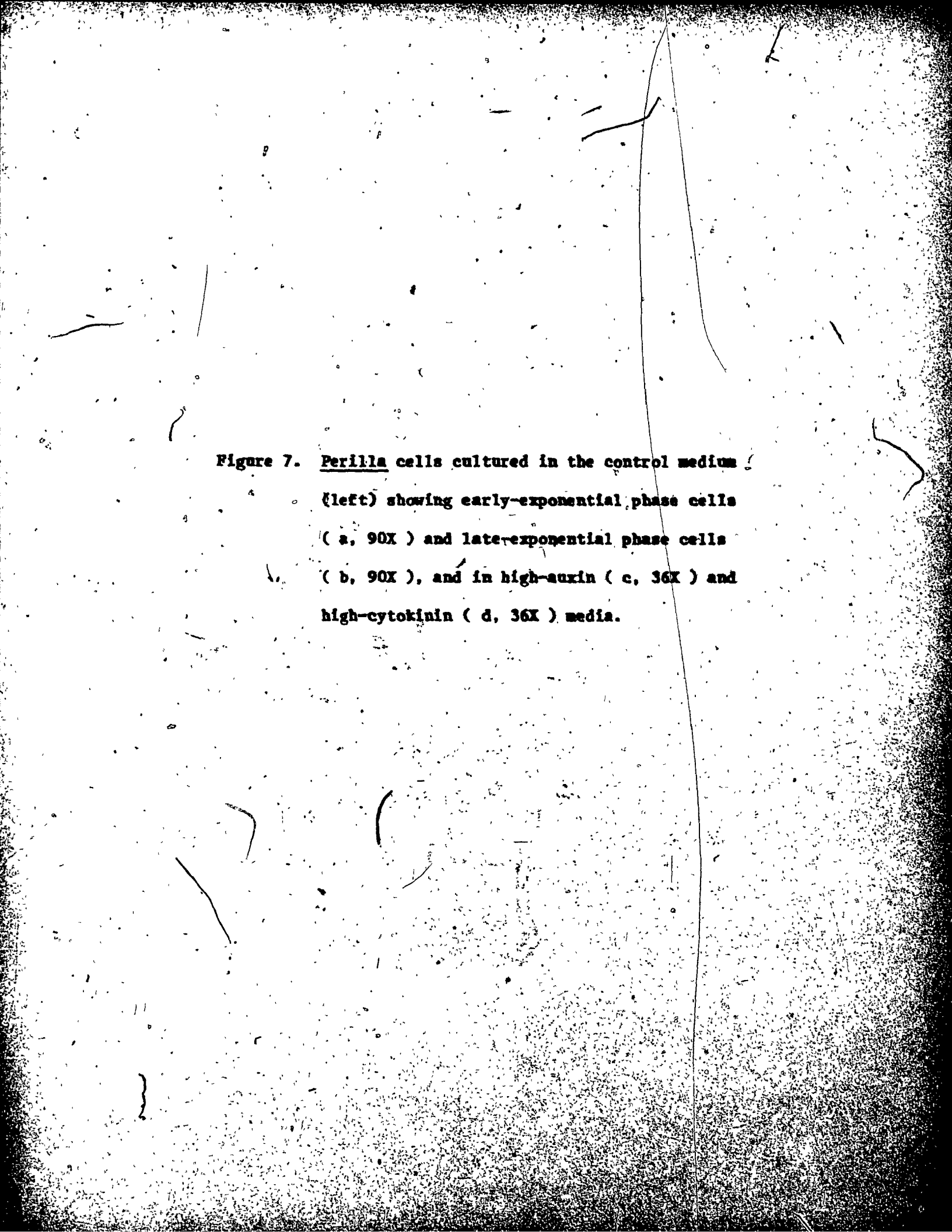
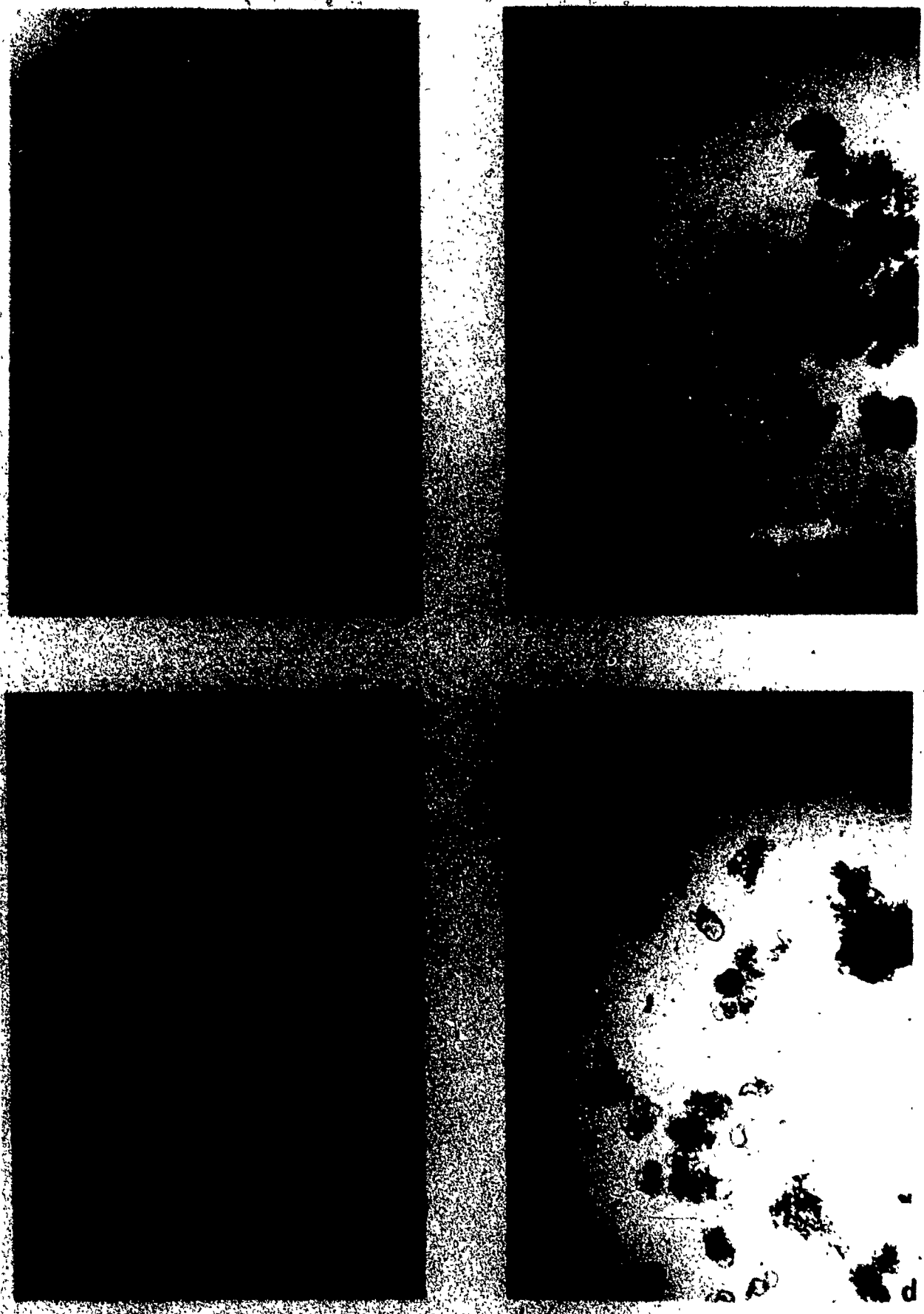


Figure 7. *Perilla* cells cultured in the control medium (left) showing early-exponential phase cells (a, 90X) and late-exponential phase cells (b, 90X), and in high-auxin (c, 36X) and high-cytokinin (d, 36X) media.


d

reveal its phenolic constituents. The phenolic pattern (Fig. 8) was found to consist of two major fluorescent compounds, 1,1' and 2,2'. Three minor spots were also visible in UV-light, however their small concentration did not allow for their identification.

