https://theses.gla.ac.uk/

Theses Digitisation:

https://www.gla.ac.uk/myglasgow/research/enlighten/theses/digitisation/

This is a digitised version of the original print thesis.

Copyright and moral rights for this work are retained by the author

A copy can be downloaded for personal non-commercial research or study, without prior permission or charge

This work cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given

Enlighten: Theses
https://theses.gla.ac.uk/
research-enlighten@glasgow.ac.uk

The regulation of caffeine production in *Camellia sinensis* L. cv. Iranian and Darjeeling Tea

By

Abdullah Hatamzadeh-Dehboneh B.Sc and M.Sc

Thesis submitted to the University of Glasgow
for the degree of Doctor of Philosophy in Plant Biochemistry.

Division of Biochemistry and Molecular Biology
Institute of Biomedical and Life Sciences
University of Glasgow

May 1998

© **A. Hatamzadeh.**

ProQuest Number: 10390920

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.

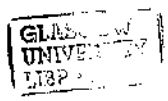
ProQuest 10390920

Published by ProQuest LLC (2017). Copyright of the Dissertation is held by the Author.

All rights reserved.
This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

GLASGOW UNIVERSITY
LIBRARY
11210 (copy 2)



Acknowledgements

My God ! to you is due all praise, always and forever, eternally, increasing, not diminishing, as you like and please.

I would like to thank my supervisor Dr. Alan Crozier for his helpful and guidance in supervising my research. I am also grateful to Prof. J. R. Coggins for his assistance in letting me use facilities in the Division of Biochemistry and Molecular Biology.

Thanks would also go to Mrs. Allison Sutculiffe for her technical assistance, especially in helping me tame the HPLC, and Dr. P. Dominy for his help in computing and use of facilities in his lab.

I would especially like to thank Professor Hiroshi Ashihara of Ochanomizu University, Tokyo, Japan, for his encouragement and helpful advice. Thanks are also due to Professor Takeo Suzuki in the Department of Applied Biology, Kyoto Institute of Technology, Japan for reading some parts of my thesis on metabolism of purine alkaloids and the effect of phytohormone in the purine nucleotides metabolism.

I wish to appreciate Mr. Hadipour, the Director of Institute Research of Horticulture Lahijan, Iran, for the generous supply of the tea seeds for my project.

I would also like to thank the Iranian Government, Ministry of Culture and Higher Education and Guilan University for providing me with research scholarship.

Last but not least, I am extremely grateful to my wife and my children, for their help and patience during the past few years.

This thesis is dedicated to my wife with love and I greatly appreciate that I could not do without her support.

Contents

Declaration

I hereby declare that the work presented in the following thesis is my own. No part of this work has been previously presented for any other degree.

Abdullah Hatamzadeh

May 1998

Summary

In the first part of this project, the distribution of caffeine and associated purine alkaloids in tea seedlings (*C. sinensis* cv. Iranian tea No. 100, and *C. sinensis* cv. Darjeeling tea) were investigated. Analysis was carried out by high performance liquid chromatography. The major purine alkaloid in tea seedling is caffeine. More than 90% of the caffeine was detected in the leaves. The highest amounts of caffeine were found in apical bud of Iranian and Darjeeling tea both of which contained ca. 38 mg caffeine per gram fresh weight. The lowest levels of caffeine were found in roots and cotyledons. Theobromine, a precursor of caffeine biosynthesis, was found in leaves, but in amounts far lower than those of caffeine. Trace amounts of paraxanthine were detected occasionally in leaves of Iranian tea.

The second part of this project investigated the biosynthesis of caffeine in tea leaves using ^{14}C-labelled adenine and guanine. Both substrates were converted to caffeine but adenine was a more effective precursor than guanine. Time-course and pulse-chase studies showed a transient incorporation of label into theobromine, the immediate precursor of caffeine, prior to a more substantial rise in [^{14}C]caffeine. Biosynthesis of caffeine occurred more extensively in young leaves, declining substantially in mature and aged leaves. Although the exact pathway was not elucidated in this study, the available data supports the operation of an AMP → IMP → xanthosine → → → theobromine → caffeine pathway. [8-^{14}C]Caffeine was degraded only slowly to $^{14}CO_2$, via the purine catabolism pathway, which explains the accumulation of endogenous caffeine in tea leaves. Caffeine catabolism was, however, more extensive in leaves incubated in light than in darkness.

In the third section of this project, the effects of the phytohormones, abscisic acid, indole-3-acetic acid, gibberellic acid and zeatin, on the metabolism of [8-^{14}C]adenine and caffeine by the first leaves of Iranian and Darjeeling tea in light and darkness, were investigated. Overall the phytohormones had little effect on the conversion of adenine to caffeine. However, incubations with [8-^{14}C]caffeine showed that in darkness the phytohormones, most notably abscisic acid and indole-3-acetic acid, increased the rate of catabolism of caffeine after 12 h. In light the effect was less marked with caffeine degradation being enhanced by zeatin, and to a lesser degree abscisic acid and gibberellic acid, but only after in incubation period of 24 h.

Abbreviation

ABA	Abscisic acid.
Ad	Adenine.
Ado	Adenosine.
ADP	Adenosine -5′ -diphosphate.
All	Allantoin.
Aln	Allantoic acid.
AMP	Adenosine -5′ -monophosphate.
AN	Adenine nucleotides.
ATP	Adenosine -5′ - triphosphate.
°C	Degrees centigrade.
Cf	Caffeine.
Ci	Curie.
c.p.m	count per minute.
cm	centimetre.
cm^2	square centimetre.
DNA	Deoxyribonucleic acid.
dpm	disintegration per minute.
D.W.	Dry weight.
F. W.	Fresh weight.
g	gram(s).

g^{-1}	per gram.
GA_3	Gibberellic acid.
GDP	Guanosine 5′ - diphosphate.
GMA	γ-glutamylmethylamide.
GMP	Guanosine 5′ - monophosphate.
GTP	Guanosine 5′ - triphosphate.
Gu	Guanine.
Guo	Guanosine.
Hyp	Hypoxanthine.
Jan	January.
IAA	Indole-3-acetic acid.
IMP	Inosine 5′ - monophosphate.
Ino	Inosine.
kdpm	10^3 dpm.
l	Litre.
Ln	logarithm.
m	Meter.
Mar	March.
mg	milligram.
min	Minute.
ml	Millilitre.
Mol	Mole.

MSE	Methanol - soluble extract.
NA	nucleic acid.
nm	nanometer.
NMT	*N*-methyltransferase.
Nov	November.
PA	Purine alkaloid.
PCA	Perchloric acid.
PN	Purine nucleotide.
PPFD	Photosynthetic photo-flux density.
PRPP	5-phosphoribosyl-1-phyrophosphate.
RNA	ribonucleic acid.
s	second.
SAH	S-adenosyl-homocysteine.
SAM	*S* - adenosyl - *L* - methionine.
Sept	September.
SD	Standard deviation.
SE	Standard error of the mean.
Tb	Theobromine.
TLC	Thin layer chromatography.
Tp	Theophylline.
µCi	Micro-Curie.
µg	Microgram.

µmol	Micromole.
v/v	volume to volume.
w/v	weight to volume.
WSE	Water - soluble extract.
Xan	Xanthine.
Xao	Xanthosine.
XMP	Xanthosine 5′ -monophosphate.

CHAPTER 1

INTRODUCTION

1.1. Taxonomy and economic importance of tea

Tea, *Camellia sinensis* (L.) O. Kuntze belongs to the *Thea* section of the *Camellia* genus in *Theaceae*. The tea plant is a tree, 9 -15 m in height under natural conditions and about 1.5 m when cultivated. The cultivated varieties are divided into two main groups based on foliar and growth characteristics. Chinese teas, *Camellia sinensis* var. sinensis, are slow growing, dwarf trees, with small, narrow, dark green leaves. They are resistant to cold weather. Assam teas, *C. sinensis* var. assamica, are taller quick-growing trees with large leaves that are also resistant to low temperature. It is a diploid containing 30 chromosomes (Kato, 1989; Takeo, 1992). Tea is of special interest from a commercial view point because of its use as a beverage while to the biochemist it is of interest because it is one of ca.100 species that contain purine alkaloids (**Fig. 1.1**).

According to Sealy (1958) the genus *Camellia*, which includes 82 species, is classified into 12 subgeneric sections. *Thea* is one of these sections and includes five species, three of which, namely *C. sinensis* (tea), *C. irrawadiensis* and *C. taliensis*, contain purine alkaloids (Nagata, 1991). Tea is the most important of all *Camellia* spp. because of its widespread use as a beverage. Taxonomy plays a major role in the identification of true teas among the *Camellia* spp. listed for commercial exploitation (Banerjee, 1992).

Caffeine (1,3,7-trimethylxanthine) (**Fig. 1.1, I**) is the major purine alkaloid in *C. sinensis*. Tea was the first plant to be used to produce a caffeine containing beverage (Eden, 1976) and today it is one of the most important agricultural products in the international market and many countries are involved in its production, trade, and consumption. The most common type is black (fermented) tea. In China and Japan

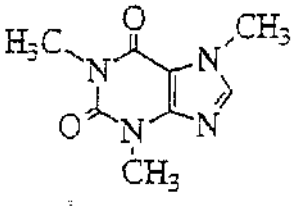
green (non-fermented) and Oolong (semi-fermented) tea are consumed, while in most other countries black tea is preferred. The major tea producing countries are India, China, Japan, Sri Lanka, Indonesia and Central Africa (Kato, 1989; Finger et al., 1992).

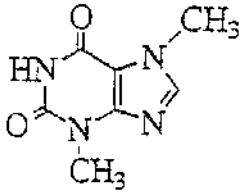
Generally, green tea is produced from var. sinensis, while black tea is made from var. assamica. The variety assamica is not used for green tea production because its much greater flavanol content would result in a brew that is excessively bitter. Fermentation of tea involves the hydrolysis and oxidation of leaf constituents by endogenous enzymes. In the case of green tea, the enzymes in fresh leaves are inactivated immediately after picking by steaming or pan-firing. In the manufacture of Oolong tea, fresh leaves are withered under sunlight for a short period. (see Takeo, 1992).

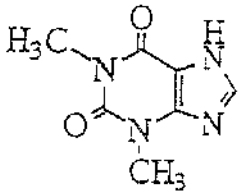
The 1994 Food Agriculture Organization year book reported that world tea production was more than 2.6 million tons of which over 2.1 million tons were produced in Asia. India, with more than 0.7 million tons, was the next biggest tea producer. The United Kingdom is the largest importer of tea with over 20% of world production. The second biggest importer is the United States (Kato, 1989).

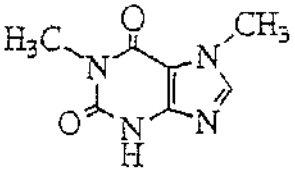
Most of the world's population is exposed to caffeine to varying degrees since it occurs in several plants that are used in the preparation of widely consumed drinks. Caffeine has a number of limited therapeutic effects (Timson, 1977). In medicine, it is used as a stimulant for the central nervous system while along with the related purine alkaloid theobromine (3,7-dimethylxanthine, **II**) it is also a diuretic (Janick, 1986). In eukaryotic organisms, post-treatments with caffeine have been found to enhance chromosome damage induced by chemical and physical mutagens. (Kihlman, 1977).

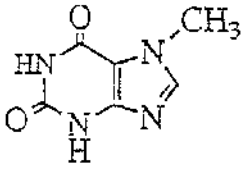
Also, caffeine and other methylxanthines may be useful in insect control by protecting young tissues from predators (Nathanson, 1984).

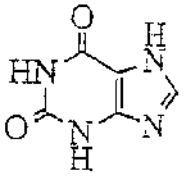

Caffeine (I)

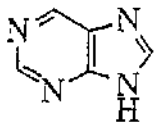

Theobromine (II)

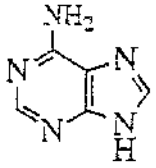

Theophylline (III)


Paraxanthine (IV)


7-Methylxanthine (V)


Xanthine (VI)


Purine (VII)


Adenine (VIII)

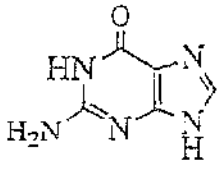

Guanine (IX)

Fig. 1.1. Structure of purine derivatives.

1.2. Discovery and general chemistry of purine alkaloids

According to Kihlman (1977), caffeine was isolated from coffee beans (*Coffea arabica*) independently by Friedlieb Ferdinand Runge and F. Von Giese in the early 1820s. Caffeine was found in tea leaves by Oudry in 1827 and in guarana nuts (*Paullinia sorbilis* or *P. cupana*) by Martius in 1826. Stenhouse detected caffeine in mate' leaves (*Ilex paraguayensis*) in 1843, and it was found in cola nuts (*Cola acuminata*) by Daniel in 1865. Theobromine was isolated from cocoa beans (*Theobroma cacao*) in 1842 by Woskresensky and theophylline (1,3-dimethylxanthine, **III**) was found in tea leaves in 1888 by Kossel. The third of the three isomeric dimethylxanthines, paraxanthine (1,7-dimethylxanthine, **IV**) was first discovered in human urine by Salomon in 1883 and identified much later as a constituent of coffee (Chou and Waller, 1980; Baumann and Frischknecht, 1988a). 7-Methylxanthine (**V**) was found in *Beta vulgaris* by Bresler in 1904 and was later isolated from tea callus tissue along with caffeine and theobromine by Ogutuga and Northcote (1970a).

Research on the chemistry of caffeine is linked closely with studies on purine derivatives in general and the nucleic acid components, adenine (**VIII**) and guanine (**IX**) in particular. (Kihlman, 1977).

1.3. Distribution of purine alkaloids in higher plants

Purine alkaloids have been detected in more than 100 species (Viani, 1991) belonging to about 30 genera (Baumann and Frischknecht, 1988a,b), with the highest concentrations in the genera *Camellia, Coffea*, *Cola*, *Ilex*, *Paullina* and *Theobroma* (Waller, et al., 1993).

Accumulation of purine alkaloids in plants of the genus *Camellia* has been found only in species of section *Thea* and in one strain of *C. kissi* of the section *Paracamellia* (Nagata and Sakai, 1985a,b; Nagata, 1986). The major purine alkaloid found in *C. sinensis* and *C. taliensis* of the section *Thea* is caffeine whereas *C. irrawadiensis* of the same section contains mainly theobromine and smaller amounts of caffeine (Nagata and Sakai, 1985a). The caffeine content of Assam tea shoots, expressed as a percentage of the dry weight (d.w) is as follows: apical bud, 4.7%; first leaf, 4.2%; second leaf, 3.5%; third leaf, 2.9%; upper stem, 2.5% and lower stem 1.4% (Harler, 1963). Theobromine is present in *C. sinensis* leaves in much smaller amounts than caffeine, typically less than 0.2%. In contrast to *C. sinensis,* the leaves of *C. japonica* and *C. sasanqua* contain negligible quantities of purine alkaloids (Nagata and Sakai, 1984, 1985a; Nagata, 1986; Suzuki and Waller, 1988) while *C. irrawadiensis* leaves contain ca. 0.5% theobromine and much lower levels caffeine (Nagata and Sakai, 1985a).

These observations indicate that the metabolism of purine alkaloids differs among species of the genus *Camellia* (Ashihara and Kubota, 1987). In fruit of *C. sinensis*, the pericarp contains the highest concentration of purine alkaloids with caffeine and theobromine representing 1-2% and 0.05-0.1% respectively of the fresh weight. Lower, but still appreciable amounts occur in the seeds, especially in the coats (Suzuki and Waller, 1985a, 1988).

Cordell (1981) reported the following levels of purine alkaloids in seeds: coffee 1-2% caffeine; cola 3.5% caffeine and traces of theobromine; guarana 2.5-5% caffeine; cocoa 0.9-3% theobromine. The leaves of mate' contain up to 2% caffeine while the caffeine content of *C. sinensis* leaves ranges from 1-4%. The patterns of the purine

alkaloids, caffeine and theobromine in leaves of coffee and mate' are similar to those in tea leaves (Frischknecht et al., 1982, 1986).

1.4. Biosynthesis of purine alkaloids in plants

1.4.1 Precursors of the purine ring system

Studies on the origin of the carbon and nitrogen atoms incorporated into the purine ring system in animals indicate that formate provides C-2 and C-8, and that C-6 comes from carbon dioxide. Glycine is incorporated into the purine as an intact molecule at C-4, C-5 and N-7 (Sonne et al., 1948; Buchanann et al., 1948) (Fig. 1.2).

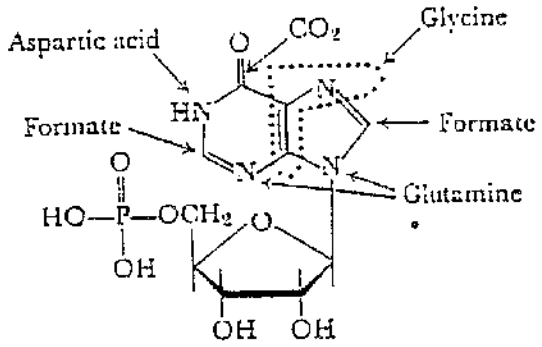


Fig. 1.2. Origins of the purine ring. From Ulbricht, 1964.

With the use of ^{15}N-labeled substrates, the α-amino group of asparatic acid was found to be incorporated into the N-1 position of the purine ring (Sonne et al., 1956), while the amide nitrogens of glutamine were shown to be the source of N-3 and N-9

(Levenberg, et al., 1956; Ulbricht, 1964; Garrett and Grisham, 1995). Therefore, each of the four nitrogen atoms in the purine ring arises from an amino acid (Lehninger, 1982). Anderson and Gibbs (1962) and Proiser and Serenkov (1963) showed that the same precursors are incorporated into the purine ring of caffeine by *C. arabica* and *C. sinensis* plants.

1.4.2 Biosynthesis and metabolic pathways

The biosynthesis of caffeine begins with the degradation of purine nucleotides (Suzuki and Takahashi, 1976c; Ashihara and Kubota, 1986, 1987; Negishi et al., 1992). Since purine nucleotides play important roles in nucleic acid synthesis and energy metabolism, the biosynthesis of purine nucleotides should be correlated closely with the growth of plant cells (Shimazaki and Ashihara, 1982). Purine nucleotides are synthesized by two different pathways. One involves de novo biosynthesis, in which purine nucleotides are synthesized from amino acids and other small molecules. The ribose of nucleotides is derived from 5-phosphoribosyl-1-pyrophosphate (PRPP). The second route is the salvage pathway whereby purine nucleotides are synthesized from pre-existing nucleoside and nucleotide bases. In the case of nucleoside salvage, the phosphate group of ATP is transfered to the nucleosides, while in the case of nucleobase salvage, the phosphoribosyl group of PRPP is transferred to nucleobases to generate the nucleotides (Nobusawa and Ashihara, 1983; Ashihara et al. 1988). Purine bases, such as adenine, guanine and hypoxanthine and their nucleosides are precursors in the synthesis of both nucleotides and ureides in higher plants and they can serve as precursors of caffeine after conversion of their respective nucleotides (Suzuki and Takahashi, 1977; Thomas and Schrader; 1981; Nobusawa and Ashihara, 1983; Waller et al., 1993).

Biosynthesis of nucleotides in the tea and coffee plants is similar to that in other plants, which do not contain caffeine (Suzuki et al., 1991a; 1992). There are several lines of evidence obtained with higher plants to suggest the existence of both the biosynthesis of adenine nucleotides de novo and their production via salvage pathways, as shown in other organisms, such as mammals and bacteria. Also, almost all exogenously supplied adenine and adenosine are salvaged by adenine phosphoribosyltransferase and adenosine kinase respectively, and AMP is formed (Ross, 1981; Hirose and Ashihara, 1984a,b; Yabuki and Ashihara, 1991; Ashihara and Fujimori, 1993). AMP is the initial product of adenylate that is synthesized during the biosynthesis of purine nucleotides de novo and by the salvage pathways of adenine and adenosine (Henderson and Paterson, 1973). Furthermore, it is the major purine nucleotide in green tea products followed by ADP and GMP (see Waller et al., 1993).

IMP is the first product of the purine nucleotide biosynthesis de novo with a complete purine ring structure (Atkins, 1980). IMP is the precursor of AMP and GMP. The conversion of IMP to AMP requires the replacment of the keto group at position 6 by an amino group. In the formation of GMP, IMP is first dehydrogenated to XMP, which is then converted to GMP (Anderson, 1979; Barankiewicz and Paszkowki, 1980; Coddington, 1985a,b). Both XMP and GMP are converted to xanthosine which through the action of nucleosidase is metabolized to xanthine which degraded further via the purine catabolism pathway to CO_2 and NH_3 (**Fig. 1.3**). IMP dehydrogenase converts IMP to XMP, the immediate precursor of GMP and is regulated by NAD. XMP is converted to GMP by GMP synthetase, which is regulated by ATP (Anderson, 1979).

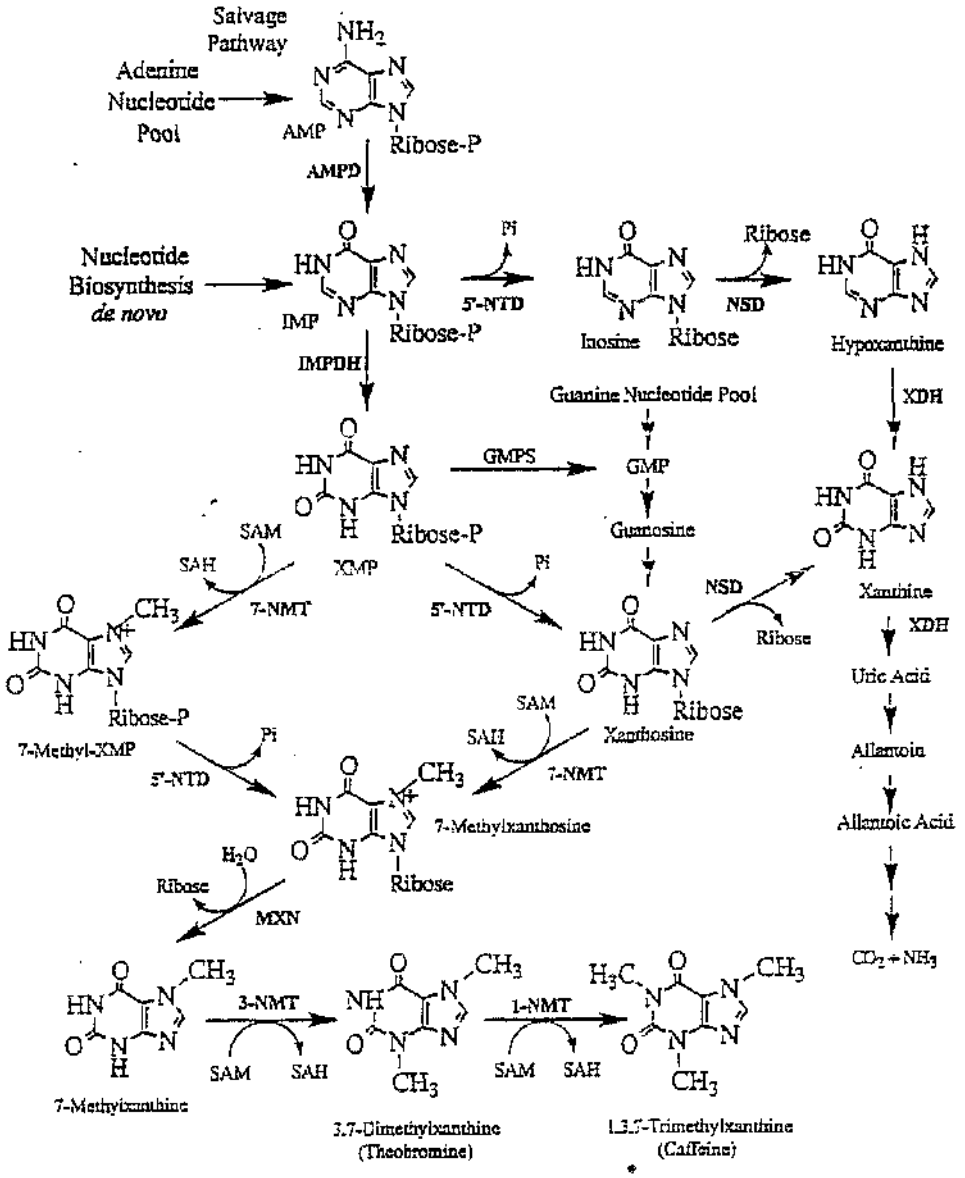


Fig. 1.3. Metabolic pathways illustrating both the biosynthesis of caffeine from purine nucleotides and the catabolism of purine nucleotides. AMP deaminase (AMPD), 5′ - nucleotidase (5′-NTD), nucleosidase (NSD), 7-methylxanthosine nucleosidase (MXN), IMP dehydrogenase (IMPDH), 1-N-methyltransferase (1-NMT), 3-N-methyltransferase (3-NMT), 7-methyltransferase (7-NMT), xanthine dehydrogenase (XDH) and GMP synthetase (GMPS).

