CELL SUSPENSION CULTURE STUDIES OF THE *COFFEA ARABICA* L.

BY

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ABSTRACT

Cultured tissues derived from the coffee plant, Coffea arabica L., were grown in vitro in the form of both callus and suspension cultures. The suspension cultures grew rapidly and appeared healthy. Microscopic examination showed that the cells characteristically grew in long filamentous chains.

Suspension cultures were examined for the presence of three components - free amino acids, caffeine and chlorogenic acid. By examining these components the species specificity could be determined.

The free amino acids of the coffee bean are thought to be one of the major precursors of coffee aroma on roasting. The coffee suspension cultures were shown to contain a similar pattern of free amino acids although the total content was much higher in the cultures than in the intact green coffee bean. Aspartic acid, glutamic acid, phenylalanine, alanine, valine, threonine, serine, and glycine were the predominant amino acids present in the coffee suspension culture. Threonine, serine, glycine, alanine and phenylalanine were the major free amino acids in the green coffee bean. The free amino acid content in the suspension culture exhibited an initial rise, decreased during active growth, then increased rapidly to the maximum level during the decline of the culture.

Roasted coffee bean extracts were investigated to ascertain whether one solvent could in preference extract some of the major precursors of coffee aroma. Methanol was found to extract material from green coffee beans which on roasting produced coffee aroma.

Caffeine was detected in the cell suspension cultures. However, problems with the analytical methods gave rise to questionable results. The suspension cultures, at maximum

caffeine yield, contained 0.03% caffeine (dry weight) whereas the green coffee bean contained considerably more caffeine (1.15%, dry weight). The caffeine content of the tissues increased during the lag phase, decreased during the rapid phase and then increased again in the stationary phase and ultimately production levelled off during the deline phase of growth.

The cell cultures produced chlorogenic acid in low concentrations at the maximum 0.14% dry weight in contrast to the green coffee bean which contains 6.5% dry weight. The production or accumulation of chlorogenic acid followed a similar pattern to that of the cell caffeine production over the growth curve. Caffeic acid was also detected.

The cell suspension cultures of Coffea arabica L. were shown to be species specific in their biochemical capabilities.

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INTRODUCTION

The concept of cultivating isolated plant cells was conceived by Haberlandt in 1902 (White, 1963; Nickell, 1962). Since then, the field of plant tissue cultures has been gradually expanding although it is still at the fundamental stages of development. Plant cell suspension cultures can be grown in liquid medium in a manner similar to microbial cultures. Unlike the microbial cultures, however, the cell suspensions are composed generally of a heterogenous population of cell types (Willmer, 1963) and as a consequence growth and subsequent development tend to be somewhat difficult to control.

The isolated plant cell is theoretically biochemically totipotent; that is, the cell has the full biochemical potential found in the intact parent plant (Krikorian, 1965). Cultures have been shown to produce carbohydrates, amino acids and proteins readily (Klein, 1960; Staba, 1963). Secondary metabolites such as alkaloids have been detected in very low quantities (Puhan and Martin, 1971). The extent to which the plant cell's biochemical potential can be controlled is still unknown. At the present time in most cases the products produced by the plant tissue cultures can be obtained and/or produced more economically from the intact plant.

Tissue cultures derived from a Coffea arabica L. plant were used in this study to determine if they could serve as a possible potential for producing 'coffee'. Coffee, as the consumer recognizes it, is the roasted product of Coffea species beans. The roasting process through a series of 'browning reactions' transforms the bean from a green colour with a disagreeable odour to a chocolate brown product with the characteristic coffee flavour and aroma. Numerous authors have suggested that the major precursors of coffee flavour and aroma exists in the amino acids, proteins and carbohydrate classes of compounds (Feldman et al, 1969; Merritt and Angeline, 1971).

If this theory is correct and the isolated plant cell is species biochemically totipotent, then it may be possible to produce a coffee flavour and aroma on roasting of the coffee tissue cultures (Townsley and Buckland, 1972).

Several components, free amino acids, caffeine and chlorogenic acid, found normally in the green coffee bean were investigated in the Coffea arabica L. suspension cultures.

The free amino acids of green coffee bean are thought to be one of the major precursors of roasted coffee aroma. Caffeine and chlorogenic acid, while they are not thought to contribute significantly to roasted flavour and aroma, are normally secondary metabolites found in the coffee bean. These three components were examined to ascertain whether a coffee suspension culture is still species specific in its biochemical potential.

Roasting of green coffee bean extracts was also investigated. The aim was to find if one of the solvents used would extract enough of the major precursors of coffee aroma.

The aim of this study was to compare some of the recognized chemical constituents of the green coffee bean with those found in coffee cell suspension cultures.

TERMINOLOGY

Articles dealing with plant tissue culture use varying terminology which often, unfortunately, makes it difficult to determine exactly what type of tissue culture has been used. The specific terminology to be used in the present study to describe the various types of plant tissue cultures is:

1. undifferentiated growth - an embryonic type of cell growth in which all or most of the cells present are the same or closely related in structure and function, so are not specialized for organized functions such as the formation of leaves and roots (Routien *et al.* 1956).

2. (plant) tissue cultures - a general term encompassing all forms of plant tissues cultured *in vitro* such as callus and cell suspension cultures.

3. callus cultures - homogenous masses of undifferentiated cells 'to all outward appearances' grown on solid medium (Tulecke, 1961).

4. (single cell) suspension cultures - a submerged culture containing single cells and very small cell aggregates. The cells have no secondary wall thickening (Willmer, 1963).

LITERATURE REVIEW

1. History of plant tissue culture.

As early as 1902, Haberlandt envisaged culturing isolated plant cells on artifical medium. He based his 'outrageous' ideas upon Schwann's (1839) concept of cellular totipotency. Haberlandt (1902) proposed that studies could be done more readily on organization and cellular relationships within the plant if isolated plant cells and tissues could be cultivated. He assumed that there was no limit to cell divisibility. He, however, was unsuccessful in his attempts to cultivate single cells or tissues mainly as his choice of experimental material was wrong rather than his techniques (Nickell, 1962; White, 1963). Later he stated (translated by White, 1963) "at any rate, the cultivation of isolated cells in nutrient solution would make possible an experimental approach to many important problems from a new point of view".

It was not until the early 1930's that continuous growth *in vitro* of plant segments was achieved. White (1934) and Gautheret (1934) were both successful in obtaining elongation of isolated root tips *in vitro*.

In 1939, the first publication appeared describing the successful cultibation of both carrot and tobacco cambial callus cultures over prolonged periods of time (Gautheret, 1939; Nobecourt, 1939; White, 1939). Since then the field of tissue culture has greatly expanded.

In 1954, Muir *et al* produced cell suspensions from *Tagetes erocta* and *Nicotiana tabacum* calluses. These liquid suspensions contained both single cells and small clumps of cells. Since this first successful attempt at single cell suspensions, single cell suspensions have been obtained from most dicotyledonous

and a few monocotyledonous plants. Klein (1960) has stated that almost all tissues can be grown although he questioned the actual value of doing so.

2. The potential of cells.

"If the zygote receives all the genetic information which is essential for the whole organism, and if its subsequent divisions are as consistently equational as they seem to be, then the original totipotency of the zygote should persist in the array of derivative parenchyma cells despite their variation in form and metabolism" (Blakely and Steward, 1964). Thus meristematic (undifferentiated) cells isolated from the parent plant and cultures should have the biochemical potential of the parent plant (Krikorian, 1965).

Plant cells, however, cultured *in vitro* tend towards a common cell type which is simple and undifferentiated in nature. The cells have a narrow range of detectable enzymes and a narrow range of metabolic activity (Tulecke and Nickell, 1960). Weinstein *et al* (1962) stated that the tissue cultures were cytologically, physiologically, and biochemically different from the parent plant. Staba (1963) concluded that single cell cultures were similar biochemically to lower forms of plant life. He summarized the commercial potential of plant cell cultures as follows: "carbohydrates, amino acids and proteins can be produced by plant suspension cultures. To what extent it is economically practical to grow and regulate such cultures remains to be seen". Staba (1969) later, however, stated that just because this cell type appeared to be 'geared up' for active growth and protein production, it should not be concluded that the cell is not potentially able to produce the desired products.

Numerous plant products have been isolated from callus and single cell suspension cultures. These include alkaloids, pigments, proteins, enzymes, organic acids, phenolics, saponins, steroids, and terpenoids (Puhan and Martin, 1971). It has

been found, however, in tissue cultures the level of the products detected was below the level in the parent plant (Puhan and Martin, 1971; Tomita, 1971).

3. The coffee plant.

The coffee found on the retail shelf is a highly refined product of the Coffea species. It is well known for its pleasing flavour and aroma and for its stimulating effects on animal metabolism (Sivetz, 1963). The Coffea species is a tropical evergreen which originated in the African highlands of Kenya and Abyssinia (Thompson, 1971).

Coffee is grown commercially today in most of the tropical countries of Africa, Asia, Oceania, and South and Central America. In the 1971-1972 season the coffee world produced 9,398.4 million pounds of green coffee and exported 6,930 million pounds to non-coffee producing countries. For the 1972-1973 season it is forecast that 9,603 million pounds of green coffee will be produced and 7,035.6 million pounds will be exported. Brazil is the top coffee producing and exporting country with Columbia following (Foreign Agricultural Service, 1971 and 1972).

The Coffea species is a widely variable plant. Coffea arabica L. and its varieties account for nearly all of the commercial market with Coffea liberica and Coffea robusta accounting for the rest (Thompson, 1971).

Three species of the Coffea family (C. arabica L., C. canephora Robusta, and C. liberica Bull. ex Hiern) have been grown in vitro. The callus cultures obtained were of two types. The first was white and spongy in appearance and the second was more compact. Only the Robusta variety callus showed embryoidal development in the callus tissues (Staritsky, 1970).

4. Free amino acids in plant tissue cultures and green coffee beans.

Intact plants and plant tissue cultures have been shown to contain the usual spectrum of free amino acids and amides (Synge, 1955; Steward et al, 1958; Weinstein et al, 1959). The plant tissue cultures, however, have a much lower content of total free amino acids and amides (Steward et al, 1958; Weinstein et al, 1959) (Table 1). For example, intact potato tissues and potato callus tissues contain 1,281 and 452 micrograms of nitrogen per gram fresh weight respectively (Steward et al, 1958); carrot plants and carrot callus cultures contain 801 and 124 micrograms of nitrogen per gram fresh weight respectively (Steward et al, 1958); and brittleweed (Happlopappus gracilis) plants brittleweed callus cultures contain 468.8 and 122.6 micrograms nitrogen per gram fresh weight respectively (Krikorian, 1965). Steward et al (1958) concluded that several amino acids and amides, especially asparagine, glutamine, and arginine, were lower in content in the plant tissue cultures than in the parent plant while γ-aminobutyric acid was higher in content. Krikorian (1965) noted that generally there was little difference between the cultures and the parent plant in the free amino acid content except for specialized amino compounds which the cultured tissues tended to be lacking.

In tobacco suspension cultures, Koiwai et al (1971) found that the total free amino acid content tended to decrease a little during the lag phase and then to increase rapidly in the log phase of growth. The principal amino acids were glutamine, asparagine, and γ-aminobutyric acid, which varied in content with the age of the culture. The glutamine content was low during the lag phase of growth and

Table 1. Comparison of the free amino acids in intact tissue and callus cultures of potato, carrot and brittleweed plants.

Amino acids	potato† intact	potato† callus	carrot† intact	carrot† callus	brittleweed* intact	brittleweed* callus
aspartic acid	11.22*	1.64	25.90	1.20	5.20	3.20
glutamic acid	16.95	2.73	42.00	4.50	1.40	7.40
serine	8.81	7.28	17.00	1.50	3.50	2.50
glycine	5.15	8.00	1.70	0.40	1.30	5.70
asparagine	291.50	0.00	153.00	0.00	present	
threonine	11.83	4.50	11.80	0.70	3.20	trace
alanine	20.15	21.50	93.00	10.40	3.40	21.20
glutamine	579.00	15.72	77.80	0.00	present	
histidine	-	-	-	-	3.30	2.5
lysine	12.10	2.62	0.50	0.00	11.90	20.10
arginine	114.50	15.46	35.20	0.00	-	-
methionine	7.74	0.00	2.61	0.00	trace	
proline	0.00	0.00	4.20	0.10	2.30	5.70
valine	29.10	6.31	8.60	0.90	6.40	2.70
leucines	11.15	8.05	7.80	0.70	9.60	4.70
phenylalanine	11.71	6.18	13.30	2.00	trace	
γ-aminobutyric acid	40.75	88.30	18.40	2.30	6.30	4.60
β-alanine	-	-	-	-	0.50	0.00
pipecolic acid	-	-	-	-	3.20	trace
total	1281.0	452.0	801.0	124.0	468.8	122.9

† Steward et al, 1958;

* Krikorian, 1965.

"all values expressed as micrograms nitrogen per gram fresh weight - no values given for that individual amino acid."

then decreased slightly after the peak of growth (day 6). The asparagine content increased during the lag phase, decreased very slightly during the log phase and then rapidly increased again during the stationary phase of growth. γ-aminobutyric acid was found to increase markedly during the lag phase, and then decrease gradually after rapid growth began (Koiwai et al, 1971).

The content of glutamic acid, glycine and lysine decreased during the lag phase of growth and then increased during the log phase of growth. The content of alanine, serine, leucines, and proline increased during the lag phase and then decreased in content during the log phase. The other amino acids were found to have no recognizable pattern although most of them were elevated during the stationary phase of growth (Koiwai et al, 1971).

γ-aminobutyric acid was the only amino acid found in plant tissue cultures consistently in a higher content than in the parent plant, although other amino acids have been shown to be higher in the plant culture than in the intact plant (Stewart et al, 1958; Bove et al, 1957). Koiwai et al (1971) found that γ-aminobutyric acid increased in content in the plant tissue cultures only during the lag phase of growth and thereafter gradually decreased.

Wickremasinghe et al (1963) showed that γ-aminobutyric acid accumulated in rose, bean, sycamore, and brittleweed cultures when the air supply was limited. γ-aminobutyric acid is thought to be an intermediate in the oxidation of glutamic acid to organic acids of the citric acid cycle. A suppression of the oxidation as a result of a limited supply of air would cause an accumulation of γ-aminobutyric acid (Koiwai et al, 1971).

The free amino acids and proteins were both found to vary in content and composition with the age of the culture (Steward et al, 1958). A Paul's scarlet rose suspension culture was found to contain 96 micrograms of nonprotein nitrogen per gram fresh weight and 915 micrograms of protein nitrogen per gram fresh weight after four days of culturing. After twelve days of cultivation the cultures were found to contain 84 micrograms of nonprotein nitrogen per gram fresh weight and 219 micrograms of protein nitrogen per gram fresh weight (Fletcher and Beavers, 1970). The relative composition of the cell protein did not reflect the relative composition of the free amino acids present. This seems to indicate that more than just simple condensation of the free amino acids occurs in protein synthesis (Steward et al, 1958).

Changes in the environmental conditions caused changes in the soluble and insoluble (protein) amino acid content and composition. When potassium was added to the medium the amount of free amino acids present in the callus cultures was strongly influenced (Tulecke, 1961). Light caused a slight increase in total amino acid content of peanut callus cultures. The light favoured increases in aspartic acid, serine, threonine, and valine content, while cultures grown in dark favoured higher levels of proline and glycine (Krikorian, 1965; Krikorian and Steward, 1969).

Green coffee beans contain appreciable amounts of nonprotein amino containing compounds (Underwood and Deatherage, 1952). The free amino acids of green coffee beans (var. Columbia) were analysed quantitatively (Table 2). Wolfrom et al (1960) found ten free amino acids plus γ-aminobutyric acid and Walter et al (1970) found five additional free amino acids on further analysis.

Table 2. The free amino acids present in green coffee beans (var. Columbia) (Walter *et al*, 1970).

amino acid	percent concentration
aspartic acid	0.33
serine	0.12
asparagine	0.30
glutamic acid	0.49
proline	0.14
glycine	0.02
alanine	0.24
valine	0.02
isoleucine	0.03
leucine	0.03
tyrosine	0.04
phenylalanine	0.08
γ-aminobutyric acid	0.30
lysine	0.04
histidine	0.04
arginine	0.04

5. Roasting of green coffee beans.

The flavour and aroma of the green coffee bean is not very appealing and it is only with roasting that the characteristic flavour and aroma develop. During the roasting process there occurs a mild pyrolysis of the bean's constituents accompanied by the gradual formation of volatile substances (Gianturco, 1967; Gianturco, 1965; Gianturco et al, 1964). Pyrolysis is a chemical change occurring at elevated temperatures which results in the degradation and synthesis of products (Sivetz, 1963).

The coffee beans are generally roasted at approximately 220°C (Feldman et al, 1969). The bean initially loses its free water (70%) and then, as the internal temperature of the bean begins to rise, the bound water is lost until the water content is reduced to one to two percent. The internal temperature of the coffee bean has then reached approximately 400°F and the absorption of heat by the bean is supplemented by the liberation of heat from internal pyrolytic reactions. This strong exothermic reaction is accompanied by the sudden expansion or puffing of the bean as well as internal rupturing of the cell layers. The drastic hydrolysis of proteins and other plant constituents allows the development of volatiles and the release of carbon dioxide. The reactions occurring in the bean must be stopped abruptly by cooling rapidly at the desired end point as the reactions once initiated occur in a few seconds. The colour of the bean changes rapidly from green to dark brown during the final minutes of roasting (Sivetz, 1963; Feldman et al, 1969; Keable, 1910; Furia, 1971).

The intact bean has been likened to a small autoclave in which the chemical constituents react and interact under restricted conditions (Keable, 1910). The possibilities of reactions among the different chemical classes present in the green coffee bean under pyrolytic conditions are virtually

unlimited but there is some selectivity evident as expressed by the greatly varying number of compounds formed (Gautschi et al, 1967).

The nature of the reactants and their analysis before and after roasting indicate that the Maillard reaction, Strecker degradation, base catalyzed sugar reaction, etc. occur perhaps along uncommon pathways governed by low water content, localized buffer systems, and a fluccuating balance of reaction products.