The major compounds were identified as a caffeoyl (3,4-dihydroxycinnamoyl) ester and free caffeic acid, respectively. Each compound usually appeared as two spots when chromatographed in aqueous solvents due to isomerization into trans- (low R_f) and cis- (high R_f) forms. Whereas the bound form of caffeic acid (spot 1,1') was susceptible to mild alkaline hydrolysis and yielded free caffeic acid, hydrolysis with 2N HCl, for 10-15 min did not liberate the phenolic moiety. Further hydrolysis resulted in destruction of the caffeoyl residue. This indicates an esterified, rather than glycosylated, nature of this compound. The alkaline hydrolysis products were chromatographed in several solvents in order to identify the esterifying moiety. This was done by examination of the TLC plates in UV-light and the use of specific spray reagents. Apart from caffeic acid, no other phenolic moiety, cyclic organic acid (quinic, shikimic) or sugar was found among the hydrolysis products. The possibility of its being chlorogenic (3-O-caffeoylquinic) acid was also eliminated by co-chromatography with an authentic sample. It was concluded that this caffeoyl

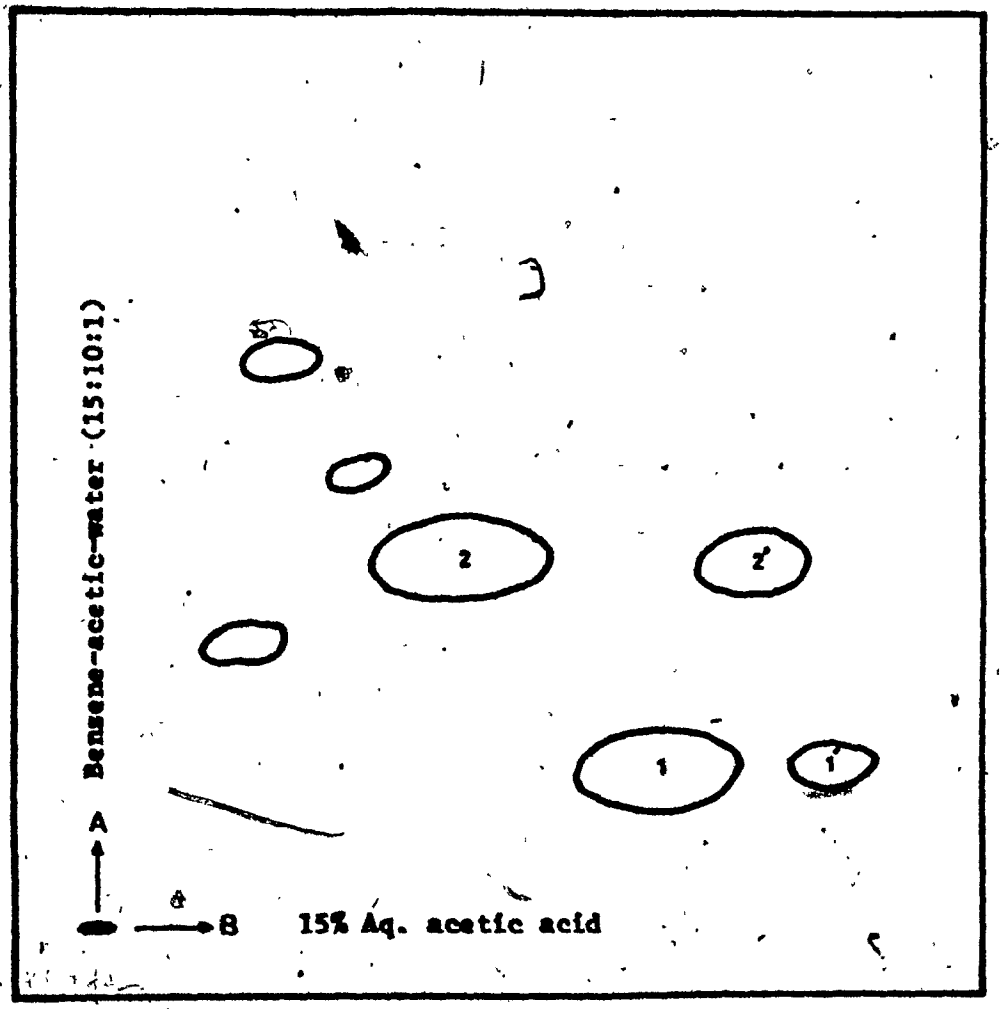


Figure 8. Diagram of a chromatoplate showing two-dimensional separation of the phenolic constituents of Perilla cells.

compound is possibly a glucose ester where the sugar moiety would be destroyed during hydrolysis with alkali. Furthermore, its high R_f values in aqueous solvents also supports this view.

Caffeic acid was identified by (a) co-chromatography with an authentic sample (Light's Chemicals, Bucks, England) in five different solvent systems, (b) fluorescence in UV-light, (c) colour reaction with diazotized sulfanilic acid reagent, (d) UV-absorption spectra in methanol and spectral shifts with sodium acetate and boric acid (Fig. 9) and its infra-red spectrum (Fig. 10). These results are summarized in Table 4.

Further evidence for the identity of both compounds was obtained by administering L-phenylalanine-U-^{14}C and trans-cinnamic acid-2-^{14}C to one-week-old suspension cultures under aseptic conditions for 24 hours. Almost 80% of the label taken up by the cells was incorporated into both compounds in a ratio of approximately 2:1 for the ester and free acid, respectively. The dynamics of their biosynthesis is presented in a later section (see Section IV, 8).

5. Degradation of Cell Wall Residue

The extractive-free cell wall residue was degraded using the alkali-nitrobenzene oxidation method in an attempt to identify any phenolic compounds that may be incorporated