In tea and coffee plants, xanthosine, as well as being converted to xanthine, also enters the caffeine biosynthesis pathway which involves a xanthosine → 7-methylxanthosine → 7-methylxanthine → theobromine → caffeine route (**Fig. 1.3**) (Suzuki and Takahashi, 1975a,b, 1976a,c, 1977; Looser et al., 1974; Cordell, 1981; Roberts and Waller, 1979; Baumann, et al., 1978; Waller et al., 1983; Negishi et al., 1985a,b,c, 1992; Suzuki and Waller 1985b; Waller and Suzuki, 1989; Fujimori et al., 1991; Suzuki et al., 1992; Fujimori and Ashihara, 1994). 7-Methylxanthine was first reported to be produced from the xanthosine in tea leaves by Jones and Robins (1963). Later, Ogutuga and Northcote (1970b) and Suzuki and Takahashi (1976c) reported that 7-methylxanthine is produced by both de novo and salvage pathways in tea. Many investigators have shown that 7-methylxanthosine is the first methylated compound in the biosynthesis of caffeine, and that xanthosine is the direct precursor of 7-methylxanthosine both *in vivo* and *in vitro* (Suzuki and Takahashi, 1975b, Suzuki et al., 1991a; 1992; Negishi et al., 1985a,b,c). Recently Schulthess and Baumm (1995b) and Schulthess et al. (1996) have reported that the methylation of XMP, rather than xanthosine, is the crucial step leading from primary metabolism to caffeine biosynthesis in coffee. According to this hypothesis XMP is converted to 7-methyl-XMP which does not accumulate in detectable quantites as it is metabolized rapidly to 7-methylxanthine (**Fig. 1.3**). This is different from the conclusions of Suzuki and Takahash (1975b) and Negishi et al., (1985a) that caffeine biosynthesis in tea starts with the methylation of xanthosine to yield 7-methylxanthosine. A large number of investigators have agreed that 7-methylxanthine is not formed directly from xanthine (**VI**) (Suzuki and Takahashi, 1975b; Roberts and Waller, 1979; Negishi et al., 1985a; Schulthess and Baumann,1995b; Schulthess et al., 1996).

Nucleosides may be converted to their respective nucleotides via the salvage pathway (Negishi et al., 1988). Ashihara et al. (1991c) reported that adenine nucleotides synthesized from exogenously supplied adenosine and adenine are efficiently converted to guanine nucleotides, while guanine nucleotides formed from guanosine and guanine are not converted to adenine nucleotides. Negishi et al. (1991) showed that the radioactivity of ^{14}C-labeled adenosine, inosine and guanosine fed to excised tea shoots was incorporated into caffeine at a high rate after the rapid labeling of free nucleotides, whereas the radioactivity of ^{14}C-labeled xanthosine was incorporated directly into 7-methylxanthosine, theobromine and caffeine. Also, Negishi et al. (1994) demonstrated that the reaction from guanosine to xanthosine, which is converted to 7-methylxanthosine, proceeds faster than the reaction from guanine to xanthosine in tea leaves. They believed that part of the guanosine was converted directly to xanthosine, although most was incorporated into guanine nucleotides.

The biosynthesis of caffeine from purine nucleotides was investigated recently by Ashihara et al. (1995a,b, 1996a,b) and Crozier et al. (1995) who demonstrated that caffeine is synthesized from adenine nucleotides and guanine nucleotides via 7-methylxanthosine, 7-methylxanthine and theobromine the latter being the immediate precursor in caffeine in both coffee and tea leaves (see **Fig 1.3**).

Nazario and Lovat (1993a,b,c) presented data indicating that theobromine, theophylline and caffeine are the products of different separate pathways in leaves of *C. arabica*. They proposed that two separate de novo and salvage purine pools are involved in the biosynthesis of theobromine but not caffeine in leaves of coffee. They suggested that theobromine is synthesized from adenine nucleotides, theophylline from guanine nucleotides and caffeine from the degradation of purines via hypoxanthine to xanthine

with paraxanthine the immediate precursor of caffeine. Ashihara et al (1995a,b, 1996a, b) rejected the proposal of Nazario and Lovat (1993a,b,c) that theobromine is not an immediate precursor of caffeine in *C.arabica* leaves as in detailed studies they were unable to confirm the findings of the American investigators.

In addition, there is evidence that paraxanthine is an active methyl acceptor *in vitro* and may participate in the biosynthesis of caffeine in higher plants. Using cell-free preparations from tea leaves, Suzuki and Takahashi (1975b) and Poulton (1981) demonstrated that paraxanthine is a more active methyl acceptor of *N*-methyltransferase (NMT) than other methylxanthines (1-methylxanthine, 3-methylxanthine, 7-methylxanthine, theobromine, theophylline and hypoxanthine) xanthine, xanthosine and XMP. However, the synthesis of caffeine via paraxanthine from 1-methylxanthine is unlikely *in vivo*, because the rate of synthesis of paraxanthine from 1- and 7-methylxanthine was negligible *in vitro* (Kato et al. 1996). Presumably, therefore, the synthesis of caffeine from 1-methylxanthine via paraxanthine is of little importance in tea leaves. A similar conclusion was reached by Roberts and Waller (1979) with cell-free extracts from immature green fruit of coffee. Looser et al. (1974) and Suzuki and Takahashi (1976b) have confirmed that 7-methyxanthine and 7-methylxanthosine are precursors of caffeine, but theophylline is not involved in the biosynthesis of caffeine. Ashihara et al. (1995a) demonstrated that *C. arabica* leaves convert guanine to theobromine and caffeine, but not into either theophylline or paraxanthine.

Kato et al. (1996) confirmed that the main route of the biosynthesis of caffeine is from xanthosine via 7-methyxanthine and theobromine, and also proposed the existence of several minor pathway which may be involved in the biosynthesis of caffeine in tea leaves. One of these minor pathways in tea leaves is 7-methylxanthine → paraxanthine

→ caffeine while a second route is xanthine → 3-methylxanthine → theophylline → caffeine pathway (**Fig. 1.4**). Kato et al. (1996) demonstrated that the metabolism of 7-methylxanthine to theobromine is 10-fold faster than its conversion to paraxanthine.

In tea plants, caffeine is distributed in leaves, flowers and fruits, and theobromine is also found in the young organs of these tissues. The biosynthetic capacity for the synthesis of caffeine from adenine is very high in young leaves (Ashihara and Kubota, 1986; Suzuki and Waller, 1988; Fujimori and Ashihara 1990; Ashihara et al. 1991a, b; Fujimori et al., 1991). In contrast, theobromine levels and the rate at which it is metabolised are both very low (Ashihara and Kubota, 1986). The production of purine alkaloids differs among *Camellia* species (Ashihara and Kubota, 1987). It has also been established that the biosynthesis of caffeine from [8-^{14}C]adenine and [8-^{14}C]hypoxanthine takes place in the young leaves of *Camellia sinensis* (Suzuki and Takahashi, 1975a,b, 1976c; Negishi et al. 1985a,b; Ashihara and Kubota 1986), but in *C. irrawadiensis* only theobromine is synthesized (Ashihara and Kubota, 1987). Both the caffeine content and its synthesis from adenine are extremely low in tissue culture and stem tissue of tea (Ashihara and Kubota, 1986).

Ashihara et al. (1995b) investigated the metabolism of caffeine in high and low caffeine containing cultivars of *Camellia sinensis* and concluded that caffeine biosynthesis from AMP was operative via theobromine in both types of cultivar. They suggested that the caffeine biosynthesis from [8-^{14}C]adenine in the caffeine-rich cultivars is faster than in low caffeine cultivars, while the rate of degradation of both adenine nucleotides and caffeine into CO_2 was greatest in cultivars with a low endogenous caffeine content.

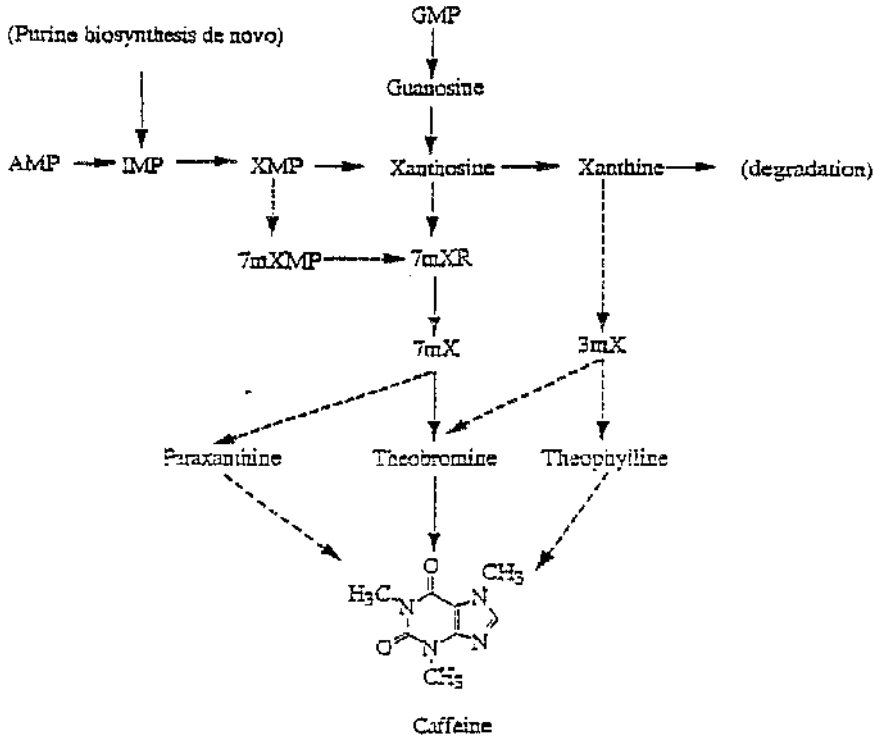


Fig. 1.4. Proposed pathways for the biosynthesis of caffeine from purine nucleotides in young leaves of *C. Sinensis*. Solid arrows indicate main pathway and dashed arrows minor routes. 3-Methylxanthine (3mX), 7-methylxanthine(7mX), 7-methylxanthosine (7mXR), 7-methylxanthosine-5'-monophosphate (7mXMP). From Kato et al.(1996).

In addition, Ashihara et al. (1995a) demonstrated the rate of biosynthesis of caffeine from adenine, guanine and xanthosine in the young leaves of coffee is higher than in aged leaves. Also, they proposed that the reduced rate of incorporation into caffeine is probably an indirect consequence of enhanced purine catabolism in the mature and aged leaves which restricts the availability of xanthosine as a substrate for caffeine biosynthesis.

Neuenschwander and Baumann (1991) suggested that mature somatic embryos, like adult coffee plants, have capacity to produce purine alkaloids. They showed, that while the caffeine content increases during embryo germination, the theobromine level is low at all stages. Ashihara (1993) reported that in the metabolism of [8-^{14}C]guanosine by young mate' leaves, more than half of radioactivity was recovered as catabolites of purines. It was noted that guanosine was an intermediate compound on the main pathway for the catabolism of GMP in plants, whereas adenine is not incorporated directly into the catabolic pathway of purine nucleotides (Yabuki et al., 1991). Ashihara (1993) reported that the biosynthesis of purine alkaloids did not take place in the mature leaves of mate', whereas the precursors of purine nucleotides were incorporated into caffeine and theobromine by the young leaves. Suzuki et al. (1991b) demonstrated that [8-^{14}C]adenine was incorporated into purine alkaloids and the capacity for biosynthesis of caffeine increased in young leaves of tea in spring and diminished as the leaves matured in summer in Japan. These investigators suggested that the biosynthesis of caffeine is dependent primarily upon the activity of *N*-methyltransferases. Compared with young tea leaves (Fujimori et al., 1991), the rate of synthesis of caffeine from adenine and especially the rate of conversion of theobromine to caffeine, is very low in the stamens of flower buds in tea (Fujimori and Ashihara, 1993). Suzuki and Waller (1982) established that theobromine, theophylline and caffeine accumulate in coffee cell suspension cultures.

The production and accumulation of purine alkaloids are associated with the developmental stage of tissues (i.e. leaves, flower, fruits and seeds) and with seasonal changes, especially in the teas grown in temperate climates (Suzuki et al., 1991a; 1992). Cloughley (1982) reported that the caffeine content of tea leaves decreased significantly

during the cold winter months of June and July in Central Africa. Similarly, Suzuki and Waller (1985c and 1986) demonstrated that the concentration of caffeine and theobromine in leaves and shoots of tea in Japan decrease significantly in August, October and November while total nitrogen increases appreciably in November and December. The alkaloids, thus, appear to play no role in the storage of nitrogen in tea leaves during winter months (Cloughley, 1982; Suzuki and Waller, 1985c, 1986, 1988). The caffeine content of *C. sinensis* leaves is reported to be high in the spring and low in the winter in Japan (Fujimori et al., 1991; Suzuki et al., 1991b). Fujimori and Ashihara (1990) reported that the rate of synthesis of purine alkaloids from [8-^{14}C]adenine in flowers of *C. sinensis* declined after flowering. Also, levels of caffeine and theobromine, in the fruit of tea increase during the growing season until the fruit is ripe and dried. Fujimori et al. (1991) reported that incorporation of radioactivity of [8-^{14}C]adenine into nucleic acid and catabolites of purine nucleotides was found throughout the year, but incorporation into theobromine and caffeine was found only in the young tea leaves harvested from April to June.

1.5. Degradation of caffeine

Although there is little information on the biodegradation of caffeine in tea, the early investigations showed that caffeine is degraded via ureides. Ureides are a class of cyclic or acyclic nitrogenous organic compounds derived from or structurally related to urea. The representatives of this class of compounds which have been found in plants include uric acid, allantoin and allantoic acid (Schubert and Boland, 1990).

Inoue and Adachi (1962). suggested that caffeine in tea leaves may be degraded to methylurea or amalic acid. Biodegradation of caffeine to xanthine, which is further

metabolized to uric acid by the purine catabolism pathway, was first shown occuring slowly in ageing leaves of *Coffea arabica* by Kalberer (1965). He found that caffeine was first degraded to 3-and/or 7-methylxanthine, xanthine and uric acid, which was further degradation to allantoin, allantoic acid and urea. Kalberer (1965) believed that allantoin is the precursor of allantoic acid and urea. Urea is hydrolyzed by urease to CO_2 and NH_3.

A large number of investigators demonstrated that caffeine is catabolized very slowly in the coffee plants (Suzuki and Waller, 1984a,b; Waller and Suzuki, 1988; Crozier et al., 1995; Ashihara, et al., 1996a). The caffeine content of tea leaves declines both in light and darkess, but the decline in light is appreciably faster than in darkess. However, the rate of caffeine degradation is low even in light (Suzuki and Waller, 1985a).

Mazzafera et al. (1991, 1994) suggested that caffeine degradation proceeds via two distinct N-demethylations, routes involving either theobromine or theophylline. The low caffeine content in leaves and endosperms of immature fruits of *C. dewevrei* may be due to slow biosynthesis and fast degradation. In contrast, the high caffeine content in leaves and endosperms of *C. arabica* appears to be a consequence of a rapid rate of production coupled with a slow degradation rate.

Theophylline is as the first biodegradation product of caffeine in immature and mature seeds of *C. arabica*. The biodegradation products of [8-^{14}C]theophylline are 3-methylxanthine, xanthine, uric acid, allantoin, allantoic acid and urea. It is now clear that theophylline is associated primarily with caffeine biodegradation and not caffeine biosynthesis. In coffee fruits, caffeine is synthesized via theobromine and degraded very

slowly via theophylline (Suzuki and Waller, 1984a, b). Also, Crozier et al. (1995) and Ashihara, et al. (1996a) have shown that theophylline is catabolized very rapidly to 3-methylxanthine, xanthine and trace amounts of 7-methylxanthine. Xanthine is then degraded to CO_2 and NH_3 via uric acid, allantoin and allantoic acid by the purine catabolism pathway. In the presence of allopurinol which blocks entry into the purine catabolism pathway, xanthine is converted primarily to 7-methylxanthine in coffee leaves (Crozier et al., 1995; Ashihara, et al., 1996a). (**Fig. 1.5**).

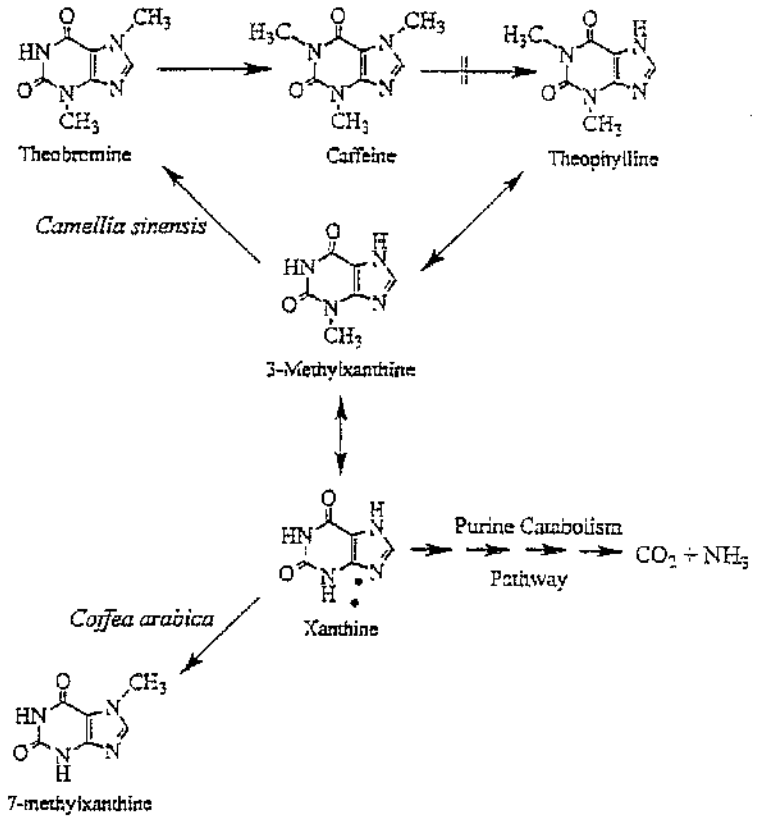


Fig. 1.5. Purine alkaloid catabolism and salvage pathways operating in tea and coffee plant. Arrow with two vertical bars repesents a blocked conversion. Double headed arrows indicate reversible conversions.

Ashihara et al. (1997) and Ito et al. (1997) have recently demonstrated that theophylline is catabolised to xanthine via 3-methylxanthine in mature, aged and young leaves of tea. It was shown that a significant amount of [8-^{14}C]theophylline is salvaged for the synthesis of caffeine via 3-methylxanthine in young leaves of tea as illustrated in **Figure 1.5**. Ashihara et al (1997) indicated that trace amounts of xanthine are salvaged for caffeine biosynthesis and this is increased when purine catabolism is blocked by allopurinol. Xanthine was converted to caffeine via 3-methylxanthine and theobromine in young leaves of tea.

1.6. The influence of light and dark on caffeine levels

Ogutuga and Northcote (1970a) showed that in young tea callus the accumulation of caffeine in the dark is greater than in light, and proposed that light has an inhibitory effect on caffeine biosynthesis. Konishi et al. (1972) demonstrated that the caffeine content of shoots of tea seedlings was higher in darkness than in light. In contrast, Frischknecht et al. (1977a,b) reported that light stimulates caffeine biosynthesis in tissue cultures of coffee and that the caffeine content is twice as high in light that in darkness. To further complicate matters. Baumann and Gabriel (1984) reported that caffeine levels increased during the germination of *Coffea arabica* seed and that the biosynthesis of caffeine is more rapid in darkness than in light.

1.7. The role of phytohormone in the metabolism of purine alkaloids

There are few studies on the effects of phytohormone in the metabolism of purine alkaloids. Schulthess with co-workers (1991) have demonstrated that ethephon, which releases ethylene, stimulates caffeine biosynthesis in suspension cultures of coffee cells. In addition, increasing concentrations of ethephon in darkness increased caffeine

production. Although light and low concentrations of ethephon stimulate caffeine production, with ethephon concentrations higher than 10 mM, purine alkaloid synthesis is inhibited under photoperiodic conditions. Subsequently, Schulthess and Baumann (1995a) observed that the caffeine biosynthesis is accelerated rapidly by ethephon in suspension cultured coffee cells without the size of the various purine pools being affected. These investigators confirmed that ethephon is an effective stimulator of caffeine biosynthesis in dark.

1.8. Aims of the project

The aims of this project are to examine the levels and metabolism of caffeine in Iranian and Darjeeling teas as summarized below:

i) Distribution of purine alkaloids in 5.5-15 month-old seedlings of Iranian No.100 and Darjeeling tea was investigated using reverse-phase high performance liquid chromatography (HPLC). The levels of caffeine and related methylxanthines in different parts of the tea plants were determined.

ii) The biosynthesis of caffeine and associated compounds was studied by feeding radiolabelled adenine and guanine to leaves of in Iranian and Darjeeling tea. In addition, the biosynthesis of caffeine was investigated in tea leaves incubation in light and in darkness.

iii) *In vivo* studies were carried out on the degradation of [^{14}C]caffeine in young, mature and aged leaves of tea.

iv) The effects of phytohormones, abscisic acid (ABA), gibberellic acid (GA_3), indole-3-acetic acid (IAA) and zeatin, on the metabolism of [^{14}C]caffeine were investigated in tea leaves incubated in light and darkness.

CHAPTER 2

MATERIALS AND METHODS

2.1. Plant material

Seeds of tea (*Camellia sinensis* cv. *No. 100* of Iran and *C. sinensis* cv. Darjeeling) were obtained from the Institute of Tea Research, Lahijan, Iran. After breaking the coats, the seeds were germinated in a compost medium (Fison Levington, Multi-purpose compost, pH 5, Thomas Daggs and Son, Glasgow UK) without any additional nutrients. Individual germinating seeds were then grown in a similar medium in plant-pots under a natural photoperiod in a greenhouse at Garscube Estate, University of Glasgow during 1993-1995.

2.2. Sources of radiochemicals

The source and specific activities of radiochemicals used in the present study were as follows: Amersham International plc, Little Chalfont, England: [8-^{14}C]Adenine (specific activity 1.96 and 2.26 GBq mmol^{-1}) and n-[1-^{14}C]hexadecane (2.11 GBq mmol^{-1}); New England Nuclear, Boston, MA, USA: [8-^{14}C]guanine hydrochloride (1.85 and 2.1 GBq mmol^{-1}); Moravek Biochemicals Inc., Brea, Cal, USA: [8-^{14}C]caffeine (52 mCi mmol^{-1}), [2-^{14}C]theobromine (2.07 MBq. μmol^{-1}, and [2-^{14}C]xanthine (1.94 MBq. μmol^{-1}); American Radiolabelled Chemicals Inc., St. Louis, Mo., USA: [8-^{14}C]theophylline (2.04 MBq. μmol^{-1}) .

2.3. Analysis of endogenous purine alkaloids

2.3.1 Sample preparation

The initial aim of this study was to determine the distribution of purine alkaloids, such as caffeine, theobromine and other methylxanthines, in different parts of tea seedlings of 5.5, 7, 9, 11, 13 and 15 months of age (May, July, Sept, Nov 1993, Jan and Mar 1994).

Seedlings were divided into apical bud, first leaf and the next eight leaves were collected individually as indicated in **Fig. 2.1**. The 10^{th} leaf and older leaves were collected as one sample. The first to 9^{th} internodes were harvested individually (**Fig. 2.1**) while lower internodes were collected as one sample. Cotyledons when present, and roots were also harvested. All tissues were frozen in liquid nitrogen, ground to a fine powder with a pestle and mortar and then weighted. Powdered tissue was placed in a glass tube, 5-10 ml distilled water was added, and the tube was heated in boiling water for 10 min to extract the purine alkaloids. The aqueous extract was filtered through Whatmann No.1 filter paper and stored at -20 °C prior to analysis.

2.3.2 High performance liquid chromatography

Solvents were delivered at a flow rate of 1 ml min^{-1} by an Altex 332 liquid chromatograph (Altex Scientific Inc., Berkeley, CA, USA). Samples were introduced off-column via an Altex 210 sample injection valve. Reverse-phase HPLC utilized a 5µm ODS Hypersil column (250 x 4.6 mm i.d.) (Capital HPLC Specialists Bathgate, Lothian, UK), packed in house. Samples were eluted from the column with a 25 min gradient of 0-40% methanol in 50 mM sodium acetate (pH 5.0). Column elute was directed to an LC 871 absorbance monitor (Pye Unicam, Cambridge, UK) operating at 272 nm, which was linked to a chart recorder. The gradient elution reverse phase HPLC system separated nine different purine derivatives (**Fig. 2.2**). Identification of purine alkaloids in tea extracts was based on co-chromatography with the appropriate standard while quantification was achieved by reference of peak heights to standard curves.

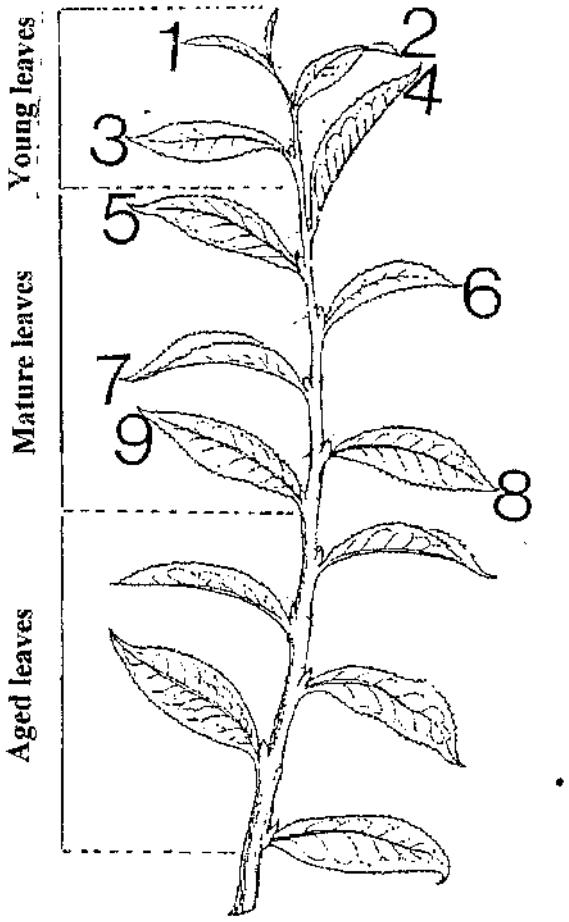


Fig. 2.1. A shoot of tea seedling, that shows the number of leaves and internodes that were harvested for purine alkaloid analysis.