The Maillard type reactions, if permitted to go to completion, produce both volatiles and browning of the product (Gautschi et al, 1967). This type of reaction involves the interaction of reducing sugars and amines. The free amino acids of the product would be expected to react more rapidly than the protein amino acids with the carbohydrates (Feldman et al, 1969). There are excellent reviews written on the mechanisms of browning reactions in foods (Hodge, 1963 and 1967; Danehy and Pigman, 1951; Reynolds, 1963, 1965 and 1969).

The aroma of coffee is extremely complex in nature and as a result much work has been done in this field. There are several review articles which give a fairly comprehensive coverage of the field (Winter et al, 1967; Friedel et al, 1971). The number of compounds isolated from the aroma sample is affected by the method of its acquisition. Using a head space sample, Watanabe (1969) isolated and identified 313 compounds but concluded that his list was not complete. Weidmann and Mohr (1970) isolated 363 compounds from coffee aroma. They identified 131 acyclic compounds, 73 isocyclic compounds, and 159 heterocyclic compounds. Gautschi et al (1967) and Weidmann and Mohr (1970) concluded that aroma arises mainly from a large number of volatile compounds blended together rather than from an individual compound. Reymond et al, (1963) concluded that the aroma was also influenced by

nonvolatile components present. The aroma produced on roasting is dependent upon numerous factors, for example the roasting method, temperature of the roast, the water content of the beans, and the final grind (Furia, 1971).

The difference between light (mild) and dark (French) roast coffee is reflected by differences in the volatiles present and their concentrations. The possibility of specific volatiles having two or more precursors of different thermal stabilities has been demonstrated. Moreover, the formation of specific volatiles can occur along two or more pathways which have different energy requirements (Self, 1963; Gianturco, 1965 and 1969). Thus the length of the roast is very important to the production of volatiles.

The pyrolysis of amino acids, proteins and peptides has been related to the flavour and aroma of coffee after roasting (Merritt and Angeline, 1971). The degradation and interaction products of amino compounds are thought to be the main source of volatiles in roasting coffee (Gianturco *et al*, 1967). The study, however, of the precursors of coffee flavour and aroma has been largely ignored (Gianturco *et al*, 1967).

Russwurm (1969) found that extracts obtained from coffee beans would not produce coffee aroma on roasting unless the fractions containing sugars and amino acids were combined. Erdman (1902) demonstrated coffee aroma caused by nitrogenous compounds. Although he roasted a mixture of coffee tannic acid and raw sugar only to obtain a burnt smell, he was able to obtain coffee aroma on roasting when caffeine was added to the mixture before roasting.

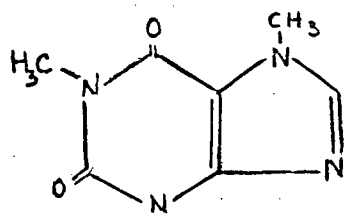
The primary aroma of cocoa is produced by the interaction of flavonoids, sugars and amino acids. By roasting a methanolic extract of fermented cocoa beans, cocoa aroma was obtained.

The methanol solution extracts flavonoids, sugars and amino acids from the cocoa bean (Rohan and Stewart, 1965; Rohan, 1964). The free amino acids present have been shown to vary greatly in composition and content between cocoa plant varieties and thus it is not surprising to obtain differences between varieties in the roasted aroma. Also different amino acids react at different rates and to different extents under the roasting conditions (Rohan and Stewart, 1966). The cocoa bean is fermented before roasting to achieve the desirable aroma and flavour. Roasting an unfermented bean results in an aroma resembling roasted broad beans (Rohan, 1964).

Coffee substitutes have been produced from non coffee plant origins. General Foods Corporation (1963 and 1966) produced a 'coffee' beverage by heating an aqueous (10%) mixture of proteinaceous material such as peanuts, and a reducing sugar and then diluting the roasted product. Calcium carbonate was added to the mixture to maintain the pH between 4.5 and 5.2 (General Foods Corporation, 1966). Broderick (1968) concluded that a synthetic instant coffee with full fresh flavour and aroma was practically impossible to synthesize.

6. Caffeine.

Another nonprotein source of nitrogen in green coffee beans and roasted coffee beans is caffeine. It is well known for its stimulatory effect of animal metabolism. It is a xanthine alkaloid with the formulation of:



(Clarke, 1969; Sivetz, 1963). Caffeine is present in most coffee varieties in varying amounts (1.2% in Coffea arabica; 2.0% in C. robusta; and none in wild varieties) (Lehman, 1971; Feldman et al, 1969). Roasting of the bean causes a gradual decrease in concentration of caffeine mainly as a result of sublimation (Sharka and Telepcak, 1964).

Plant alkaloids are generally produced during high metabolic activity (Hamerslag, 1950), and decrease in content with increasing age of the tissue (Hamerslag, 1950; Beauden-Dufour and Mueller, 1971; Wanner and Kalberer, 1966; Kaeber, 1965). Ogutuga and Northcote (1970a and 1970b) studied caffeine biosynthesis in tea callus cultures. The calluses after thirty days growth contained 400 micrograms caffeine per gram dry weight (Ogutuga and Northcote, 1970a) whereas the intact tea plant contains approximately 3.5% caffeine (Fecak and Struhar, 1970). In the callus medium caffeine was also found to increase rapidly near the end of the rapid growth phase of the culture (Ogutuga and Northcote, 1970a). The relative proportion of caffeine in the callus and in the medium varied with the age of the culture. Caffeine production is thought to be followed either by irreversible discharge from the cell or release as a result of cellular autolysis (Ogutuga and Northcote, 1970a).

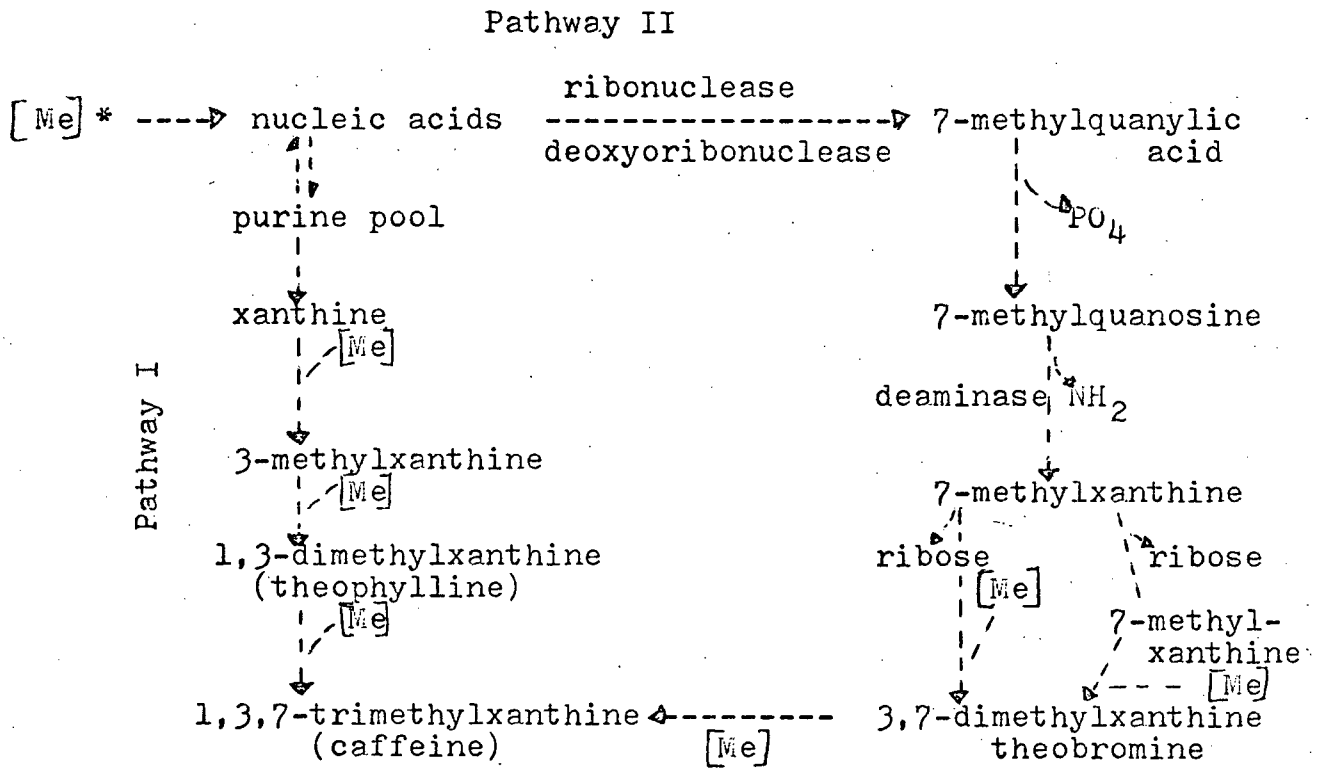
Caffeine determination has also been determined on sycamore and bean callus cultures (Ogutuga and Northcote, 1970a). The results showed that these two cultures do not synthesize caffeine. The lack of caffeine in the sycamore and the bean callus cultures and the presence of caffeine in the tea callus cultures demonstrate the genus specificity of the products produced by the callus cultures (Ogutuga and Northcote, 1970a).

Caffeine is synthesized in intact plants along the pathways for normal purine synthesis, followed by methylation of the

purine ring (Proiser and Serenkov, 1963; Anderson and Gibbs, 1962). Ogutuga and Northcote (1970a and 1970b) proposed two pathways for the synthesis of caffeine (Figure 1). Pathway II is thought to be the major pathway as an increase in caffeine content occurs during tea leaf fermentation probably caused by a catabolic breakdown of nucleic acids. When ribonucleic acid was added to the callus tissue medium an increase in caffeine content followed (Ogutuga and Northcote, 1970a).

Light was found to enhance purine formation and thus caffeine synthesis (Anderson and Gibbs, 1962). Ogutuga and Northcote (1970b), however, reported light had an inhibitory effect upon caffeine production of the tea callus. The highest yields were obtained in calluses grown in complete darkness (1,500 micrograms caffeine per gram dry weight). Precursors of the methylated purine ring such as ammonium formate were also found to cause marked increases in caffeine production (Ogutuga and Northcote, 1970a).

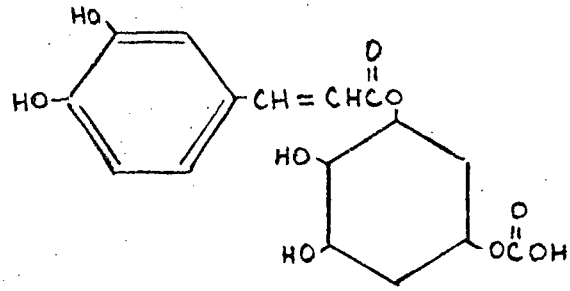
Figure 1. The pathways proposed for the synthesis of caffeine (Ogutuga and Northcote, 1970a).



* [Me] - indicates a methyl group.

7. Chlorogenic acid.

Chlorogenic acid is an acidic phenol found normally in green coffee beans and their roasted products. Chlorogenic acid is composed of two simple acids, quinic acid and caffeic acid. The formulation for chlorogenic acid is:



(Sivetz, 1963). Several isomers of chlorogenic acid have also been isolated from green coffee in small amounts. These isomers are isochlorogenic acid and neochlorogenic acid (Chassevant, 1969; Smith, 1963).

In green coffee beans the content of chlorogenic acid varies between varieties (6.5% dry weight in Coffea arabica and 7.7% in C. robusta) (Lehman, 1971) and between reports (7.3% dry weight in C. arabica) (Feldman et al, 1969). Lehman et al (1967) reported that chlorogenic acid was present in green coffee beans from 5.5% to 7.5% dry weight.

Chlorogenic acid and its hydrolysis products, quinic acid and caffeic acid, do not contribute to coffee aroma but only to the flavour (Lee, 1962). Roasting of the coffee bean causes a decrease in the content of chlorogenic acid probably as a result of the hydrolysis of the molecule. Lehman et al (1967) reported after roasting of coffee beans that chlorogenic acid

content had dropped to 3.6% and 4.5% of the dry weight. Feldman et al (1969), however, reported only 0.3% chlorogenic acid was present after roasting of coffee beans. The content of chlorogenic acid varied greatly with the extent of roasting (Sivetz, 1963).

Chlorogenic acid has been detected in tissue cultures of potato (Gamborg, 1967) and tobacco (Bergman, 1963). In the tobacco tissue cultures the level was found to be increased with the addition of 10% kinetin to the medium. This also increased the content of other alkaloids present (Bergman, 1963). Light also had a stimulatory effect on the production of chlorogenic acid by the tissue culture with continuous lighting producing the highest level. Higher phenolic contents were also obtained in callus cultures when α-NAA was added to the medium (Leonova et al, 1970).

Two possible pathways for the biosynthesis of chlorogenic acid have been demonstrated. The major pathway is:

phenylalanine ---▷ cinnamic acid ---▷ ρ-coumarate ---▷
---▷ ρ-coumarolylquinic acid ---▷ chlorogenic acid

(Zucker, 1963; Steck, 1968). The secondary pathway which has been shown to exist is:

phenylalanine ---▷ cinnamic acid ---▷ ρ-coumarate ---▷caffeine
---▷ chlorogenic acid

(Zucker, 1963; Steck, 1968). According to Zucker (1963) cinnamic acid is not only an intermediate in chlorogenic acid biosynthesis but also stimulates chlorogenic acid synthesis. Colonna and Boudet (1971) concluded that chlorogenic acid is not an inert product which accumulates but that it plays an active metabolic role in the plant cell.

METHODS AND MATERIALS

1. Preparation of the cultures.

Healthy vigorously growing young shoots were taken from a Coffea arabica L. plant growing in the University Horticultural greenhouses. The shoots were stripped of leaves, cut into two inch lengths to facilitate easier handling and sterilized by submersion in 5% sodium hypochlorite solution for approximately twenty minutes. All procedures thereafter were performed aseptically to avoid contamination. Following sterilization, the two inch lengths were rinsed in distilled water, cut into pieces or explants approximately three millimeters long and placed on solid medium (Table 3). The implanted flasks of medium were incubated in darkness at 25°C.

When the explants were growing well the callus tissue clumps were broken into fragments which were placed into flasks of liquid medium (Table 3). These flasks were incubated at 25°C in darkness on a horizontal rotary shaker (New Brunswick Gyrotory Shaker Model G10) at approximately 160 rpm (Figure 2).

2. Maintenance of the cultures.

The callus cultures on solid medium were transferred to fresh medium (Tables 3 and 4) every four to eight weeks to maintain sufficient moisture and nutrients for continuous growth of the cultures. The transfer was effected by breaking the growing callus clumps into pieces and placing these pieces individually onto fresh medium.

The liquid cultures were transferred to fresh liquid medium (Tables 3 and 4) every two weeks until satisfactory growth was well established and thereafter transfer was repeated every eight to ten days to maintain the vigour of the cultures.

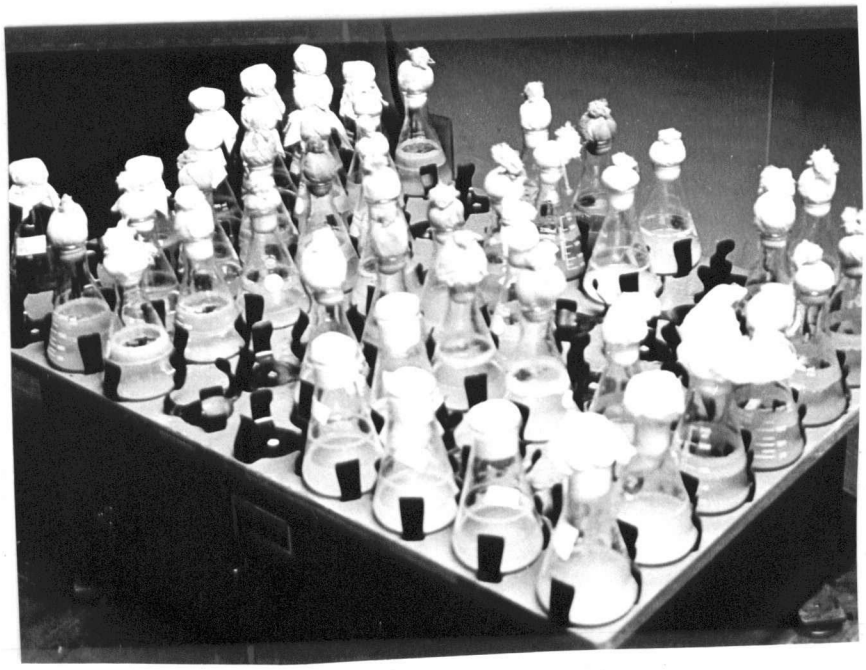


Figure 2. The horizontal shaker used for aeration of the suspension cultures - containing cultures of different ages.

Table 3. The composition of the PRL-4-C-CM medium, liquid and solid* (Gamborg et al, 1968).

$NaH_2PO_4.H_2O$	90 milligrams/liter
Na_2HPO_4	30
KCl	200
$(NH_4)_2SO_4$	200
$MgSO_4.7H_2O$	250
KNO_3	1,000
$CaCl_2.2H_2O$	150
KI	0.75
2,4-D	2.0
sucrose	20,000
N-Z amine type A**	500
coconut milk	10 milliliters/liter
vitamin stock solution	10
iron stock solution	5
micronutrient stock solution	1

* for solid medium - 10 grams per liter of agar-agar was added. The solution was heated until the agar-agar had melted and then the medium was dispensed.
** enzymatically hydrolyzed casamine.

Vitamin stock solution (stored in a plastic bottle)

nicotinic acid	10 milligrams/100 milliliters
thiamine HCl	100
pyridoxine HCl	10
myo-inositol	1,000

Iron stock solution (kept frozen)

$FeSO_4.7H_2O$	278 milligrams/100 milliliters
Na_2EDTA	372

Micronutrient stock solution (kept frozen)

$MnSO_4.H_2O$	1,000 milligrams/100 milliliters
H_3BO_3	300
$ZnSO_4.7H_2O$	300
$Na_2MoO_4.2H_2O$	25
$CuSO_4$	25
$CoCl_2.6H_2O$	25

Table 4. The composition of the B5 medium, liquid and solid* (Gamborg et al, 1968).