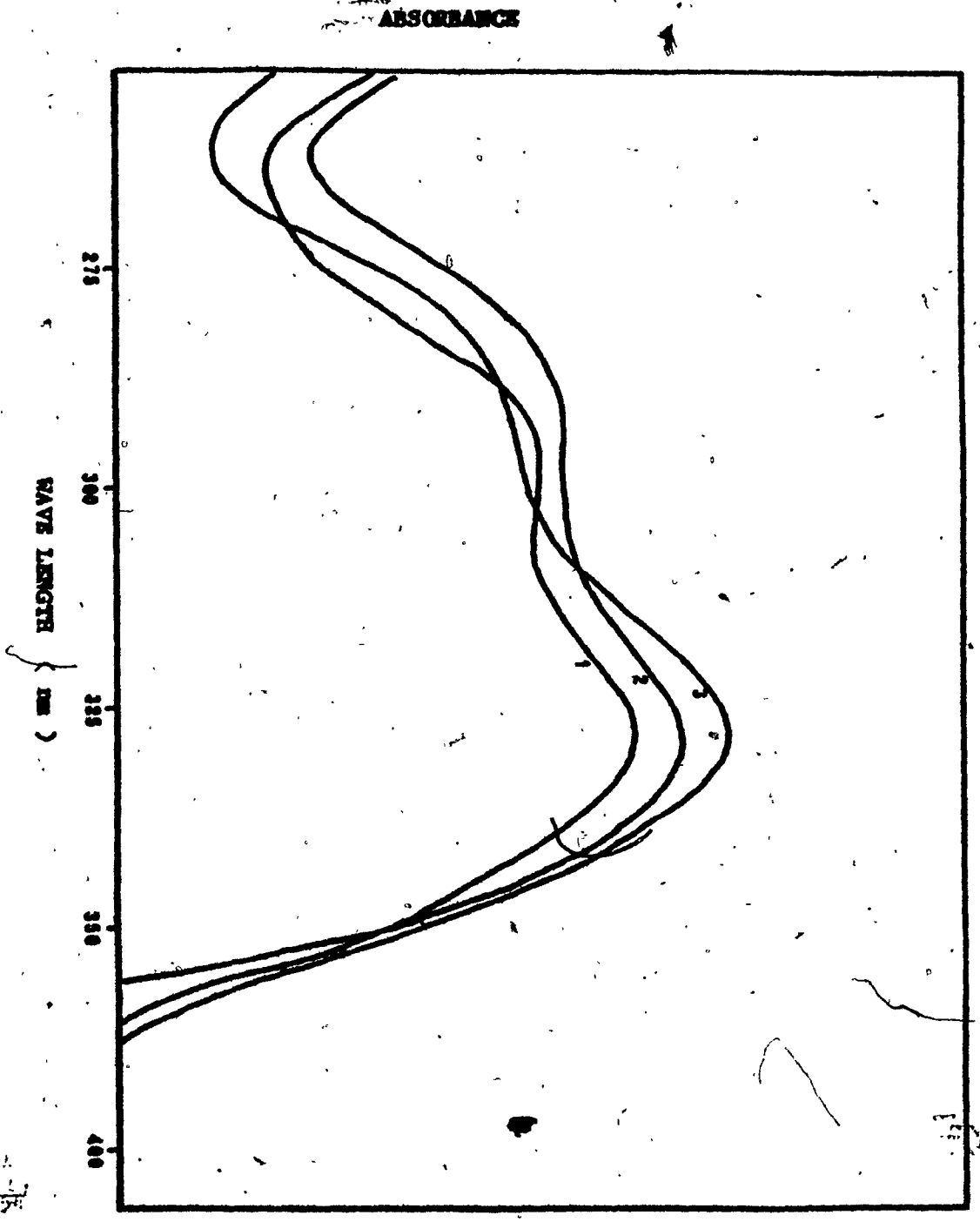


Figure 9. Ultraviolet absorption spectra of 1, authentic caffeic acid; 2 and 3, caffeoyl ester and caffeic acid respectively, isolated from Perilla cells.

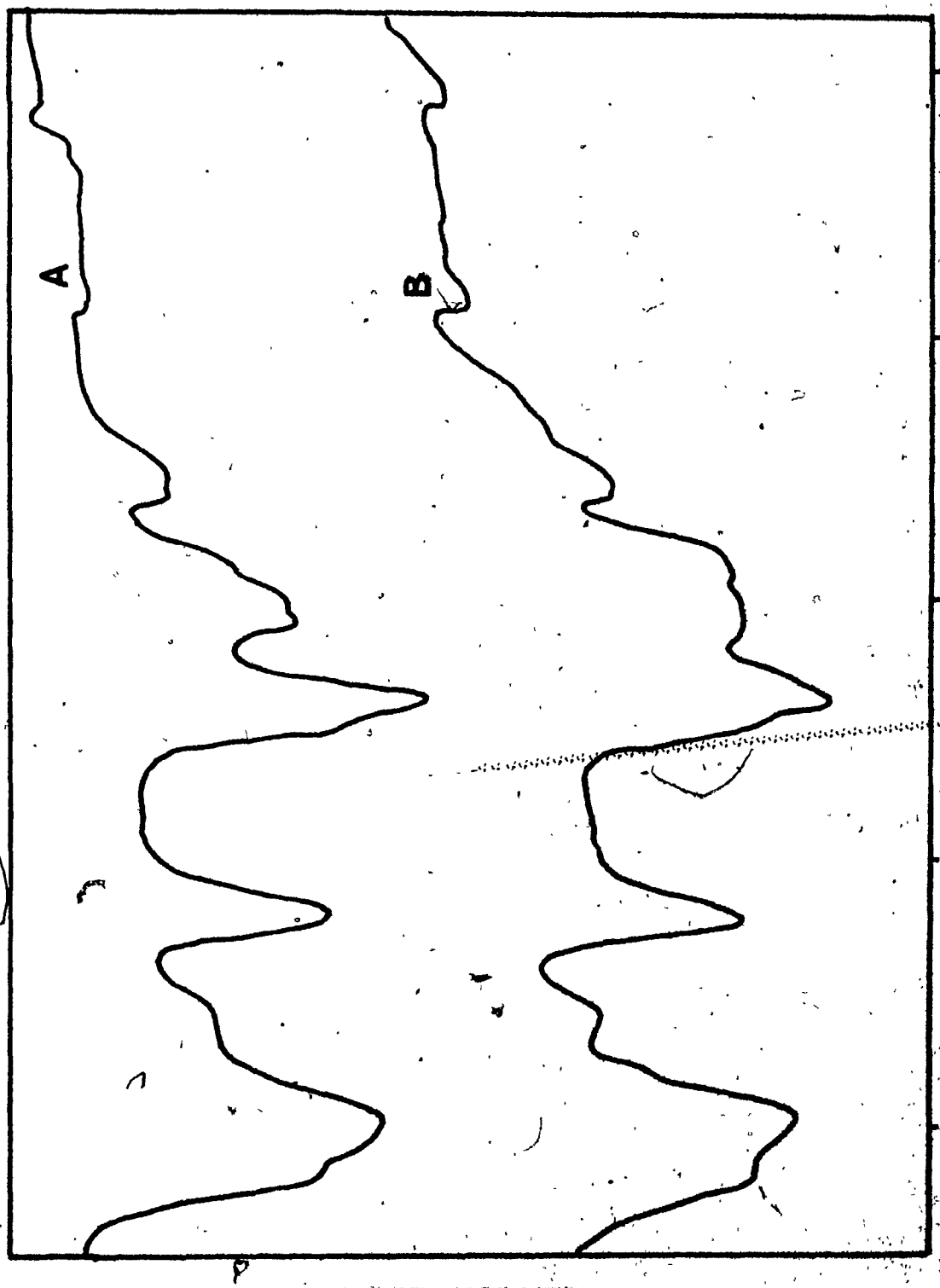


Figure 10. Infra-red spectra of standard caffeic acid (curve A) and a 1:1 mixture of caffeic acid and caffeoyl ester (curve B) isolated from Perilla cells.

Table 4: Comparison of the chromatographic and spectral characteristics of caffeic acid, isolated from Perilla cultures with those of an authentic sample.

Characteristics	Alkaline hydrolysis product (spot 1)	Caffeic acid (spot 2)	Authentic caffeic acid
R_f values (X 100) in*:			
Solvent A	46	46	46
Solvent B	16	16	16
Solvent C**	58,75	58,75	57,75
Solvent D	91	91	91
Solvent E	78	78	78
UV-maxima (nm)			
- MeOH	329,291^{sh}	328,290^{sh}	328,293^{sh}
MeOH + H_3BO_3	360,315	360,315	360,315
IR-maxima (cm^{-1})	1590 1440 1275	1590 1440 1275	1600 1440 1275
Colour in UV-light (366 nm)			
- NH_3 vapors	Blue	Blue	Blue
+ NH_3 vapors	Bright blue	Bright blue	Bright blue
Colour reaction with:			
Diaz. sulfanilic acid	Yellow	Yellow	Yellow
Diaz. p-nitroaniline/ NaOH	Blue	Blue	Blue

* Solvent systems (v/v): A, Benzene-acetic acid-water (15:10:1); B, same as in A (125:72:3); C, 15% Aqueous acetic acid; D, tert-Butanol-2N HCl-acetic acid-water (6:1:1:2); E, n-Butanol-acetic acid-water (4:1:2.2).

**Trans- and cis-isomers respectively.

into cell wall material. After a number of attempts no typical lignin degradation products, namely vanillin and syringaldehyde, were obtained. Evidently, the absence of ferulic (3-methoxy-4-hydroxycinnamic) acid (Fig. 2) in the soluble pool of phenolic compounds excludes the possibility of recovering vanillin from the cell wall degradation products.

The cell wall residue was also hydrolyzed with 4N NaOH at room temperature in order to liberate any phenolic compounds that may be esterified with cell wall polysaccharides. Trace amounts of caffeic acid were observed on chromatograms of hydrolyzed extracts. However, the incorporation of caffeoyl residues into cell wall material has been reported in a number of species (Brown and Neish, 1956; Taylor and Zucker, 1966).