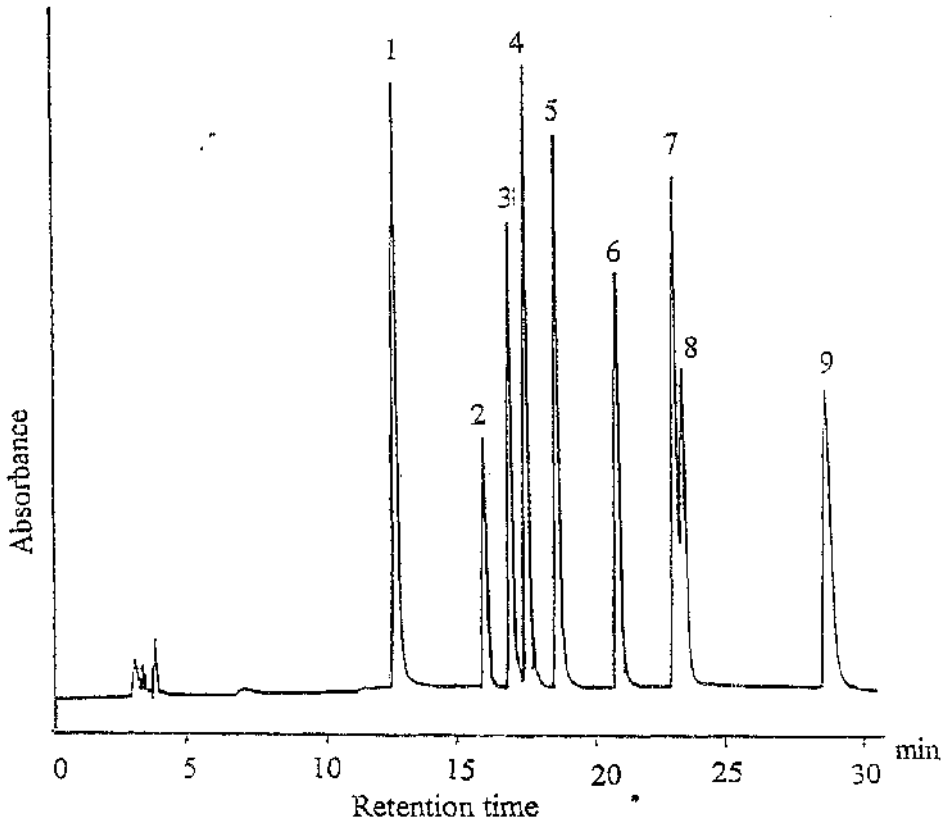


Fig. 2.2. Reversed-phase HPLC separation of purine alkaloids standards on a 25 min, 0 - 40% gradient of methanol in 50 mM sodium acetate (pH 5.0). Flow rate of 1 ml min^{-1}. Column: ODS Hypersil 5µm, 250 × 4.6 mm i.d. Detector: absorbance monitor at 272 nm. Sample: 250 ng of (1) xanthine (2) xanthosine (3) 7-methylxanthine (4) 3-methylxanthine (5) 1-methylxanthine (6) theobromine (7) paraxanthine (8) theophylline (9) caffeine.

2.4. Metabolism of [8-^{14}C] adenine

1st, 2nd ,3rd, Mature and aged leaves were selected from Darjeeling and Iranian tea seedlings. The leaves were washed with distilled water and cut into small pieces approximately 0.4 cm^2 after discarding the main vein. About 100 mg F.W. of leaf sections was incubated in 1.3 ml of distilled water, 0.5 ml of 100 mM potassium phosphate buffer (pH 5.6), 0.2 ml of 0.2 M sucrose and 1100 k dpm (0.5 μCi) of [8-^{14}C]adenine (sp.ac. 1.96 GBq $mmol^{-1}$) in a 25 ml Erlenmeyer flask. The Erlenmeyer flask had a centre well containing a small glass tube into which was inserted a piece of Whatmann No.1 filter paper wetted with 100 μl of 20% KOH. In these experiments in natural light, the flasks were shaken gently on a horizontal rotary shaker (100 strokes min^{-1}) at 25°C for 6, 12, 24 and 48 h.

2.4.1 Determination of $^{14}CO_2$

$^{14}CO_2$ released during the incubation period was absorbed by the KOH solution on the filter paper in the centre well. At the end of the incubation period, the filter paper was placed in a 50 mL flask containing 4 ml H_2O and the radioactivity in duplicate 0.2 ml aliquots determined by channel-ratio liquid scintillation counting using Ultima-Flo Af (Packard) scintillation cocktail.

2.4.2 Methanol soluble extract

At the end of incubation period, the tea leaf segments, separated from the incubation medium by filtering through a tea strainer, were washed with 50 mL of distilled water. They were then crushed and homogenised in washed sand with 80% methanol containing 20 mM sodium diethyldithiocarbamate (Sigma Chemical Company Ltd,

Poole, UK) using a chilled porcelain mortar and pestle. The resultant tissue homogenate was centrifuged at >10000 g for 5-10 min. The supernatant and pellet were separated and the pellet was re-extracted and centrifuged two further times. The supernatant fractions containing the methanol-soluble extract (MSE) were combined to yield a total volume of 10 ml. The radioactivity in the MSE was measured by liquid scintillation counting using triplicate 100 µl aliquots. The MSE was reduced to dryness in vacuo and redissolved in 0.5 ml of 80% MeOH prior to analysis by HPLC and/or thin layer chromatography (TLC).

2.4.3 Hydrolysis of Nucleic Acids

After removal of the MSE, the tissue residue was treated with 4 ml of 6% perchloric acid (PCA) in boiling water for 15 min to hydrolyse purine residues of RNA and DNA to purine bases (adenine and guanine). This nucleic acid (NA) extract was then centrifuged at 10000 g for 5 min, the supernatant collected and the pellet washed with 4 ml of cold 6% PCA prior to re-centrifugation and further extraction. All the PCA-soluble extracts were combined (total volume 8 ml) and 0.5 ml aliquots used for estimation of radioactivity by liquid scintillation counting. The PCA-soluble extract was neutralised with 20% KOH, filtered to remove precipitated perchlorate before being reduced to dryness in vacuo. The NA extract was then redissolved in 0.4 ml 80% methanol prior to analysis by TLC.

2.5. Analysis of radiolabelled compounds

2.5.1 High performance liquid chromatography

The liquid chromatograph column and solvent conditions used were the same as described in Section 2.3.2 except that after passing through the absorbance monitor, column eluate was directed to a radioactivity monitor (Reeve Analytical, Glasgow UK) with a 200 µl heterogeneous flow cell packed with a cerium-activated lithium glass scintillator. This facilitated the detection of ^{14}C peaks of >1000 dpm (Sandberg et al. 1987). Typically, 20 k dpm aliquots of MSE were analysed.

2.5.2 Thin layer chromatography

Because nucleotides are retained on the reversed-phase HPLC column, radiolabelled components in MSE and NA extracts were analysed by TLC using 200 × 200 mm sheets of microcrystalline cellulose (Spot film, Tokyo Kasei Kogyo Co., Tokyo, Japan). The solvent system used was n-butanol/acetic acid/water (4:1:2, v/v). After approximately 8 h, the developed TLC plate was dried and placed in a casette in contact with Kodak X-OMAT AR film (18×24 cm, Hyper film-MP, Amersham) in dark room. Radiolabelled spots, detected after 2-3 weeks exposure, were scraped off the TLC plates, eluted from the cellulose support with distilled water and the radioactivity measured by liquid scintillation counting.

For detection of allantoin and allantoic acid spots on TLC plates, the following reagent was used: 2g of p-dimethylaminobenzaldehyde dissolved in 80 ml 2N HCl in 88% ethanol. This reagent was sprayed to the TLC plate and allantoin and allantoic acid appeared as yellow spots.

2.6. Metabolism of [8-^{14}C]adenine and [8-^{14}C]guanine in pulse chase experiments

1st and 2nd Leaves used in these experiments were selected from Darjeeling tea seedlings and incubations of leaf segments were set up in the presence of either 1μCi [8-^{14}C]adenine or 1μCi [8-^{14}C]guanine hydrochloride, as described in Section 2.4. The incubation medium was replaced with fresh medium devoid of radiolabelled substrate, after an initial 4 h pulse. Samples were collected and processed as described Section 2.4 after 4, 8, 12, 24, and 48 h.

2.7. Metabolism of [8-^{14}C]adenine in light and darkness

1st and 2nd Leaf of Iranian tea were used in these experiments. Leaf samples were prepared in 0.5 μCi [8-^{14}C]adenine, as described Section 2.4, and incubated in darkness or in light (Osram Warm White 20w fluorescent tubes, ca 200 μ mol. $m^{-2}.s^{-1}$ ppfd ~ 400-700 nm) for 12, 24 and 48 h after which $^{14}CO_2$, MSE and NA fraction were obtained.

2.8. Metabolism of [8-^{14}C]caffeine in light and darkness

1st Leaf of Iranian tea was used in metabolism studies with [8-^{14}C]caffeine in light and dark. The methods for preparation of leaf segments and medium were the same as described in Section 2.6 with leaf segments being incubated with 1μCi [8-^{14}C]caffeine for 4, 12, 24 and 48 h. The radioactivity of samples were processed as described in Section 2.4.

2.9. Effects of phytohormones on the metabolism of [8-^{14}C]adenine and [8-^{14}C]caffeine in light and darkness

Iranian and Darjeeling tea leaves, were incubated in light and darkness using the system described in Section 2.4. In the presence and absence of 10^{-6} M ABA, GA_3, IAA and zeatin. The leaves were preincubated with phytohormone for 4 h prior to the addition of either 0.5 μCi [8-^{14}C]adenine or 1μCi [8-^{14}C]caffeine. Samples were collected after 12 and 24 h and metabolites were analyzed as described in Section 2.4.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Endogenous purine alkaloids

HPLC was used to detect and quantify endogenous purine alkaloids in Iranian and Darjeeling tea seedlings and to investigate age/seasonal variations in the levels of these compounds.

3.1.1. HPLC analysis

Figure 3.1.1 illustrates HPLC traces obtained in the analysis of aliquots of an aqueous extract from the apical bud of a 7 month-old seedling of Iranian tea. The main components in the extract is caffeine with a smaller amount of theobromine. Co-chromatography with authentic standards was used to confirm the identity of both compounds. Although additional peaks were present in the apical bud extract, none had retention times that corresponded with those of purine alkaloid standards.

3.1.2. Accumulation of methylxanthines in Iranian tea seedlings

Seedlings were divided into individual leaves and internodes, as described in section 2.3.1 of the Materials and Methods, prior to extraction and analysis by HPLC. The information presented in **Table 3.1.1** shows the amounts of caffeine and theobromine present in the different parts of a 5.5 month-old Iranian tea seedling harvested in May 1993. Leaves contained much more caffeine than internodes with the highest amounts, 4.6 and 5.1 mg, being found in the fourth and fifth leaves. When the data are expressed on a mg g^{-1} fresh weight basis a different picture emerges and it is evident that there is a strong caffeine concentration gradient within the seedling. The apical bud and first leaf

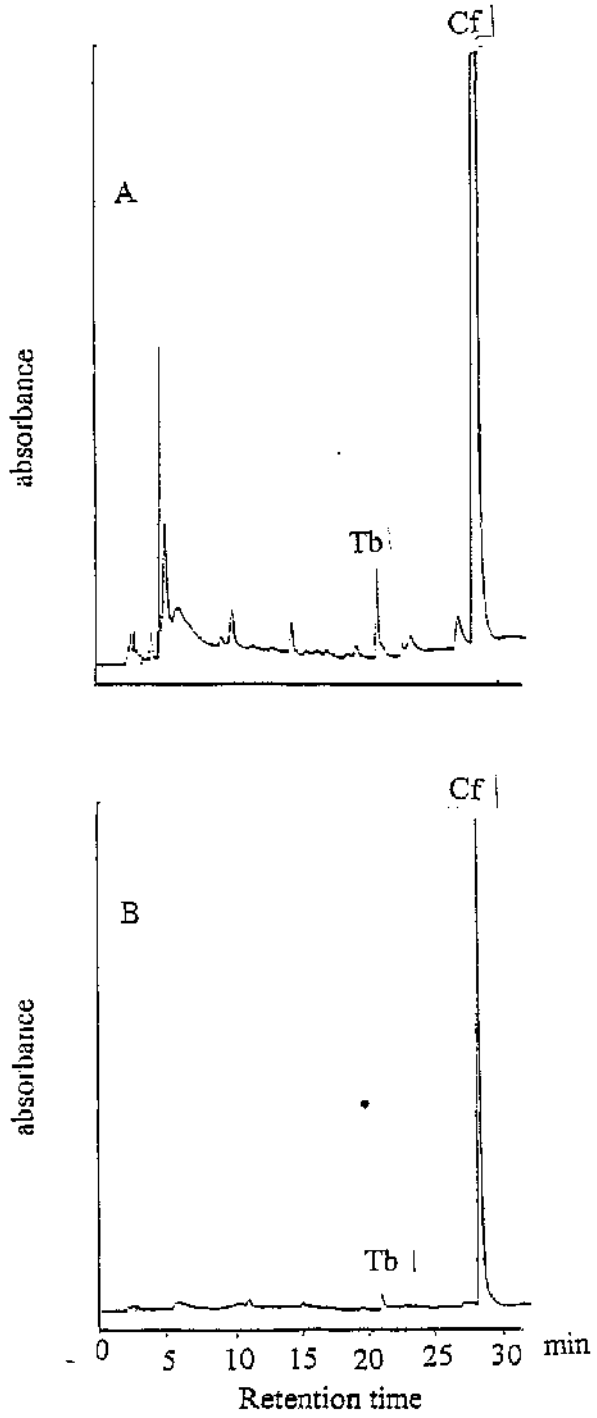

Cf
A
absorbance
Tb
Cf
B
absorbance
Tb
0
5
10
15
20
25
30
min
Retention time

Fig. 3.1.1. Gradient HPLC analysis of purine alkaloids in Iranian tea. Column: 250 × 4.6 mm i.d. 5µm ODS Hypersil. Gradient: 25 min, 0-40% methanol in 50 mM sodium acetate (pH 5.0). Flow rate: 1 ml min^{-1}. Detector: Absorbance monitor at 272nm. Cf, caffeine, Tb- theobromine. Sample (A) 50µl aliquot of a 5ml extract of 10 mg of apical buds from 7 month-old seedlings (July 1993), (B) as A but 10µl aliquot.

contain >30 mg caffeine g^{-1} and the concentration declines gradually as the age of the leaf increases, with 4.3 mg g^{-1} being present in the ninth leaf. The concentration of caffeine in the internodes was relatively low, except for the young, elongating first internode, between the apical bud and the first leaf, which contained 23.9 mg caffeine g^{-1}. The amounts of theobromine present in the same tissues were much lower than the levels of caffeine although, as with caffeine, the highest concentration were present in the apical bud, first leaf and first internode (**Table 3.1.1**). Only trace levels of caffeine were present in the roots and cotyledons which did not contain detectable quantities of theobromine.

3.1.3. Distribution of purine alkaloids in Iranian tea seedlings

Information on the distribution of purine alkaloids in 5.5 to 15 month-old Iranian tea seedlings (May 1993 to March 1994) is presented in **Tables 3.1.1** to **3.1.6**. Caffeine was the major compound detected with lower levels of theobromine and, occassionally paraxanthine. This is the first reported detection of paraxanthine in tea leaves. Paraxanthine is much better substrate for the 3-*N*-methyltransferase from tea leaves than theobromine in vitro (Kato et al. 1996) which may explain why it does not accumulate to any extract in vivo. In all instances, as discussed in the previous section, a strong gradient of caffeine was present within the seedling, with highest concentrations being found in the apical bud and the young expanding first leaf. In the case of the 15 month old seedlings triplicate samples were analysed (**Table 3.1.6**) and it is evident that the method is acceptably precise and that the overall trends of the mean values are similar to those obtained with the younger seedlings where only one sample was analysed (**Table 3.1.1 - 3.1.5**).

Table. 3.1.1. Distibution of caffeine and theobromine in the seedlings of **Iranian** tea. Age **5.5** months (May 1993). nd - not detected.

	Caffeine		Theobromine	
Plant material	mg	mg g^{-1} (F.W.)	µg	mg g^{-1} (F.W.)
Apical bud	1.20	34.3	43	1.23
1st Leaf	1.88	31.3	24	0.40
2nd Leaf	1.83	18.7	17	0.17
3rd Leaf	3.77	11.5	35	0.11
4th Leaf	4.57	7.1	39	0.06
5th Leaf	5.10	7.0	28	0.04
6th Leaf	1.12	4.7	21	0.09
7th Leaf	2.19	4.0	28	0.05
8th Leaf	2.46	4.6	56	0.11
9th Leaf	2.95	4.3	37	0.05
Other Leaves	2.65	2.9	56	0.06
1st Internode	0.19	23.9	6	0.75
2nd Internode	0.08	4.7	3	0.19
3rd Internode	0.07	3.0	2	0.09
4th Internode	0.08	1.7	3	0.06
5th Internode	0.05	1.0	2	0.04
6th Internode	0.10	1.0	3	0.03
7th Internode	0.11	0.7	6	0.04
8th Internode	0.10	0.8	6	0.05
Other Stem	0.37	0.4	27	0.03
Roots	0.05	0.01	nd	nd
Cotyledons	0.004	0.01	nd	nd

Table. 3.1.2. Distribution of caffeine and theobromine in the seedlings of **Iranian** tea. Age 7 months (July 1993). nd - not detected

	Caffeine		Theobromine	
Plant material	mg	mg g^{-1} (F.W.)	µg	mg g^{-1} (F.W.)
Apical bud	0.44	44.4	13	1.30
1st Leaf	0.63	35.1	12	0.67
2nd Leaf	0.78	12.9	9	0.15
3rd Leaf	1.35	12.0	19	0.17
4th Leaf	3.20	9.0	67	0.19
5th Leaf	2.18	7.9	39	0.14
6th Leaf	1.98	5.0	145	0.36
7th Leaf	1.29	5.6	41	0.18
8th Leaf	1.18	5.0	55	0.23
9th Leaf	1.51	4.1	73	0.20
Other Leaves	2.95	2.9	128	0.13
1st Internode	0.10	25.0	1	0.18
2nd Internode	0.08	11.9	1	0.15
3rd Internode	0.08	13.3	1	0.17
4th Internode	0.10	7.9	3	0.27
5th Internode	0.16	4.8	6	0.19
6th Internode	0.20	3.5	10	0.17
7th Internode	0.13	3.0	8	0.18
8th Internode	0.13	3.5	6	0.17
Other Stem	0.71	0.9	42	0.06
Roots	0.06	0.01	nd	nd
Cotyledons	0.01	0.02	nd	nd

Table. 3.1.3. Distribution of caffeine, theobromine and paraxanthine in the seedlings of **Iranian** tea. Age **9** months (September 1993). nd - not detected

	Caffeine		Theobromine		Paraxanthine	
Plant material	mg	mg g^{-1} (F.W.)	µg	mg g^{-1} (F.W.)	µg	mg g^{-1} (F.W.)
Apical bud	0.40	39.7	2	0.18	18	1.80
1st Leaf	0.89	23.5	20	0.53	55	1.45
2nd Leaf	1.65	11.8	25	0.18	154	1.10
3rd Leaf	2.03	10.9	32	0.17	172	0.93
4th Leaf	3.14	9.8	42	0.13	208	0.65
5th Leaf	2.22	9.7	26	0.11	126	0.55
6th Leaf	0.60	2.9	45	0.22	109	0.53
7th Leaf	0.62	2.2	62	0.23	116	0.42
8th Leaf	0.85	2.2	27	0.07	155	0.40
9th Leaf	0.39	1.5	34	0.13	123	0.46
Other leaves	1.15	0.7	179	0.12	173	0.11
1st Internode	0.06	14.0	1	0.33	3	0.75
2nd Internode	0.07	4.6	5	0.31	7	0.44
3rd Internode	0.07	3.2	8	0.35	8	0.34
4th Internode	0.10	3.1	12	0.35	8	0.25
5th Internode	0.08	3.6	11	0.49	3	0.14
6th Internode	0.08	3.9	10	0.48	2	0.10
7th Internode	0.12	4.0	18	0.61	3	0.11
8th Internode	0.13	2.2	33	0.54	5	0.08
Other Stem	0.20	0.02	105	0.11	nd	nd
Roots	0.07	0.01	nd	nd	nd	nd
Cotyledons	0.01	0.02	nd	nd	nd	nd

Table. 3.1.4. Distribution of caffeine, theobromine and paraxanthine in the seedlings of **Iranian** tea. Age **11** months (November 1993). nd - not detected

	Caffeine		Theobromine		Paraxanthine	
Plant material	mg	mg g^{-1} (F.W.)	µg	mg g^{-1} (F.W.)	µg	mg g^{-1} (F.W.)
Apical bud	0.83	32.1	11.0	0.43	17	0.65
1st Leaf	0.14	9.8	1.0	0.09	7	0.50
2nd Leaf	0.72	3.3	13.0	0.06	100	0.45
3rd Leaf	0.17	1.7	5.0	0.05	31	0.30
4th Leaf	0.34	0.5	26.0	0.04	155	0.23
5th Leaf	0.17	0.5	11.0	0.03	117	0.34
6th Leaf	0.52	0.9	18.0	0.03	165	0.30
7th Leaf	0.04	0.5	4.0	0.06	14	0.20
8th Leaf	0.09	0.2	15.0	0.04	62	0.16
9th Leaf	0.03	0.1	9.0	0.02	16	0.04
Other Leaves	0.20	0.4	20.0	0.04	60	0.11
1st Internode	0.02	3.6	0.4	0.08	0.3	0.06
2nd Internode	0.06	2.5	1.0	0.05	0.8	0.03
3rd Internode	0.05	1.2	2.0	0.05	1.0	0.02
4th Internode	0.03	0.5	1.0	0.02	1.0	0.02
5th Internode	0.02	0.6	1.0	0.03	1.0	0.03
6th Internode	0.02	0.4	0.4	0.08	0.8	0.02
7th Internode	0.02	0.5	1.0	0.03	0.8	0.02
8th Internode	0.04	0.4	2.0	0.03	2.0	0.02
Other Stem	0.25	0.3	8.0	0.01	nd	nd
Branch, 9th node	0.30	2.1	11.0	0.08	38	0.26
Branch, 10th node	0.20	3.5	2.0	0.04	4	0.07
Branch, 11th node	0.17	0.2	27.0	0.03	173	0.15
Roots	0.08	0.01	nd	nd	nd	nd

Table. 3.1.5. Distribution of caffeine, theobromine and paraxanthine in the seedlings of **Iranian** tea. Age **13** months (January 1994). nd - not detected

	Caffeine		Theobromine		Paraxanthine	
Plant material	mg	mg g^{-1} (F.W.)	µg	mg g^{-1} (F.W.)	µg	mg g^{-1} (F.W.)
Apical bud	0.36	22.3	20	1.25	15	0.94
1st Leaf	0.11	2.3	19	0.41	39	0.85
2nd Leaf	0.10	1.8	16	0.30	38	0.72
3rd Leaf	0.13	1.4	19	0.20	64	0.67
4th Leaf	0.12	0.6	18	0.09	95	0.48
5th Leaf	0.10	0.5	8	0.04	78	0.38
6th Leaf	0.12	0.4	21	0.06	82	0.22
7th Leaf	0.14	0.3	24	0.05	80	0.18
8th Leaf	0.09	0.3	31	0.09	112	0.31
9th Leaf	0.14	0.5	22	0.08	82	0.28
Other Leaves	0.56	0.3	102	0.06	395	0.23
1st Internode	0.07	2.1	2	0.08	10	0.32
2nd Internode	0.07	1.6	2	0.04	1	0.03
3rd Internode	0.04	1.5	1	0.04	1	0.03
4th Internode	0.07	1.7	2	0.05	2	0.04
5th Internode	0.03	0.7	1	0.02	1	0.02
6th Internode	0.04	0.7	1	0.02	2	0.03
7th Internode	0.06	0.6	1	0.01	2	0.02
8th Internode	0.05	0.4	2	0.01	2	0.02
Other Stem	0.35	0.2	17	0.01	14	0.01
Branch, 5th node	0.26	0.6	32	0.07	107	0.22
Branch, 6th node	0.36	1.0	37	0.10	101	0.27
Branch, 7th node	0.39	0.7	34	0.06	121	0.22
Branch, 8th node	0.33	2.2	19	0.12	47	0.31
Branch, 10th node	0.21	1.1	22	0.12	39	0.21
Branch, 12th node	0.18	1.8	13	0.13	54	0.53
Roots	0.12	0.01	nd	nd	nd	nd

Table. 3.1. 6. Distribution of caffeine and theobromine in the seedlings of **Iranian** tea. Age **15** months (March 1994). Three samples analysed, data expressed as mean values ± standard error. nd - not detected

	Caffeine		Theobromine	
Plant material	mg	mg g^{-1} (F.W.)	µg	µg g^{-1} (F.W.)
Apical bud	0.4 ± 0.1	26.7 ± 1.7	22.0 ± 3.0	1470 ± 170
1st leaf	2.4 ± 0.8	11.9 ± 1.7	35.0 ± 15.7	190 ± 60
2nd leaf	2.7 ± 0.4	11.8 ± 1.8	47.3 ± 11.4	200 ± 40
3rd leaf	1.8 ± 0.8	8.1 ± 3.3	33.3 ± 7.9	150 ± 10
4th leaf	1.4 ± 1.0	3.0 ± 1.9	68.0 ± 0.6	160 ± 10
5th leaf	0.4 ± 0.2	0.8 ± 0.4	39.3 ± 16.3	80 ± 30
6th leaf	0.1 ± 0.0	0.6 ± 0.3	27.3 ± 10.5	70 ± 20
7th leaf	0.2 ± 0.1	0.5 ± 0.3	23.7 ± 7.8	60 ± 20
8th leaf	0.2 ± 0.1	0.6 ± 0.2	18.3 ± 9.6	50 ± 20
9th leaf	0.1 ± 0.0	0.2 ± 0.1	11.3 ± 2.2	20 ± 3
Other leaves	0.5 ± 0.3	0.3 ± 0.1	61.7 ± 30.2	40 ± 7
1st Internode	0.1 ± 0.0	9.5 ± 1.2	7.7 ± 4.2	460 ± 120
2nd Internode	0.1 ± 0.0	7.4 ± 0.4	6.0 ± 2.5	310 ± 50
3rd Internode	0.1 ± 0.0	4.7 ± 1.0	5.0 ± 1.0	260 ± 40
4th Internode	0.1 ± 0.1	3.4 ± 0.6	9.0 ± 1.5	240 ± 50
5th Internode	0.1 ± 0.0	1.6 ± 0.3	7.3 ± 2.3	120 ± 20
6th Internode	0.1 ± 0.0	1.5 ± 0.5	7.3 ± 2.2	130 ± 50
7th Internode	0.1 ± 0.0	1.6 ± 0.1	5.7 ± 0.3	150 ± 10
8th Internode	0.1 ± 0.0	1.4 ± 0.0	6.7 ± 1.3	170 ± 30
Other stems	1.1 ± 0.3	0.4 ± 0.1	132.7 ± 30.4	60 ± 20
Branch, 5th node	3.0 ± 0.1	13.2 ± 0.3	111.0 ± 35.5	500 ± 170
Branch, 6th node	5.8 ± 1.8	12.1 ± 0.2	99.7 ± 28.0	240 ± 60
Branch, 7th node	2.6 ± 0.4	11.3 ± 0.3	78.7 ± 28.0	320 ± 80
Branch, 8th node	2.6 ± 0.2	5.8 ± 0.4	58.0 ± 4.0	130 ± 20
Branch, 9th node	4.3 ± 1.5	5.8 ± 0.2	180.0 ± 99.0	210 ± 70
Branch, 10th node	4.9 ± 1.2	5.0 ± 0.5	163.7 ± 79.0	150 ± 50
Branch, 11th node	5.6 ± 1.1	6.2 ± 0.2	99.7 ± 28.1	110 ± 10
Branch, 12th node	4.6 ± 0.7	6.1 ± 0.4	123.7 ± 31.9	170 ± 50
Branch, 13th node	4.3 ± 0.4	5.7 ± 0.2	103.7 ± 21.1	140 ± 30
Roots	0.2 ± 0.1	0.03 ± 0.0	36.0 ± 21.4	20 ± 10

In order to examine the data in more detail, the information in **Tables 3.1.1** to **3.1.6** was reformulated so variations in purine alkaloid levels in the different parts of the plant could be evaluated more fully.