$NaH_2PO_4 \cdot H_2O$	150	milligrams/liter
KNO_3	2,500	
$(NH_4)_2SO_4$	134	
$MgSO_4 \cdot 7H_2O$	250	
$CaCl_2 \cdot 2H_2O$	150	
KI	0.75	
sucrose	20,000	
2,4-D	0.2	
vitamin stock solution**	10	milliliters/liter
iron stock solution**	5	
micronutrient stock solution**	1	

* for solid medium - 10 grams agar-agar per liter of medium. The solution was heated until the agar-agar had melted and then the medium was dispensed.

** for stock solution compositions see Table 3.

The transfer of the liquid cultures was accomplished by placing a ten milliliter of cell suspension aliquot into one hundred milliliters of fresh medium.

3. Growth and sampling of cultures for analysis.

In preparing the cell suspension cultures for analysis, seven to ten day old growing suspension cultures were filtered aseptically through miracloth (supplied by Calbiochem) and allowed to drain for approximately fifteen minutes. Flasks containing one hundred milliliters and five hundred milliliters of fresh medium were then inoculated with 0.5 grams and 2.5 grams of these filtered cells, respectively.

For each analysis, sufficient flasks of each size were inoculated to allow removal of one to three flasks each sampling day over a period of twenty-five days. Twenty-five days was chosen for the length of the sampling period as the growth, measured by dry weight, had peaked and was beginning to decline by the twenty-fifth day of growth. On sampling days, the required number of flasks were randomly removed from the shaker, and the contents filtered through miracloth. The residue of cells was washed with approximately twenty-five milliliters of distilled water and allowed to drain for an additional fifteen minutes. The cells were weighed to establish the fresh weight of the cells and then freeze dried using a Virtis freeze drier at approximately five microns vaccuum. When dry, the cells were reweighed to establish their dry weight. The dried cells were stored in a desicator over Tel-tale (W.R. Grace and Comp., Davison Chemical Division) until use to present spoilage and maintain a constant moisture content.

All cell analyses were carried out using the dried cells as the moisture content was more uniform throughout thus making the dry weight more reliable than the fresh weight.

The pH of the medium was measured on samples of the filtrate obtained after removing the cells from the growing culture. This filtrate was later discarded.

4. Media.

Plant tissue cultures, like microorganisms, require certain substances to promote and maintain growth in vitro. Essential requirements may vary depending upon the type of the plant and culture desired. In general, a carbohydrate solution fortified with minerals and vitamins is required. For initiation and promotion of rapid growth, a growth stimulant may be added in low concentrations.

The solid nutrient medium initially used to stimulate callus growth and to maintain the cultures was solidified PRL-4-C-CM (Gamborg et al, 1968; Table 3). After several months growth, the cultures were transferred to solid B5 medium (Gamborg et al, 1968; Table 4). Both media were dispensed into 250 milliliter erlenmeyer flasks in 100 milliliter amounts. The callus cultures were maintained throughout the study to ensure a ready supply of callus material in case of contamination or loss of cell vigour in the suspension cultures.

The liquid nutrient medium initially used for the cell suspension cultures was liquid PRL-4-C-CM (Gamborg et al, 1968; Table 3). After the cultures became well established on this medium, the medium was then changed to liquid B5 medium (Gamborg et al, 1968; Table 4). The liquid media, both the PRL-4-C-CM and the B5, were dispensed in one hundred milliliter aliquots into 250 milliliter and 1000 milliliter erlenmeyer flasks respectively. The amount used depended upon the dry weight required for the planned analyses of the cells.

The change in media composition was made mainly for chemical reasons. Once the cultures were well established, a lower concentration of growth stimulator (just 2,4-D) maintained the cultures in an active growing state. The PRL-4-C-CM medium, solid or liquid contained coconut milk and casein hydrolysate, both of which have an unknown chemical composition. On the other hand, the liquid or solid B5 medium is completely synthetic in composition. Thus all constituents added are known and also it is cheaper to prepare. Chemical analyses for this study were simplified and facilitated by the use of the artifical medium. The switch to the artifical medium did not noticeably affect the cell growth.

The flasks containing both solid and liquid media were stoppered with non absorbent cotton wool plugs wrapped in several layers of cheesecloth to allow transfer of gases between the atmosphere and flask, and to minimize the possibility of contamination.

5. Coffee bean preparation.

Green coffee beans were prepared for analyses and used as a control standard in each set of tests performed. The beans were imported from San Salvador and obtained through the courtesy of Nabob Foods Limited, Vancouver, B.C. To prepare the beans for analyses, the beans were ground fine enough to pass through a forty mesh seive using a Wiley mill (intermediate model, Arthur H. Thomas Company). Dry ice was added to keep the green coffee from becoming pasty during grinding and to control heat generation in the machine and the coffee during grinding (Horwitz (AOAC), 1970; Schaller, 1972). To reduce the rate of deterioration and to avoid pastiness of the powder during storage, the ground coffee was stored in the freezer at-4°C until used.

6. Amino acid analysis.

Generally, free amino acid determination was carried out by extracting the tissue culture or medium with 70 to 80% ethanol, filtering the extract, drying the extract *in vacuo* and then passing the resulting residue through a cation exchange resin. The resulting product was then analyzed on an amino acid analyzer (Simpkins and Street, 1970). With the coffee samples, especially the bean, chlorogenic acid interference occurred to such an extent that it became necessary to eliminate the interferring factor(s).

Samples of both ground coffee and dry cells were analyzed similarly for their free amino acids.

a. Preparation of the samples for analyses.

The samples were prepared using the following steps. The filter paper used in this analysis was Whatman number one which had been well washed with 95% alcohol, then dried under a gentle stream of air to remove residual alcohol. This alcohol treatment was used to remove any free amino acids present in the filter paper.

i. Extraction of the free amino acids from the samples (Simpkins and Street, 1970; Brenner et al, 1963).

One gram of each sample to be analyzed was extracted by homogenizing with one hundred milliliters of boiling 80% ethanol three to five minutes at high speed on an Osterizer (Galaxie VIII) and then left overnight to ensure complete extraction. The samples were then filtered through ethanol washed filter paper and the residue washed with 80% ethanol. The residue was then discarded.

The filtrate was dried *in vacuo* using a Rotovat (Model R-Buchi) at 45°C and approximately fifteen millimeters vacuum to remove the ethanol.

ii. Hydrolysis of the filtrate (Fitzpatrick and Porter, 1966).

The filtrates were hydrolyzed under mild conditions to lower the chlorogenic acid interference.

The dry residue was taken up in one hundred milliliters of 4N hydrochloric acid and heated at 120°C in an oil bath for three hours. After hydrolysis, the solutions were cooled and filtered through prepared filter paper to remove any precipitate that had formed during hydrolysis. The filtrates were evaporated to dryness *in vacuo* at 65°C to remove hydrochloric acid. This was repeated three times by the addition of distilled water to the dry residue.

iii. Desalting of the filtrates (Brenner et al, 1963).

The samples were desalted to remove any sugars and minerals present. A cation exchange column (Biorad AG 50 - 8x; 100 - 200 mesh; H form), one by twenty centimeters in dimensions, was used for the desalting procedure. The column was regenerated after use and maintained at approximately pH 3.25 with a citrate buffer (0.15 molar) of that pH.

The residue left after removal of the hydrochloric acid was taken up in approximately ten milliliters of a citrate buffer pH 2.2 and then applied to the prepared column. The flow rate was two to three milliliters per minute. Following the sample approximately fifteen milliliters of citrate buffer pH 2.2 was applied to wash out all extraneous material. The amino acids were then eluted from the column with twenty to thirty milliliters of 2N ammonium hydroxide. The eluent

was collected and dried *in vacuo* at 45°C. To remove some of the excessive ammonia, the residue was dried at least three times after each addition of ten milliliters of distilled water.

iv. Ammonia removal (Beveridge, 1972).

As the ammonia content was still to high for good separations on the amino acid analyzer, further ammonia removal was necessary.

Using solutions of 0.5M ammonium sulphate adjusted over a range of pH 8 to pH 12 with 1M sodium hydroxide, it was found that the lowest alkaline pH at which ammonia readily volatilized was pH 10. Ammonia volatilizes more readily at higher pHs but the alkalinity was kept as low as possible to avoid possible alkaline hydrolysis.

To determine whether this treatment would have any adverse effects upon the amino acid patterns or resolution, samples of coffee cells and of ground coffee beans were used. These samples were split in two and one half was treated. The other half was left untreated. The samples were run on the amino acid analyzer and the results compared. No differences were noted between the treated and untreated samples except that in the treated samples, the ammonia concentration was lower and histidine was more readily calculated.

The dried desalted residue resulting from the protocal of the previous section was then transferred quantitatively to large (150 milliliter) beakers, using twenty-five to fifty milliliters of distilled water. The pH of these solutions was adjusted to approximately pH 10 using 1M sodium hydroxide. The beakers were placed in a vacuum desiccator containing

concentrated sulphuric acid. The desiccator was then pumped down to approximately 0.2 microns vacuum with care being taken to avoid 'bumping' of the samples. After one and a half hours under vacuum, the samples were removed and their pH adjusted to approximately pH 6.

The samples were frozen to prevent spoilage. When the samples were to be analyzed, the samples were defrosted and dried *in vacuo* at 60°C.

v. Analysis on the amino acid analyzer (Moore and Stein, 1954).

The dried samples were taken up quantitatively in five milliliters of citrate buffer pH 2.21, filtered, if required, and then stored in the freezer until needed. The samples were run on a dual column arrangement on a Pheonix amino acid analyzer.

b. Dry weights of the samples (Beveridge, 1972).

The free amino acids were calculated in terms of dry weight of the ground coffee bean and the dry cells. The dry weight was determined by weighing a sample of 250 milligrams into an aluminium foil cup, placing it in an oven at 104° C, and reweighing after five hours in the oven and every hour after until no further change in weight was detected. This final weight was used as the dry weight of the sample.

c. Calculations (Phoenix Instrument Book).

The amount of free amino acids in each sample was calculated on the dry weight of that sample. The calculations for each amino acid involved the measuring of the area enclosed by its corresponding peak on the chromatogram using the height

times width (HW) method of integration.

d. Protein content of the samples (Gornall et al, 1949).

The acid hydrolysis involved in the amino acid sample preparation could also hydrolyze to a limited extent any protein present after the ethanolic extraction. A biuret reaction was therefore performed on part of some sample solutions of ground coffee beans and coffee cells before and after hydrolysis to ensure that the protein content was minimal.

The samples were dried *in vacuo*, each was then mixed with ten milliliters of distilled water and five milliliters of the solution was mixed well with one milliliter of biuret reagent. The absorbance of the solutions was then determined at 583 millimicrons on a Beckman spectrophotometer. As no noticeable reaction occurred, the protein content of the sample solutions was less than one percent.

e. Thin layer chromatography of the free amino acids (Jones and Heathcote, 1960).

Several of the samples run on the amino acid analyzer were also chromatographed on Cellulose Mn 300 plates for further identification. The samples were applied to the plates in the lower left hand corner and dried. The plates were then run first in isopropanol : water : formic acid (40 : 10 : 2), dried, turned and then run in tertiary butanol : methyl ethyl ketone : ammonia (88% by volume) : water (25 : 15 : 6.25 : 3.75) in the second dimension. The plates were visualized using ninhydrin spray (0.3 grams ninhydrin, 20 milliliters glacial acetic acid, and 5 milliliters collidine in 75 milliliters absolute ethanol). The Rf values and the colours of visible spots were noted.

7. Roasting of the green coffee bean, ground coffee, and coffee bean extracts.

The green coffee bean produces the familiar aroma, flavour and colour on roasting. It should thus be possible to roast coffee extracts to obtain coffee aroma.

a. Roasting of the green coffee bean.

The green coffee bean was placed in a Coors porcelain flat dish and roasted at 220°C in a muffle furnace (Thermolyte) for fifteen minutes and then rapidly cooled in an ice bath. The beans were then crushed to obtain a coffee aroma.

b. Roasting ground green coffee.

The green beans were ground in a Wiley mill to pass through a twenty mesh sieve. One gram portions were placed into Coor's porcelain crucible dishes and roasted in a muffle furnace at 220°C for varying lengths of time. A dish of water was placed in the oven in some cases to raise the oven's humidity and thus slow down the evaporation of the sample's moisture. Upon removal from the oven, the samples were covered with a watch glass to retain any aroma escaping and cooled rapidly in an ice bath.

Efforts to obtain coffee aroma from the ground bean pellets were made using a Paar's pellet machine. The pellets were made in 0.5, 1.0, and 1.5 gram portions. These were roasted at 220°C in the presence of a dish of water until the pellets were dark brown externally. The pellets were then cooled rapidly and disrupted. The internal colour and aroma were noted.

c. Roasting coffee bean extracts.

i. Preparation of the samples.

Ten grams of ground coffee (40 mesh) were extracted with one hundred milliliters of boiling solvent of each of distilled

water, methanol, and ethanol by homogenization at high speed for three to five minutes using an Osterizer. The solutions were left overnight then filtered through Whatman filter paper and the residue washed with the appropriate solvent (approximately twenty milliliters). The methanol and ethanol solutions were reduced in volume at 45°C in vacuo to remove excesses of the solvent. The solutions were then diluted slightly by the addition of water. This raised the freezing point closer to the freezing point of water. All the solutions were then freeze dried and stored in a desiccator until use.

ii. Roasting of coffee bean extracts.

The extracts (0.5 grams) and one milliliter of water per sample were mixed together and roasted until brown in Coor's porcelain crucible dishes in a muffle furnace at 220°C. When brown, coffee extracts were removed from the oven, a watch glass placed over top, and then the dishes were cooled rapidly in ice. A dish of water was present in the oven for most of the test mixtures. Various additions were made to the samples. These additives were calcium carbonate, dextrose, caffeine, tannic acid and chlorogenic acid. They were added at fifty milligrams each per sample. In some cases 0.5 N hydrochloric acid or 0.5 N sodium hydroxide were used in place of water. The colour and aroma of the samples were noted after roasting.

8. Caffeine in the green coffee bean and the coffee cells.

The caffeine content of green coffee and coffee cells was determined using a modification of the micro Bailey - Andrew method (Horwitz (AOAC), 1970).

a. Determination of caffeine (Horwitz (AOAC), 1970).

One gram of ground coffee or of coffee cells was mixed with five grams of powdered magnesium oxide and approximately

100 milliliters of water. The solution was heated to boiling and boiled forty-five minutes, swirling occasionally. The solution was then cooled and filtered through a Whatman number one filter paper into a 125 milliliter separatory funnel. Four milliliters of sulphuric acid (in a ratio of one milliliter of acid to nine milliliters of water) was added and the solution was mixed well. The solution was then extracted five times with ten milliliter portions of chloroform. Each extraction was shaken vigorously for one minute and then left to sit until the emulsion broke. The chloroform layer (the bottom layer) was drained into a second 125 milliliter separatory funnel. When the extraction was completed, five milliliters of 1% potassium hydroxide were added to the chloroform extract. The solution was shaken vigorously for one minute and then left until the emulsion broke. The chloroform layer was then drained off through a cotton plug into a kjeldahl flask. The chloroform extract was digested on a digestion rack after the addition of 1.30 grams of potassium sulphate, 40 milligrams of mercuric oxide, and two milliliters of concentrated sulphuric acid. The samples were allow to digest for one hour after the solutions became clear, and then were cooled.

Ammonium content of the samples was determined by Nessler's reaction. Aliquots of 0.05, 0.1, and 0.2 milliliters of digest were mixed well with five milliliters of Nessler's reagent (Fisher) in duplicate. The solutions were read at 500 millimicrons on a Beckman spectrophotometer. Standards containing twenty, thirty, and fifty micrograms of nitrogen were also run (Fitzsimmons and Mason, 1972).

Calculation of the caffeine content was performed using the following equation:

$$\left(\frac{10}{\text{average O.D. of standard} \,/\, \text{10 micrograms nitrogen}}\right)\left(\text{optical density of sample}\right)(\text{volume factor})$$

The volume factors were as follows: for 0.2 milliliter sample the volume factor was 1; for 0.1 milliliter sample - 2; and for the 0.05 milliliter sample - 4.

b. Paper chromatography of caffeine (Ogutuva and Northcote, 1970).

Several samples of medium coffee cells and ground coffee beans were run on paper chromatography. The chloroform extract of these samples was spotted on paper chromatograms and allowed to dry. A standard of caffeine (Fisher) was run with the samples. The papers were developed by descending chromatography for twenty hours in the upper phase of n-butanol : water (86 : 14 v/v). Caffeine was detected by ultra violet light.

9. Chlorogenic acid in the green coffee bean and the coffee cells.

The chlorogenic acid content of ground coffee and dry coffee cells was determined following the AOAC method (Horwitz, 1970).

a. Determination of chlorogenic acid.

i. Preparation of the sample solution.

Samples of 700 milligrams of both ground coffee bean and dry coffee cells were placed in centrifuge tubes and twenty-five

milliliters of petroleum ether was added and mixed in thoroughly. The samples were then centrifuged at 5,000 rpm for ten minutes in a Servall angle centrifuge (Ivan Sorvall Inc) and the supernatant decanted off and discarded. This extraction with petroleum ether was repeated two more times to ensure complete removal of any lipids present. The remaining residue was placed on a watch glass and left to dry until the odour of petroleum ether was no longer detectable (approximately half an hour).

The samples were then transferred with small amounts of water to erlenmeyer flasks (300 milliliter size for the dry cells and one liter size for the coffee bean). Because of the low concentration of chlorogenic acid present in the dry cells, and because of the shortage of experimental material, it was necessary to reduce the total volume of the sample solution of the dry cells to increase the chlorogenic acid concentration sufficiently for detection.

Boiling distilled water was then added to the dry residues in the erlenmeyer flasks - 400 milliliters to the ground coffee bean and 90 milliliters to the dry coffee cells. The solutions were then rapidly brought to a boil and boiled gently for fifteen minutes. The flasks were swirled frequently to keep the sample submerged. Flasks were cooled rapidly to room temperature. The cooled solutions were transferred to volumetric flasks and made up to volume - 500 milliliters for the ground coffee and 125 milliliters for the dry cells. The solutions were then filtered through Whatman number one filter paper discarding the first twenty-five to thirty milliliters of filtrate. The remaining filtrate was used for the sample solution.

ii. Determination of the chlorogenic acid.

(a). Untreated determination.