6. The Content of Caffeoyl Compounds During Culture Growth

The content of both caffeoyl compounds, in cultures grown in the control medium, was determined as µmoles caffeic acid per culture (Fig. 11) or per gram fresh weight of cells (Fig. 12), and was expressed in relation to growth parameters. These experiments were repeated at least twice and both the patterns of growth and phenolic contents were found to be consistent.

Figure 11 shows that active accumulation of caffeoyl compounds begins during the lag phase and continues throughout

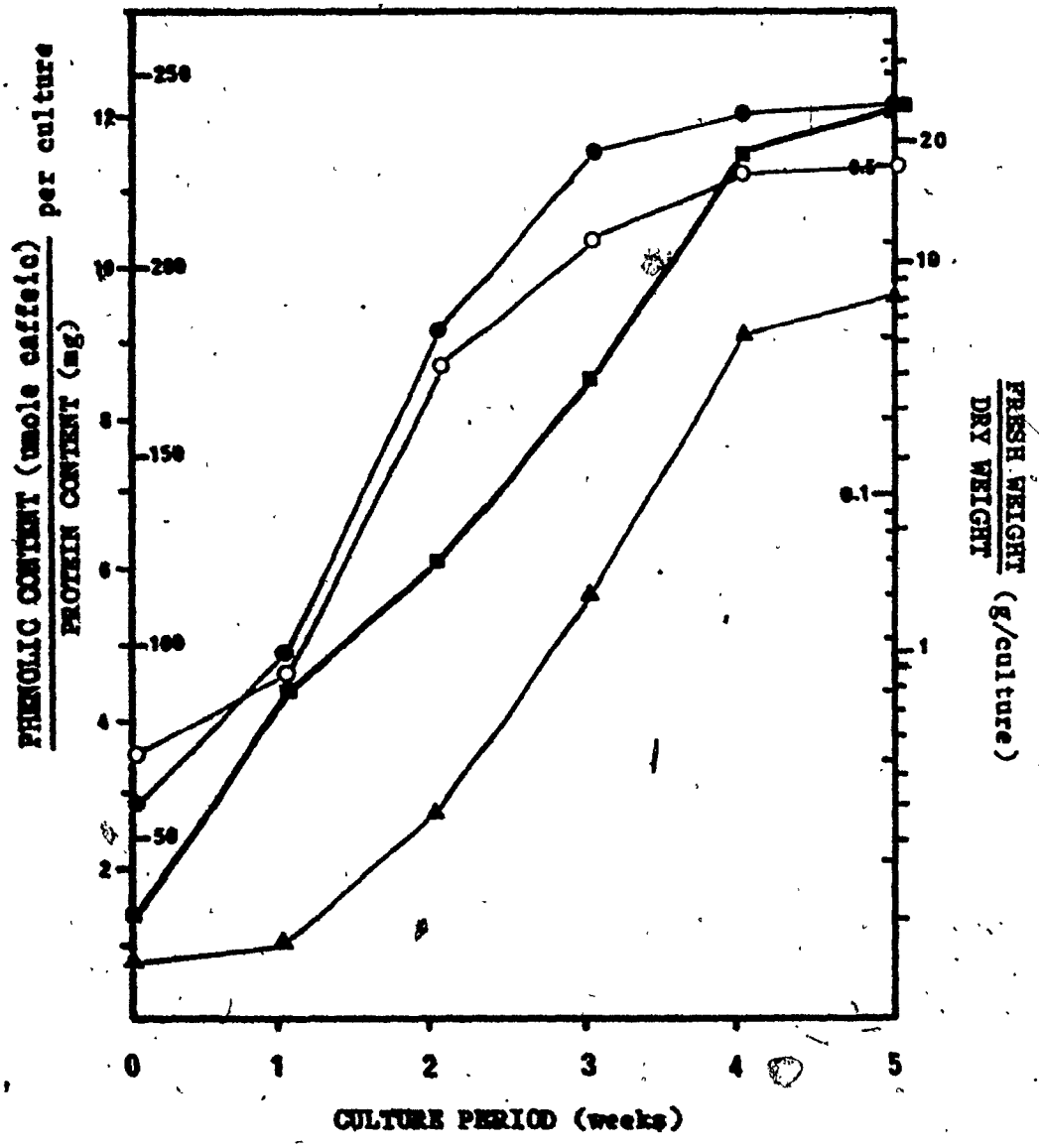


Figure 11. Phenolic content (-■-) of Perilla cells in relation to fresh weight (-○-), dry weight (-●-) and protein content (-▲-), all caculated per culture.

exponential growth. There is approximately three-fold increase in the rate of accumulation after one week in culture as compared with a 3-fold increase during the rest of culture growth. This rate of accumulation closely parallels the growth rate of the suspension culture. Stationary phase cells exhibit not only a slow rate of phenolic synthesis, but apparently begin to break and lose some phenolics to the external medium. Trace amounts of caffeoyl compounds were detected in the culture medium of stationary cells.

When both protein and phenolic contents are expressed on fresh weight basis (Fig. 12) a different relationship appears with respect to growth. Again, the content of caffeoyl compounds reaches its maximum at the early exponential growth, preceding that of protein. There is a sharp decline however, during mid-exponential phase, followed by a low, but steady rate of accumulation throughout the remaining growth cycle. When the increased tissue yield during exponential growth is taken into consideration, then such apparently low rate of accumulation, actually represents a state of active phenolic synthesis.

The characteristic feature of both situations (Figs. 11 and 12) is that initiation of caffeoyl synthesis takes place during the lag phase and the maximum phenolic content is attained during the early exponential phase of culture growth, when both tissue yields and protein content continue