3.1.3.1. Shoot

The shoot was sub-divided in to upper (apical bud, leaves 1-3, internodes 1-3), middle (leaves 4-9, internodes 4-8) and lower shoot (leaves and internodes below leaf 9). The estimated concentration of caffeine and theobromine in the three parts of the shoot in 5.5 to 15 month-old Iranian tea seedlings are presented in **Figure 3.1.2**. It is evident that the concentration of caffeine was always higher in the upper shoot than in the middle and lower shoot. The concentration of caffeine also appears to change during the course of the year. While this could be an effect of the age of the seedlings, it is more likely to be a reflection of seasonal changes. The concentration of caffeine in the upper shoot in May and July 1993 was ca. 16 mg g^{-1}. From this high point it declined to ca. 4 mg g^{-1} in November 1993 and January 1994 before increasing to ca. 11 mg g^{-1} in March 1994 at the beginning of spring. Thus, caffeine levels in the upper shoot appear to be highest in summer and lowest in winter. This trend was also evident, but less exaggerated, in the middle and lower shoots of Iranian tea seedlings (**Fig. 3.1.2**).

Theobromine concentrations in the three parts of the shoot were much lower than those of caffeine. However, as with caffeine, the concentrations were highest in the upper shoot and similar seasonal variations were apparent with a decline in winter and a rise early spring.

The upper shoot is very important for black tea production. The seasonal variations in caffeine and theobromine concentrations in the different parts of the upper shoot are presented in **Fig.3.1.3**. The major purine alkaloid, caffeine, was found in highest concentrations, 44.4 mg g^{-1}, in the apical bud of 7 month-old seedlings in July 1993. The levels in the apical bud then decreased to a low point of 21.3 mg g^{-1} in January 1994 before increasing in March to 26.7 mg g^{-1}. The caffeine content of the first leaf was 35.1 mg g^{-1} in July 1993 and declined, far more rapidly than the caffeine content of the apical bud, to a mere 2.3 mg g^{-1} in January 1994 before increasing to 11.9 mg g^{-1} in March 1994. Similar, but smaller seasonal changes were observed in the caffeine content of the second and third leaves which contained substantially lower than the apical bud and first leaf.

3.1.3.2. Leaves

From the data presented in **Tables 3.1.1** to **3.1.6.**, it is evident that more than 90% of the caffeine in Iranian tea seedlings is present in the leaves. When the leaves classified as young (apical bud, 1st to 3rd leaf), mature (4th - 9th leaf) and ages (below 9th leaf), it is clear that the highest concentrations of caffeine are found in the young leaves (**Fig. 3.1.4**) and that the seasonal variations in caffeine and theobromine, not surprisingly, mirror those presented in **Figure 3.1.2** for the upper shoot.

3.1.3.3. Stem

Variations in the caffeine and theobromine content of the upper (internodes 1-3), middle (internodes 4-8) and lower (below 8 th internode) stem of seedlings harvest between May 1993 and March 1994 are presented in **Figure 3.1.5**. The concentration of caffeine

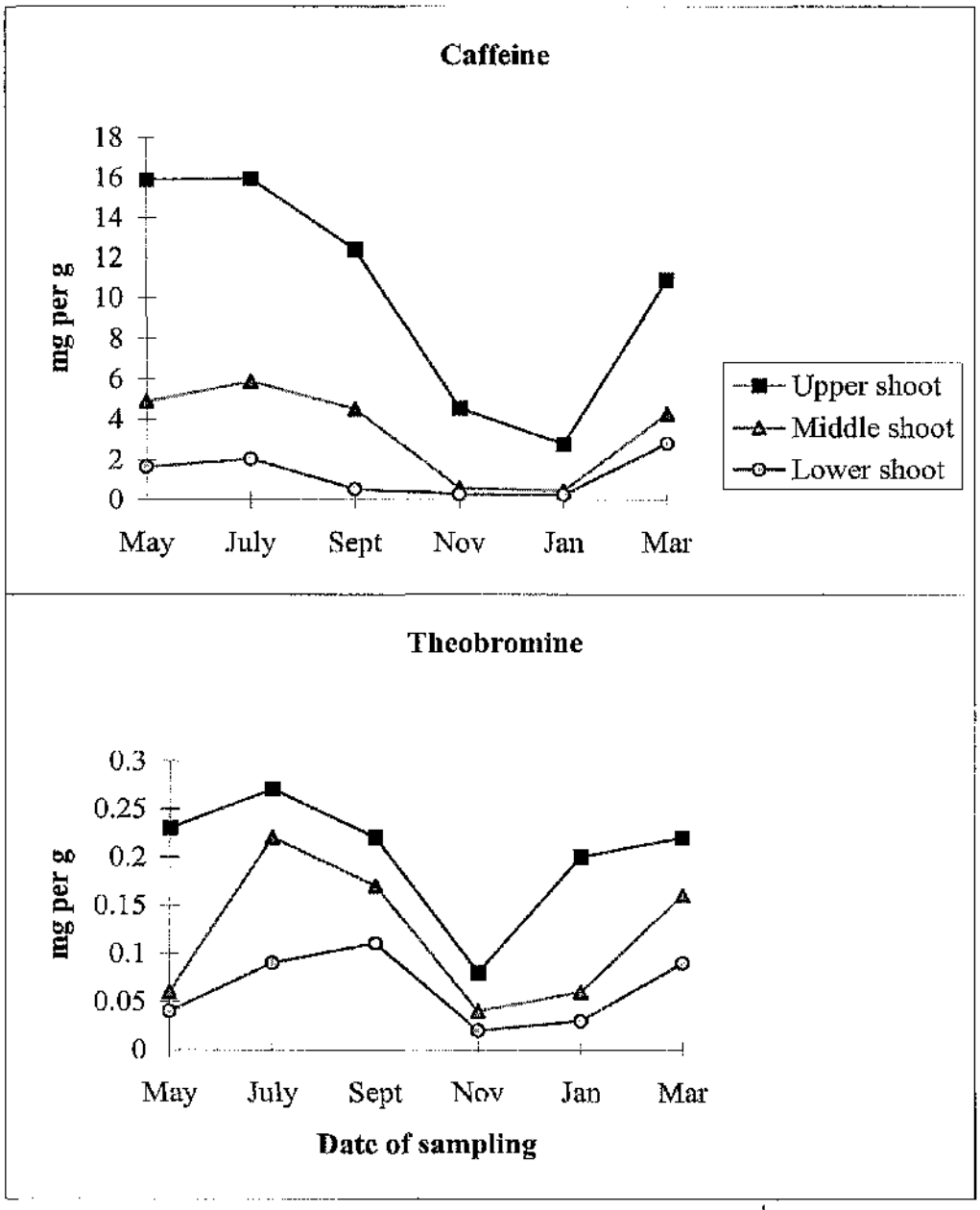


Fig. 3.1.2. Concentration of caffeine and theobromine (mg g^{-1}) in the upper, middle and lower shoot of 5.5-15 month-old Iranian tea seedlings (May 1993-March 1994).

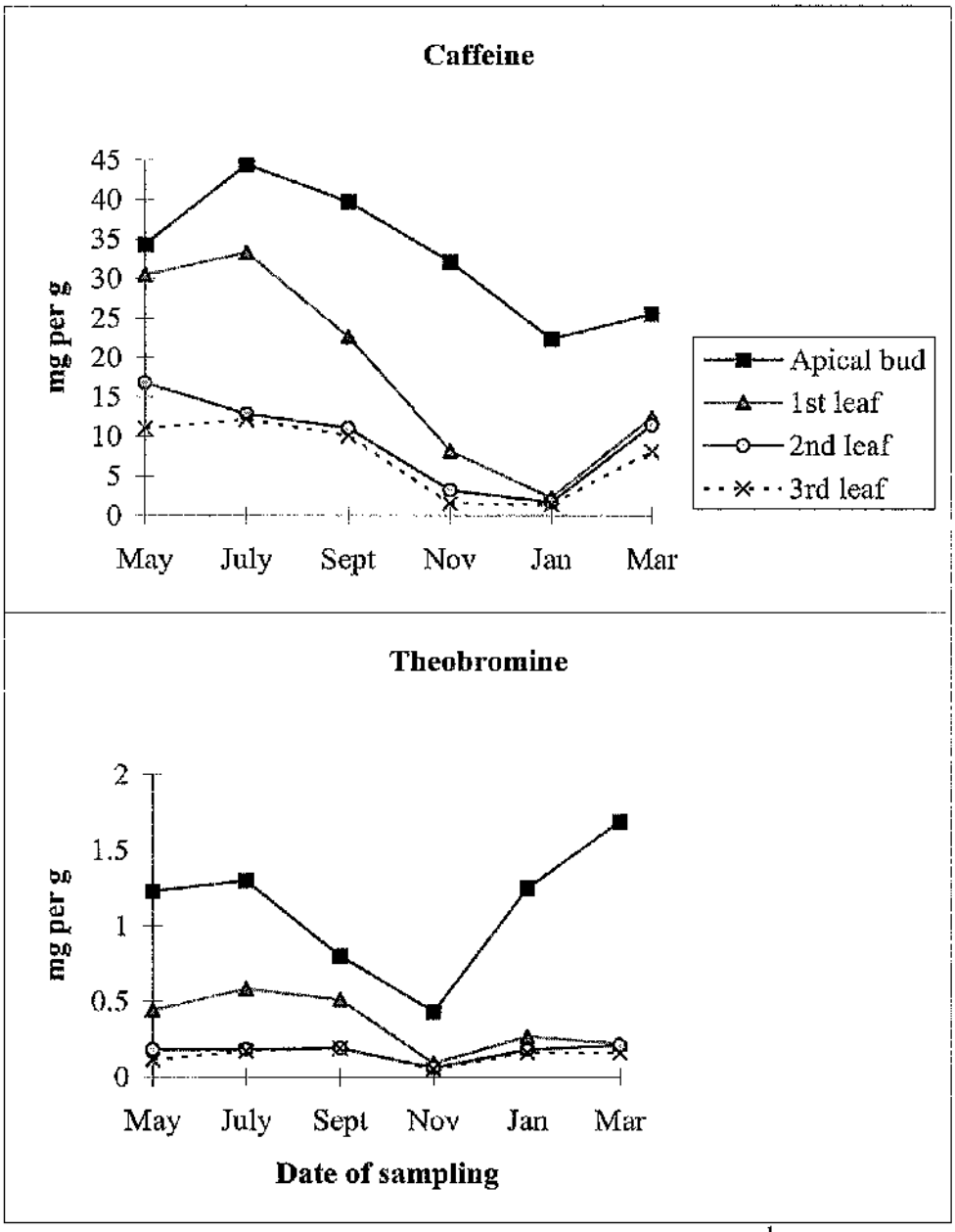


Fig. 3.1.3. Concentration of caffeine and theobromine (mg g^{-1}) in the apical bud, 1st leaf, 2nd leaf and 3rd leaf of 5.5-15 month-old Iranian tea seedlings (May 1993- March 1994).

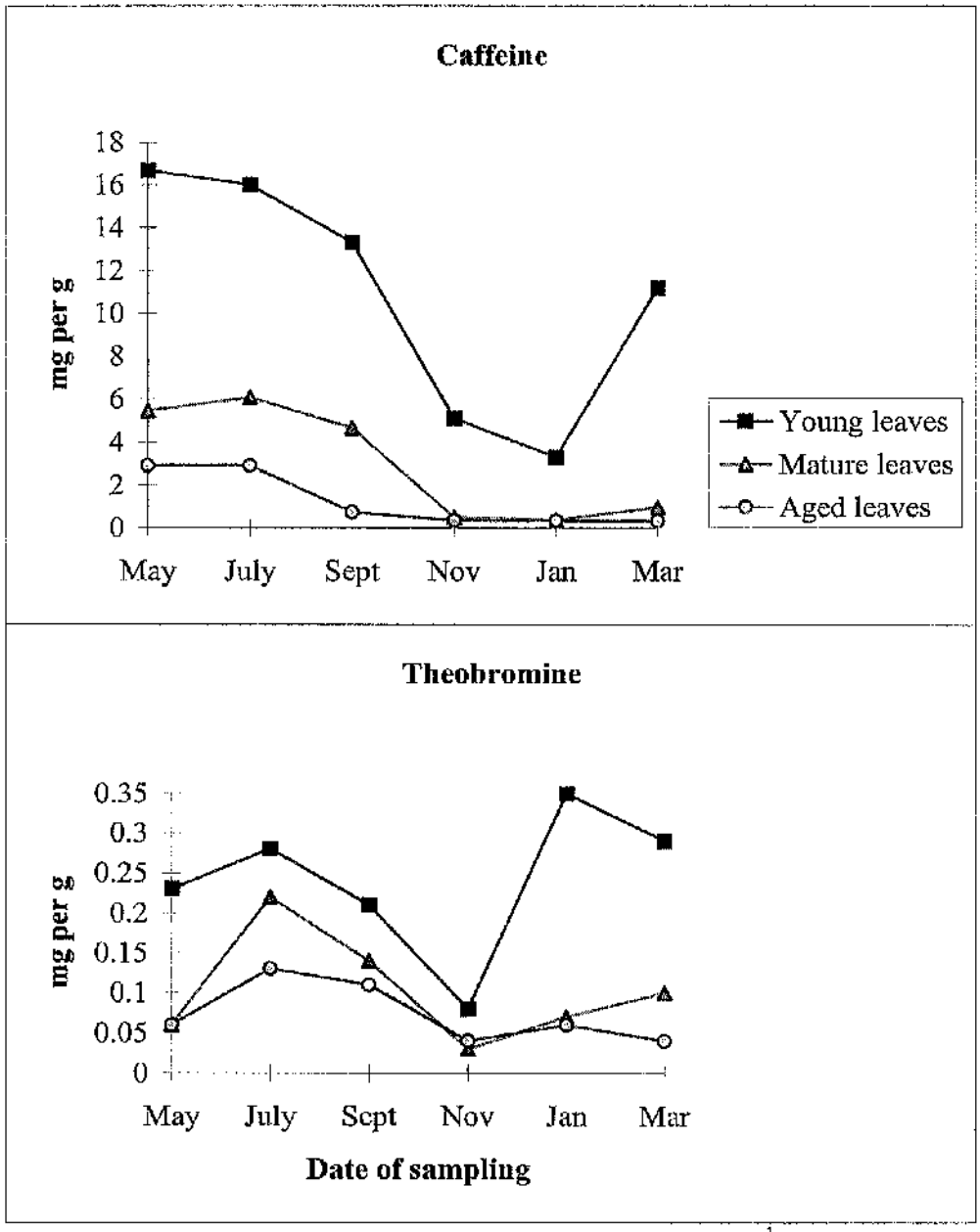


Fig. 3.1.4. Concentration of caffeine and theobromine (mg g^{-1}) in the young, mature and aged leaves of 5.5-15 month-old Iranian tea seedlings (May 1993 - March 1994).

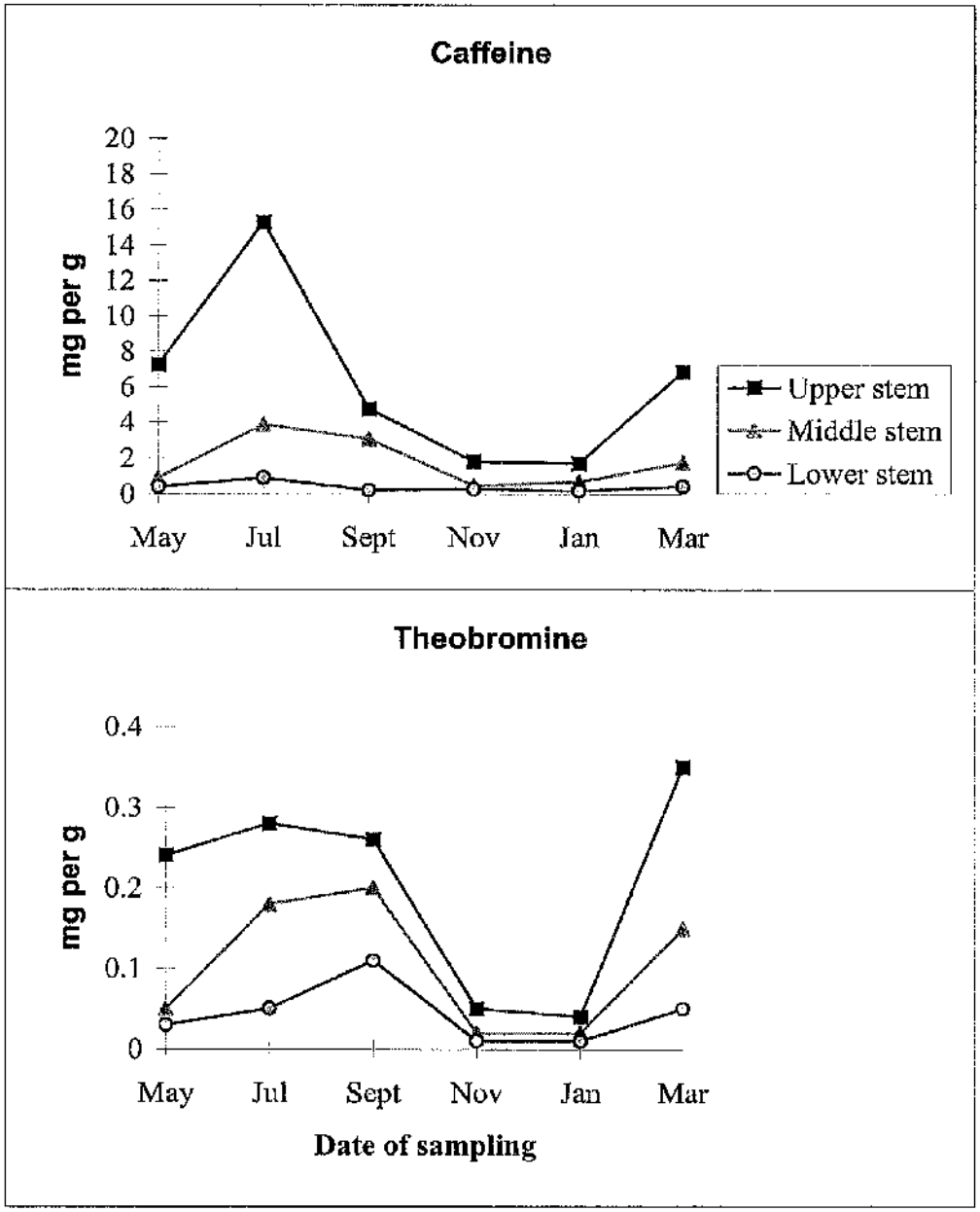


Fig. 3.1.5. Concentration of caffeine and theobromine (mg g^{-1}) in the upper, middle and lower stem of 5.5-15 month-old Iranian tea seedlings (May 1993-March 1994).

is higher in the upper stem than in the middle and lower stem and similar seasonal fluctuations to those observed in leaves, also occur. Once again theobromine is present in much lower concentrations than caffeine, and in winter months it was not detected in some samples.

3.1.3.4. Root and cotyledons

Although not of economic importance, the purine alkaloid content of roots and cotyledons of Iranian tea seedlings were investigated. The cotyledons, which were lost from plants older than 9 months, contained only minor levels of caffeine and theobromine was below the limit of detection. Roots, similarly, contained only trace quantities of caffeine (**Tables 3.1.1 - 3.1.6**).

3.1.3.5. Branches

As the seedlings grew older branches began to appear and these too were analysed to determine their purine alkaloid content (**Tables 3.1.4 -3.1.6**). Once again caffeine was the major component with only trace amounts of theobromine being detected. In November 1993 and January 1994 the concentration of caffeine in branches was less than 3 mg g^{-1}, however this increased in all branches in plants harvested in March 1994 with the upper three branches containing more than 10 mg g^{-1}.

3.1.3.6. Paraxanthine in Iranian tea

Paraxanthine (1,7-dimethyxanthine) which has been found only rarely in plants, was detected in the present study in extracts of Iranian tea seedlings harvested in September and November 1993 and January 1994 (**Tables 3.1.3-3.1.5**). Like caffeine, the concentration of paraxanthine was the higher in young leaves than mature and aged

leaves and stem tissue. The highest concentration of paraxanthine, 1.8 mg g^{-1}, was detected in the apical bud from 9 month-old seedlings harvested in September 1993 (**Table 3.1.3**). The HPLC traces obtained with this sample and when it was co-chromatographed with a paraxanthine standard are illustrated in **Figure 3.1.6**. These results together with those of Kato et al. (1996) suggest (i) paraxanthine may be a precursor rather than a degradation product of caffeine and (ii) caffeine biosynthesis in young leaves in winter may be different from than in spring-summer leaves.

3.1.4. Summary of the distribution of caffeine and theobromine in Iranian tea seedlings

Caffeine and theobromine content of key parts of Iranian tea seedling harvested between May 1993 and March 1994 are summarised in **Table 3.1.7**. The purine alkaloid levels are expressed as a percentage of the fresh weight. The data show clearly that caffeine is the major purine alkaloid and that it is present in highest concentrations in the upper part of the shoot, most notably the apical bud and expanding first leaf. The information presented in **Table 3.1.7** also provides evidence for seasonal variations in the caffeine and theobromine content of tea seedlings, with highest levels being detected in summer month and lowest in winter.

Fig. 3.1.6. Gradient HPLC analysis of purine alkaloids in the apical bud Iranian tea seedlings (September 1993). Column: 250 × 4.6 mm i.d. 5μm ODS Hypersil. Gradient: 25 min, 0 - 40% methanol in 50 mM Sodium acetate (pH5). Flow rate: 1ml min^{-1}. Detector: absorbance monitor at 272nm. Sample: (A) 100μl aliquot of a 5 ml aliquots extract of 10 mg of apical buds from 9 month-old of tea seedling (September 1993). (B) A co-chromatographed with 2 ng of caffeine (Cf), theobromine (Tb) and paraxanthine (Pa).

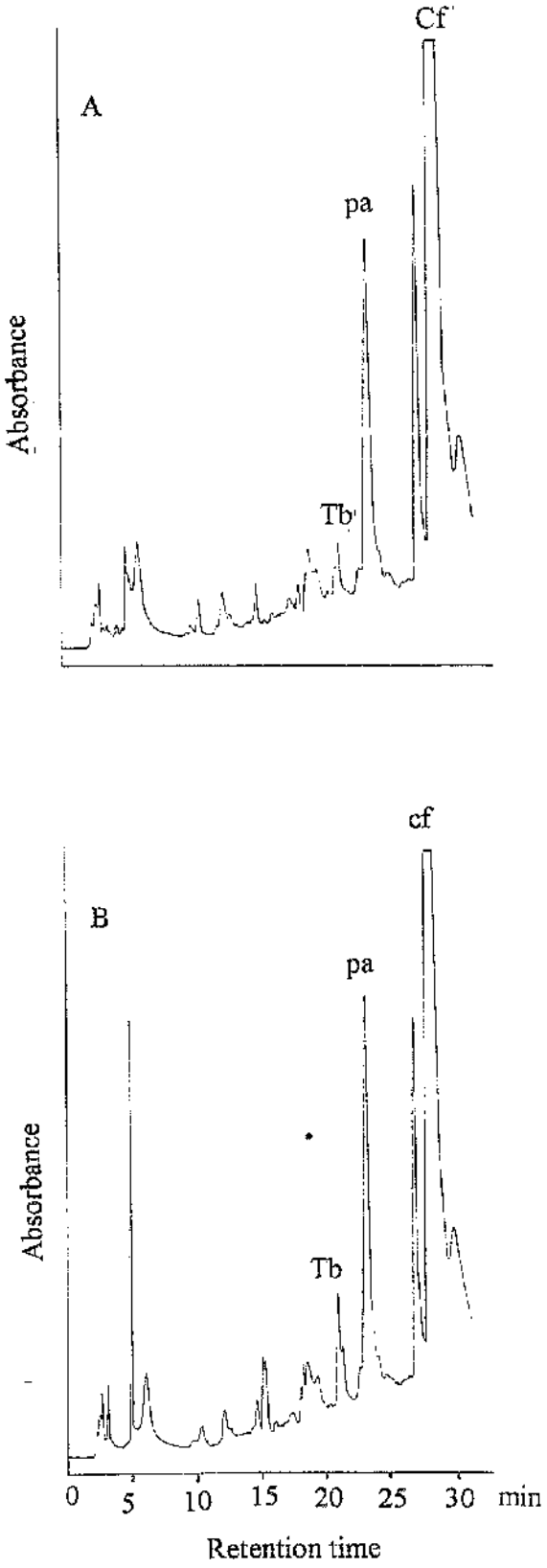

A
Cf
pa
Tb
Absorbance
B
cf
pa
Tb
Absorbance
0
5
10
15
20
25
30
min
Retention time

Table. 3.1.7. Distribution of caffeine and theobromine in **Iranian** tea seedlings over an 11 month period from May 1993 to March 1994. Data expressed as a percentage of fresh weight. Cf - caffeine, Tb - theobromine, nd - not detected.