Ten milliliters of the sample solution was transfered to a one hundred milliliter volumetric flask and diluted to volume

with distilled water. The absorption of the solution was measured at 324 millimicrons against distilled water using a D.B. spectrophotometer (Beckman).

(b). Lead treated determination.

Fifty milliliters of sample solution were transferred to a one hundred milliliter volumetric flask. One milliliter of a saturated potassium acetate solution and five milliliters of a basic lead acetate solution ($Pb[OAc]_2$ with a specific gravity of 1.25) were added to the sample solution while swirling the flask. The resulting solution was placed in a boiling water bath for five minutes and then cooled rapidly to room temperature using tap water. The solution was then placed in an ice bath and mechanically stirred. After one hour the flask was removed from the ice bath, and the stirrer washed down with distilled water. The solution was warmed to room temperature using tap water, diluted to volume with distilled water and then filtered through Whatman number one filter paper discarding the first twenty to thirty milliliters of filtrate. The absorbance was read on the remaining filtrate immediately at 324 millimicrons on the D.B. spectrophotometer.

iii. Preparation of the standard curves.

A standard solution of chlorogenic acid (supplied by J.T. Baker Co.) was made (40 milligrams per liter) and from this solution a series of standards were derived. The series was made by transferring a given aliquot (one milliliter to 20 milliliters) to a one hundred milliliter volumetric flask and diluting the solution to volume using distilled water. The absorption of this series was read at 324 millimicrons on a D.B. spectrophotometer against distilled water.

iv. Calculation of the concentration of chlorogenic acid in the sample solutions.

The optical readings obtained for the untreated and the lead treated sample solutions were converted to apparent concentrations using the standard curve for chlorogenic acid. The corrected concentrations for the samples were then calculated using the following formula:

corrected concentration = Co - (Ci - 0.00045)/5

Co - apparent concentration of chlorogenic acid in solution taken for absorbance measurement without lead treatment.

Ci - apparent concentration of chlorogenic acid in the filtrate after lead treatment.

0.00045 - correction factor for the solubility of lead chlorogenate after the lead acetate treatment in milligrams per milliliters.

b. Paper chromatography of the chlorogenic acid (Fitelson, 1969).

Paper chromatograms were run on the extracts of dry coffee cells and the coffee beans. One gram of sample was extracted with 80% boiling ethanol, homogenized at high speed on an Osterizer and then allov i to stand overnight to ensure complete extraction. The sample solutions were then filtered through Whatman number one filter paper and the filtrate evaporated to dryness in vacuo at 45°C. The residue was taken up in a minimal amount of water and applied to a paper chromatogram (Whatman number one). A reference solution containing chlorogenic acid, caffeic acid, malic acid, tartaric acid, oxalic acid and citric acid was run with the sample solutions.

The chromatogram was developed in the upper phase of n-butanol : water : acetic acid (4 : 5 : 1) for fourteen hours. The paper was then dried and examined under ultra violet light. The paper was then sprayed with an aniline furfural spray reagent (0.3 milliliters of aniline, and 0.3 milliliters of furfural, in one hundred milliliters of methanol).

RESULTS AND DISCUSSION

1. The growth of the cultures.

a. Callus cultures.

Undifferentiated growth became visible on one or both ends of the coffee stem explant after approximately one month of incubation. The initial growth was slow and brownish in colour. The morphology of the callus was firm, smooth and compact. After several months of incubation, the growth pattern and morphology of the callus cultures had changed markedly. The calluses were pale grey in colour and grew very rapidly. The morphology of the calluses was irregularly lobed and very friable (Figure 3). This type of growth was maintained during the study. These callus cultures appeared to be very similar to the friable coffee cultures obtained by Staritsky (1970).

b. Suspension cultures.

Single cell growth was apparent approximately three weeks after inoculating the liquid medium with callus fragments. The growth of the cultures was observed by a gradual increase in the medium's opaqueness and by the accumulation of debris and cells at the high liquid line (Figure 2). Growth could also be observed by microscopic observation.

Initially, growth of the suspension cultures was slow and erratic with obvious variation between cultures inoculated from the same flask. Small calluses were formed during the early stages of the suspension cultures. These were gradually eliminated over time by transferring only the cell suspension (that is only the material which remained in suspension after allowing the contents to settle for a few minutes).

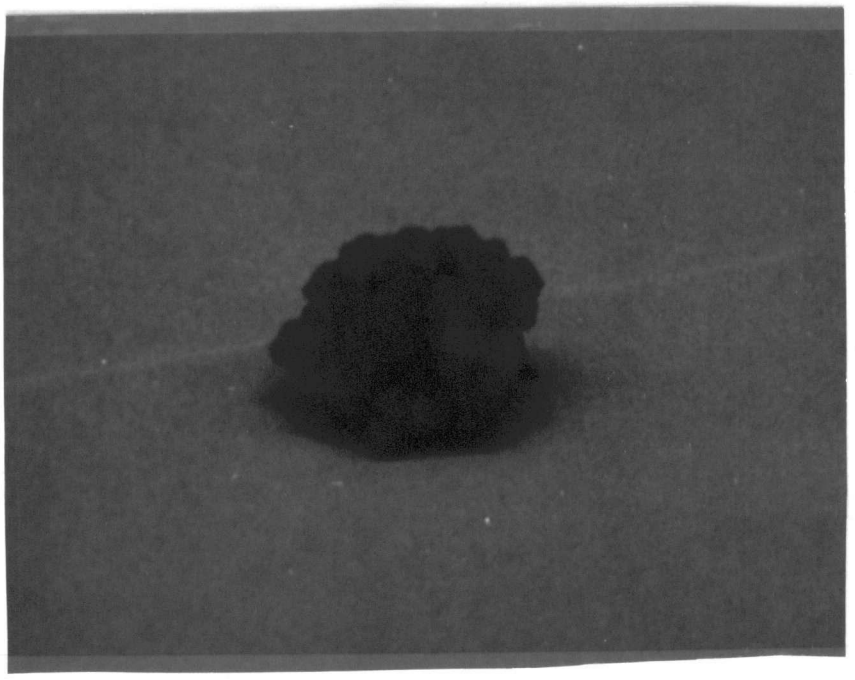


Figure 3. A Coffea arabica L. callus culture grown on B5 medium.

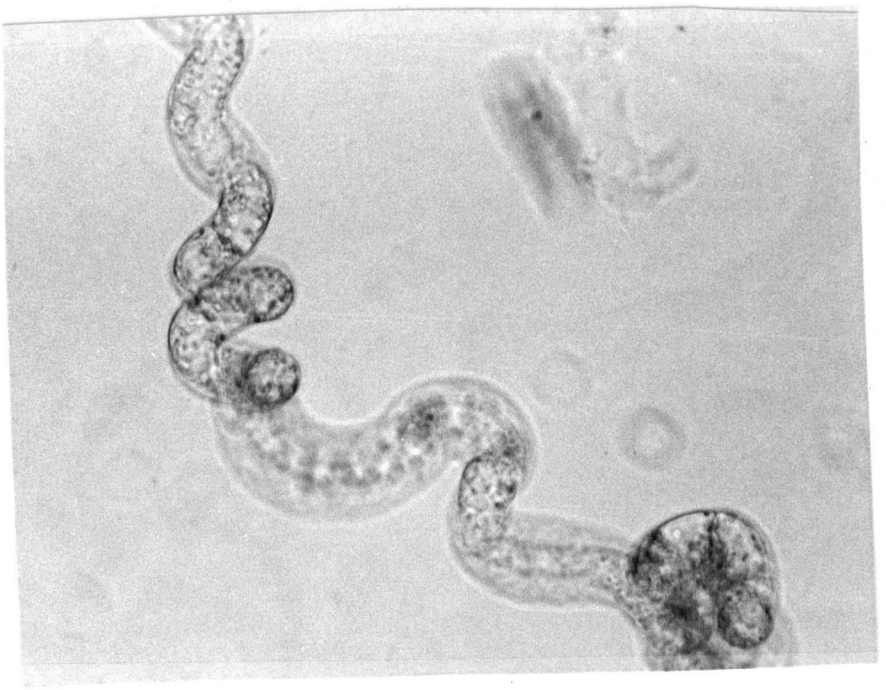
The growth rate of the cell suspension cultures increased with continual transfer and uniformity became more apparent between the cultures and the growth rate from the same source of inoculum. Once uniform growth was obtained the cultures were grown for study.

The cell suspension cultures were composed mainly of long filament-like strand of cells with minor amounts of single cells and small cell aggregates (clumps) present. Generally plant cell suspension cultures are composed mainly of single cells and small cell aggregates (Nishi and Sugano, 1970; Townsley and Buckland, 1972; Rose, 1972).

The long filamentous type of growth observed is very similar to algae filaments (Figures 4 and 5). Spiralling of the filaments either singly (Figure 4) or in pairs (Figure 5) was frequently observed. Occasionally variation of cell size within the chains was observed (Figure 5). This variation in size may be related to the stage of elongation or division of the individual cell. Elongation of the cells was generally unidimensional and division occurred only in a plane perpendicular to the long axis of the cell, thus producing long filaments.

Nishi and Sugano (1970) reported the occurrence of long filamentous growth in carrot suspension cultures grown on liquid medium containing IAA. Carrot cultures grown on the same liquid but containing different growth hormones differred in cellular morphology and in the mode of cellular division. Differences in morphology and cell division have been attributed previously mainly to nutritional differences although differences between cell cultures on the same medium can be caused by cell clone (line) selection (Martin, 1972; Townsley and Buckland, 1972).

In bacterial cultures it has been demonstrated that limiting levels of certain nutrients such as divalent cations,



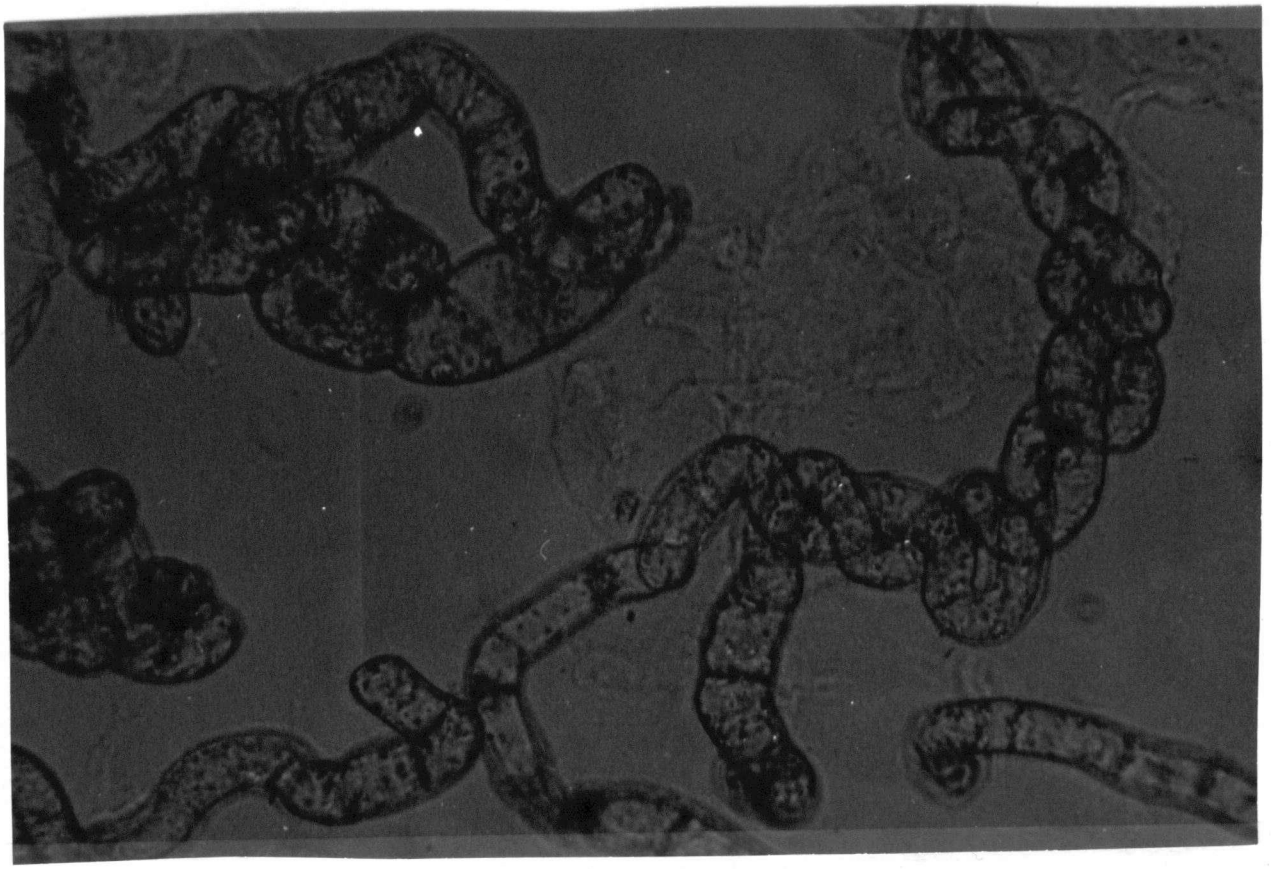
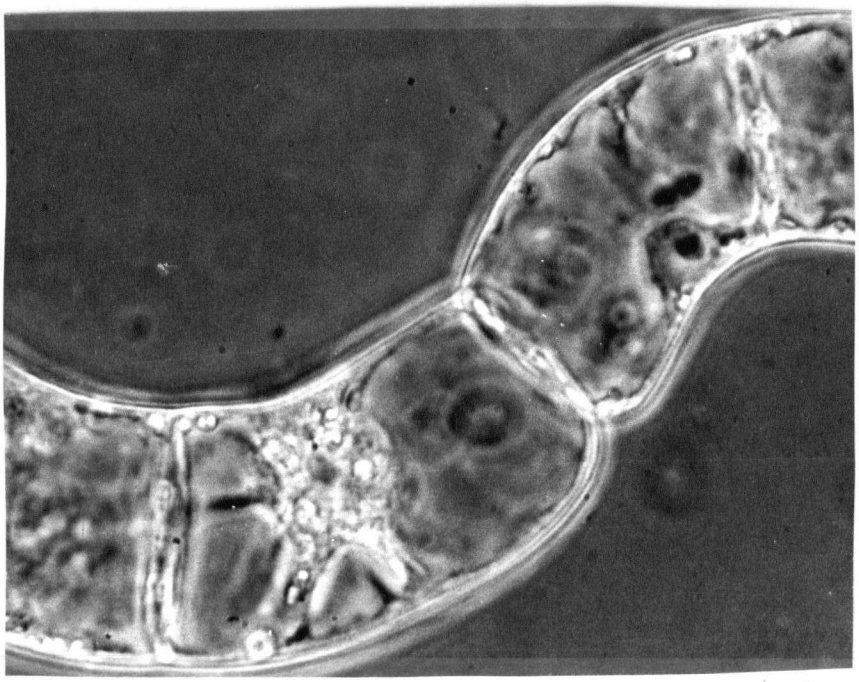


Figure 4. A filament from a Coffea arabica L. suspension culture showing filament spiralling (100x magnification) from a twelve day old culture.

Figure 5. Cell suspension from Coffea arabica L. culture showing filaments spiralling together and varying cell size within filaments (100x magnification) from a twelve day old culture.

and glutamate can cause filament formation (Macdonald, 1971). The limiting levels of these nutrients does not allow normal separation of the cells after division. Separation is normally caused by the action of extracellular autolytic enzymes (Macdonald, 1971). This latter bacterial mechanism is not known to operate in plant cell suspension cultures. However, it is possible that the separation of the plant cells in vitro is mediated by a similar mechanism dependent upon the growth hormone present and the level of certain nutrients.

During the 'log phase' of growth the individual cell appeared to be healthy and quite active metabolically when observed under the microscope. The nucleus, nucleoli and cytoplasmic strands were generally distinguishable (Figures 6 and 7). Movement along the cytoplasmic strands was also observed frequently. Division of the cells within a chain was occasionally noted. In the older cells, small bodies of unknown composition began to appear in large numbers (Figures 8 and 9). These bodies were still visible after the cell had died (Figure 9). Possibly, these bodies were some type of starch granule. Some layering within these structures can be observed in Figure 9. Layering is commonly observed in most types of starch granules. These bodies did not react to the application of iodine which turns blue - black on contact with amylose starch. Amylopectin starch turns a faint red brown with the iodine test and is sometimes difficult to detect. These bodies could, therefore, be starch granules composed mainly of amylopectin. Starch granules have been previously observed in suspension cultures of potato (Gamborg, 1967) and in other plant suspension cultures (Rose, 1972). Rose (1972) has observed starch granules in several plant suspension cultures similar to the ones observed in the coffee suspension culture (Figures 8 and 9).