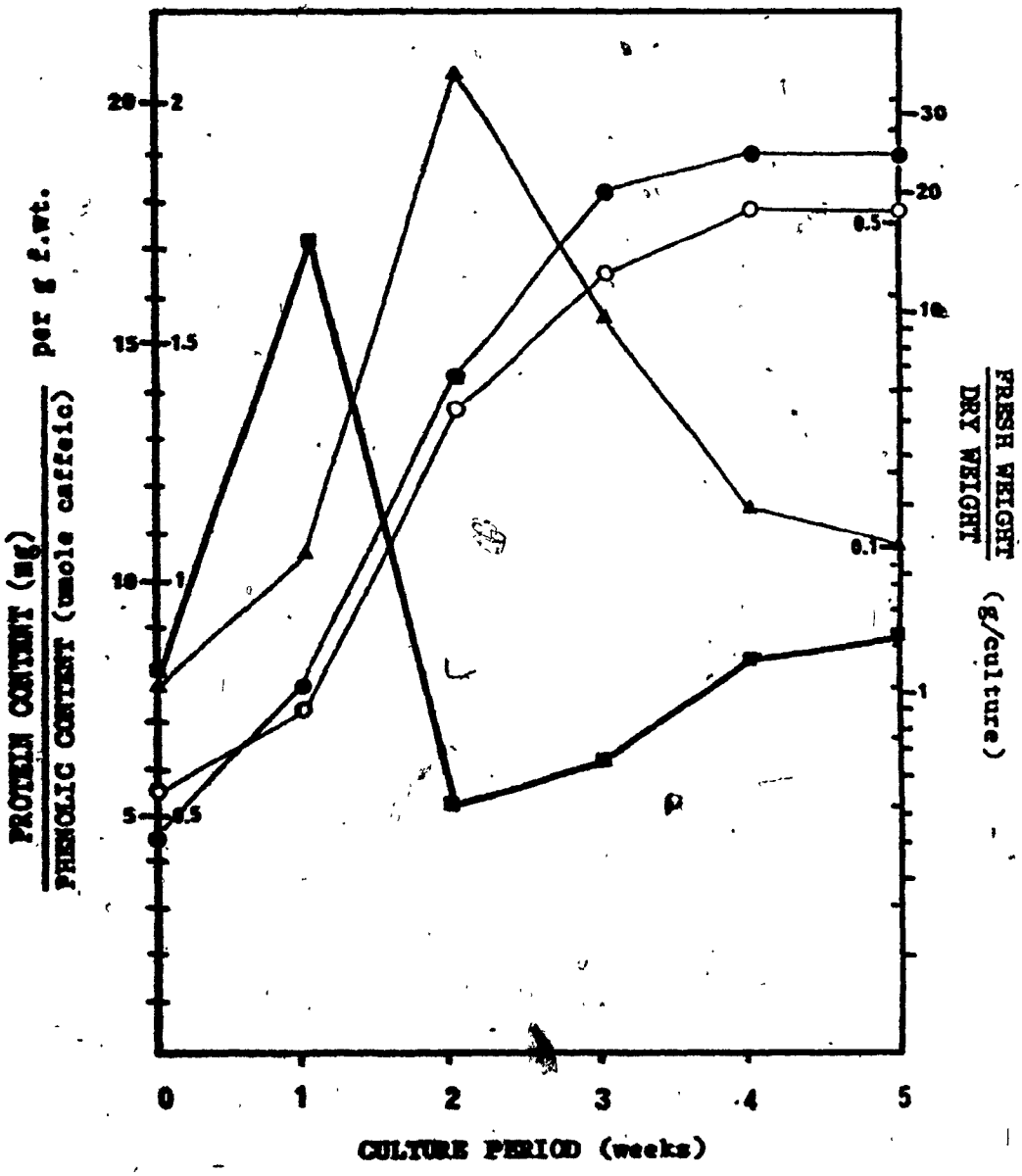


Figure 12. Phenolic content (-■-) of Perilla cells in relation to fresh weight* (-○-), dry weight* (-●-) and protein content (-▲-), calculated on fresh weight basis (*, per culture).

to increase. It should be noted, however, that the presentation of results on the basis of fresh weight offers more realistic expression of metabolite formation in relation to culture growth.

7. The Effects of NAA and Kinetin on the Content of Caffeoyl Compounds During Culture Growth

The effect of high levels of growth substances on the content of both caffeoyl compounds is shown in Figure 13. The data in this figure has been obtained from two sets of experiments involving duplicate samples and was found to be reproducible. Cultures grown with high kinetin (9.4×10^{-6}M) exhibited a significant increase in both caffeoyl compounds over that of the control (4.7×10^{-7}M) after one week in culture. Furthermore, HK resulted in the extension of the period over which synthesis of the ester takes place, especially during exponential growth of the culture.

A high concentration of NAA (2.7×10^{-5}M), on the other hand, resulted in a 60% reduction in the amount of caffeoyl ester during the first week of culture growth. However, it maintained a higher rate of accumulation that of the control (5.4×10^{-6}M) during exponential growth of the culture. It is noticeable that, in almost all cases, the amount of caffeoyl ester is usually higher than that of the free acid by a factor of 3 to 5, especially during the period of maximum accumulation.

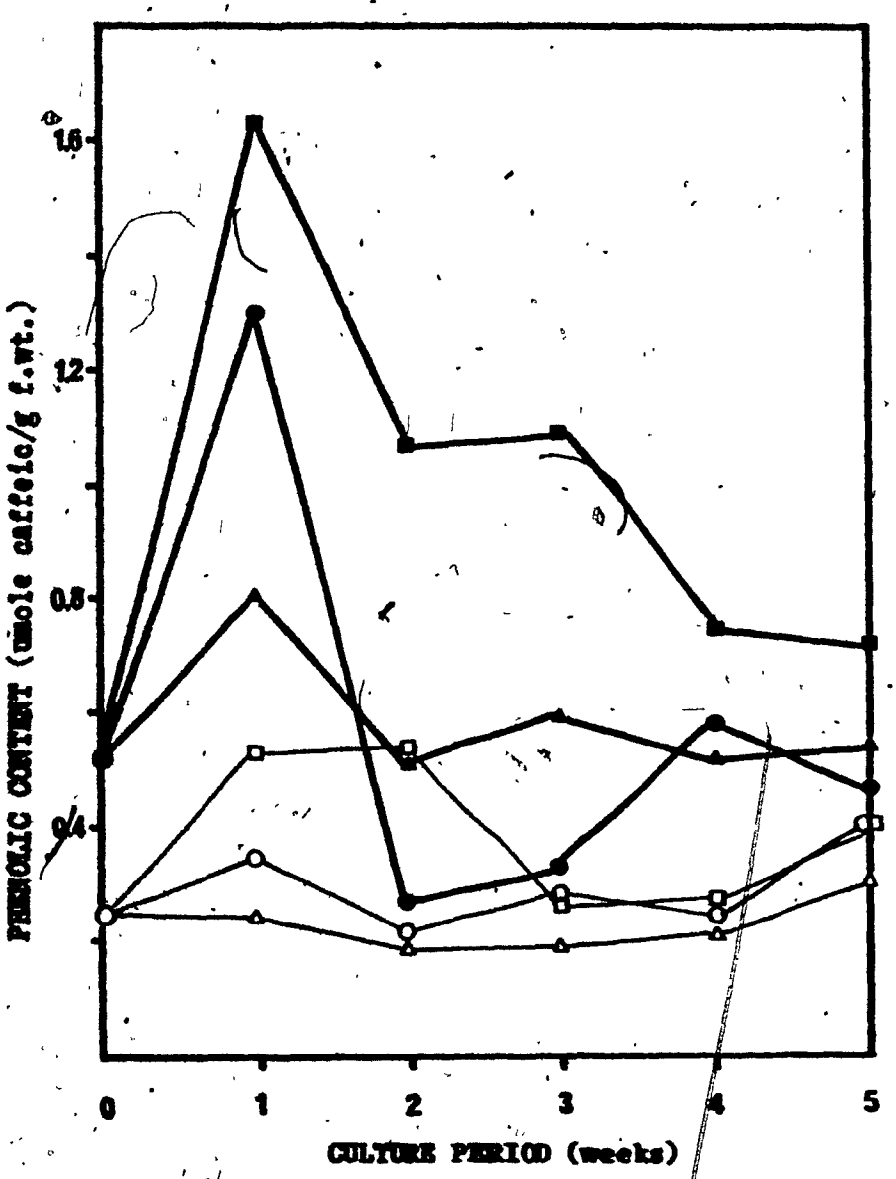


Figure 13. Caffeoyl ester (solid symbols) and caffeic acid (open symbols) contents of Perilla cells cultured in control (○), high-auxin (△) and high-kinin (□) media.