	May		July		September		November		January		March	
Structure	**Cf**	**Tb**	**Cf**	**Tb**	**Cf**	**Tb**	**Cf**	**Tb**	**Cf**	**Tb**	**Cf**	**Tb**
Apical bud	3.43	0.12	4.44	0.13	4.00	0.08	3.21	0.04	2.23	0.13	2.55	0.17
Upper shoot	1.59	0.02	1.60	0.03	1.24	0.02	0.45	0.01	0.28	0.02	1.10	0.02
Middle shoot	0.49	0.01	0.59	0.02	0.45	0.02	0.06	nd	0.04	0.01	0.43	0.02
Lower shoot	0.16	nd	0.21	0.01	0.05	0.01	0.03	nd	0.02	nd	0.28	0.01
Young leaves	1.67	0.02	1.60	0.03	1.33	0.02	0.51	0.01	0.33	0.04	1.12	0.03
Mature leaves	0.55	0.01	0.61	0.02	0.47	0.01	0.05	nd	0.04	0.01	0.10	0.01
Aged leaves	0.29	0.01	0.29	0.01	0.07	0.01	0.04	nd	0.03	0.01	0.03	nd
Upper stem	0.72	0.02	1.53	0.03	0.47	0.04	0.18	0.01	0.17	nd	0.68	0.04
Middle stem	0.09	0.01	0.39	0.02	0.31	0.05	0.05	nd	0.07	nd	0.18	0.02
Lower stem	0.04	nd	0.09	0.01	0.02	0.01	0.03	nd	0.02	nd	0.04	0.01
Root	0.001	nd	0.001	nd	0.001	nd	0.001	nd	0.001	nd	0.002	nd

3.1.5. Accumulation of methylxanthines in Darjeeling tea seedlings

Tables 3.1.8 provides information on the amounts of caffeine and theobromine present in the different parts of 5.5 month-old Darjeeling tea seedlings harvested in May 1993. As with Iranian tea seedlings, caffeine was the predominant methylxanthine with much lower levels of theobromine being detected. Once again caffeine was present in highest concentrations in the apical bud and first leaf with lower levels in the older leaves and internodes although the first internode did contain more caffeine than the older, lower internodes. These plants had lost their cotyledons and the roots contained only trace amounts of caffeine, while theobromine was not present in detectable quantities.

3.1.6. Distribution of purine alkaloids in Darjeeling tea seedlings

Information on the distribution of purine alkaloids in 5.5 to 15 month old Darjeeling tea seedlings is presented in **Tables 3.1.8** to **3.1.13**. Caffeine was the major compound detected along with lower levels of theobromine. In contrast to Iranian tea, paraxanthine was not detected in any of the samples of Darjeeling tea seedlings that were analysed. There was again evidence for the presence of a caffeine gradient of within the plant, with highest concentrations being found in the apical bud and the young expanding first leaf. In the case of the 15 month old seedlings triplicate samples were analysed (**Table 3.1.13**) and this demonstrates the precision of the analytical procedures and also indicates that the overall trends of the mean values are similar to those obtained with the younger seedlings where only one sample was analysed (**Tables 3.1.8 - 3.1.12**).

Table. 3.1.8. Distribution of caffeine and theobromine in the seedlings of **Darjeeling** tea. Age **5.5** months (May 1993). nd - not detected.

	Caffeine		Theobromine	
Plant material	mg	mg g^{-1} (F.W.)	µg	mg g^{-1} (F.W.)
Apical bud	0.40	26.7	10	0.67
1st Leaf	1.51	11.6	27	0.20
2nd Leaf	1.68	5.2	37	0.12
3rd Leaf	1.99	4.1	30	0.06
4th Leaf	2.60	3.8	34	0.05
5th Leaf	1.77	3.4	32	0.06
6th Leaf	0.97	4.1	15	0.06
7th Leaf	1.39	4.3	18	0.06
8th Leaf	2.62	4.2	21	0.03
9th Leaf	3.04	4.2	20	0.03
Other Leaves	2.38	2.8	20	0.02
1st Internode	0.08	3.0	1	0.05
2nd Internode	0.07	1.7	2	0.05
3rd Internode	0.06	1.4	2	0.04
4th Internode	0.07	0.6	3	0.02
5th Internode	0.07	0.6	3	0.03
6th Internode	0.05	0.8	3	0.04
7th Internode	0.04	0.8	2	0.04
8th Internode	0.04	0.5	2	0.02
Other Stem	0.26	0.4	13	0.02
Roots	0.07	0.02	nd	nd

Table. 3.1.9. Distribution of caffeine and theobromine in the seedlings of **Darjeeling** tea. Age 7 months (July 1993).

	Caffeine		Theobromine	
Plant material	mg	mg g^{-1} (F.W.)	µg	mg g^{-1} (F.W.)
Apical bud	0.24	30.3	5	0.63
1st Leaf	0.75	12.6	10	0.16
2nd Leaf	2.50	6.5	69	0.20
3rd Leaf	3.55	6.6	90	0.17
4th Leaf	2.80	5.8	90	0.18
5th Leaf	2.54	6.0	74	0.18
6th Leaf	2.18	4.1	110	0.20
7th Leaf	1.87	4.4	86	0.20
8th Leaf	2.03	4.8	70	0.17
9th Leaf	1.39	3.8	48	0.13
Other Leaves	1.61	3.7	38	0.08
1st Internode	0.11	18.4	2	0.30
2nd Internode	0.22	4.8	6	0.14
3rd Internode	0.29	3.0	8	0.09
4th Internode	0.27	3.5	10	0.12
5th Internode	0.23	3.0	8	0.11
6th Internode	0.21	3.0	8	0.11
7th Internode	0.17	1.5	8	0.07
8th Internode	0.22	2.0	10	0.08
Other Stem	0.43	0.3	14	0.01
Roots	0.06	0.01	23	0.003

Table. 3.1.10. Distribution of caffeine, theobromine and paraxanthine in the seedlings of **Darjeeling** tea. Age **9** months (September 1993). nd - not detected.

	Caffeine		Theobromine	
Plant material	mg	mg g^{-1} (F.W.)	µg	mg g^{-1} (F.W.)
Apical bud	0.30	24.6	12.0	1.00
1st Leaf	0.19	12.8	4.0	0.30
2nd Leaf	0.91	3.8	32.0	0.13
3rd leaf	0.59	2.3	28.0	0.11
4th Leaf	0.38	1.7	20.0	0.09
5th Leaf	0.41	1.5	24.0	0.09
6th Leaf	0.46	1.2	29.0	0.07
7th Leaf	0.42	1.0	21.0	0.05
8th Leaf	0.10	0.6	6.4	0.04
9th Leaf	0.19	0.9	17.0	0.08
Other Leaves	0.71	0.7	42.0	0.04
1st Internode	0.08	7.9	1.6	0.16
2nd Internode	0.04	2.8	1.0	0.07
3rd Internode	0.04	1.4	1.6	0.05
4th Internode	0.08	1.3	4.0	0.06
5th Internode	0.10	1.4	5.0	0.07
6th Internode	0.08	1.1	4.0	0.06
7th Internode	0.07	0.8	4.0	0.06
8th Internode	0.07	0.6	3.0	0.03
Other Stem	0.14	0.2	5.0	0.01
Roots	0.070	0.01	nd	nd
Cotyledons	0.002	0.1	nd	nd

Table. 3.1.11. Distribution of the caffeine and theobromine in the seedlings of **Darjeeling** tea. Age **11** months (November 1993). nd - not detected.

	Caffeine		Theobromine	
Plant material	mg	mg g^{-1} (F.W.)	µg	mg g^{-1} (F.W.)
Apical bud	0.21	21.0	6	0.60
1st Leaf	0.41	11.4	7	0.20
2nd Leaf	0.76	2.8	43	0.16
3rd Leaf	0.09	0.3	21	0.08
4th Leaf	0.13	0.6	14	0.07
5th Leaf	0.07	0.6	9	0.01
6th Leaf	0.16	0.3	24	0.04
7th Leaf	0.06	0.2	19	0.05
8th Leaf	0.05	0.1	14	0.03
9th Leaf	0.04	0.2	9	0.03
Other Leaves	0.09	0.2	37	0.09
1st Internode	0.09	6.3	4	0.30
2nd Internode	0.04	2.4	2	0.13
3rd Internode	0.07	2.0	3	0.10
4th Internode	0.04	1.5	2	0.08
5th Internode	0.05	1.1	4	0.10
6th Internode	0.07	1.0	9	0.14
7th Internode	0.06	0.8	8	0.10
8th Internode	0.05	0.6	8	0.10
Other Stem	0.34	0.3	22	0.02
Branch, 7th node	1.13	3.8	80	0.27
Branch, 8th node	0.54	3.6	42	0.28
Branch, 9th node	0.27	3.4	6	0.08
Branch, 10th node	1.05	5.5	29	0.15
Roots	0.11	0.01	nd	nd

Table. 3.1.12. Distribution of caffeine and theobromine in the seedlings of **Darjeeling** tea. Age **13** months (January 1994). nd - not detected.

	Caffeine		Theobromine	
Plant material	mg	mg g^{-1} (F.W.)	µg	mg g^{-1} (F.W.)
Apical bud	0.22	18.3	7	0.58
1st Leaf	1.22	3.9	40	0.13
2nd Leaf	1.52	2.6	61	0.11
3rd Leaf	0.91	1.8	46	0.11
4th Leaf	0.64	1.8	29	0.08
5th Leaf	0.90	1.5	42	0.07
6th Leaf	0.60	0.9	48	0.09
7th Leaf	0.26	0.7	16	0.04
8th Leaf	0.18	0.4	19	0.04
9th Leaf	0.16	0.3	26	0.05
Other Leaves	0.95	0.4	190	0.08
1st Internode	0.09	5.6	2	0.15
2nd Internode	0.03	1.6	2	0.10
3rd Internode	0.01	0.8	2	0.13
4th Internode	0.02	0.6	4	0.10
5th Internode	0.02	0.4	18	0.14
6th Internode	0.02	0.3	9	0.14
7th Internode	0.02	0.2	9	0.12
8th Internode	0.01	0.2	5	0.06
Other Stem	0.49	0.2	192	0.08
Branch, 13th node	0.44	3.4	24	0.17
Branch, 15th node	1.04	2.6	81	0.20
Branch, 16th node	1.05	2.4	44	0.10
Branch, 17th node	0.60	1.9	30	0.10
Roots	0.23	0.04	nd	nd

Table.3.1.13. Distribution of caffeine and theobromine in the seedlings of **Darjeeling** tea. Age **15** months (March 1994). Three samples analysed, data expressed as mean values ± standard error. nd - not detected.

Plant material	Caffeine		Theobromine	
	mg	mg g^{-1} (F.W.)	µg	µg g^{-1} (F.W.)
Apical bud	0.3 ± 0.1	28.3 ± 0.9	16.3 ± 5.4	1330 ± 240
1st leaf	1.2 ± 0.5	7.9 ± 1.3	33.3 ± 20.9	200 ± 80
2nd leaf	1.9 ± 0.8	6.4 ± 0.8	43.0 ± 24.4	130 ± 40
3rd leaf	1.9 ± 0.8	5.3 ± 0.5	44.3 ± 25.9	120 ± 30
4th leaf	1.7 ± 0.7	5.1 ± 0.7	39.0 ± 21.4	100 ± 30
5th leaf	0.8 ± 0.5	1.3 ± 0.6	58.7 ± 37.7	90 ± 40
6th leaf	0.8 ± 0.7	1.1 ± 0.7	51.3 ± 23.7	90 ± 10
7th leaf	0.6 ± 0.4	1.0 ± 0.6	37.0 ± 7.8	90 ± 20
8th leaf	0.6 ± 0.4	0.9 ± 0.6	57.0 ± 26.1	90 ± 30
9th leaf	0.6 ± 0.5	0.8 ± 0.6	58.3 ± 26.2	80 ± 30
Other leaves	3.2 ± 1.3	0.9 ± 0.3	258.0 ± 116.0	70 ± 30
1st Internode	0.1 ± 0.0	8.0 ± 0.5	15.7 ± 7.1	660 ± 100
2nd Internode	0.1 ± 0.1	3.6 ± 0.7	8.0 ± 2.9	300 ± 100
3rd Internode	0.1 ± 0.0	2.9 ± 0.7	6.3 ± 2.3	280 ± 100
4th Internode	0.1 ± 0.0	2.6 ± 0.6	9.0 ± 4.0	300 ± 60
5th Internode	0.1 ± 0.0	2.1 ± 0.3	8.7 ± 0.7	270 ± 90
6th Internode	0.1 ± 0.0	1.7 ± 0.1	7.0 ± 1.2	190 ± 50
7th Internode	0.1 ± 0.0	1.5 ± 0.2	6.7 ± 0.9	140 ± 30
8th Internode	0.1 ± 0.0	1.6 ± 0.2	9.7 ± 3.8	220 ± 50
Other stems	0.1 ± 0.0	0.3 ± 0.0	225.0 ± 105.0	60 ± 20
Branch, 7th node	2.3 ± 0.8	6.4 ± 1.2	61.3 ± 14.8	170 ± 3
Branch, 8th node	3.3 ± 1.5	3.9 ± 0.2	102.3 ± 39.7	130 ± 10
Branch, 9th node	3.0 ± 0.9	5.6 ± 0.6	85.3 ± 23.2	160 ± 10
Branch, 10th node	3.6 ± 2.1	4.7 ± 0.7	78.7 ± 37.2	110 ± 20
Branch, 11th node	4.3 ± 0.5	4.9 ± 0.5	87.3 ± 37.7	140 ± 20
Roots	0.04 ± 0.01	0.02 ± 0.01	nd nd	nd nd

3.1.6.1. Shoot

The concentration of caffeine and theobromine in the upper, middle and lower shoot of 5.5 to 15 month-old Darjeeling tea seedlings (May 1993 to March 1994) are presented in **Figure 3.1.7**. The upper shoots had a higher content caffeine than the middle and lower shoots. As with Iranian tea, the concentration of caffeine in the shoot tissues, especially the upper and middle shoot, change during the course of the year with highest levels in summer months and lowest in winter. This trend was not reflected in the, albeit, very low concentrations of theobromine.

The seasonal variations in caffeine and theobromine concentrations in the different parts of the upper shoot are presented in **Figure 3.1.8**. Caffeine, was found in highest concentrations, 30.3 mg g^{-1}, in the apical bud of 7 month-old seedlings in July 1993. The levels in the apical bud then declined to 18.3 mg g^{-1} in January 1994 before increasing in March to 28.3 mg g^{-1}. The lower concentrations of caffeine in the first, second and third leaves followed a similar pattern.

3.1.6.2. Leaves

Like Iranian tea seedlings, more than 90% of the caffeine in Darjeeling tea is present in the leaves. The highest concentrations are found in the young leaves and the seasonal variations (**Fig. 3.1.9**) parallel those observed in the upper shoot (**Fig. 3.1.7**). The very low concentrations of theobromine in leaves appear to fluctuate independently of the seasonal variations in caffeine content.

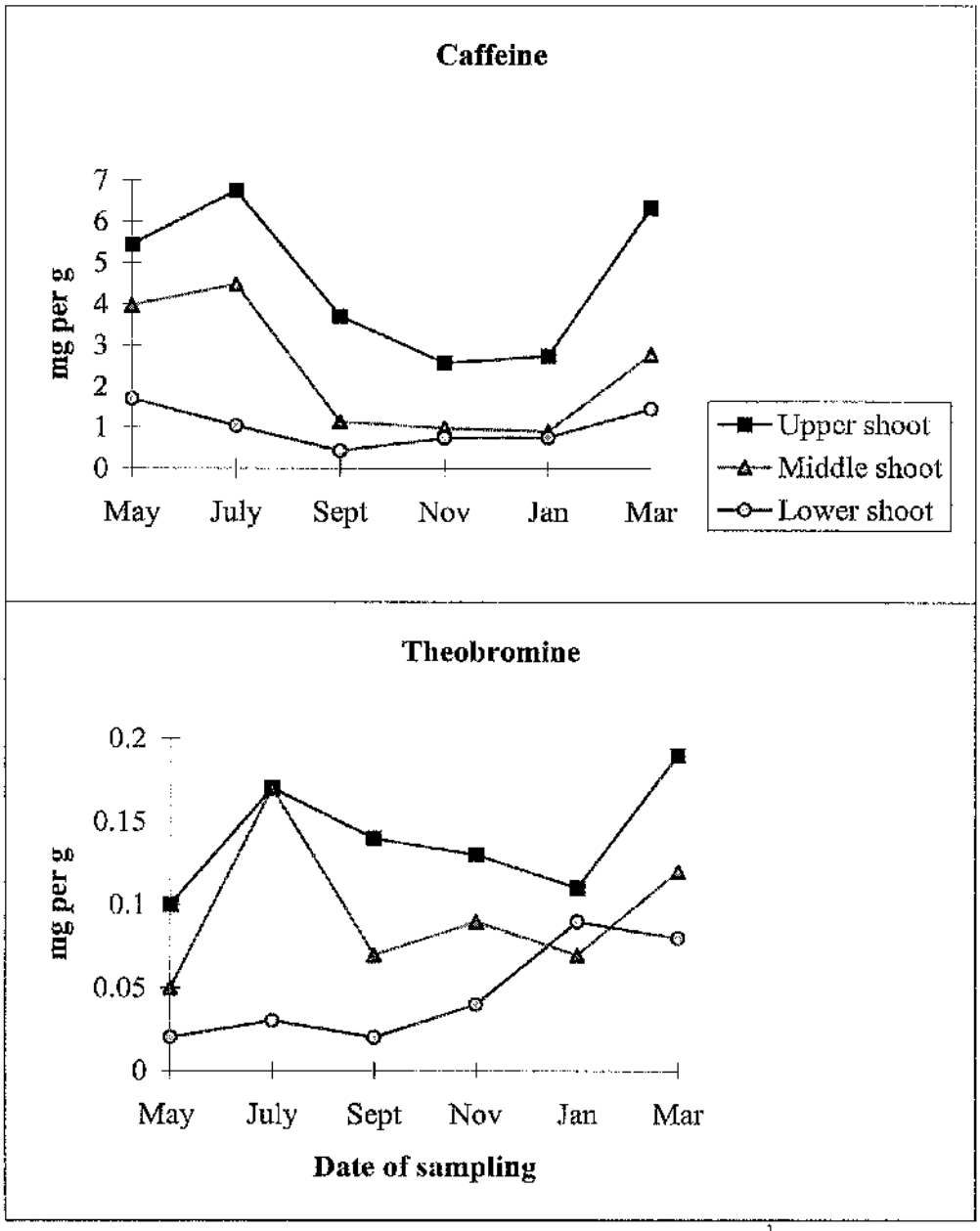


Fig. 3.1.7. Concentration of caffeine and theobromine (mg g^{-1}) in the upper, middle and lower shoot of 5.5-15 month-old Darjeeling tea seedlings (May 1993-March 1994).

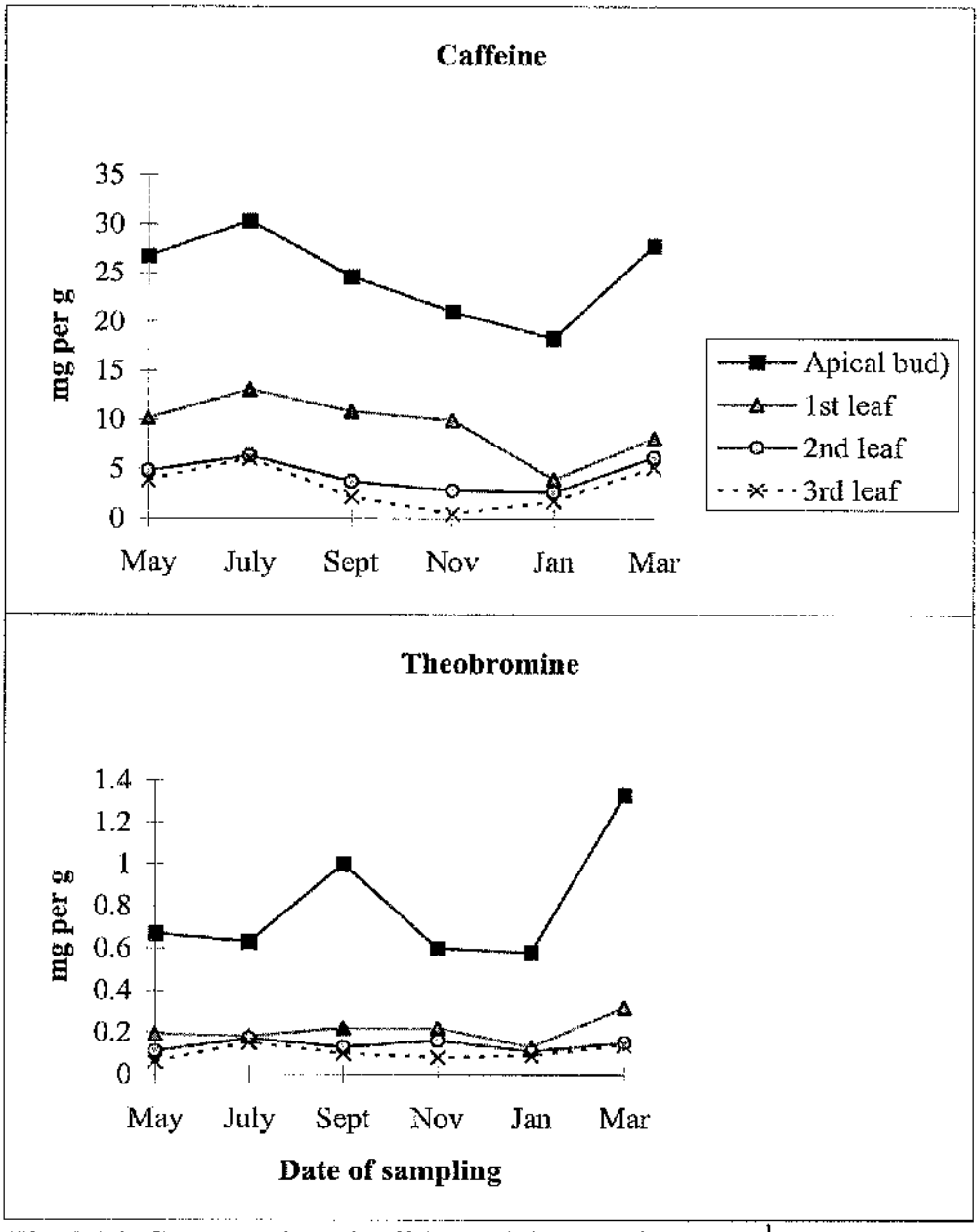


Fig. 3.1.8. Concentration of caffeine and theobromine (mg g^{-1}) in the apical bud, first, second and third leaf of the 5.5-15 month-old Darjeeling tea seedlings (May 1993-March 1994).

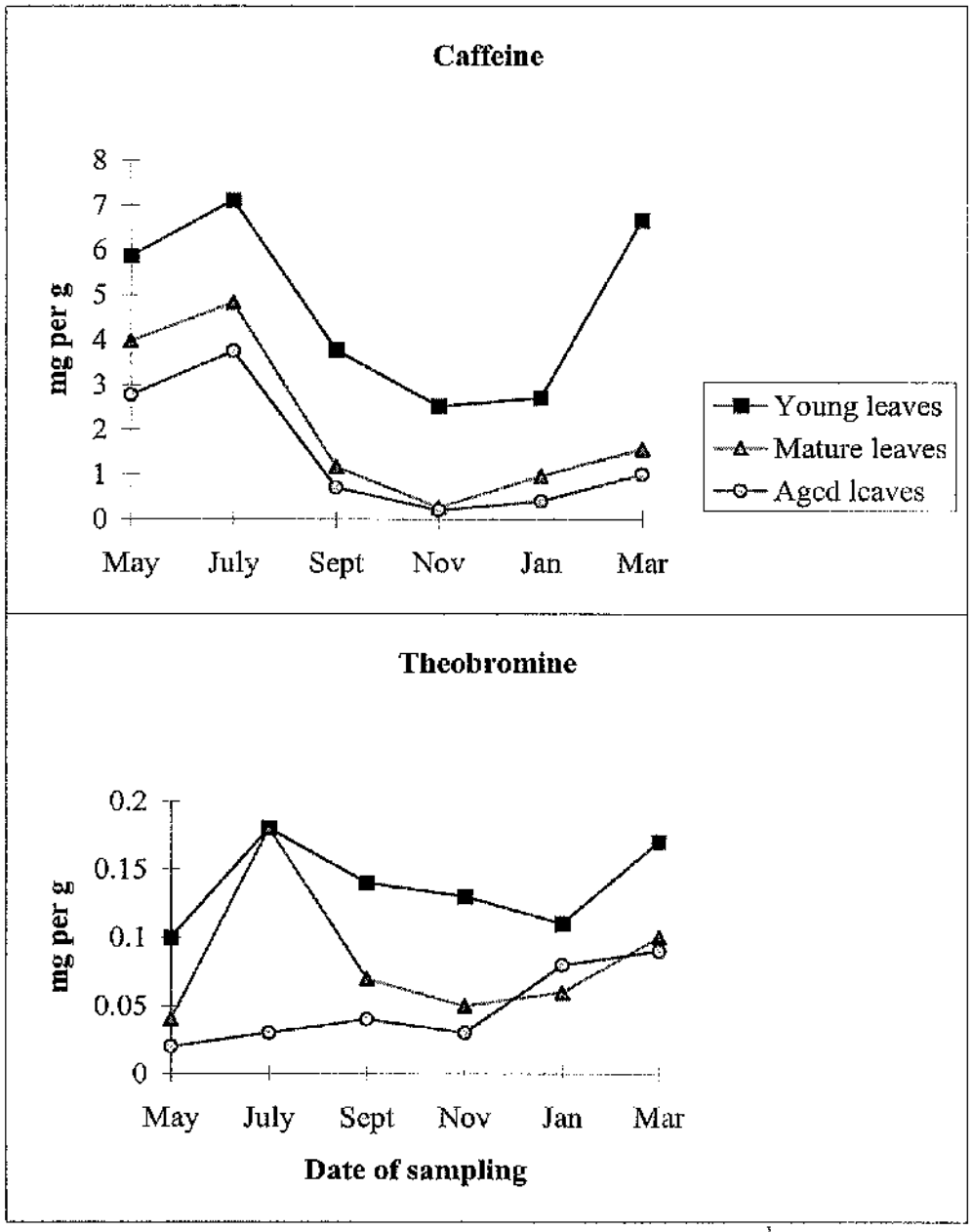


Fig. 3.1.9. Concentration of caffeine and theobromine (mg g^{-1}) in the young, mature and aged leaves of 5.5-15 month-old Darjeeling tea seedlings (May 1993-March 1994).