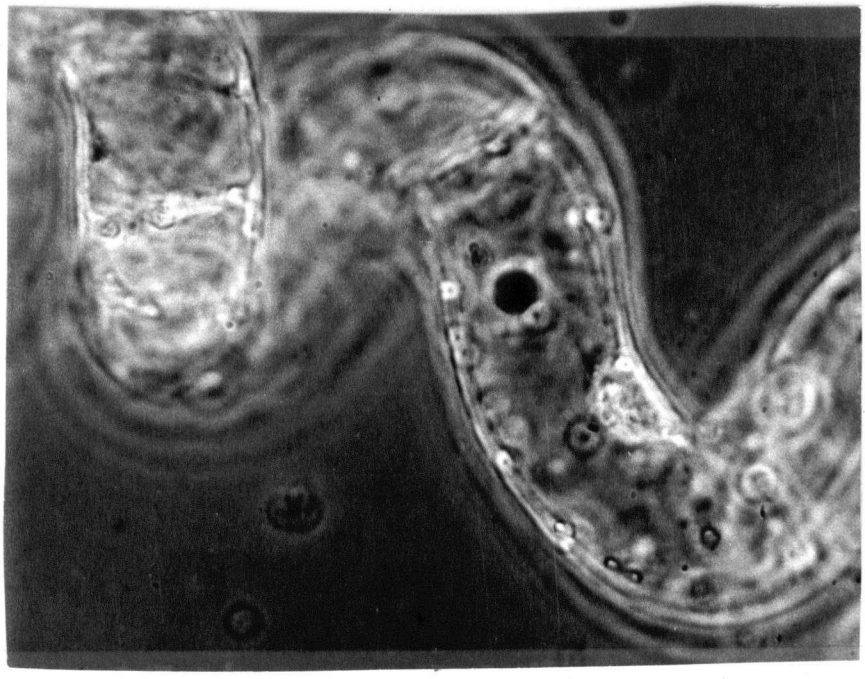
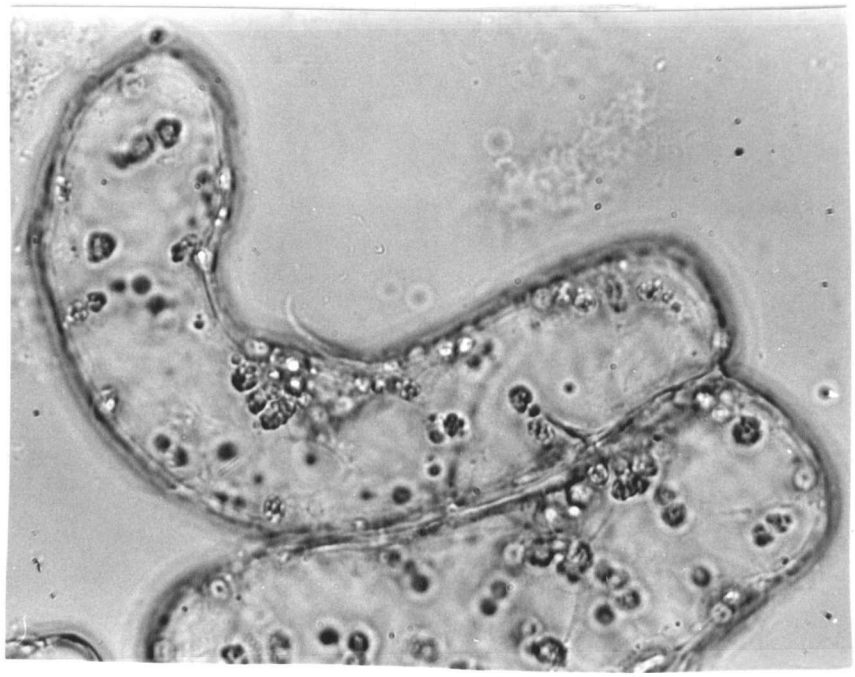


Figure 6. Healthy Coffea arabica L. cells showing nucleus, nucleolus, and cytoplasmic strands (magnification - 400x) from a ten day old culture.

Figure 7. A healthy Coffea arabica L. cell within a filament spiral (magnification - 400x) from a fourteen day old culture.



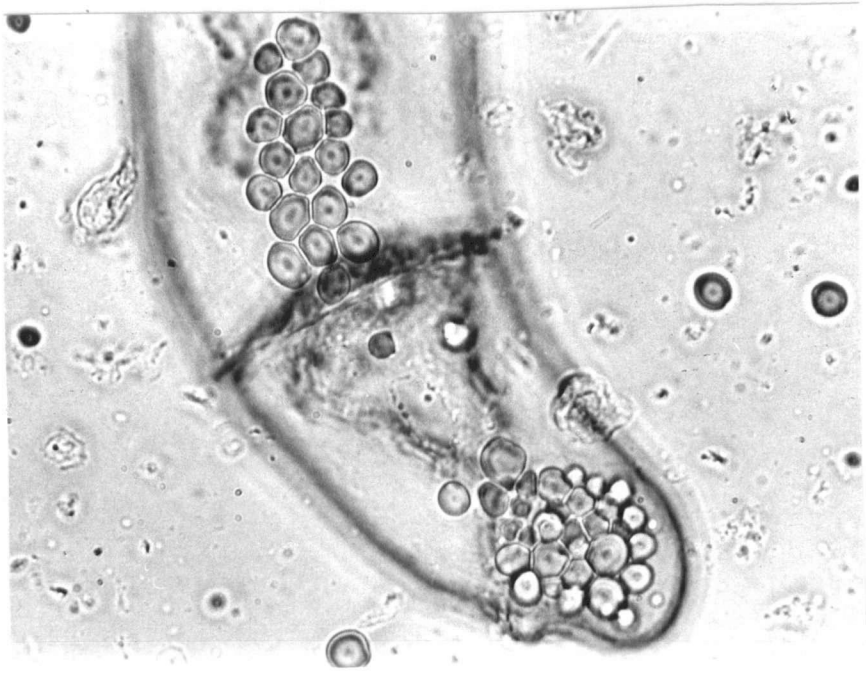


Figure 8. Coffea arabica L. cells containing unknown bodies throughout the cell (magnification - 400x) from a fourteen day old culture.

Figure 9. Dead cells of Coffea arabica L. cultures containing unknown bodies within the cell wall (magnification - 400x) from a twenty-five day old culture.

c. Growth of the suspension culture.

Growth of the suspension cultures was measured in grams fresh weight and grams dry weight of the cells. The pH value of the medium was determined on the filtrate following the removal of cells. The fresh weight of the cells is not a reliable value as actively dividing cells show an increase in water uptake (Figure 10). Thus, the value obtained may vary greatly from actual cell weight. The dry weight of the cells is a more accurate representation of actual cell growth (Figure 10). The pH of the medium indicates the utilization of nitrate and possibly the release of cell metabolites into the medium.

The growth curves obtained were similar to plant cell growth curves obtained by other authors (Simpson and Street, 1970; Townsley, 1972). The initial lag phase of the culture averaged about four days and was followed by a period of rapid growth. Rapid growth (generally termed log phase of growth) started at approximately day eight and continued for six days. Growth continued fairly rapidly peaking at day eighteen. Exponential growth was not obtained during the rapid phase of growth. Several authors have interpreted a log phase of growth in plant suspension cultures on an arithmetic plot only (Gamborg, 1967; Simpson and Street, 1970; Nishi and Sugano, 1971). Rose (1972), however, does not accept the concept of exponential growth as being applicable to the rapid growth phase in plant cell cultures.

The stationary phase of growth exists while the rate of anabolic growth of the cell population is approximately equivalent to the rate of catabolic growth. This phase averaged only four days in these cultures. The stationary phase was then followed by a decline in both fresh and dry

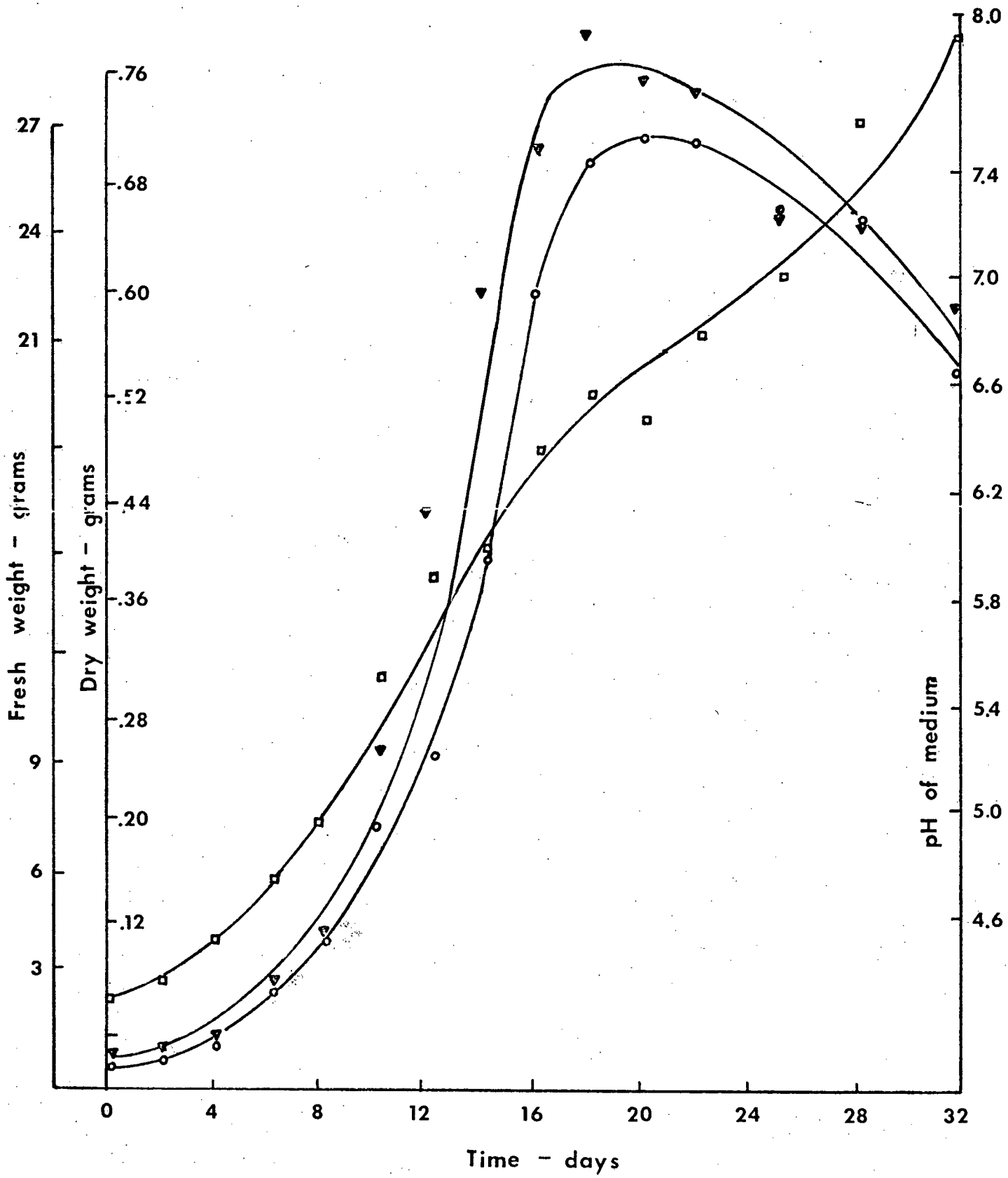

Fresh weight - grams
Dry weight - grams
pH of medium
Time - days
27
24
21
9
6
3
.76
.68
.60
.52
.44
.36
.28
.20
.12
8.0
7.4
7.0
6.6
6.2
5.8
5.4
5.0
4.6
0
4
8
12
16
20
24
28
32

Figure 10. Growth of the Coffea arabica L. cell suspension cultures.

Points are average of eight to ten samples (values given in Table 5).

symbols:

□——□ pH of medium

○——○ dry weight of filtered cells

▽——▽ fresh weight of filtered cells

Table 5. The measurement of growth in Coffea arabica L. cell suspension cultures

time (days)	fresh weight of cells* (grams)	dry weight of cells* (grams)	pH of medium*
0	0.50	-	4.30
2	0.75	0.025	4.39
4	1.12	0.037	4.53
6	2.23	0.078	4.76
8	4.05	0.113	4.99
10	7.32	0.262	5.51
12	9.26	0.431	5.90
14	14.88	0.595	5.99
16	22.42	0.698	6.37
18	25.90	0.784	6.58
20	26.40	0.758	6.49
22	26.29	0.743	6.80
25	25.67	0.668	7.02
28	25.77	0.664	7.50
32	20.40	0.585	7.93

---

*values given are average of eight to ten samples.

weight of the cells. This decline occurs when the internal rate of catabolism exceeds the rate of anabolism (Figure 10 and Table 5).

The stationary phase is generally more extended (tobacco - Nichi and Sugano, 1971; soya bean - Gamborg, 1967) than was observed in these coffee cultures. The rapid decline soon after the rapid cell growth would seem to indicate the presence of material(s) which cause the inhibition of cell growth or death of the cells. This material is possibly a secreted cell metabolite or a limiting medium constituent. Dead cells, observed early in the culture's growth, increased in number with the increase in age of the culture (Figure 9) especially after rapid growth had ceased.

The pH of the medium rose with the increase in growth (Figure 10 and Table 5). Generally the pH of the medium (both PRL-4-C-CM and B5) decreases or remains at a constant level once rapid growth has ceased; for example, in rose (Wegg, 1972) and bean (Buckland, 1972) cultures. The rapid increase in the pH value of the medium after growth had ceased indicates release of cell metabolites into the medium either by secretion or by cell lysis. In either case, it is probably the dead cells which release the compound(s) which cause the increase in pH with the decline in growth.

The cell yield of the cultures was fairly good although higher yields have been obtained by other authors using different suspension cultures (Nishi and Sugano, 1971; Buckland, 1972). The cell growth was quite healthy and uniform throughout the study. No contamination by mold or other microorganisms was observed.

2. Feee amino acids in Coffea arabica L. cell suspension cultures green coffee beans.

Free amino acids were detected and analyzed in suspension culture and green beans of Coffea arabica L. The most noteable difference was in total content of the free amino acids. The coffee suspension culture contained 301.7 micromoles of amino acids per gram dry weight of tissue at the maximum whereas the coffee bean contained 25.3 micromoles of amino acids per gram dry weight of tissue. There were also differences in the composition of amino acids present.

a. Determination of free amino acids - methods.

The procedure followed in preparing the samples for analysis involved a mild hydrolysis of the samples. This hydrolysis was essential to rid coffee bean samples of interfering substances. The same procedure was carried out with the suspension culture samples to allow direct comparison of the results. The hydrolysis was very effective since the abberant behavior of coffee bean samples on amino acid analysis was eliminated.

The mild acid hydrolysis also hydrolyzed the amides to their respective amino acids (glutamine to glutamic acid and asparagine to aspartic acid). This was concluded to be the reason no evidence of amides present was found on the resulting chromatogram (Figure 11) whereas there were indications of the presence of amides when the samples were run on thin layer chromatograms.

γ-aminobutyric acid was not detectable on the amino acid analyzer used for analysis. A standard run containing a fairly high concentration of γ-aminobutyric acid failed to show where this amino acid would elute.

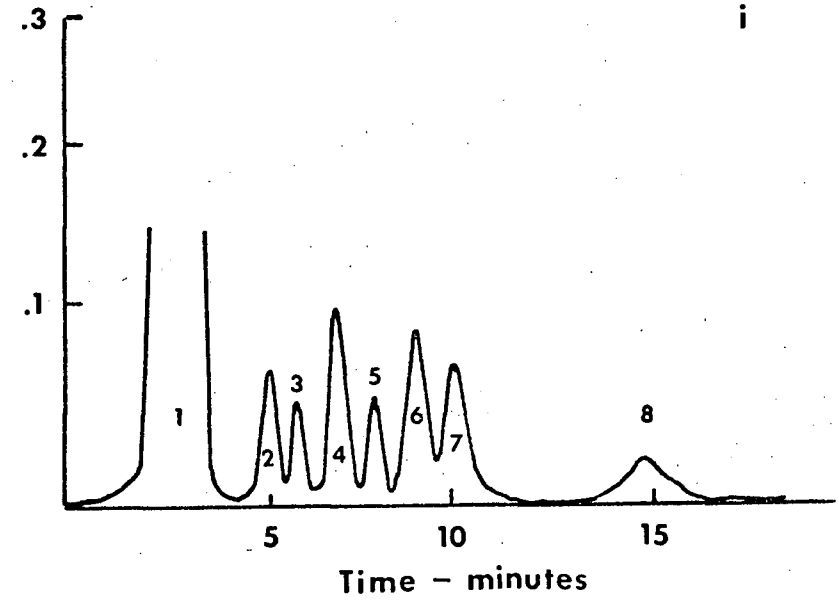
Sampling for amino acid analyses was started six days after inoculation, but three samples for neutral and acidic amino acids were lost (Table 6).

b. Free amino acids in Coffea arabica L. suspension cultures.

The total free amino acid content increased with the age of the culture from 32.0 micromoles of amino acids per gram dry weight (day 6) to 301.7 micromoles of amino acids per gram dry weight (day 25) (Table 6 and Figure 12). The concentration of free amino acids increased rapidly during active growth and again during culture decline. The total acidic and neutral amino acids followed the same pattern as total amino acids and the basic amino acids remained relatively stable in concentration, elevating slightly in the stationary and decline phase (Figure 12).

There was a marked drop in total amino acids and total acidic and neutral amino acid content at day twenty-two. It was less marked in the basic amino acids but still present. This drop occurred just after the maximum cell growth had been attained (day 20). The free amino acids were rapidly synthesized during active growth and with completion of active growth the synthesis decreased. The rise at day twenty-five in the content of amino acids may be a result of cellular breakdown accompanying cell and culture death (Figure 12).