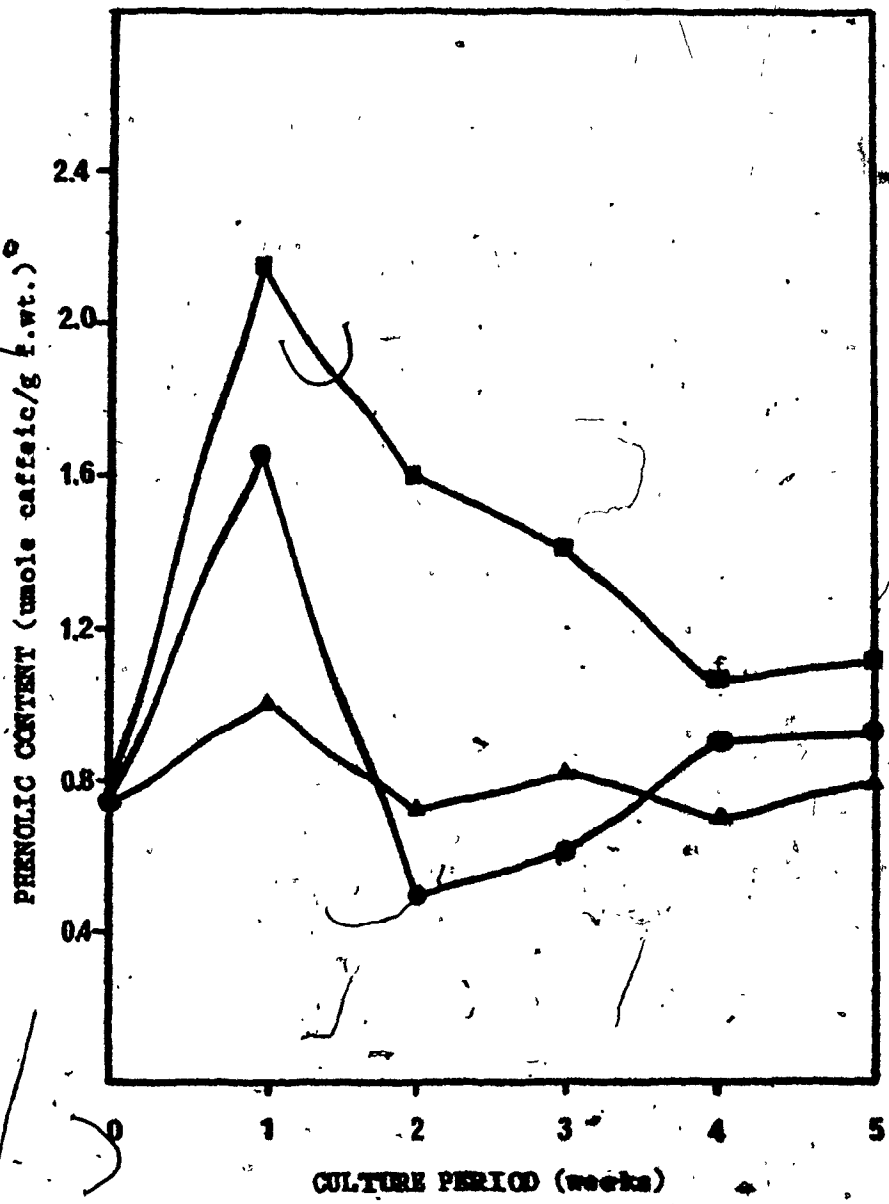


Figure 14. Total caffeoyl content of Perilla cells cultured in control (—●—), high-auxin (—△—) and high-kinin (—■—) media.

The overall effect of increasing the levels of growth substances (Fig. 14) is that auxin reduced, and cytokinin increased, the caffeoyl content of the cells during early exponential growth over that of the control culture. Furthermore, treatment with HK maintained a higher level of caffeoyl compounds, 2 to 3 times over that of the control, during mid- and late exponential growth. It should be noted, however, that regardless of the quantitative changes there was no alteration in the pattern of accumulation of these compounds in relation to culture growth.

8. Incorporation of Cinnamic acid-2-^{14}C Into Various Fractions of Perilla Cultures

In this experiment, the labelled precursor was administered to duplicate suspension cultures at different stages of growth and were incubated for 24-hour-periods. The cultures were then analyzed for incorporation of label into the soluble fraction and cell wall residue, as well as the specific activities of caffeoyl compounds. The results, presented in Figure 15, show that there was a significant increase of label incorporation into the alcohol-soluble fraction during the lag phase of cell growth which reached its maximum after one week in culture. This was followed by a sharp drop in activity, then a slow, but steady decrease throughout the remaining culture period. Such a pattern of

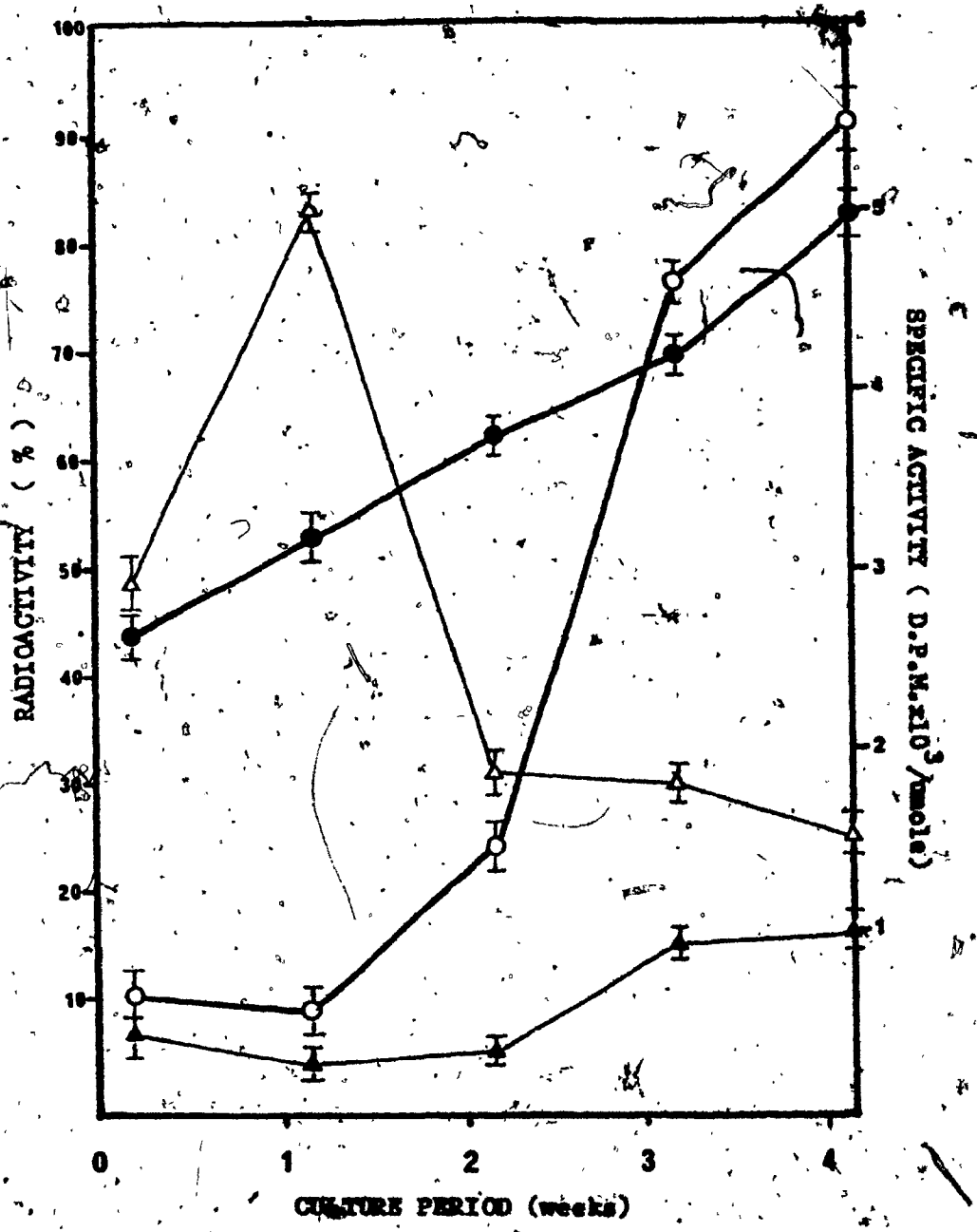


Figure 15. Incorporation of label from trans-cinnamic acid-2-^{14}C into alcohol-soluble (-△-) and alcohol-insoluble (-▲-) fractions; and the specific activities of caffeoyl ester (-●-) and caffeic acid (-○-) in Perilla cells.

label distribution appears to be consistent with the accumulation of caffeoyl compounds during culture growth (see Section IV, 6 and Fig. 12).

In contrast with the high incorporation of label into the soluble fraction, the alcohol-insoluble residue exhibited a consistently low level of radioactivity, except for a slight increase near the end of growth. This may not be unexpected, since the growth of Perilla cultures proceeds without significant cell differentiation or organization. The nature of phenolic compounds which contribute to the radioactivity of the cell wall fraction could not be determined, since alkaline nitrobenzene oxidation of the alcohol-insoluble residue yielded none of the known lignin aldehydes. However, the recovery of small amounts of caffeic acid after alkaline hydrolysis of the wall fraction indicates incorporation of phenylpropanoid moieties into cell wall material.