3.1.6.3. Stem

The caffeine and theobromine content of the upper, middle and lower stem of Darjeeling tea seedlings harvest between May 1993 and March 1994 are presented in **Figure 3.1.10**. Theobromine was below the limit of detection in many instances and the concentration of caffeine was higher in the upper stem than in the middle and lower stem. Variations in the levels of both compounds were not clearly related to seasonal factors.

3.1.6.4. Roots and cotyledons

When present cotyledons contained low levels of caffeine and trace amounts of theobromine **(Table 3.1.10)** while roots were found to contain only very low concentrations of caffeine **(Table 3.1.8 - 3.1.13)**.

3.1.6.5. Branches

Once again caffeine was the major component with much lower amounts of theobromine being detected. Although the trend was less clear cut than with Iranian tea, the data indicate that the caffeine content of the branches increased between January and March **(Tables 3.1.11 - 3.1.13)**.

3.1.7. Summary of the distribution of caffeine and theobromine in Darjeeling tea seedlings

Caffeine and theobromine content of key parts of Darjeeling tea seedling harvested between May 1993 and March 1994 are summarised in **Table 3.1.14**.

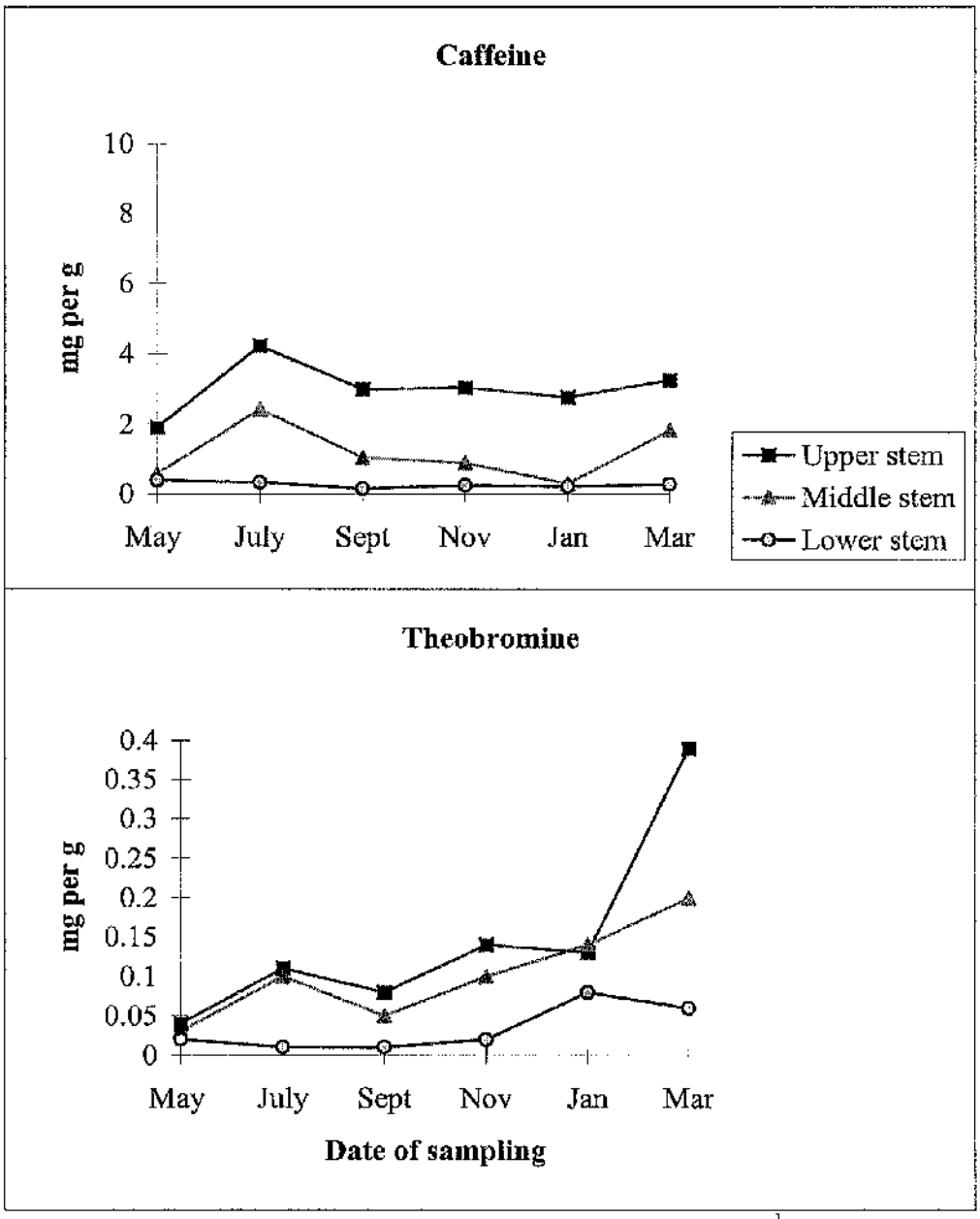


Fig. 3.1.10. Concentration of caffeine and theobromine (mg g^{-1}) in the upper, middle and lower stem of 5.5-15 month-old Darjeeling tea seedlings (May 1993-March 1994).

Table. 3.1.14. Distribution of caffeine and theobromine in **Darjeeling** tea seedlings over an 11 month period from May 1993 to March 1994. Data expressed as a percentage of fresh weight. Cf - caffeine, Tb - theobromine, nd - not detected.

	May		July		September		November		January		March	
Structure	**Cf**	**Tb**	**Cf**	**Tb**	**Cf**	**Tb**	**Cf**	**Tb**	**Cf**	**Tb**	**Cf**	**Tb**
Apical bud	2.67	0.07	3.03	0.06	2.46	0.10	2.10	0.06	1.83	0.06	2.78	0.13
Upper shoot	0.55	nd	0.64	0.02	0.37	0.01	0.26	0.01	0.27	0.01	0.63	0.02
Middle shoot	0.35	nd	0.45	0.02	0.11	0.01	0.10	0.01	0.09	0.01	0.28	0.01
Lower shoot	0.17	nd	0.12	nd	0.04	nd	0.07	nd	0.08	0.01	0.15	0.01
Young leaves	0.59	0.01	0.71	0.02	0.38	0.01	0.25	0.01	0.27	0.01	0.67	0.02
Mature leaves	0.40	nd	0.48	0.02	0.12	0.01	0.03	0.01	0.10	0.01	0.16	0.01
Aged leaves	0.28	nd	0.37	nd	0.07	nd	0.02	nd	0.04	0.01	0.10	0.01
Upper stem	0.19	nd	0.42	0.01	0.3	0.01	0.3	0.01	0.28	0.01	0.32	0.04
Middle stem	0.06	nd	0.24	0.01	0.1	0.01	0.10	0.01	0.03	0.01	0.18	0.02
Lower stem	0.04	nd	0.03	nd	0.02	nd	0.02	nd	0.02	0.01	0.03	0.01
Root	0.002	nd	0.001	nd	0.001	nd	0.001	nd	0.002	nd	0.0002	nd

The purine alkaloid levels are expressed as a percentage of the fresh weight. Caffeine is the predominant purine alkaloid and it is present in by far the highest concentrations in the apical bud and expanding first leaf. The information presented in **Table 3.1.14** also provides evidence for seasonal variations in the caffeine content of tea seedlings, with highest levels being detected in summer months and lowest in winter. However, especially in tissues containing low concentrations of caffeine, the trend is not as clear-cut as it was in seedlings of Iranian tea.

3.1.8. Comparison of the purine alkaloids content of Iran and Darjeeling tea seedlings

Table 3.1.15 is a compilation of information from **Tables 3.1.6** and **3.1.13** which contain data obtained in replicate analyses of the caffeine and theobromine content of 15 month old Iranian and Darjeeling tea seedlings harvested in March 1994. This shows that the purine alkaloid content of the two types of tea is very similar with the caffeine being present in much higher concentrations than theobromine, its immediate precursor. In both instances, highest concentrations of caffeine and theobromine were detected in the apical bud and there is a gradient within the plant with purine alkaloid concentrations declining substantially as the tissues increase in age. Although the caffeine content of the apical bud of the two teas is similar, there is a tendency for lower caffeine concentration in Darjeeling tea, the upper, middle and lower shoot of which contains 6.3 ± 0.5, 2.6 ± 0.1 and 1.6 ± 0.4 mg g^{-1} respectively compared to 10.8 ± 1.7, 4.3 ± 0.2 and 2.9 ± 0.4 mg g^{-1} for the same tissues in Iranian tea seedling.

Table. 3.1.15. Distribution of caffeine and theobromine in Iranian and Darjeeling tea seedlings at 15 months (March 1994). Data expressed as mg g^{-1} mean values ± standard error (n = 3), nd- not detected.

	Iranian tea		Darjeeling tea	
Structure	Cf	Tb	Cf	Tb
Apical bud	26.7 ± 1.9	1.5 ± 0.2	28.3 ± 0.9	1.3 ± 0.2
Upper shoot	10.8 ± 1.7	0.2 ± 0.04	6.3 ± 0.6	0.2 ± 0.02
Middle shoot	4.3 ± 0.2	0.2 ± 0.02	2.2 ± 0.08	0.1 ± 0.003
Lower shoot	2.9 ± 0.4	0.1 ± 0.02	1.5 ± 0.4	0.04 ± 0.02
Young leaves	11.1 ± 2	0.2 ± 0.04	6.6 ± 0.6	0.15 ± 0.03
Mature leaves	1.0 ± 0.3	0.07 ± 0.01	1.6 ± 0.43	0.1 ± 0.02
Aged leaves	0.3 ± 0.1	0.04 ± 0.01	0.9 ± 0.25	0.07 ± 0.03
Upper stem	6.7 ± 0.2	0.3 ± 0.1	3.9 ± 0.8	0.5 ± 0.15
Middle stem	1.9 ± 0.3	0.15 ± 0.02	1.8 ± 0.16	0.2 ± 0.05
Lower stem	0.4 ± 0.1	0.06 ± 0.02	0.3 ± 0.04	0.06 ± 0.02
Root	0.03 ± 0.01	nd	0.02 ± 0.01	nd

3.2. Metabolism of purine nucleotide

3.2.1. Metabolism of [8-^{14}C]adenine by young, mature and aged leaves of Iranian tea

The metabolism of [8-^{14}C]adenine in the first, second, third, mature and aged leaves of Iranian tea was investigated in a time course study. At the end of incubation period, radioactivity in the MSE and NA fraction was measured together with the amount of $^{14}CO_2$ released. The methanolic extract was analysed by TLC and the position of radiolabelled bands determined by autoradiography (see **Figure 3.2.1**). Radiolabelled zones detected by autoradiography were scrapped off the TLC plate, the microcrystalline cellulose eluted with water and the level of radioactivity measured by liquid scintillation counting.

A summary of the data obtained with 6, 12, 24 and 48 h incubation periods are presented in **Tables 3.2.1 - 3.2.5**. There was higher uptake of radioactivity by the younger leaves than the mature and aged leaves. In the first, second and third leaves, [^{14}C]adenine was taken up rapidly and within 6 h was converted to adenine nucleotides and nucleic acids. The proportion of radioactivity in these pools, and that associated with [^{14}C]adenine, declined with further incubation. In 6 h incubations with the first leaf, the level of incorporation of label into theobromine (8.6 ± 0.8%) was similar to that associated with caffeine (7.6 ± 0.8%), but there after the proportion of labelled theobromine declined while incorporation of ^{14}C into caffeine increased and after 24 h and 48 h represented 48.7 ± 0.8% and 34.6 ± 3.0% respectively of the recovered radioactivity. Only trace levels of radioactivity were associated with theophylline, which is in keeping with the fact that endogenous caffeine levels in young tea leaves are high

as a consequence of the 1-*N*-demethylation step, responsible for the catabolism of caffeine to theophylline, being blocked (Ashihara et al. 1997). Although relatively little label was associated with the ureides, allantoin and allantoic acid, increasing amounts of label entered the purine catabolism pathway as indicated by the extent of $^{14}CO_2$ release. Similar metabolic profiles were obtained when [8-^{14}C]adenine was incubated with the first, second and third leaves of Iranian tea seedlings.

In mature and aged leaves, much higher levels of [^{14}C]adenine were present after 6 h than with the younger leaves and, in marked contrast to the younger tissues, there was no detectable incoporation of label into adenine nucleotides (**Tables 3.2.4** and **3.2.5**). Particularly in the aged leaves, there was also a greater incorporation of radioactivity into the nucleic acid fraction. After 48 h this represented 71.1 ± 0.1% of the recovered radioactivity compared with 20.9 ± 1.6% in incubations with the first leaf. The level of radioactivity associated with purine alkaloids was also reduced in incubations with mature and aged leaves, most dramatically so in the case of caffeine. The amount of $^{14}CO_2$ released was much lower in aged leaves indicating that less of the substrate was entering the purine catabolism pathway, something that is also implied by an absence of incorporation of label into allantoin and allantoic acid.

The data obtained in these initial metabolism experiments demonstrate that adenine is an effective substrate for caffeine biosynthesis and there are large differences in the metabolism of adenine by young, mature and aged leaves of Iranian tea. The young, first, second and third leaves are much more active sites of caffeine biosynthesis than mature and aged leaves.

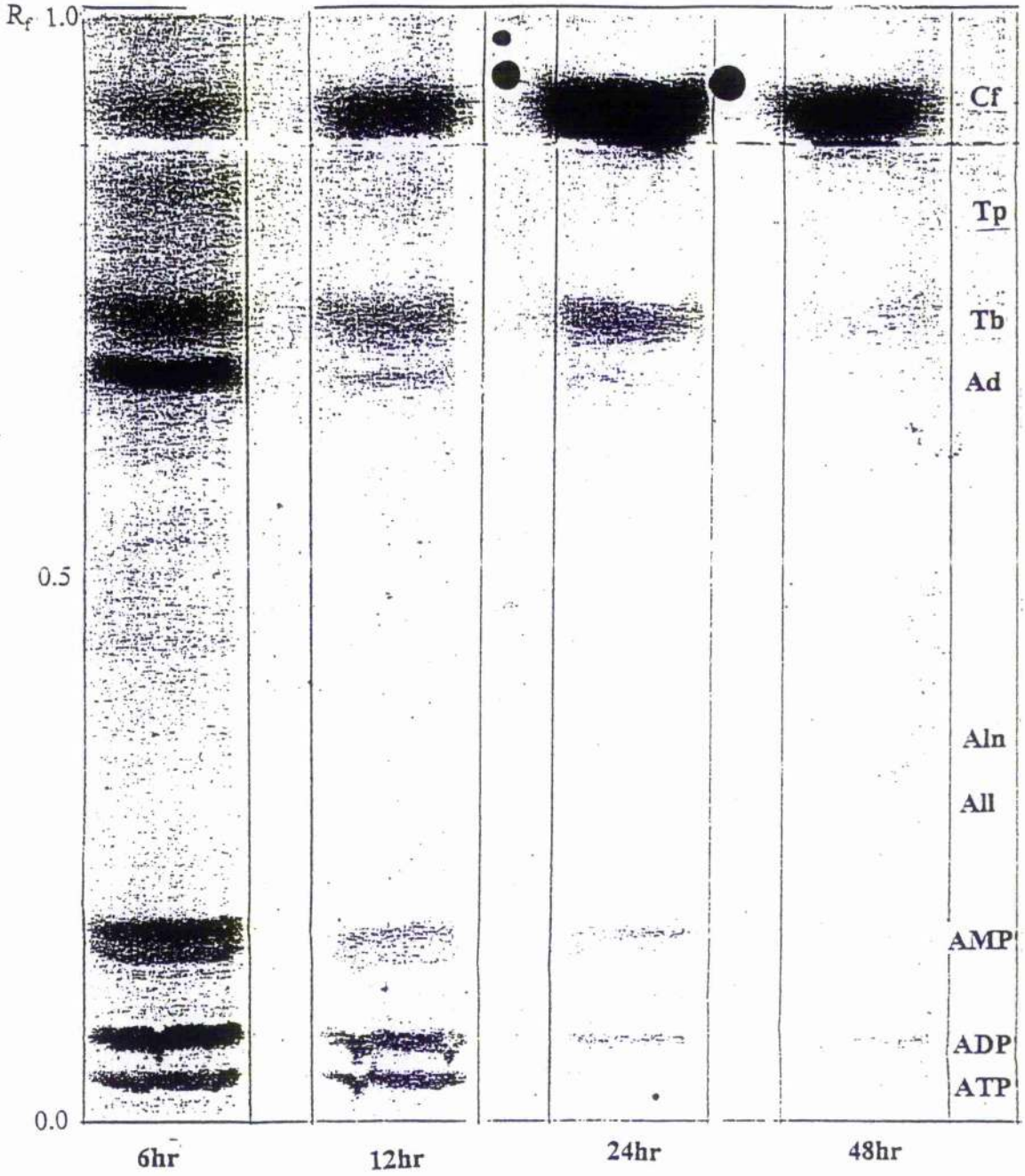


Fig. 3.2.1. An autoradiogram of the methanol-soluble fraction. Segments adminstered [8-^{14}C] adenine were extracted with MeOH and fractionated by TLC in n-butanol-acetic acid- water (4:1:2; v/v). The figure was obtained from the experiments using 1st leaf of Iranian tea. Cf-caffeine, Tp- theophylline, Tb-theobromine, Ad-adenine, Aln-allantoic acid, All-allantoin, AMP-adenosine-5′-monophosphate, ADP-adenosine-5′-diphosphate and ATP-adenosine-5′-triphosphate.

Table. 3.2.1. Summary of the metabolism of [8-^{14}C]adenine (0.5 μCi) by 100 mg of the first leaf of Iranian tea seedlings after incubation for 6, 12, 24 and 48 h in a natural photoperiod.

Time→ Metabolites↓	**6h**	**12h**	**24h**	**48h**
ATP+ADP	17.8 ± 1.2	11.8 ± 2.6	3.3 ± 0.6	2.6 ± 0.6
AMP	12.2 ± 1.6	5.8 ± 0.9	2.3 ± 0.1	1.0 ± 0.6
All+Aln	1.0 ± 0.1	1.9 ±0.1	1.6 ± 0.7	1.1 ± 0.6
Ad	13.6 ± 0.7	4.0 ± 1.1	1.4 ± 0.2	1.0 ± 0.3
Tb	8.6 ± 0.8	7.6 ± 0.8	5.2 ± 0.1	2.9 ± 0.1
Tp	1.9 ± 0.3	1.5 ± 0.8	1.2 ± 0.6	0.2 ± 0.2
Cf	7.7 ± 0.6	22.3 ± 0.4	48.7 ± 0.8	34.6 ± 3.0
NA	36.8 ± 2.1	40.8 ± 0.4	20.3 ± 1.3	20.9 ± 1.6
$^{14}CO_2$	0.7 ± 0.0	4.3 ± 0.4	16.2 ± 0.7	35.9 ± 0.8
Total radioactivity recovered	297 ± 1.9	231 ± 21	313 ± 13	201 ± 3

Levels of residual [8-^{14}C]adenine (Ad) and radiolabelled metabolites, adenosine-5′-triphosphate (ATP), adenosine-5′-diphosphate (ADP), adenosine-5′-monophosphate (AMP), allantoin (All), allantoic acid (Aln), theobromine (Tb), theophylline (Tp), caffeine (Cf), nucleic acid (NA) fraction and $^{14}CO_2$ expressed as % of the total radioactivity recovered ± standard error (n = 2). Total radioactivity recovered expressed as k dpm ± standard error (n = 2).

Table. 3.2.2. Summary of the metabolism of [8-^{14}C]adenine (0.5 μCi) by 100 mg of the second leaf of Iranian tea seedlings after incubation for 6, 12, 24 and 48 h in a natural photoperiod.

Time→ Metabolites↓	6h	12h	24h	48h
ATP+ADP	15.5 ± 0.7	8.3 ± 1.4	3.3 ± 0.5	1.6 ± 0.1
AMP	20.1 ± 0.8	4.0 ± 0.5	2.0 ± 0.4	1.0 ± 0.3
All+Aln	1.2 ± 0.2	0.9 ± 0.0	1.2 ± 0.2	1.3 ± 0.2
Ad	9.3 ± 0.4	2.4 ± 0.3	1.3 ± 0.2	0.5 ± 0.0
Tb	8.9 ± 0.6	9.2 ± 0.1	4.7 ± 0.7	2.2 ± 0.2
Tp	0.6 ± 0.2	0.5 ± 0.2	0.4 ± 0.1	0.3 ± 0.1
Cf	8.0 ± 0.4	27.2 ± 0.1	38.5 ± 1.1	37.0 ± 0.6
NA	35.3 ± 1.3	37.9 ± 2.3	34.3 ± 0.1	24.4 ± 0.9
$^{14}CO_2$	1.3 ± 0.1	9.7 ± 0.1	14.6 ± 0.3	29.8 ± 0.3
Total radioactivity recovered	361 ± 16	514 ± 18	502 ± 0.1	470 ± 5

Levels of residual [8-^{14}C]adenine (Ad) and radiolabelled metabolites, adenosine-5′-triphosphate (ATP), adenosine-5′-diphosphate (ADP), adenosine-5′-monophosphate (AMP), allantoin (All), allantoic acid (Aln), theobromine (Tb), theophylline (Tp), caffeine (Cf), nucleic acid (NA) fraction and $^{14}CO_2$ expressed as % of the total radioactivity recovered ± standard error (n = 2). Total radioactivity recovered expressed as k dpm ± standard error (n = 2).

Table. 3.2.3. Summary of the metabolism of [8-^{14}C]adenine (0.5 μCi) by 100 mg of the third leaf of Iranian tea seedlings after incubation for 6, 12, 24 and 48 h in a natural photoperiod.

Time→ Metabolites↓	6h	12h	24h	48h
ATP+ADP	14.2 ± 1.0	6.6 ± 0.8	2.1 ± 0.0	1.2 ± 0.2
AMP	19.4 ± 0.2	4.4 ± 0.9	2.6 ± 0.4	0.9 ± 0.0
All+Aln	1.2 ± 0.1	1.2 ± 0.2	1.1 ± 0.3	1.3 ± 0.3
Ad	6.6 ± 0.2	1.7 ± 0.1	1.1 ± 0.2	0.6 ± 0.1
Tb	11.4 ± 0.1	14.3 ± 1	9.2 ± 1.1	3.5 ± 0.2
Tp	2.4 ± 0.7	2.4 ± 1.0	0.7 ± 0.1	0.3 ± 0.0
Cf	3.7 ± 0.3	17.7 ± 3.2	34.3 ± 0.0	30.6 ± 2.4
NA	39.8 ± 0.2	34.6 ± 1.0	31.3 ± 0.7	26.5 ± 0.5
$^{14}CO_2$	1.5 ± 0.2	17.3 ± 1.8	17.6 ± 0.7	35.3 ± 1.3
Total radioactivity recovered	408 ± 12	615 ± 21	670 ± 26	666 ± 23

Levels of residual [8-^{14}C]adenine (Ad) and radiolabelled metabolites, adenosine-5′-triphosphate (ATP), adenosine-5′-diphosphate (ADP), adenosine-5′-monophosphate (AMP), allantoin (All), allantoic acid (Aln), theobromine (Tb), theophylline (Tp), caffeine (Cf), nucleic acid (NA) fraction and $^{14}CO_2$ expressed as % of the total radioactivity recovered ± standard error (n = 2). Total radioactivity recovered expressed as k dpm ± standard error (n = 2).

Table. 3.2.4. Summary of the metabolism of [8-^{14}C]adenine (0.5 μCi) by 100 mg of the mature leaf of Iranian tea seedlings after incubation for 6, 12, 24 and 48 h in a natural photoperiod.