The content of individual amino acids varied with the age of the cultures. A typical chromatogram of an eighteen day culture can be seen in Figure 11. The free amino acids, leucine, tyrosine, proline, arginine, and unknown one and two increased gradually in content during rapid growth of the culture, and then levelled off or decreased slightly during the decline in growth of the culture. These amino acids were present in the culture in relatively low concentrations (Table 6).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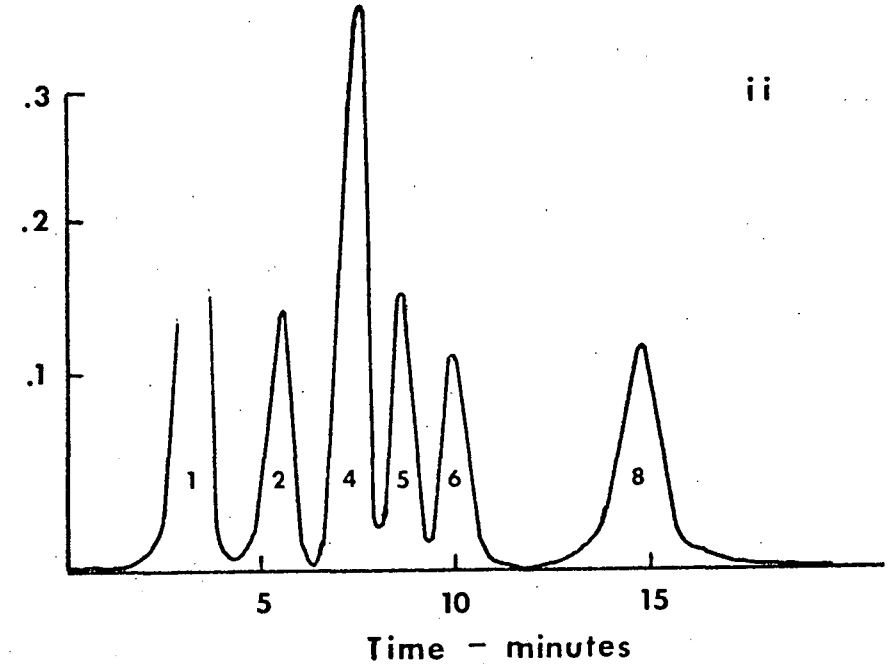
Time – minutes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Time – minutes

Figure 11. Chromatographic separation on the amino acid analyzer of the free amino acids in Coffea arabica L. green beans and cell suspension culture.

a. Separation of basic amino acids

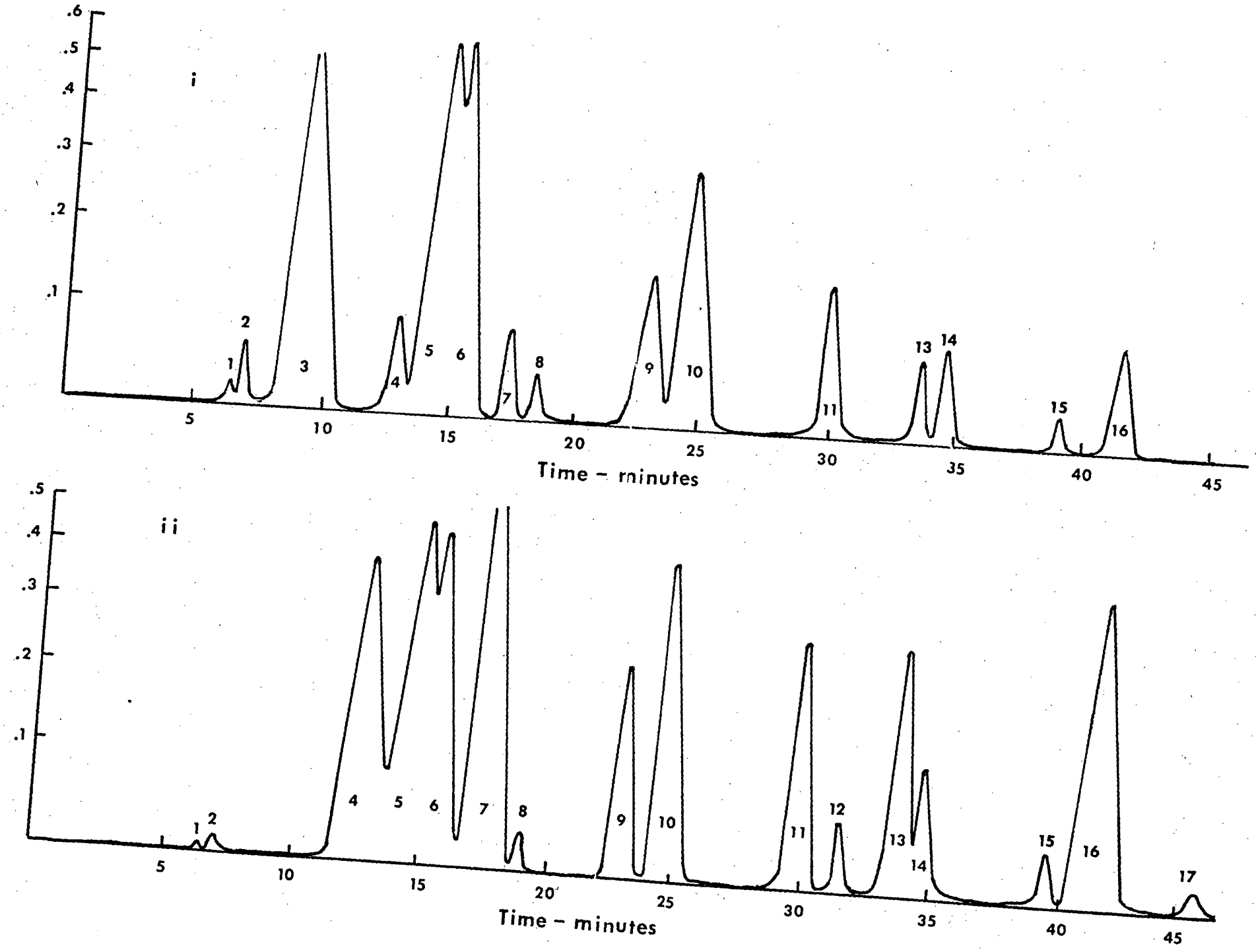
i. Green coffee bean - sample application contained 0.075 gram dry weight of sample.

ii. Coffee cell suspension culture (18 days old) - sample application contained 0.0375 grams dry weight of sample.

identity of peaks

1. acidic and neutral amino acids
2. unknown 1 - possibly tryptophan
3. unknown a*
4. lysine
5. histidine
6. ammonia
7. unknown b*
8. arginine

*unknown a and b were not calculated individually in tabulation of amino acids (Table 6).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40
45
Time – rninutes
ii
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6
7
8
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10
11
12
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14
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16
17
5
10
15
20
25
30
35
40
45
Time – minutes

Figure 11. Chromatographic separation on the amino acid analyzer of the free amino acids in Coffea arabica L. green beans and cell suspension cultures.

b. Separation of neutral and acidic amino acids.

i. Green coffee bean - sample application contained 0.0625 grams dry weight of sample.

ii. Coffee cell suspension culture (18 days old) - sample application contained 0.0175 grams dry weight of sample.

Identity of peaks

1. unknown c*
2. unknown d*
3. unknown e*
4. aspartic acid
5. threonine
6. serine
7. glutamic acid
8. proline
9. glycine
10. alanine
11. valine
12. methionine
13. isoleucine
14. leucine
15. tyrosine
16. phenylalanine
17. unknown 2

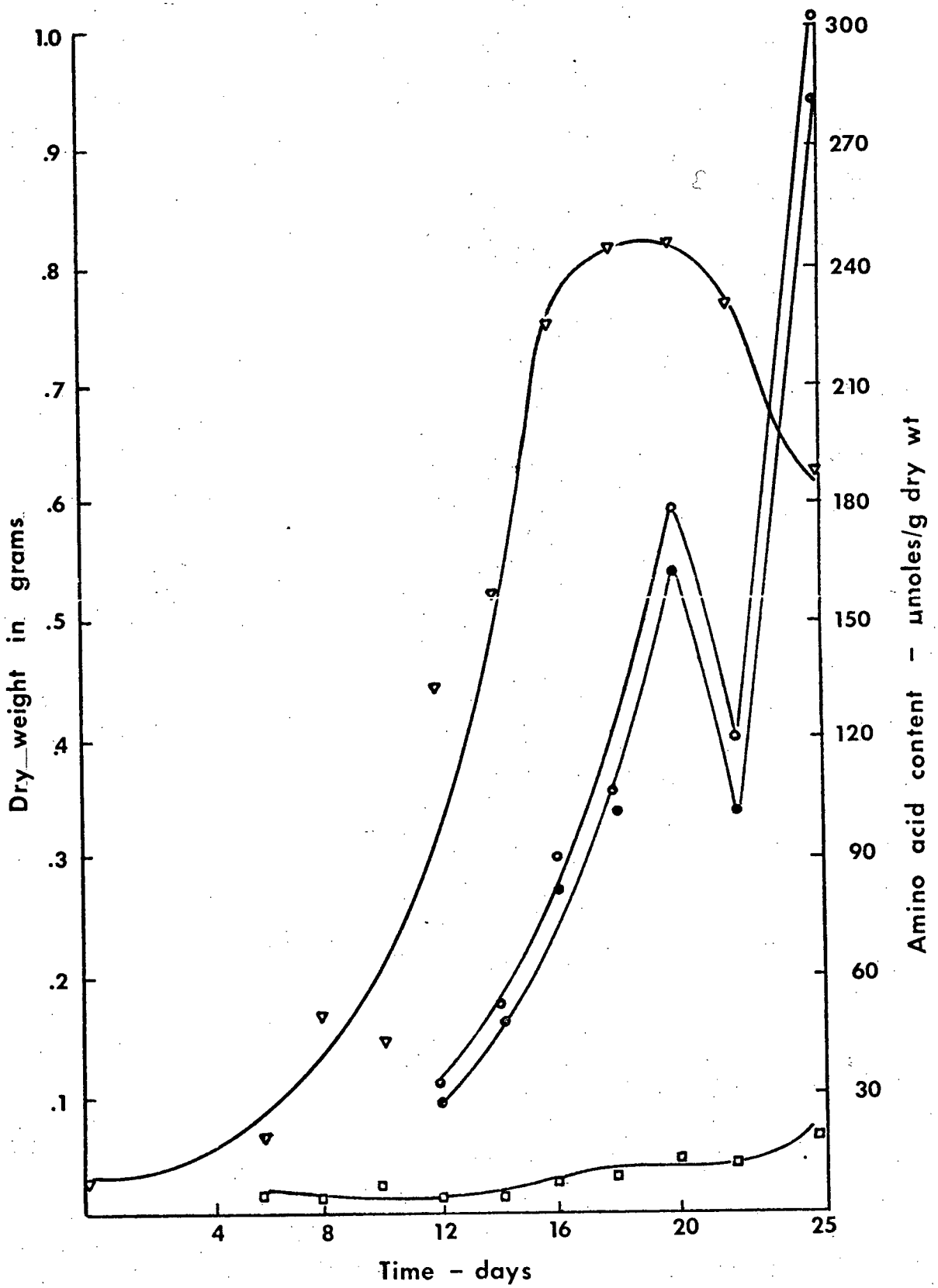
*unknowns c, d, and e were not calculated individually in tabulation of amino acids (Table 6).

Table 6. Free amino acids present in Coffea arabica L. suspension cultures and green coffee beans.

	Coffea arabica L. suspension cultures (days after inoculation)										green coffee beans
amino acids	6	8	10	12	14	16	18	20	22	25	
aspartic acid	-	-	-	0.512	-*	0.470	7.265	32.496	20.451	91.998	0.321
threonine	-	-	-	1.133	3.502	6.957	9.260	12.418	7.420	14.575	5.764
serine	-	-	-	1.373	7.265	11.888	7.757	9.306	7.653	19.866	4.500
glutamic acid	-	-	-	6.372	5.972	13.962	20.825	24.832	15.292	36.242	0.474
proline	-	-	-	0.056	0.101	0.178	0.155	0.424	0.175	0.568	0.178
glycine	-	-	-	1.889	4.867	4.836	5.175	6.349	5.173	11.352	1.058
alanine	-	-	-	6.569	9.453	5.758	11.858	17.164	11.014	27.266	3.209
cysteic acid	-	-	-	-*	-*	-*	+	+	0.854	4.097	-*
valine	-	-	-	1.478	2.079	3.152	6.139	12.828	7.741	20.933	0.888
methi nine	-	-	-	0.330	+	0.633	1.376	2.571	1.411	3.639	-*
isoleucine	-	-	-	0.611	0.937	2.348	6.670	10.877	5.783	10.585	0.517
leucine	-	-	-	0.761	1.722	2.056	3.233	3.409	1.610	2.664	0.656
tyrosine	-	-	-	0.342	0.880	1.458	1.926	2.315	1.405	2.364	0.559
phenylalanine	-	-	-	3.168	5.423	19.574	17.317	25.779	13.455	31.559	1.147
lysine	1.204	1.120	1.534	0.933	1.129	0.882	2.710	4.676	7.646	8.743	0.410
histidine	1.043	0.628	0.697	0.489	0.625	2.421	1.624	3.912	3.142	6.457	0.199
arginine	0.662	0.700	1.014	1.796	0.486	1.181	1.466	0.943	0.593	3.350	0.117
unknown 1	1.331	1.672	2.188	1.251	0.858	0.960	0.824	3.535	1.358	1.163	0.212
unknown 2	-	-	-	0.216	0.273	0.566	0.458	0.320	-*	0.640	-*
total basics°	4.220	4.441	5.433	4.605	3.097	5.444	6.625	13.065	12.737	19.692	1.436
total acidics and neutrals°	-	-	-	27.419	46.508	80.326	99.414	161.852	101.285	281.966	23.867
total°	-	-	-	32.024	49.605	85.770	106.039	174.917	114.022	301.658	25.303

- not analyzed; -* none detected; + trace present only; °totals include minor unknown amino acids not listed.

values given are the average of two samples - expressed in micromoles amino acid per gram dry weight of sample.


1.0
.9
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Dry weight in grams
300
270
240
210
180
150
120
90
60
30
Amino acid content - µmoles/g dry wt
4
8
12
16
20
25
Time - days

Figure 12. Free amino acid content of Coffea arabica L. suspension culture.

points are averages of two samples
(values given in Table 6)

symbols:

▽——▽ dry weight of filtered cells
(values from Table 5)

○——○ total free amino acid content of cultures

●——● total free acidic and neutral amino acid content of cultures

□——□ total free basic amino acid content of cultures

Cysteic acid was not detectable in the culture until the stationary phase and then only in low concentrations. The concentration of cysteic acid was elevated during the decline phase of growth. It would appear the apparent synthesis of cysteic acid is depressed during the rapid phase of growth although it may actually be utilized faster than it is produced.

Alanine, threonine, serine, histidine and phenylalanine increased in concentration during the rapid phase and continued to increase through the stationary and decline phases of growth. The rest of the amino acids increased rapidly during the rapid growth and stationary phases of the culture and remained elevated through the decline phase of growth (Table 6).

The major free amino acids present were aspartic acid, glutamic acid, phenylalanine, alanine, and valine. The predominant amino acid was aspartic acid. Asparagine has been shown to be the predominant amino acid in potato and carrot callus tissue cultures grown in darkness while glutamine predominates in tissue cultures grown in light (Steward et al, 1958). Asparagine has also been shown to increase rapidly during the stationary phase of growth in tobacco suspension cultures (Koiwai et al, 1971).

A comparison between the free amino acids detected in coffee suspension cultures and tobacco suspension cultures (Koiwai et al, 1971) showed that the free amino acid patterns were not the same. In the coffee suspension cultures, aspartic acid, glutamic acid, glanine, valine and phenylalanine predominated while in the tobacco suspension cultures glutamine, asparagine, threonine, glutamic acid, phenylalanine, and γ-aminobutyric acid predominated. This demonstrates a species difference between the cultures.

c. Free amino acids in green coffee.

Although the free amino acids detected in the green coffee bean were in higher concentrations (Table 6) than had previously been reported (Walter et al, 1970; Table 2) the same amino acids were detected. Threonine was an exception. Threonine was not only found to be present in the sample used for this study but was also considered to be one of the major amino acids (5.8 micromoles per gram dry weight) present. There was no γ-aminobutyric acid detected in the samples analyzed. The differences in composition of the amino acid profile from those obtained by Walter et al (1970) and by Buckland (1972) may be a result of numerous factors such as differences between varieties, cultivation, harvesting and post harvesting practises.

Threonine, serine, alanine, phenylalanine and glycine were the major amino acids present in the sample analyzed. There was no trace of methionine or cysteic acid. Proline, histidine and arginine were present in low but detectable levels (Table 6 and Figure 11).

d. Protein content of samples used for analysis of free amino acids.

A biuret reaction was performed on samples of the ethanol extract of both green bean and coffee suspension cultures to determine the protein content. No protein was detectable. Therefore less than one percent protein was present in the samples before and after hydrolysis (Beveridge, 1972). This check was done to ensure that as little protein as possible was being hydrolyzed during preparation of samples.

e. Comparison of the free amino acid content of coffee bean and coffee suspension cultures.

Where were much higher concentrations of total and individual amino acids in the suspension culture samples, especially near the end of the growth cycle, than in the coffee bean. Cysteic acid and methionine were both found in the suspension culture but were not detectable in the green bean. Cysteic acid was very low in concentration and appeared only late in the growth cycle while methionine was present at low concentrations throughout the growth of the cells.

The major amino acids present in the suspension cultures were aspartic acid, glutamic acid, alanine, valine, and phenylalanine while the predominant free amino acids in the coffee bean were threonine, serine, glycine, alanine and phenylalanine (Table 6). The suspension culture contained fairly high concentrations of threonine, serine, glycine and isoleucine while the coffee bean contained valine. One would expect differences especially in the asparagine (aspartic acid) and glutamine (Glutamic acid) levels. Without including aspartic acid and glutamic acid content the major free amino acids were similar (Table 6).

Intact plant tissue has been shown to contain higher amounts of free amino acids than its callus cultures (Steward et al, 1958; Krikorian, 1965; Table 1). The content of free amino acids detected in coffee suspension cultures was in the expected range. The coffee bean, however, was very low in free amino acids. This may be a result of the non active state of the bean - that is the bean had been killed by drying

after harvest. Thus, the content found may not be representative of fresh green beans. The content, however, reflects the concentration of free amino acids in the coffee beans before roasting. The major identified free amino acids present in the bean are present as major identified free amino acids in the cell cultures.

3. Roasting of the green coffee bean and its extracts.

a. Roasting of green coffee.

i. Roasting of the green coffee bean in its entirety.

Before roasting the coffee bean is grey green in colour, very hard and has a very disagreeable odour. After roasting the bean was dark brown in colour externally, beaded with oil droplets, and had no distinguishable odour. The aroma was released on crushing the bean. The roasted bean crushed quite easily indicating a change in the bean possibly due to gas release and water evaporation as well as component reactions. The bean was dark brown in colour internally.

Longer roasting of the coffee bean caused a rapid change in colour to black, the oil droplets on the surface increased and the product smelt burnt before and after crushing. The important phase of the reaction which occurs on roasting is very rapid and thus the reaction must be stopped immediately at the right point of roasting to produce a satisfactory product.

ii. Roasting of ground green coffee beans.

Grinding the coffee bean before roasting was found to have a profound effect upon the capibility of the grounds to

roast. The grounds, on roasting fifteen to twenty minutes at 220°C, turned a deep brown colour. No oil droplets were observed on the surfaces of the grounds and no coffee aroma was detected even after attempting to crush the roasted grounds. The grounds were very hard even after roasting longer than twenty minutes. No aroma was detectable after having freshly roasted grounds in a small covered container overnight.

Roasting the grounds in the presence of water was attempted. It was hoped that the presence of a moister atmosphere in the oven would slow down water evaporation from the green coffee grounds and thus enable 'coffee roasting reactions' to occur. Although the roasting procedure was slightly slower the results were the same as roasting without water present.