Regardless of the changes in label incorporation into the soluble fraction during culture growth, it appears that the specific activity of both caffeoyl compounds exhibits a slow, but steady increase throughout culture growth (Fig. 15). This indicates active, de novo synthesis of a large pool of phenolics, especially when the rate of their accumulation during growth (Fig. 12) is taken into consideration and would result in actual dilution of label.

Whereas the specific activity of the ester form was two to four times higher than that of the free acid during the lag and exponential phases of growth, both values however, reach approximately equal levels near the stationary phase. These results reflect the pool size of caffeoyl compounds in relation to tissue yields at this stage of growth and suggest a possible turnover of both the ester and free acid during later growth stages.

V. DISCUSSION

Suspension cultures of Perilla grown in a defined nutrient medium exhibited typical growth curves consisting of the lag, exponential and stationary phases. These curves are similar to those described for other suspension cultures (Henshaw et al., 1966;Davies, 1972a, Hahlbrock et al., 1972). The doubling time for dry weight and protein content of the control culture, at mid-exponential growth, was approximately three and four days respectively.

Despite its tendency to form cell aggregates, this culture offers the advantage of undifferentiated, unorganized growth after passing through several subcultures. The morphogenetic expression of suspension cultures to form embryoids (Steward et al., 1958;1969), shoots and roots (Thomas and Street, 1970) and even plantlets (Thomas and Street, 1972) is not uncommon and depends upon, among other factors, the medium composition (Street, 1969;Tazawa and Reinert, 1969) and the number of passages through which the suspension has been subcultured (Thomas and Street, 1972). The uniformity in growth of Perilla cultures, especially in presence of high levels of growth substances, is desirable for metabolic studies.

The loss of biosynthetic potential for perillanin (Fig. 1), the characteristic pigment of the species, may not be unexpected. When cells or tissues are cultured in vitro

they are actually brought into a different physiological state characterized by resumption of growth by cell division and cell expansion. Therefore, it is conceivable that the particular physical, chemical and physiological environment of the culture medium may exert certain restraints on the biochemical expression of growing cells. Furthermore, several investigators have reported on the nuclear changes which occur in cultured tissues and cells (e.g. Patu and Das, 1959; Mitra and Steward, 1961; D'Amato, 1965, Lescure, 1970). Such changes may also be responsible for the altered biochemical expression of *in vitro* grown cultures (Krikorian and Steward, 1969).

Caffeic acid was identified with certainty in the free and conjugated (ester) forms, though the non-phenolic moiety of the latter could not be characterized. Whereas esters of caffeic acid are widely distributed in plants, chlorogenic acid (3-O-caffeoylquinic) is however, considered to be the most ubiquitous ester form (Sondheimer, 1964). Other esters have also been reported, such as caffeoyl-shikimic, dicaffeoyltartaric and 3,5-dicaffeoylquinic acids (Taylor, 1965). However, it may not be unexpected that the caffeoyl ester in *Perilla* cultures constitutes another, yet unreported conjugated form.

The presence of caffeic acid, in free and bound forms, as the only phenolic metabolite of *Perilla* cells should be considered a unique characteristic; since the

pathway of phenolic acid biosynthesis, namely, shikimic → L-phenylalanine → trans-cinnamic → p-coumaric → caffeic → ferulic → sinapic (Fig. 2) - which has been repeatedly demonstrated with several species (see reviews by Neish, 1960, 1964; Towers, 1964; Yoshida, 1969) - results in the accumulation of these compounds, as well as their C_6-C_1 analogs, and their wide distribution in the plant kingdom. In contrast with this general situation, it may not be a mere coincidence that Perilla cultures synthesize the caffeoyl moiety as the only detectable intermediate and end product in the pathway of phenolic acid biosynthesis. Furthermore, inspite of the fact that the caffeoyl residue forms ring-B of anthocyanins (Grisebach, 1957) especially perillanin (Fig. 1) the suspension culture, however, failed to produce this characteristic pigment under any of the culture conditions previously described (Sect. IV, 2).

There is reasonable evidence to suggest that some metabolic products of phenolic acid biosynthesis in higher plants may serve as effector molecules which influence induction or repression of enzymes that catalyze other biosynthetic pathways (Zucker et al., 1967) in a manner similar to that known in bacteria (Monod and Jacob, 1961). In barley, it has been clearly shown that the activity of phenylalanine ammonia-lyase (PAL), the first enzyme in phenolic biosynthesis, is inhibited by its product, cinnamic acid, and by p-coumaric

acid, a further metabolite in the pathway (Koukol and Conn, 1961). The potato enzyme was found to be even more sensitive than that of barley to cinnamate (Havir and Hanson, 1966). The fact that there were no major fluctuations in enzyme synthesis or the amount of cinnamate formed, implied both a repression of synthesis and a feedback inhibition of enzyme activity by cinnamate (Havir and Hanson, 1966). Except for PAL, which has been extensively studied, the enzymes involved in anthocyanin synthesis are not well known. Thus, it is conceivable, that some regulatory mechanism operates in Perilla which results in blocking both anthocyanin formation and further production of other hydroxycinnamic acids. The nature of such a mechanism deserves further investigation.

The results obtained in this investigation clearly indicate that the ability to synthesize caffeoyl compounds by Perilla is almost restricted to lag phase and early-exponential phase cells, although not to the complete exclusion of later phase cells (Fig. 12). This is shown by the rates of incorporation of cinnamate label into the alcohol-soluble fraction and the specific activities of both caffeoyl compounds (Fig. 15). Their maximum accumulation during the early-exponential phase, which preceded maximum growth, is impressive and indicates that such compounds may be essential for mediation of culture growth, as has been

frequently suggested (Sondheimer, 1964; Zucker et al., 1967 and references cited therein). However, the significant drop in the rate of phenolic synthesis at mid-exponential phase may be the result of continued protein formation in support of active growth. This, evidently, causes limitation on the availability of carbon for phenylpropanoid synthesis. The intimate relationship betwen nucleic acid and protein synthesis on the one hand, and phenylpropanoid biosynthesis on the other (Davies, 1972b), may provide a mechanism for regulating the flow of carbon into primary and secondary metabolites in Perilla cultures. The slow rate of accumulation of caffeoyl compounds during late-exponential and early-stationary growth is not unexpected since the sugar level in the nutrient medium becomes a limiting factor and its deficiency contributes to concomitant limitation of growth and phenolic synthesis. Similar results have been recently reported with polyphenol production in Paul's scarlet rose suspension culture (Davies, 1972a).

The degree of label incorporation into the soluble fraction and the specific activities observed in both caffeoyl compounds (Fig. 15) are indicative of active phenolic synthesis and reflect the pool size of both compounds curing culture growth. These results are consistent with the view that phenylpropanoid synthesis proceeds via the conjugated, or ester forms, rather than the free acids

(Neish, 1960, 1964; Towers, 1964; Zucker et al., 1967). The presence of free caffeic acid in Perilla cultures, therefore, may be considered a breakdown product of its conjugated form. However, the significant increase in specific activity of the former compound, as compared with that of the latter, at mid-exponential growth may suggest a state of turnover, especially when the pool sizes of both compounds in the control culture (Fig. 12) and their probable incorporation into cell wall material (see later) are taken into consideration. Turnover, if it exists, would be masked by the increased synthesis of the ester form near the end of culture growth. In order to determine the rate of turnover, pulse-labelling would be difficult to achieve with suspension cultures since thorough washing of cell suspension and their transfer to fresh media results in a state of osmotic shock.