Time→ Metabolites↓	**6h**	**12h**	**24h**	**48h**
Ad	85.4 ± 2.8	80.5 ± 0.5	40.6 ± 1.7	16.8 ± 2.9
Tb	0.5 ± 0.3	4.1 ± 3.1	3.6 ± 0.9	4.8 ± 1.1
Tp	0.2 ± 0.2	0.2 ± 0.1	1.7 ± 0.3	0.8 ± 0.8
Cf	3.1 ± 2.8	0.3 ± 0.1	1.3 ± 0.2	2.4 ± 1.0
NA	9.8 ± 0.3	16.0 ± 0.5	43.7 ± 1.9	36.7 ± 2.3
$^{14}CO_2$	1.4 ± 0.1	2.3 ± 0.1	7.0 ± 0.5	38.5 ± 2.3
Total radioactivity recovered	112 ± 2	138.5 ± 2.5	66.4 ± 4.1	95.5 ± 2.8

Levels of residual [8-^{14}C]adenine (Ad) and radiolabelled metabolites, theobromine (Tb), theophylline (Tp), caffeine (Cf), nucleic acid (NA) fraction and $^{14}CO_2$ expressed as % of the total radioactivity recovered ± standard error (n = 2). Total radioactivity recovered expressed as k dpm ± standard error (n = 2).

Table. 3.2.5. Summary of the metabolism of [8-^{14}C]adenine (0.5 μCi) by 100 mg of the aged leaf of Iranian tea seedlings after incubation for 6, 12, 24 and 48 h in a natural photoperiod.

Time→ Metabolites↓	**6h**	**12h**	**24h**	**48h**
Ad	84.2 ± 1.2	48.0 ± 0.4	13.4 ± 0.2	8.0 ± 1.1
Tb	1.1 ± 0.1	1.2 ± 0.6	2.0 ± 0.1	0.6 ± 0.5
Tp	0.6 ± 0.1	0.3 ± 0.1	0.8 ± 0.7	0.2 ± 0.1
Cf	1.1 ± 0.5	0.8 ± 0.6	1.7 ± 0.3	2.0 ± 0.5
NA	11.3 ± 0.5	47.9 ± 0.4	74.5 ± 0.5	71.1 ± 0.1
$^{14}CO_2$	1.8 ± 0.2	1.8 ± 0.1	7.6 ± 0.5	18.2 ± 0.1
Total radioactivity recovered	110.6 ± 4	182.2 ± 0.7	142.5 ± 8.3	213 ± 2

Levels of residual [8-^{14}C]adenine (Ad) and radiolabelled metabolites, theobromine (Tb), theophylline (Tp), caffeine (Cf), nucleic acid (NA) fraction and $^{14}CO_2$ expressed as % of the total radioactivity recovered ± standard error (n = 2). Total radioactivity recovered expressed as k dpm ± standard error (n = 2).

3.2.2. Metabolism of [8-^{14}C]adenine by young Iranian tea leaves in light and darkness

Sections of the first and second leaves of Iranian tea, weighing ca. 100 mg, were incubated with 0.5 μCi [8-^{14}C]adenine in light and darkness for the 12, 24 and 48 h after which the leaves were extracted and processed as summarised in the previous section. The data obtained are presented in **Tables 3.2.6** and **3.2.7** and **Figure 3.2.2** and **3.2.3**. In light there was a much greater release of $^{14}CO_2$ than in darkness and a lower level of incorporation of label into nucleic acids than in darkness. In darkness, however, more label was associated with adenine nucleotides and also with the purine alkaloids. After 12 h in darkness, 29.6 ± 1.4% of the recovered radioactivity from the first leaf was associated with theobromine compared to 6.6% in parallel incubations in light. A greater incorporation of label into theobromine in the dark was also observed in incubations with the second leaf of Iranian tea seedlings. There was also much more extensive accumulation of radioactivity associated with caffeine in incubations with first and second leaves in darkness (**Tables 3.2.6** and **3.2.7** and **Figure 3.2.2** and **3.2.3**).

The lower incorporation of label from [8-^{14}C]adenine into caffeine in light, and the reported accumulation of lower levels of endogenous caffeine in tea callus incubated in light compared to darkness (Ogutuga and Northcote, 1970a) are both suggestive of a reduced rate of caffeine biosynthesis and/or an enhanced rate of caffeine catabolism in light. It is also feasible that in light, adenine is directed preferentially to alternative pathways. The possibility is supported by the observation that the reduced production of [^{14}C]caffeine from [8-^{14}C]adenine in light compared to darkness was accompanied elevated $^{14}CO_2$ output and an increased incorporation of label into adenine nucleotides (**Fig 3.2.2** and **3.2.3**). In an earlier study, Suzuki (1972) demonstrated that light did not

influence the incorporation of radiolabel from [methyl-^{14}C]methionine into caffeine in tea leaves. This indicates that light does not effect directly the three methylation steps in the caffeine biosynthesis pathway.

3.2.3. HPLC analysis of radiolabelled metabolites

In selected instances, the methanolic extract obtained from the leaves with [8-^{14}C]adenine was analysed by HPLC-RC. Although purine nucleotides and nucleic acids did not elute from the column and the ureides were usually present in quantities below the limits of detection, this facilitated the confirmation of the TLC-based identification and quantification of caffeine and theobromine. A extract typical HPLC-RC trace obtained by analysing a 30k dpm aliquot of a methanolic from the first leaves of Iranian tea following incubation with [8-^{14}C]adenine for 48 h in darkness is illustrated in **Figure 3.2.4**. The data obtained with HPLC-RC confirmed the results of the TLC analyses and in the case of 48 h incubations which were examined in detail, confirmed that the first, second and third leaves had a much higher capacity of caffeine biosynthesis than mature and aged leaves of Iranian tea (**Figure 3.2.5**).

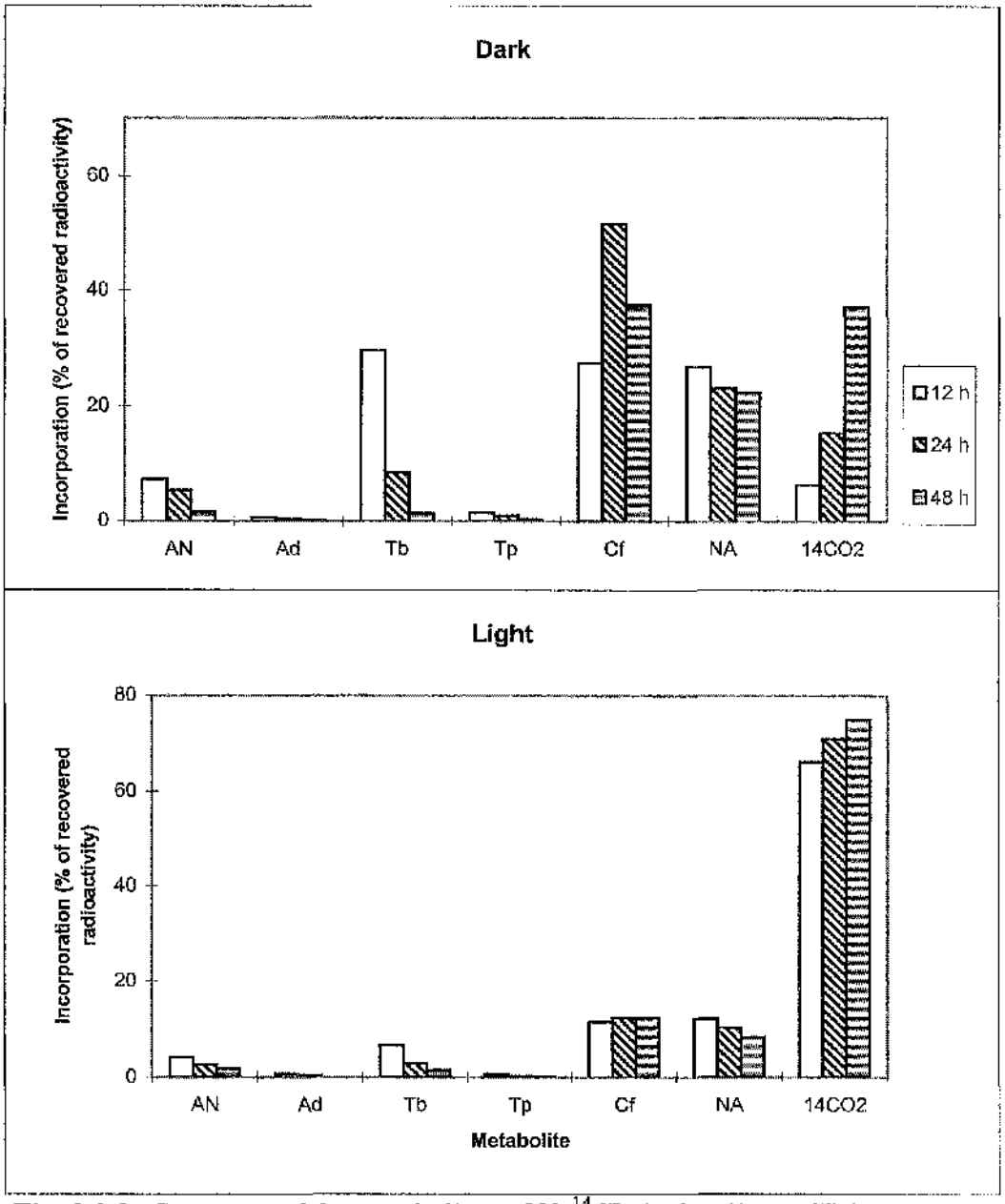


Fig. 3.2.2. Summary of the metabolism of [8-^{14}C]adenine (0.5 μ Ci) by 100 mg of the first leaf of Iranian tea seedlings after incubation in light and darkness for 12, 24 and 48 h. Incorporation expressed as a percentage of recovered radioactivity. AN- adenosine nucleotides, Ad- adenine, Tb- theobromine, Tp- theophylline, Cf-caffeine and NA- nucleic acids.

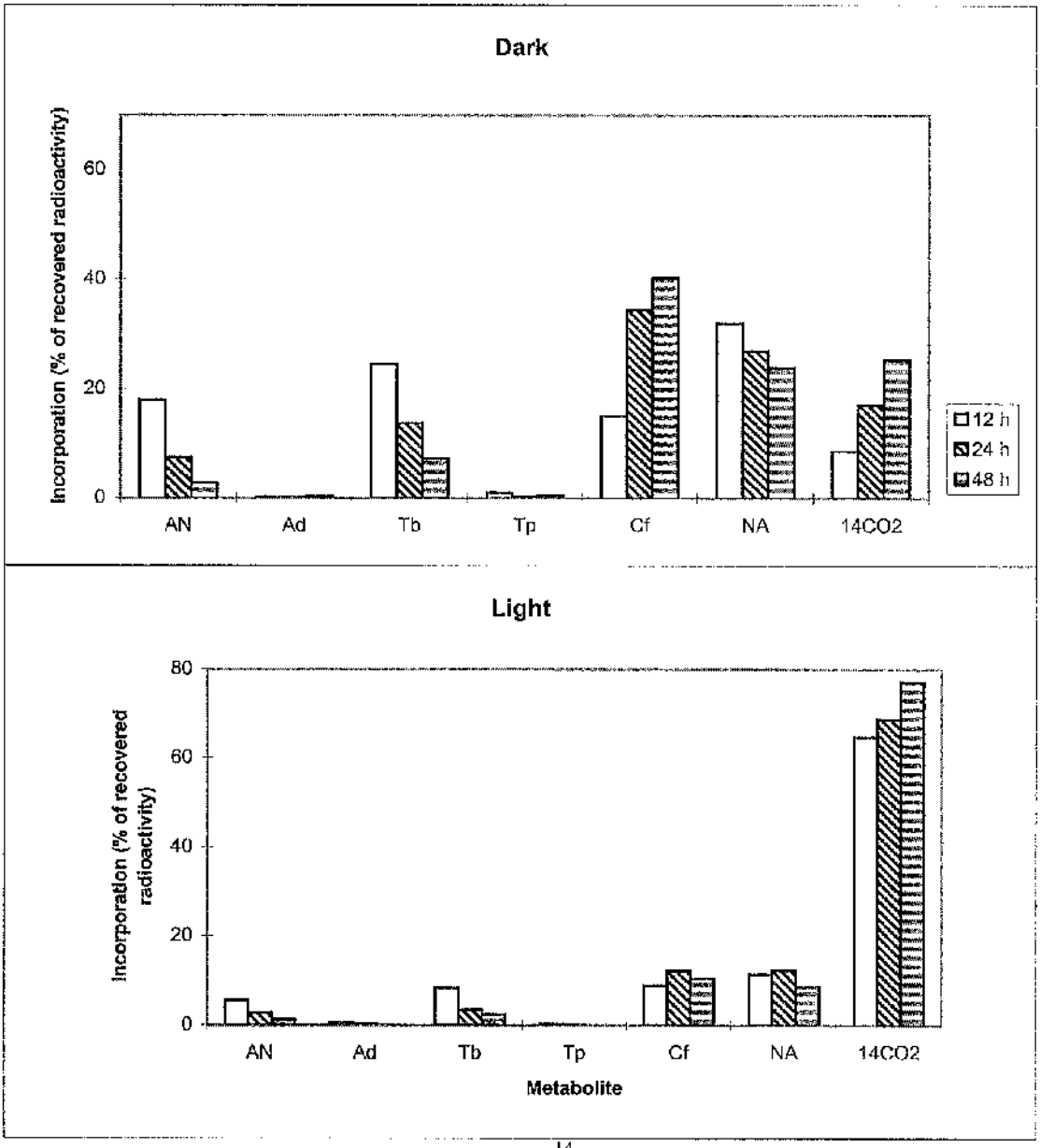


Fig. 3.2.3. Summary of the metabolism of [8-^{14}C]adenine (0.5 μ Ci) by 100 mg of the second leaf of Iranian tea seedlings after incubation in light and darkness for 12, 24 and 48 h. Incorporation expressed as a percentage of recovered radioactivity. AN- adenosine nucleotides, Ad- adenine, Tb- theobromine, Tp- theophylline, Cf-caffeine and NA- nucleic acids.

Table. 3.2.6. Summary of the metabolism of [8-^{14}C]adenine (0.5 μCi) by 100 mg of the first leaf of Iranian tea seedlings after incubation for 12, 24 and 48 h in light and dark.

	Dark			Light		
Metabolites	12 h	24 h	48 h	12 h	24 h	48 h
ATP+ADP	6.9 ± 0.5	5.1± 0.2	1.5 ±0.6	3.4 ±0.8	2.4 ±0.2	1.3 ±0.2
AMP	0.4 ±0.2	0.2 ±0.1	0.1 ±0.1	0.7 ±0.1	0.2 ±0.0	0.6±0.2
Al+ Aln	1.0 ±0.0	0.3 ±0.2	0.2 ±0.1	0.5 ±0.2	0.4 ±0.0	0.6 ±0.2
Ad	0.6 ±0.1	0.3 ±0.2	0.1 ±0.1	0.7 ±0.2	0.4 ±0.1	nd
Tb	29.6 ±1.4	8.4 ±2.3	1.3 ±0.1	6.6 ±0.5	2.8 ±0.1	1.4 ±0.1
Tp	1.4 ±0.9	0.9 ±0.2	0.3 ±0.0	0.6 ±0.2	0.2 ±0.0	0.1 ±0.0
Cf	27.4 ±1.7	51.6 ±3.5	37.5 ±0.7	11.5 ±1.1	12.3 ±1.1	12.3 ±1.2
NA	26.7 ±0.2	23.0 ±0.4	22.3 ±0.3	12.7 ±0.6	10.4 ±0.1	8.4 ±1.1
$^{14}CO_2$	6.2 ±0.5	15.2 ±0.4	37.1 ±0.9	66.2 ±2.6	71.0 ±1.3	75.1 ±0.6
Total radioactivity recovered	597 ± 29	583 ± 28	620 ± 4	379 ± 35	440 ± 23	526 ± 24

Levels of residual [8-^{14}C]adenine (Ad) and radiolabelled metabolites, adenosine-5′-triphosphate (ATP), adenosine-5′-diphosphate (ADP), adenosine-5′-monophosphate (AMP), allantoin (All), allantoic acid (Aln), theobromine (Tb), theophylline (Tp), caffeine (Cf), nucleic acid (NA) fraction and $^{14}CO_2$ expressed as % of the total radioactivity recovered ± standard error (n = 2). Total radioactivity recovered expressed as k dpm ± standard error (n = 2). nd - not detected.

Table. 3.2.7. Summary of the metabolism of [8-^{14}C]adenine (0.5 μCi) by 100 mg of the second leaf of Iranian tea seedlings after incubation for 12, 24 and 48 h in light and dark.

Metabolites	Dark			Light		
	12 h	24 h	48 h	12 h	24 h	48 h
ATP+AMP	17.1 ±1.4	7.1 ±1.5	2.5 ±0.0	4.8 ±0.8	2.0 ±0.5	1.1 ±0.1
AMP	0.8 ±0.2	0.3 ±0.2	0.2 ±0.1	0.6 ±0.2	0.7 ±0.3	0.2 ±0.2
Al+ Aln	1.0 ±0.1	0.5 ±0.3	0.2 ±0.0	1.2 ±0.2	0.5 ±0.0	0.2 ±0.2
Ad	0.2 ±0.1	0.1 ±0.0	0.3 ±0.2	0.5 ±0.3	0.4 ±0.3	0.2 ±0.2
Tb	24.5 ±1.0	13.7 ±1.9	7.2 ±0.9	8.3 ±0.0	3.4 ±0.2	2.4 ±0.3
Tp	1.0 ±0.4	0.3±0.3	0.5 ±0.2	0.3 ±0.2	0.2 ±0.2	nd
Cf	15.0 ±0.3	34.3 ±2.1	40.2 ±1.9	8.9 ±0.1	12.2 ±1.1	10.4 ±1.7
NA	31.9 ±0.9	26.9 ±0.5	23.8 ±2.1	11.3 ±0.7	12.3 ±0.2	8.5 ±0.5
$^{14}CO_2$	8.6 ±0.4	17 ±0.5	25.3 ±2.0	64.7 ±0.6	68.6 ±0.2	77.2 ±1.8
Total radioactivity recovered	563 ± 0.7	564 ± 25	566 ± 36	430 ± 2	457 ± 2	559 ± 13

Levels of residual [8-^{14}C]adenine (Ad) and radiolabelled metabolites, adenosine-5′-triphosphate (ATP), adenosine-5′-diphosphate (ADP), adenosine-5′-monophosphate (AMP), allantoin (All), allantoic acid (Aln), theobromine (Tb), theophylline (Tp), caffeine (Cf), nucleic acid (NA) fraction and $^{14}CO_2$ expressed as % of the total radioactivity recovered ± standard error (n = 2). Total radioactivity recovered expressed as Kk dpm ± standard error (n = 2). nd - not detected.

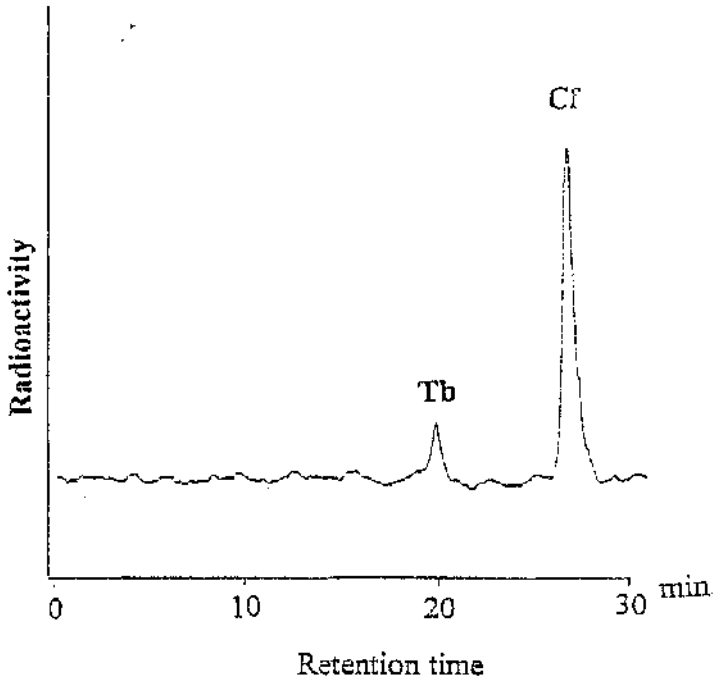


Fig. 3.2.4. Gradient reversed-phase HPLC-RC analysis of [8-^{14}C]adenine metabolites from first leaf of Iranian tea seedlings. Column: 250 × 4.6 mm i.d. 5μm ODS Hypersil. Gradient: 25min, 0-40% methanol in 50mM sodium acetate (pH 5.0). Flow rate: 1 ml min^{-1}. Detector: radioactivity monitor operating in heterogenous mode. Sample: 30 K dpm aliquot of a methanolic extract of 150 mg of first leaves of Iranian tea seedlings following a 48 h incubation with 0.25 μCi [8-^{14}C]adenine. Tb-theobromine, Cf-caffeine.

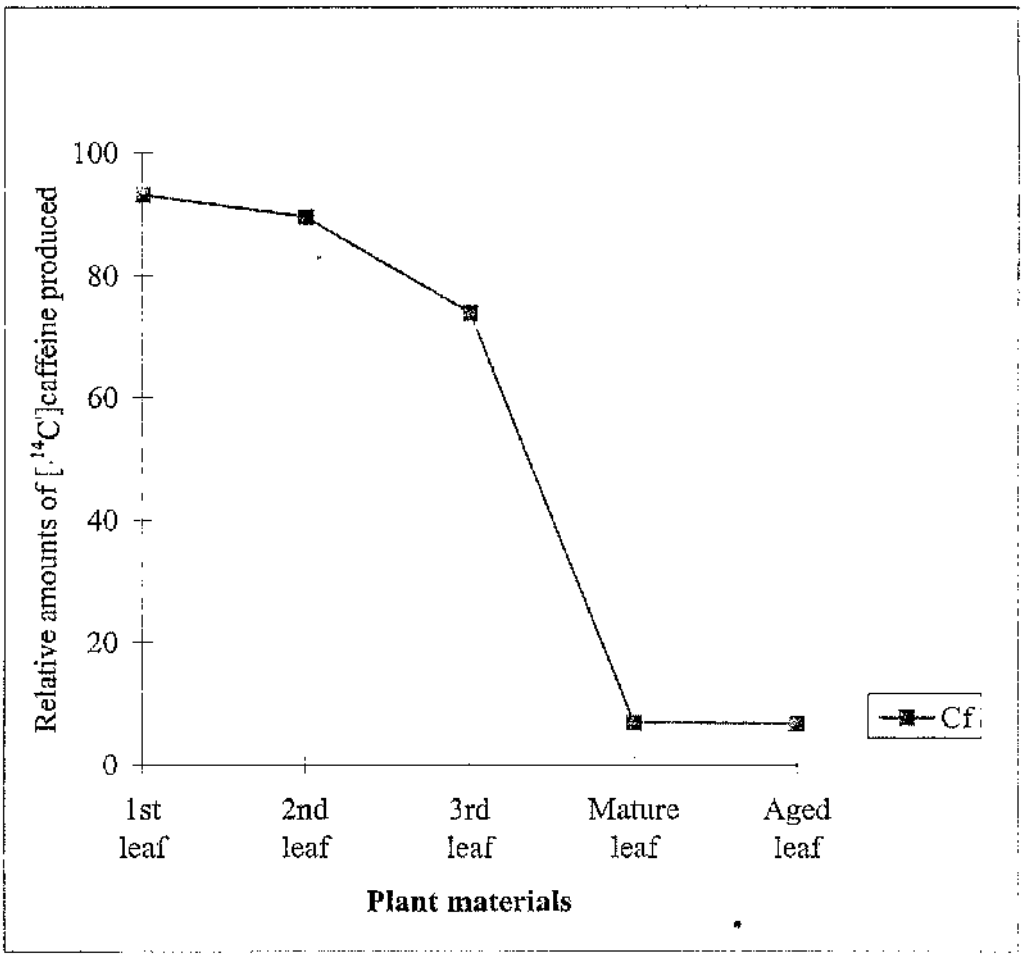


Fig. 3.2.5. Incorporation of [8-^{14}C]adenine into caffeine by first, second, mature and aged leaves of Darjeeling tea seedlings. 150mg of leaves incubated with 0.25μCi [8-^{14}C]adenine for 48 h in natural condition at 25 °C. Leaves extracted with methanol and aliquots analysed bby HPLC-RC. Incorporation in label into [^{14}C]caffeine expressed as a percentage of recovered radioactivity ± standard error (n = 3).

3.3. Pulse-chase experiment

To obtain further information on caffeine biosynthesis in young tea leaves, pulse-chase experiments were carried out in which leaves were incubated in a natural photoperiod at with either [8-^{14}C]adenine or [8-^{14}C]guanine. The incubation medium was replaced with fresh medium devoid of radiolabelled substrate after an initial 4 h pulse after which the leaves were incubated for a further 44 h. Samples were collected for analysis after 4, 8, 12, 24 and 48 h.

3.3.1. Metabolism of [8-^{14}C]adenine

The data obtained in pulse-chase experiments with [8-^{14}C]adenine and first leaves of Darjeeling tea seedlings are presented in **Table 3.3.1** and **Figures 3.3.1**. At the end of the 4 h pulse the leaves contained relatively little [^{14}C]adenine and most of the radioactivity was associated with theobromine, adenine nucleotides and nucleic acids. During the course of the subsequent chase, the level of radioactivity incorporated into the adenine nucleotides fell substantially while ^{14}C in the nucleic acid pool declined only slightly. The gradual decline in the size of the [^{14}C]theobromine pool during the chase was accompanied by increased incorporation of label in to caffeine, which after 48 h was the major radiolabelled product. Although relatively little $^{14}CO_2$ evolved during the 4 h pulse, substantially quantities, equivalent to 26.4 ± 0.2% of the recovered radioactivity, were produced by the end of the 44 h chase. In contrast to $^{14}CO_2$, the amount of radioactivity incorporated into the ureides, allantoin and allantoic acid, which are intermediates in the purine catabolism pathway, was minimal. Incorporation of label into the caffeine degradation product, theophylline, was also very low. TLC analysis of the hydrolysed NA fraction, in which purine residues of RNA and DNA have been

converted to purine bases, revealed incorporation of [8-^{14}C]adenine into not only adenine residues of nucleic acids but also guanine residues (**Fig. 3.3.2**). This indicates conversion of [^{14}C]adenine → AMP → IMP → XMP → GMP → GDP → GTP as part illustrated in **Figure 1.3**.