When the roasted grounds were ground the internal areas of individual grounds were found to be of a light roast colour or still green bean colour. No roasted coffee aroma could be detected upon grinding. The grounds were very difficult to roast evenly especially when roasted in large quantities as the individual grounds tended to stick together, and heat transfer was not even throughout the mass.

iii. Roasting of the green bean pellet.

Ground green coffee was pelleted and then roasted to determine if a larger sized object would roast more naturally. The results were similar to those obtained for the ground green coffee. After fifteen minutes of roasting at 220°C the external surfaces of the pellet were deep brown in colour. No oil droplets were present and no detectable aroma was released other than burnt or green bean aroma. The internal grounds were externally paler in colour than were the external grounds and no oil was observed on these grounds either.

The smaller pellets roasted somewhat faster and were darker internally than the larger pellets but otherwise the results were the same. In the presence of water roasting of all the pellets was slower but still produced similar results.

The pellets, on roasting, expanded mainly upward - that is, along the perpendicular axis. There was more expansion of the pellets expanded, cracking across the axis causing layering of the pellet. The apparent expansion of the pellets could be attributed to several factors, for example evaporation of internal water and/or release of gases causing internal pressure which was relieved by expansion of the pellet. This is similar to the expansion which occurs on roasting coffee beans.

The roasted pellets easily fragmented into grounds without the release of 'coffee' aroma. The individual grounds whether internally or externally located within the pellet were still very hard and on crushing did not release the 'coffee' aroma.

iv. Discussion on roasting of green coffee.

The green coffee bean roasted in the manner expected from the literature. The ground green coffee bean, however, did not produce coffee aroma and did not brown well.

The coffee bean has been likened to a miniature autoclave in which the reactions occur in a controlled environment (Keable, 1910). It has also been suggested that the selectivity of the reactions that occur is also governed by low water content, localized buffer systems and a fluctuating balance of reaction products. By grinding the coffee bean before roasting the restricted environment is destroyed and the balance that exists within the bean is upset. Roasting, however, should still produce a browning of the ground bean

although even browning throughout the samples was not obtained probably as a result of uneven thermal conductivity.

Internally the coffee bean is almost completely anaerobic restricting the reactions that occur, allowing interactions by non-volatile compounds and restraining the volatiles formed within. On roasting, the covering of the intact bean becomes impermeable to gases or volatiles trying to pass outward. Thus the volatiles are retained. Grinding of the coffee bean allows internal surfaces to be exposed to aerobic conditions and will, thus, upset the course of the roasting reactions.

The rate of water evaporation is very important as the browning reactions only occur over a narrow range of total moisture content. The grounds of green coffee beans cannot retain moisture and the water level possibly decreased too rapidly for complete browning to occur.

In summary, it would appear that while the intact bean is capable of producing a coffee colour, aroma, and flavour, the ground bean has lost this ability at least under the conditions studied.

b. Roasting of green coffee bean extracts.

Extracts obtained from green coffee were roasted in various mixtures in an attempt to isolate an extract which when roasted would produce a coffee aroma. The extract would then contain some of the major precursors of coffee aroma. The solvents used were water, methanol, and ethanol.

i. Water extracts of green coffee beans.

The water extract of the green coffee bean was a cloudy grey green before freeze drying and when lyophilized was a dull green colour. It contains water soluble proteins, free

amino acids, simple carbohydrates, some chlorophyll and other compounds. Water extracts of the coffee bean on the whole did not produce a fresh coffee aroma on roasting (Table 7a) although roasting of the extract alone or with calcium carbonate produced a faint stale coffee aroma and nice coffee brown colour. Otherwise no coffee aroma could be detected on roasting the water extract with other additives. The colour of the end products of water extracts with other additives was dark brown to black.

ii. Methanol extract of green coffee beans.

The methanol extract of green coffee was clear with no colour before freeze drying. The lyophilized product was white and flaky. The extract contains free amino acids, some peptides, and other compounds. On roasting the extract produced a fairly good coffee aroma although with not quite the full body obtained from freshly roasted coffee beans (Table 7b). The additives did not increase the coffee aroma produced on roasting but tended to mask it slightly.

iii. Ethanol extract of green coffee beans.

The ethanol extract of green coffee was clear and yellow in colour before freeze drying and after was white and flaky. The extract contained free amino acids, some carbohydrates and other compounds. This extract on roasting alone or with additives tended to produce an aroma reminiscent of roasted oil seeds (such as peanuts; Table 7c).

iv. Roasting of combined extracts of green coffee bean.

It was found that the combinations resulted in aroma reminiscent of roasted oil seeds (Table 7d).

Table 7. The aroma and colour produced upon roasting at 220°C of coffee extracts with various additives (in the presence of water).

a. roasting of water extract.

mixture roasted	colour produced	aroma produced
control (water extract only)	brown	very stale coffee
control and calcium carbonate ($CaCO_3$)	dark brown	very stale coffee
control and 1N sodium hydroxide (NaOH)	black brown	non descriptive*
control and $CaCO_3$ and 1N NaOH	black brown	non descriptive
control and dextrose	dark brown	burnt carmel
control and 1N hydrochloric acid (HCl)	black brown	sweet smelling
control and acid and destrose and $CaCO_3$	light brown	burnt and sweet

b. roasting of methanol extract.

control (methanol extract only)	brownish	coffee-cocoa
control and $CaCO_3$	light brown	coffee like
control and NaOH	black brown	non descriptive
control and NaOH and $CaCO_3$	black brown	non descriptive
control and dextrose	medium brown	sweetened coffee
control and HCl (1N)	medium brown	sweet, acidic and coffee like
control and dextrose and acid and $CaCO_3$	black brown	sweet and coffee like

c. roasting of ethanol extract.

control (ethanol extract only)	brown	roasted peanuts
control and $CaCO_3$	dark brown	roasted sunflower seeds
control and 1N NaOH	black brown	non descriptive
control and $CaCO_3$ and NaOH (1N)	black brown	non descriptive
control and dextrose	medium brown	cooked vegetable protein
control and 1N HCl	dark brown	sweet and acidic
control and dextrose and acid and $CaCO_3$	black brown	burnt sugar

d. roasting of combined extracts.

water and ethanol extracts	dark brown	roasted broad beans
water and methanol extracts	dark brown	roasted broad beans
ethanol and methanol extracts	carmel brown	bland roasted peanuts

* non descriptive - there was a strong aroma present in each case but it was difficult to relate it to anything for description. The aroma was not pleasant and was not coffee-ish at all.

v. Miscellaneous.

The addition of caffeine to a mixture of sugar and tannic acid was stated by Erdman (1902) to produce a coffee aroma. When the latter materials were roasted alone, in mixtures, or in various combinations with water, methanol, and ethanol extracts coffee aroma was not detected. A musky woody aroma was produced in all roasted mixtures containing tannic acid. Caffeine appeared to have no effect on the aroma production.

vi. Discussion of the roasting of green coffee bean extracts.

Rohan and Stewart (1965) and Rohan (1965) found that roasting a methanol extract from fermented cacao beans produced a cocoa aroma and thus contained the primary precursors of cocoa aroma. In this study methanol was also found to be the best solvent for extracting the precursors of coffee aroma from coffee beans. It would, therefore, appear that the major precursors of coffee aroma could be amino acids, sugars, and flavonoids (Rohan, 1965) although other compound classes are involved in producing a more full bodied coffee aroma (Gianturco, 1967).

4. Caffeine determination in Coffea arabica L. cell suspension cultures and green coffee beans.

Caffeine was detected in the cells of a Coffea arabica L. cell suspension culture although the results obtained were variable.

a. Caffeine determination - methods.

The usual method for caffeine determination (Horwitz AOAC , 1970) was modified slightly for microdetermination. The trial kjeldahl determinations performed were very low and variable between samples. It was hoped that Nessler's reagent would be more sensitive but a colloidal orange precipitate which formed in some cases interferred with the optical density reading.

b. Caffeine content in cell suspension.
Cultures of Coffea arabica L.

Caffeine was detected in coffee suspension cultures (Table 8). Although the apparent values were possibly higher than actual values the trend could be detected. The values were possibly higher than actual values as a result of the presence of colloidal matter. The caffeine content decreased during active growth and increased in content during nonactive growth periods (Figure 13 and Table 8).

Tea callus cultures (Ogutuva and Northcote, 1970a and 1970b) were found also to synthesize caffeine in a similar pattern - only during non active growth did caffeine accumulate. The concentration produced in the tea callus culture grown in the dark was 1,500 micrograms per gram dry weight at maximum. The coffee suspension culture was not as productive - producing only 360 micrograms per gram dry weight.

Ogutuva and Northcote (1970a) suggested that the caffeine which appeared in the latter portion of the rapid phase of growth in tea callus cultures was formed by those cells which had finished active growth. According to the latter authors some of the caffeine possibly was formed as a result of the catabolic breakdown of nucleic acids rather than from direct synthesis of purines within the cell. The caffeine produced within the cell is either secreted immediately into surrounding medium and is unable to reenter active cells or is concentrated within the cell in a membrane bound vacuole removing the caffeine from contact with the living cell (Ogutuva and Northcote, 1970a 1970b).

The caffeine in coffee cultures could be produced as it is in tea tissues. After active growth the cell may contain high amounts of nucleic acids and may route their disposal via caffeine which the cell by some means can secrete or envelop to remove it from direct contact with the cell. This process

Table 8. Caffeine content of Coffea arabica L. cell suspension cultures.

time (days)	dry weight of cultures (grams)*	caffeine content of cells* (µg/g dry wt)	pH of medium*
0	0.016	-	4.30
4	0.050	-	4.60
6	0.066	75.0	4.70
8	0.110	37.5	4.70
10	0.263	51.0	5.55
12	0.431	100.0	5.75
14	0.605	150.0	6.20
16	0.677	185.0	6.70
18	0.790	225.0	6.80
20	0.757	301.0	6.20
22	0.733	375.0	6.85
27	0.665	360.0	7.60

coffee bean

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*values given are averages of two samples (the caffeine is done in six replications of each sample).

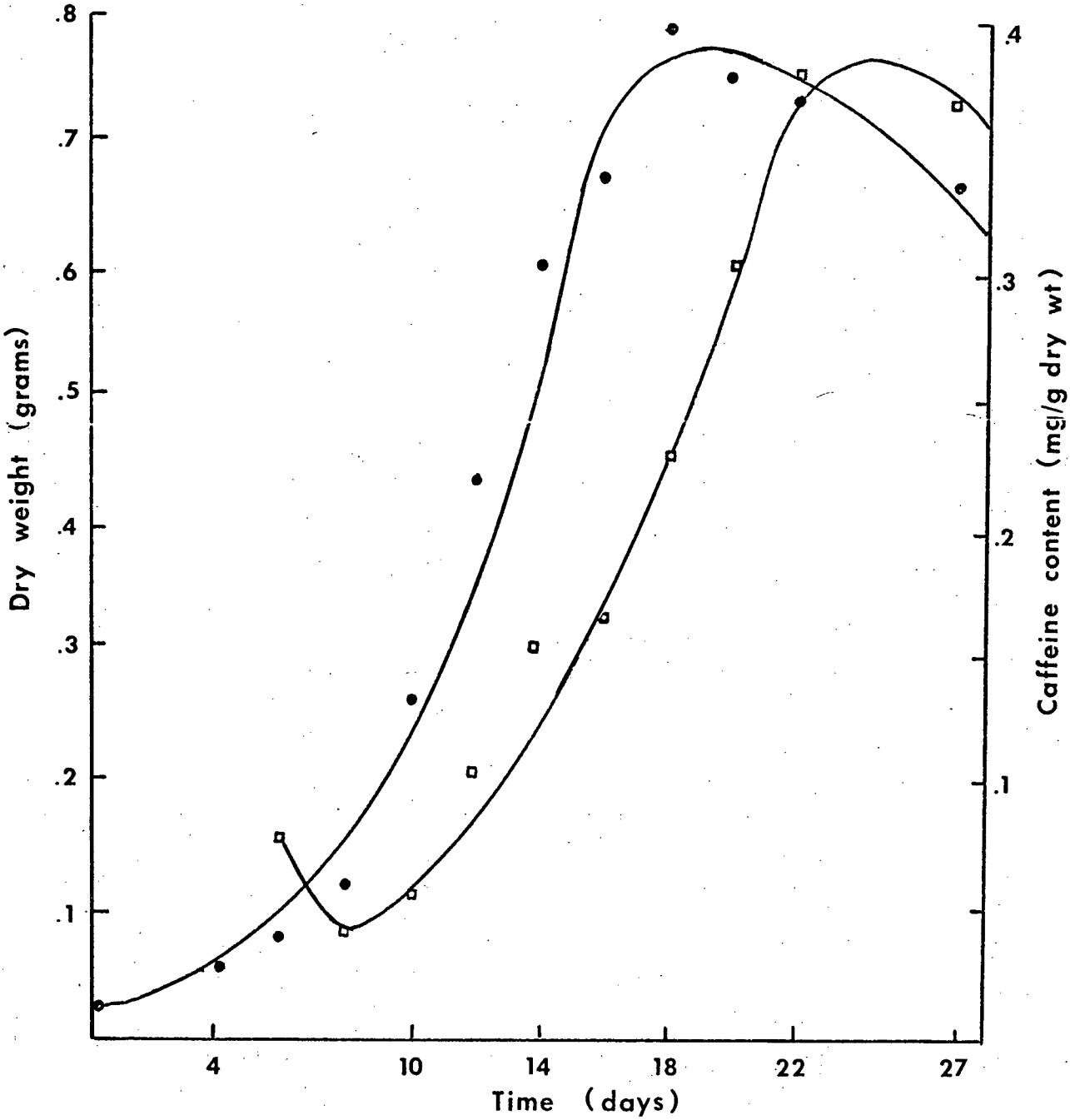

.8
.7
.6
.5
.4
.3
.2
.1
Dry weight (grams)
.4
.3
.2
.1
Caffeine content (mg/g dry wt)
4
10
14
18
22
27
Time (days)

Figure 13. Caffeine content of Coffea arabica L. cell suspensions.

Values are average of two samples and three replications of each (Values given in Table 8).

Symbols:

⊙——⊙ dry weight of Coffea arabica L. cells

□——□ caffeine content of cells

would predominately occur in older cells although some breakdown would occur even in actively dividing cells. The cell may also synthesize caffeine directly for a short space of time until back feeding of caffeine to the initial steps in purine formation inhibits the biosynthetic sequence.

c. Caffeine content in green coffee beans.

The caffeine content detected in green coffee beans was 1.15% which is fairly near the values obtained by Lehman (1971) and Feldman et al (1969).

d. Paper chromatography of caffeine.

Caffeine was detected in cell suspension cultures, culture medium and green coffee beans. Theobromine was possibly the other major spot present (Figure 14). Theobromine did not interfere in the analysis as it was removed by the addition of acidic water to the chloroform extract. The standard caffeine sample was not pure caffeine according to the paper chromatographic results.

e. Discussion.

Caffeine was produced by the coffee suspension culture at approximately 0.03% dry weight in contrast to the beans' content of 1.15% dry weight. It is obvious that one of the enzyme systems involved in caffeine production is still present. Caffeine production may be a method for the cell to dispose of non essential nucleic acids.

5. Chlorogenic acid in the Coffea arabica L. cell suspension culture and the green bean.

Chlorogenic acid was detected in the cells of a Coffea arabica L. cell suspension culture (Figure 17). As with most secondary

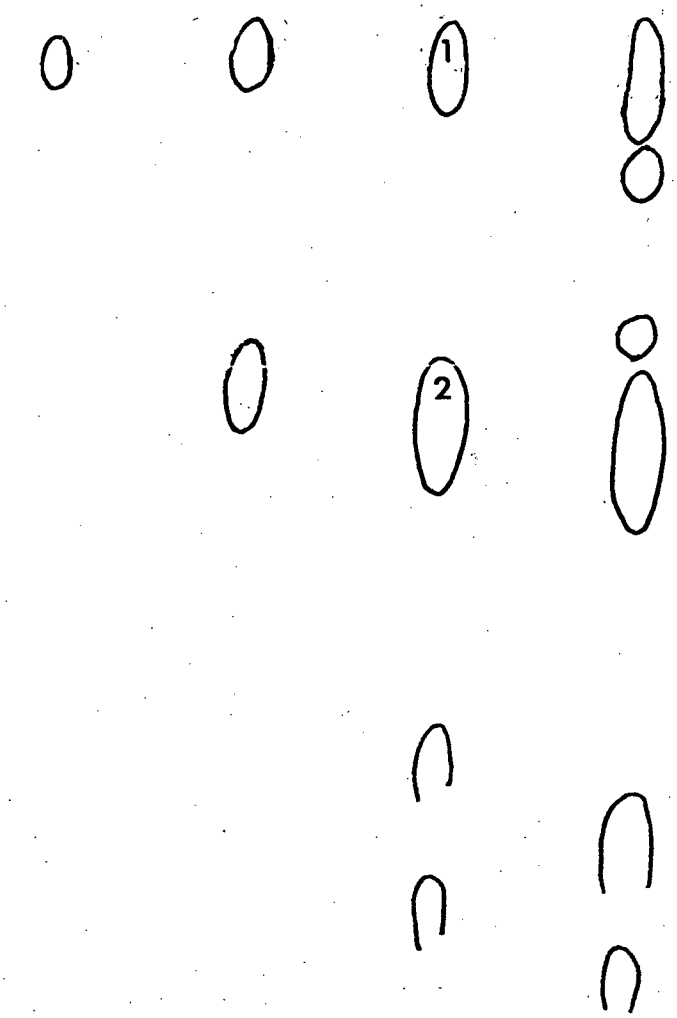

1
2
A
B
C
D

Figure 14. Paper chromatogram showing the presence of caffeine in green coffee bean and Coffea arabica L. cells and medium extracts.

A. medium extract
B. coffee cell extract
C. caffeine standard
D. coffee bean extract

spot identification

1. caffeine
2. possibly theobromine

metabolites produced in tissue cultures the maximum level detected in the cultures (0.14% dry weight) was well below the level normally present in green coffee beans (6.5% dry weight) (Table 9).