The extent of incorporation of cinnamate label into cell wall material (Fig. 15) may provide an estimate of the contribution of phenylpropanoid precursors to the non-lignified cell walls of Perilla. This poses a question as to the nature of phenolic compound(s) incorporated into the cell wall fraction. It would be inconceivable to recover any activity from the degradation products of cinnamoyl or caffeoyl moieties had they been incorporated into cell wall material, since they were labelled in C_2 of the three-carbon

side chain. Whereas lignin degradation products were not obtained from the cell wall fraction (Sect. IV, 5), however, mild alkaline hydrolysis of the extractive-free, cell wall residue yielded small amounts of caffeic acid. This indicates that caffeoyl residues actually participate, to some extent, in the formation of wall material of Perilla cells possibly by cross-linking with existing polysaccharides. Such process would also account for the turnover of caffeoyl compounds during culture growth. Similar findings have been reported with a number of intact tissues (Brown and Neish, 1956; Basyouni, et al., 1964; Taylor and Zucker, 1966). There is also the possibility that some labelled cinnamoyl residues, which are in contact with the outer surface of cells, would bind chemically to cell wall material and contribute in part, to the radioactivity of the alcohol-insoluble residue. The extent of incorporation would depend on the difference in accessibility of the exogenously supplied (cinnamoyl) and endogenously formed (caffeoyl) molecules to the sites of polymerization, namely the primary and secondary cell walls.

The effect of increased levels of growth substances on the regulation of caffeoyl synthesis in Perilla cultures proved to be of considerable interest. The increased rate of phenolic accumulation at a high cytokinin level is consistent with the reports of anthocyanin formation in;

suspension cultures of Haplopappus gracilis (Constabel et al., 1971), callus cultures of a number of acyanic species (Ibrahim et al., 1971), excised flower buds of Impatiens balsamina (Klein and Hagen, 1961), and of deoxyisoflavone synthesis in soybean callus (Miller, 1969). Whereas the literature on the regulatory effect of cytokinins in phenylpropanoid synthesis seems to be lacking, kinetin was reported to stimulate alkaloid production by increasing the rate of synthesis of methyltransferase and extending the period of steady-state synthesis by several days (Steinhart et al., 1964). The latter response is similar, in some respect, to that obtained with Perilla where a high kinetin level resulted in an extension of the period during which synthesis of the caffeoyl ester takes place (Fig. 13).

In contrast with cytokinin, the effect of an increased auxin level is to repress caffeoyl synthesis and this is most noticeable during early-exponential growth. Similar effects have been reported on polyphenol synthesis in Paul's scarlet rose suspension cultures (Davies, 1972a); production of anthocyanins in H. gracilis (Blakely and Steward, 1961; Constabel et al., 1971) as well as the accumulation of nicotine in tobacco tissue culture (Tabata et al., 1971).

A common response of Perilla cultures to high levels of either NAA or kinetin was an increase in the ratio of caffeoyl ester to the free acid, which amounted to/two- to a

three-fold during mid-exponential growth and a 40-50% increase at the stationary phase above that of the control culture (Fig. 13). Considering that the caffeoyl ester is the metabolically active form, it seems attractive to suggest, therefore, that the increased level of either growth substance may prevent, to a certain extent, the breakdown of caffeoyl ester to the free acid, especially during active growth. This observation is in contrast with the report on IAA-accelerated release of scopoletin from its glucoside, scopolin, into the medium of tobacco callus culture, whereas a high level of kinetin prevented the release of the coumarin (Skoog and Montaldi, 1961).

While the significance of phenylpropanoid compounds in plant growth and metabolism may still be a controversial subject, a speculation on the role of caffeoyl compounds in the growth of Perilla cultures is certainly appropriate. The fact that this ubiquitous phenolic moiety constitutes the only detectable intermediate and end metabolite in the pathway of phenylpropanoid biosynthesis seems to imply a functional role in growth and/or metabolism of this culture. Some regulatory mechanism might have resulted in the formation of this phenolic metabolite, which by virtue of its o-dihydroxy grouping, would regulate the activity of IAA-oxidase system in Perilla cells. The role of o-dihydric

phenols, especially caffeic acid, in the inactivation of IAA-oxidase (thus acting as IAA-synergists), is very well documented (see reviews by Sondheimer, 1964; Thimann, 1972; Galston and Davies, 1970). Furthermore, it is conceivable that the growth of Perilla may actually be regulated by its endogenous (natural) auxin, presumably IAA, rather than the synthetic auxin, NAA, added to the culture medium. In such cases, the physiological level of IAA necessary to maintain culture growth would be regulated by caffeoyl compounds. The accumulation of the latter observed at the early-exponential phase, and preceding active growth, is in support of this view.

VI. SUMMARY

1. Suspension cultures of Perilla ocymoides L. were grown in a defined medium containing sucrose (3%), NAA (1 mg/l) and kinetin (0.1 mg/l). They exhibited typical growth curves (fresh weight, dry weight and protein content) which consisted of the lag, exponential and stationary phases, and were similar to those described for other suspension cultures.
2. The growth of the culture was characterized by a light brown colour and a high proportion (approx. 9:1) of cell clumps to free cells during the end of the lag phase. This proportion gradually changes as growth proceeds, so that stationary phase cultures consist of about 80% free cells and 20% cell clumps. High levels of either auxin or cytokinin maintained a high proportion of cell clumps throughout the culture period.
3. Whereas suspension cultures of Perilla lose the biosynthetic potential for perillanin, the characteristic anthocyanin pigment of the species, however, they accumulate caffeic acid both in the free and esterified forms. Caffeoyl compounds were the only detectable intermediates and end metabolites in the pathway of phenylpropanoid biosynthesis.

4. The maximum accumulation of caffeoyl compounds occurred during early-exponential growth and the ratio of the ester to free acid amounted to 4:1. Maximum accumulation of phenolics preceded active growth of the culture, which indicates that such compounds may be essential for mediation of culture growth.
5. Trace amount of caffeic acid was obtained after mild alkaline hydrolysis of the extractive-free, cell wall residue. However, alkaline nitrobenzene oxidation of the latter gave no lignin degradation products.
6. The biosynthesis of caffeoyl compounds from trans-cinnamic acid-2-^{14}C was studied during culture growth. The degree of label incorporation into the alcohol-soluble fraction and the specific activities observed in both caffeoyl compounds indicate active synthesis of phenolic compounds and reflect their pool size during cell growth. The incorporation of label into the alcohol-insoluble residue is indicative of the participation of some phenylpropanoid moiety in cell wall formation.
7. The effect of high levels of NAA and kinetin on the regulation of caffeoyl synthesis was also investigated. The overall effect was that auxin depressed, and cytokinin increased, the phenolic content of the cells during early-exponential growth. Furthermore, a high kinetin

level resulted in an extension of the period during which synthesis of the caffeoyl ester takes place. The increase of either growth substance, however, caused an increase in the ratio of caffeoyl ester to free caffeic acid, especially during mid-exponential growth.

8. The role of caffeoyl compounds in the regulation of the endogenous level of auxin was discussed in relation to their accumulation during culture growth.

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APPENDIX A: Composition of the Nutrient Culture Medium (Murashige and Skoog, 1962).

Compound	mg/l
Macro-elements	
$CaCl_2 \cdot 2H_2O$	440
$MgSO_4 \cdot 7H_2O$	370
KNO_3	1,900
NH_4NO_3	1,650
KH_2PO_4	170
Micro-elements	
$FeSO_4 \cdot 7H_2O$	27.8
Na_2EDTA	37.3
$MnSO_4 \cdot 4H_2O$	22.3
$ZnSO_4 \cdot 4H_2O$	8.6
H_3BO_3	6.2
$Na_2MoO_4 \cdot 2H_2O$	0.25
$CuSO_4 \cdot 5H_2O$	0.025
$CoCl_2 \cdot 6H_2O$	0.025
KI	0.83

Vitamins	
Nicotinic acid	0.5
Pyridoxin-HCl	0.5
Thiamin-HCl	0.1
Glycine	2.0
Other addenda	
myo-Inositol	100