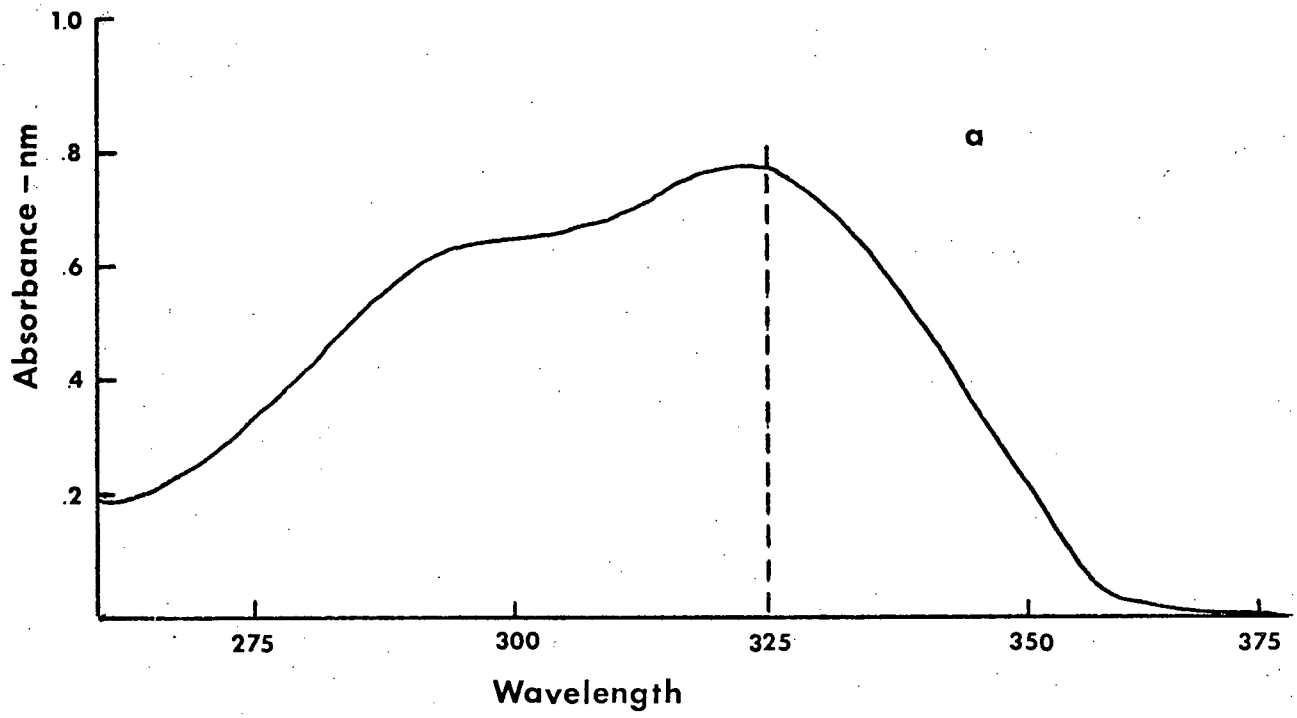
a. Chlorogenic acid determination - methods.

Chlorogenic acid analysis was not started until six days after inoculation as there was insufficient material available for the determination. The method (Horwitz (AOAC), 1970) used was modified to accomodate the amount of material available for analysis. The spectrum scan of a chlorogenic acid standard showed that the optimum absorption was at 324 millimicrons as suggested in Horwitz (1970) (Figure 15a). The calculation of the chlorogenic acid content of the samples was done on an enlargement of Figure 15b. The difference in content of chlorogenic acid between the coffee bean and the culture sample is obvious.

The method used did not distinguish among the different isomers of chlorogenic acid (chlorogenic acid, isochlorogenic acid and neochlorogenic acid) and it is possible that it determined only chlorogenic acid and not its isomers. However, no trace of the isomers was detected on paper chromatograms.

b. Chlorogenic acid in the culture medium.

The presence of chlorogenic acid was not detected using paper chromatography in the culture medium. This may have been a result of the presence of only minute quantities or the complete absence of chlorogenic acid in the medium. The pH of the culture medium rose fairly steadily during the growth cycle of the culture indicating no notable secretion of acid into the medium (Table 9). An analytical determination on the medium was not attempted as the detection of its presence by qualitative paper chromatography failed.


1.0
.8
.6
.4
.2
Absorbance – nm
a
275
300
325
350
375
Wavelength

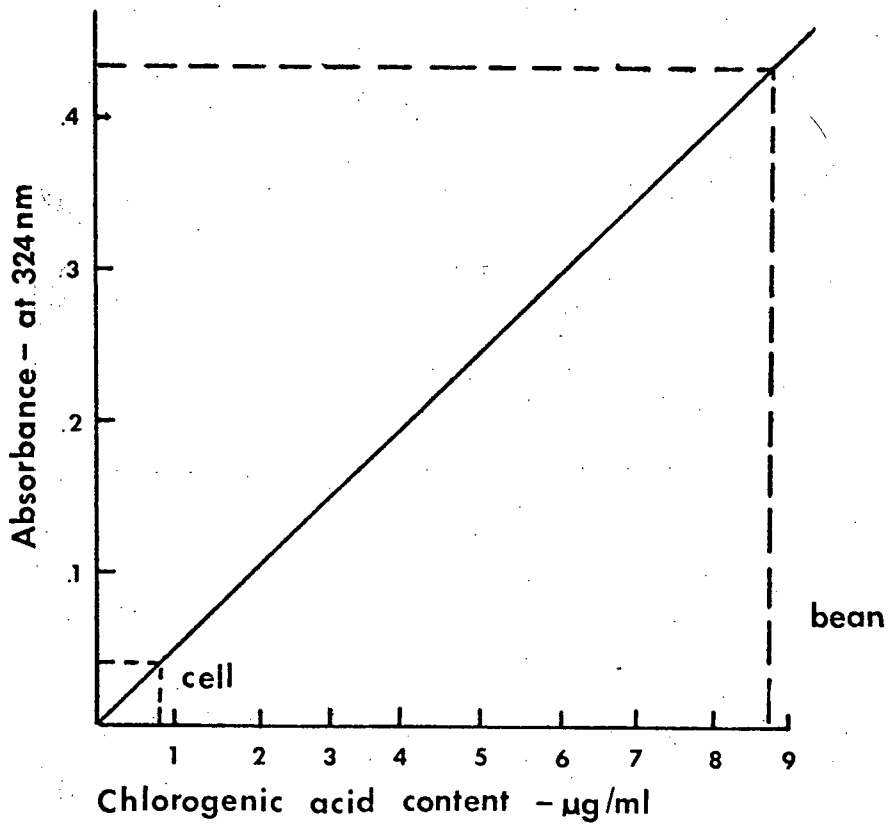

.4
.3
.2
.1
Absorbance – at 324 nm
b
bean
cell
1
2
3
4
5
6
7
8
9
Chlorogenic acid content – µg/ml

Figure 15. Standard curves for chlorogenic acid.

a. Determination of wavelength best suited for chlorogenic acid detection.

b. Standard curve for conversion of the absorbance value to a concentration value.

- a sample of green coffee bean and of a cell suspension are dotted in.

Table 9. The chlorogenic acid content of *Coffea arabica* L. suspension cultures.

time (days)	dry weight of cells* (grams)	chlorogenic acid content of cells* (mg/g dry wt)	pH of medium*
0	0.020	-	4.30
2	0.025	-	4.39
4	0.049	-	4.58
6	0.122	0.632	4.93
8	0.136	0.929	5.36
10	0.361	0.607	5.70
12	0.421	0.061	5.73
14	0.676	0.000	6.16
16	0.848	0.337	6.15
18	0.760	1.084	6.70
20	0.702	1.323	6.96
22	0.731	1.147	7.10
25	0.647	1.375	6.99
28	0.666	1.092	7.50
32	0.585	1.125	7.93
coffee bean		65.220	

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*values given are the average of four samples.

c. Chlorogenic acid content in *Coffea arabica* L. cell suspension cultures.

The chlorogenic acid content of the cultures appeared to rise and then decrease before rapid growth began. After the rapid phase of growth of the culture had ceased the content of chlorogenic acid rose rapidly levelling off during the decline of the culture. Chlorogenic acid did not appear to be produced by the culture during active growth (Figure 16). It would appear that only the older cells, not actively dividing, can synthesize chlorogenic acid as seen by the production of chlorogenic acid only during periods of little or no net culture growth (Figure 16 and Table 9). The maximum level of chlorogenic acid was obtained just after the peak of growth (as expressed in dry weight) had been reached.

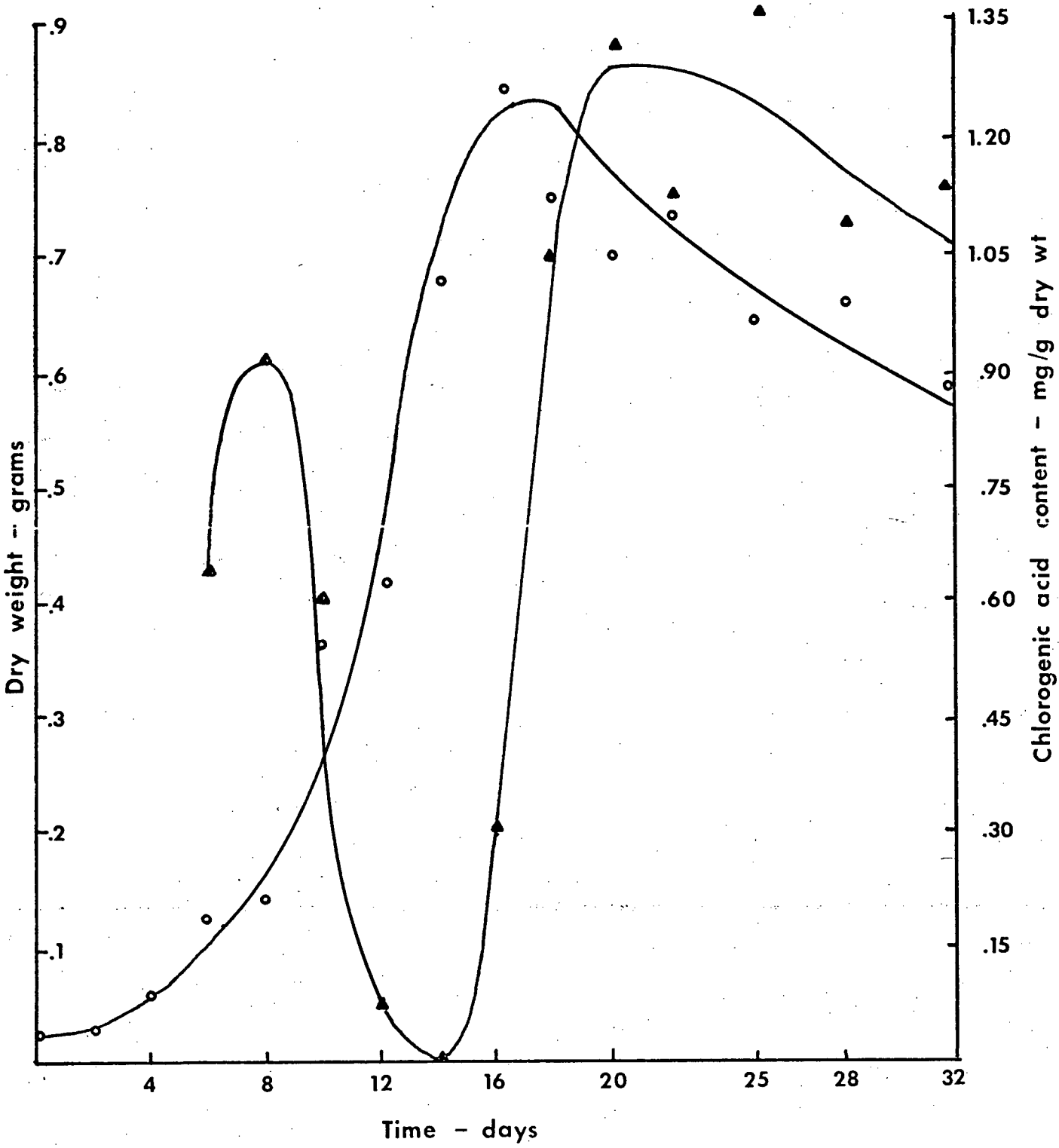
To determine if chlorogenic acid was produced normally by other cultures a bean (Buckland, 1972) and a rose (Wegg, 1972) suspension culture were analyzed. There was no chlorogenic acid detected in either sample.

d. Chlorogenic acid in green coffee beans.

The content of chlorogenic acid in the green coffee beans was 6.5% dry weight (Table 9). The normal range is 5.5 to 7.5% dry weight (Lehmann *et al*, 1967).

e. Paper chromatography of chlorogenic acid in extracts of green coffee bean and coffee tissue cultures.

Paper chromatography of extracts of the green coffee bean and the cell suspensions illustrated the presence of caffeic acid and chlorogenic acid (Figure 17). Samples from twelve, fourteen, and sixteen day old cultures failed to show the presence of chlorogenic acid although traces of caffeic acid were detected.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.2
.1
Dry weight - grams
1.35
1.20
1.05
.90
.75
.60
.45
.30
.15
Chlorogenic acid content - mg/g dry wt
4
8
12
16
20
25
28
32
Time - days

Figure 16. Chlorogenic acid production of Coffea arabica L. cell suspension culture.

Values are the average of four samples (values are given in Table 9).

Symbols:

o——o dry weight of Coffea arabica L. cell suspension cultures.

▲——▲ chlorogenic acid content of Coffea arabica L. cell suspension cultures.

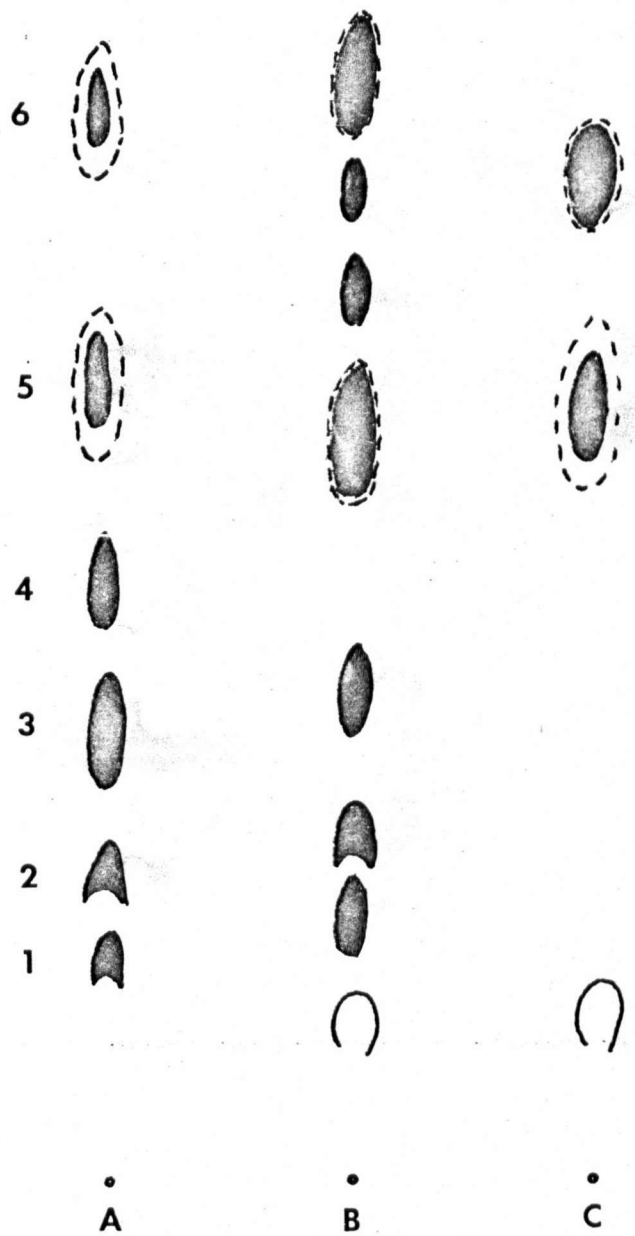
6
5
4
3
2
1
A
B
C

Figure 17. Paper chromatogram showing the presence of chlorogenic acid in extracts from green coffee and a coffee suspension culture.

Key:

A. organic acid standard

1. oxalic acid
2. tartaric acid
3. citric acid
4. malic acid
5. chlorogenic acid
6. caffeic acid

B. cell suspension culture extracts twenty day old culture

C. green coffee bean

Areas dotted in were fluorescent under ultra violet light and the darkened areas were detected using aniline-furfural reagent. The areas outlined with a solid black line appeared white using both detection methods.

f. Discussion.

Since chlorogenic acid was detected in Coffea arabica L. cultures an enzyme system responsible for chlorogenic acid production is, therefore, present in the cells. This synthesizing system would not appear to be active during cell division. However, according to Colonna and Boudet (1971) chlorogenic acid may play an active metabolic role during cell division and hence only accumulates during non active periods in the cell cycle. The enzyme system(s) for chlorogenic acid production was shown to be species specific as no chlorogenic acid was detected in the rose or bean suspension cultures whose parent tissues do not normally contain chlorogenic acid.

SUMMARY

Suspension cultures were derived from the Coffea arabica L. plant. These cultures were healthy and grew rapidly. Cells characteristically grew in long filamentous chains and in many cases contained unknown bodies which possibly could have been amylopectin starch.

The cultures were analyzed quantitatively for their content of free aminoacids, chlorogenic acid and caffeine. The total free amino acid content was found to be greater in the tissue culture cells than in the coffee bean. The major free amino acids present in the Coffea arabica L. culture cell were aspartic acid, glutamic acid, phenylalanine, alanine, valine, threonine, serine, and glycine whereas in the green coffee bean threonine, serine, glycine, alanine and phenylalanine were the predominate ones.

Roasting of green coffee bean indicated that grinding before roasting disrupts the reactions involved in browning and flavour and aroma development of the coffee bean. The inability to properly roast ground coffee bean is probably partly a result of poor thermal conductivity. The roasting of various green coffee bean extracts indicated that methanol was the best solvent for extracting major 'coffee' precursors. The roasting of the methanol extract produced a mild but stale coffee aroma.

Caffeine and chlorogenic acid were both detected in the cells of Coffea arabica L. The content was, however, well below the content normally detected in the parent plant. Determination of chlorogenic acid in other tissue cultures (rose and bean) detected no chlorogenic acid present.

In summary, Coffea arabica L. suspension culture cells were shown to be species specific in their ability to produce similar free amino acid patterns, caffeine and chlorogenic acid, the latter two in lower quantities.

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