Identical pulse-chase experiments were carried out in with [8-^{14}C]adenine was incubated with second leaves of Darjeeling tea seedlings. The data produced, which are very similar to those obtained with the first leaves, are presented in **Table 3.3.2** and **Figure 3.3.3**.

3.3.2. Metabolism of [8-^{14}C]guanine

Pulse-chase experiments were carried out in which the first leaves of Darjeeling tea were incubated with [8-^{14}C]guanine. The data obtained are presented in Table 3.3.3 and Figure 3.3.4. Although the amount of [^{14}C]caffeine produced appears slightly less than in the pulse-chase experiments with [8-^{14}C]adenine, the overall kinetics are very similar (**Table 3.3.1** and **Figure 3.3.1**). At the end of the chase, proportionally much more [^{14}C]guanine was present in the leaves than [^{14}C]adenine in the earlier experiment, also $^{14}CO_2$ production was higher than in the initial stages of the pulse-chase feeds with [8-^{14}C]adenine. During the course of the experiment, there appeared to be less label associated with guanine nucleotides than the equivalent adenine nucleotides in feeds with [8-^{14}C]adenine. Although initially lower, the nucleic acid fraction was labelled extensively in the pulse-chase experiments with [8-^{14}C]guanine than when [8-^{14C}]adenine was used as a substrate. This may reflect the fact that the cellular guanine nucleotide pool is always smaller than the adenine nucleotide pool (Suzuki et al. 1992).

Table. 3.3.1. Summary of the metabolism of [8-^{14}C]adenine in pulse-chase experiment in which 100 mg of first leaves of Darjeeling tea seedlings were incubated with 1 μCi [8-^{14}C]adenine for 4 h after which the leaves were incubated without radiolabelled substrate for a further 44 h. Samples taken for analysis 4, 8, 12, 24 and 48 h after the beginning of the incubation with [8-^{14}C]adenine.

Time→ Metabolites ↓	**4 h**	**8 h**	**12 h**	**24 h**	**48 h**
ATP+ADP	23.5 ± 4.5	11.8 ± 1.3	3.9 ± 0.6	3.3 ± 1.9	2.3 ± 1.3
AMP	10.5 ± 1.3	4.6 ± 2.0	1.4 ± 0.8	0.5 ± 0.1	0.3 ± 0.0
Al+Aln	1.2 ± 0.6	0.9 ± 0.6	0.9 ± 0.4	0.3 ± 0.1	0.1 ± 0.1
Ad	2.7 ± 1.2	1.4 ± 0.8	0.4 ± 0.2	0.1 ± 0.0	nd
Tb	20.9 ± 4.0	22.3 ± 0.8	13.0 ± 0.5	2.9 ± 0.2	1.0 ± 0.2
Tp	1.7 ± 0.5	1.0 ± 0.1	1.3 ± 0.7	0.4 ± 0.1	0.1 ± 0.0
Cf	7.0 ± 0.8	31.6 ± 0.2	49.1 ± 0.2	58.2 ± 2.6	44.6 ± 2.0
NA	35.0 ± 0.8	25.6 ± 0.3	28.9 ± 0.3	25.7 ± 0.2	25.3 ± 0.8
$^{14}CO_2$	0.7 ± 0.1	1.2 ± 0.0	1.3 ± 0.1	8.3 ± 0.5	26.4 ± 0.2
Total radioactivity recovered	358 ± 16	313 ± 11	346 ± 38	256 ± 1	327 ± 19

Levels of residual [8-^{14}C]adenine (Ad) and radiolabelled metabolites, adenosine-5′-triphosphate (ATP), adenosine-5′-diphosphate (ADP), adenosine-5′-monophosphate (AMP), allantoin (All), allantoic acid (Aln), theobromine (Tb), theophylline (Tp), caffeine (Cf), nucleic acid (NA) fraction and $^{14}CO_2$ expressed as % of the total radioactivity recovered ± standard error (n = 2). Total radioactivity recovered expressed as k dpm ± standard error (n = 2). nd, not detected.

Table. 3.3.2. Summary of the metabolism of [8-^{14}C]adenine in pulse-chase experiment in which 100 mg of second leaves of Darjeeling tea seedlings were incubated with 1 μCi [8-^{14}C]adenine for 4 h after which the leaves were incubated without radiolabelled substrate for a further 44 h. Samples taken for analysis 4, 8, 12, 24 and 48 h after the beginning of the incubation with [8-^{14}C]adenine.

Time → Metabolites↓	**4 h**	**8 h**	**12 h**	**24 h**	**48 h**
ATP+ADP	17.8 ± 1.5	10.3 ± 1.2	9.3 ± 1.4	5.4 ± 2.2	2.2 ± 0.1
AMP	12.9 ± 3.7	7.4 ± 1.3	4.2 ± 0.4	1.9 ± 0.3	0.8 ± 0.3
Al+Aln	1.8 ± 0.0	1.9 ± 1.0	1.1 ± 0.4	0.5 ± 0.1	0.4 ± 0.1
Ad	2.4 ± 0.6	1.0 ± 0.2	0.6 ± 0.1	0.1 ± 0.0	0.2 ± 0.0
Th	18.6 ± 2.0	23.9 ± 1.3	17.7 ± 0.2	5.5 ± 0.6	0.8 ± 0.2
Tp	2.8 ± 1.4	1.6 ± 0.4	1.0 ± 0.4	0.2 ± 0.1	0.2 ± 0.1
Cf	5.9 ± 0.0	19.6 ± 2.0	32.7 ± 1.1	49.7 ± 2.3	37.7 ± 3.2
NA	36.9 ± 1.4	33.4 ± 2.2	31.2 ± 1.8	27.1 ± 0.1	24.7 ± 0.4
$^{14}CO_2$	1.5 ± 0.3	2.1 ± 0.2	2.6 ± 0.1	9.8 ± 0.2	33.3 ± 2.9
Total radioactivity recovered	257 ± 13	239 ± 21	235 ± 16	259 ± 2	355 ± 23

Levels of residual [8-^{14}C]adenine (Ad) and radiolabelled metabolites, adenosine-5′-triphosphate (ATP), adenosine-5′-diphosphate (ADP), adenosine-5′-monophosphate (AMP), allantoin (All), allantoic acid (Aln), theobromine (Tb), theophylline (Tp), caffeine (Cf), nucleic acid (NA) fraction and $^{14}CO_2$ expressed as % of the total radioactivity recovered ± standard error (n = 2). Total radioactivity recovered expressed as k dpm ± standard error (n = 2).

Table.3.3.3. Summary of the metabolism of [8-^{14}C]guanine hydrochloride in pulse-chase experiment in which 100 mg of first leaves of Darjeeling tea seedlings were incubated with 1 μCi [8-^{14}C]guanine hydrochloride for 4 h after which the leaves were incubated without radiolabelled substrate for a further 44 h. Samples taken for analysis 4, 8, 12, 24 and 48 h after the beginning of the incubation with [8-^{14}C]guanine hydrochloride.

Time→ Metabolites↓	**4 h**	**8 h**	**12 h**	**24 h**	**48 h**
GTP+GDP	4.7 ± 1.0	1.8 ± 0.4	1.0 ± 0.1	1.1 ± 0.1	0.6 ± 0.2
GMP	1.4 ± 0.4	0.7 ± 0.3	0.7 ± 0.0	0.4 ± 0.1	0.4 ± 0.1
All+Aln	1.8 ± 0.1	1.3 ± 0.2	1.1 ± 0.1	0.6 ± 0.1	0.4 ± 0.1
Gu	61.3 ± 2.9	26.6 ± 0.8	20.7 ± 2.3	11.5 ± 0.6	4.7 ± 0.3
Tb	5.1 ± 1.3	13.8 ± 0.2	10.2 ± 0.3	4.6 ± 0.4	1.4 ± 0.2
Tp	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.2	0.1 ± 0.1	0.1 ± 0.0
Cf	1.7 ± 0.3	11.3 ± 3.7	21.1 ± 2.2	35.1 ± 2.2	34.8 ± 0.8
NA	19.1 ± 0.1	36.0 ± 3.0	35.7 ± 0.5	36.7 ± 0.8	35.3 ± 0.4
$^{14}CO_2$	5.2 ± 0.1	8.5 ± 0.7	9.3 ± 2.0	9.7 ± 0.5	22.5 ± 1.7
Total radioactivity recovered	344 ± 4	304 ± 19	334 ± 1	324 ± 4	272 ± 2

Levels of residual [8-^{14}C]guanine (Gu) and radiolabelled metabolites, guanosine-5′-triphosphate (GTP), guanosine-5′-diphosphate (GDP), guanosine-5′-monophosphate (GMP), allantoin (All), allantoic acid (Aln), theobromine (Tb), theophylline (Tp), caffeine (Cf), nucleic acid (NA) fraction and $^{14}CO_2$ expressed as % of the total radioactivity recovered ± standard error (n = 2). Total radioactivity recovered expressed as k dpm ± standard error (n = 2).

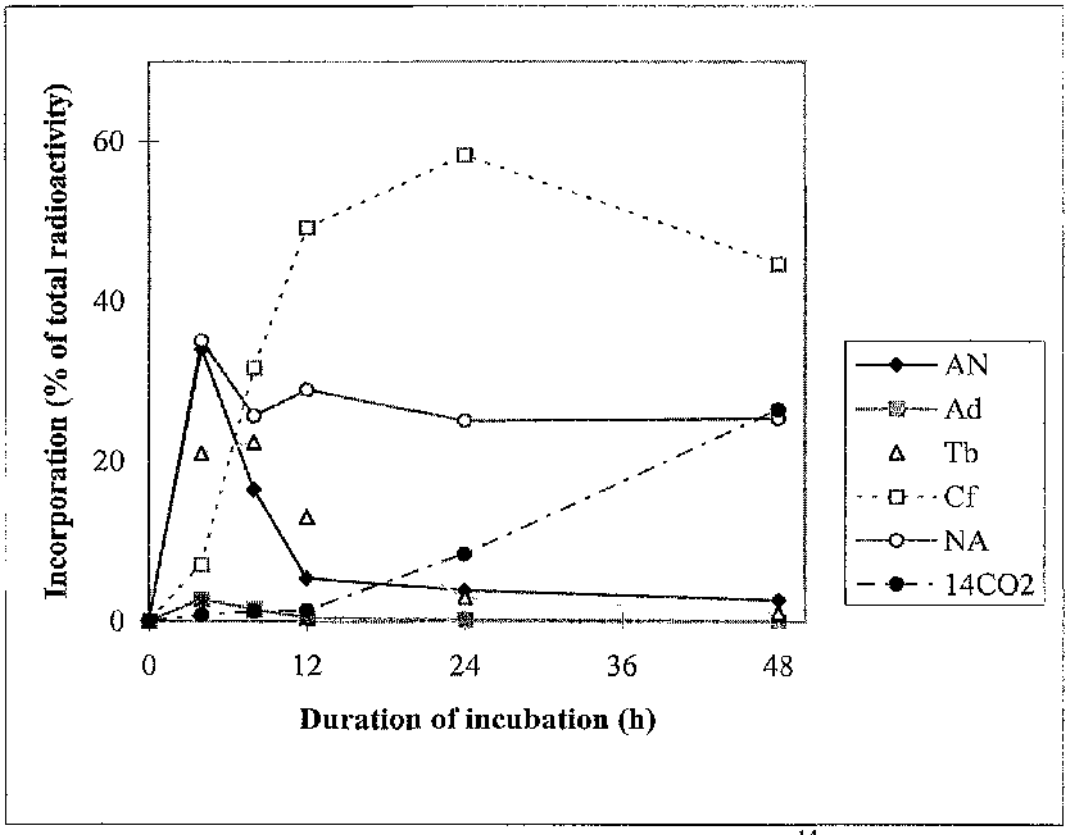


Fig.3.3.1. Distribution of radioactivity in metabolites from [8-^{14}C]adenine after pulse-chase experiment with the first leaf of Darjeeling tea seedlings. Leaves (100 mg) were incubated with 1 μ Ci [8-^{14}C]adenine for 4 h, after which the radioactivity was chased for a further 44 h. Incorporation of radioactivity expressed as a percentage of the recovered radioactivity. Ad, adenine nucleotides; Tb, theobromine; Cf, caffeine and NA, nucleic acids.

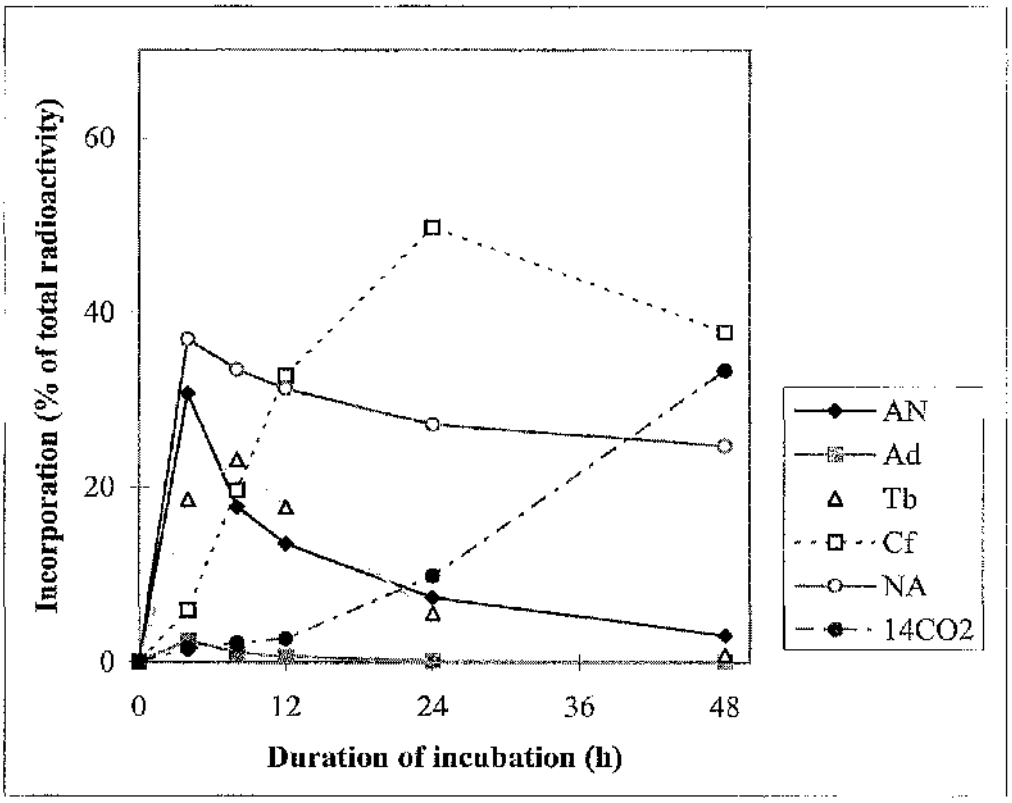


Fig.3.3.2. Distribution of radioactivity in metabolites from [8-^{14}C]adenine after pulse-chase experiment with the second leaf of Darjeeling tea seedlings. Leaves (100 mg) were incubated with 1 μ Ci [8-^{14}C]adenine for 4 h, after which the radioactivity was chased for a further 44 h. Incorporation of radioactivity expressed as a percentage of the recovered radioactivity. Ad, adenine; AN, adenine nucleotides; Tb, theobromine; Cf, caffeine; NA, nucleic acids.

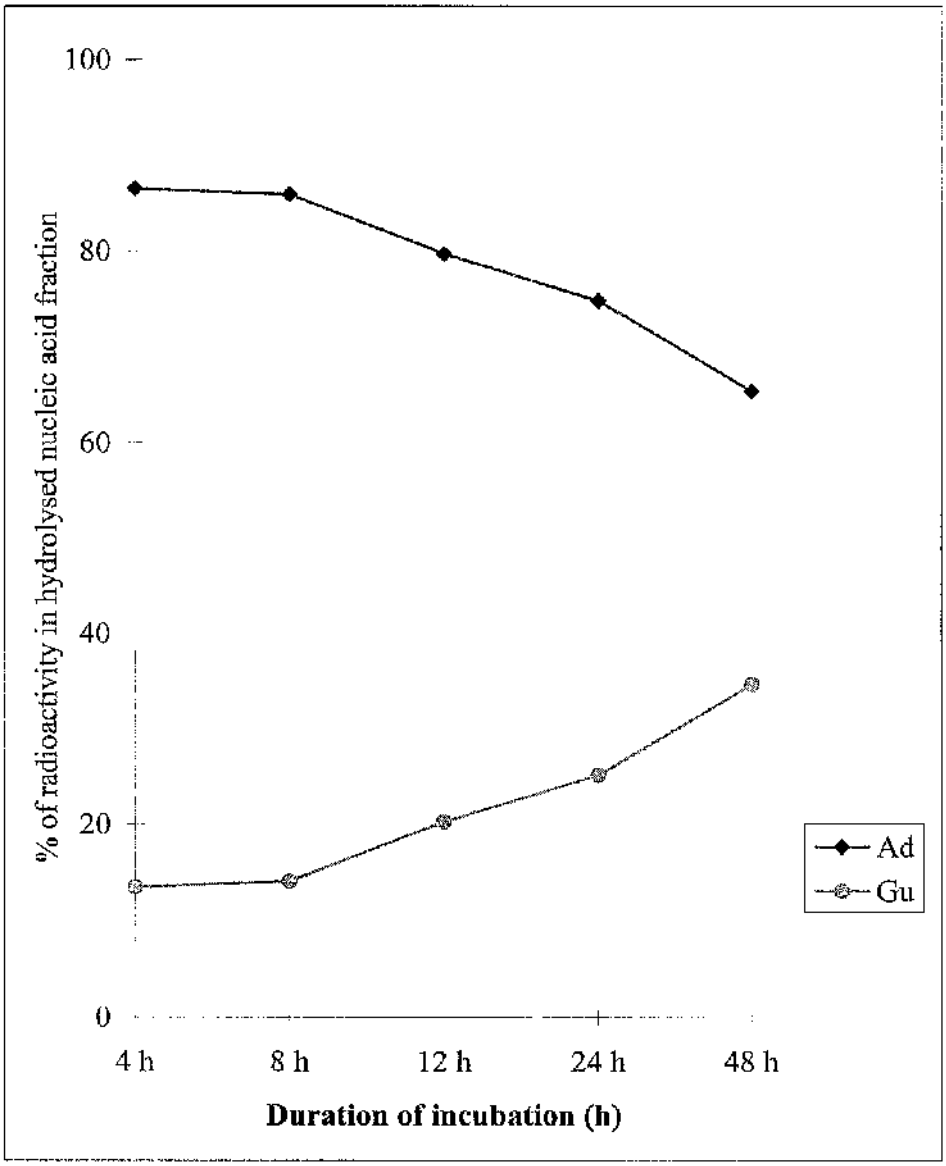


Fig. 3.3.3. Distribution of radioactivity (%) in the hydrolysed nucleic acid fraction obtained in a pulse-chase experiments in which the first leaf of Darjeeling tea seedlings were incubated with [8-^{14}C]adenine under the conditions outline in Fig.3.3.1. Ad-adenine, Gu-guanine.

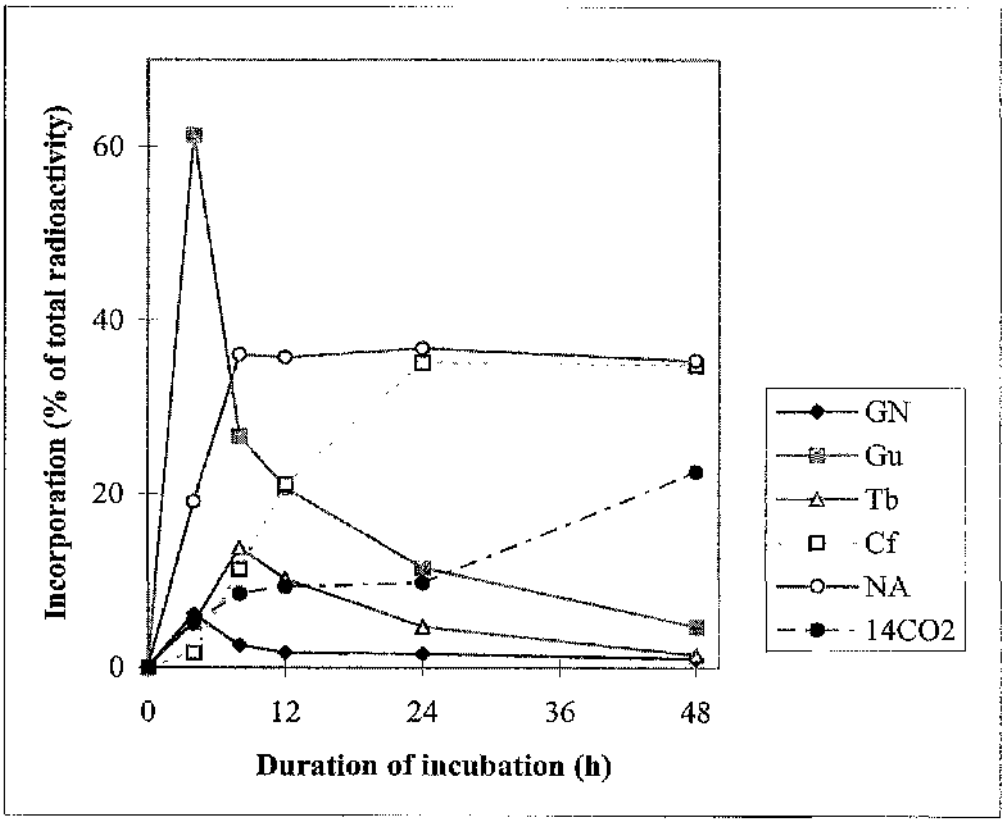


Fig.3.3.4. Distribution of radioactivity in metabolites from [8-^{14}C]guanine hydrochloride after a pulse-chase experiment with the first leaf of Darjeeling tea seedlings. Leaves (100 mg) were incubated with 1μ Ci [8-^{14}C]guanine for 4 h, after which the radioactivity was chased for a further 44 h. Incorporation of radioactivity expressed as a percentage of the recovered radioactivity. GN, guanine nucleotides; Gu, guanine; Tb, theobromine; Cf, caffeine; NA, nucleic acids.

3.4. Catabolism of [8-^{14}C]caffeine by the first leaves of Iranian tea

A recent study has shown that caffeine accumulates in tea leaves because the conversion of caffeine to theophylline is blocked. In contrast to caffeine, theophylline is metabolised rapidly to 3-methylxanthine and on to xanthine which is degraded to $^{14}CO_2$ via the purine catabolism pathway. In addition, sizeable amounts of theophylline and xanthine are salvaged for the synthesis of caffeine via theobromine (Ashihara et al. 1997) (see **Fig. 1.5**). In the present study, the catabolism of [8-^{14}C]caffeine, in light and dark, by first leaves of Iranian tea was investigated using 4, 12, 24 and 48 h incubation periods, after which the methanol extracts were analysed by TLC and $^{14}CO_2$ evolution was determined. The data obtained are presented in **Table 3.4.1** and **Figure 3.4.1**. Based on both the lower recovery of [^{14}C]caffeine and the increased production of $^{14}CO_2$ following incubation in the light for 48 h, it is clear that light enhances the rate of catabolism of caffeine. However, during the first 24 h of incubation, caffeine degradation is not affected by light. Trace levels of xanthine were detected in samples from incubations in the light and small amounts of theobromine and theophylline were present in both light and darkness.

These data demonstrate that the lower levels of incorporation of label from [8-^{14}C]adenine into caffeine in tea leaves incubated in light compared to darkness, described in Section 3.3.2, are, at least in part, a consequence of light-induced enhancement of the rate of caffeine catabolism. However, other factors would appear to be influencing caffeine metabolism, because the light-mediated enhancement of the rate of [^{14}C]caffeine degradation was not detected until 48 h after the beginning of the incubation period (**Fig. 3.4.1**) while the reduced rate of incorporation of [^{14}C]adenine into caffeine in light starts to become apparent after only a 12 h incubation (**Fig. 3.2.2**).

Table. 3.4.1. Summary of the catabolism of [8-^{14}C]caffeine (1 μCi) by 100 mg of the first leaves of Iranian tea seedlings incubated in light and darkness for 4, 12, 24 and 48 h.

Incubation time→	**Dark**			
Metabolites↓	4 h	12 h	24 h	48 h
Caffeine	99.2 ± 0.3	98.9 ± 0.3	97.4 ± 0.7	90.7 ± 0.7
Theophylline	0.2 ± 0.1	0.6 ± 0.4	1.1 ± 0.7	1.0 ± 0.5
Theobromine	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.4 ± 0.2
$^{14}CO_2$	0.1 ± 0.0	0.1 ± 0.0	1.0 ± 0.1	7.5 ± 0.2
Total radioactivity recovered	1178 ± 136	2046 ± 24	2173 ± 97	2020 ± 9

Incubation time→	**Light**			
Metabolites↓	4 h	12 h	24 h	48 h
Caffeine	97.2 ± 1.0	97.3 ± 0.3	95.6 ± 0.4	57.2 ± 1.5
Theophylline	1.1 ± 0.6	1.2 ± 0.2	0.9 ± 0.4	0.6 ± 0.6
Theobromine	0.2 ± 0.2	0.5 ± 0.1	0.4 ± 0.0	0.4 ± 0.0
Xanthine	0.5 ± 0.0	0.3 ± 0.3	0.2 ± 0.0	0.2 ± 0.1
$^{14}CO_2$	0.1 ± 0.0	0.2 ± 0.0	2.5 ± 0.0	41.1 ± 1.1
Total radioactivity recovered	1092 ± 57	1535 ± 0.1	1782 ± 9	1880 ±25

Levels of residual [8-^{14}C]caffeine and radiolabelled metabolites expressed as a percentage of the recovered radioactivity ± standard error (n = 2). Total radioactivity recovered expressed as k dpm ± standard error (n = 2).

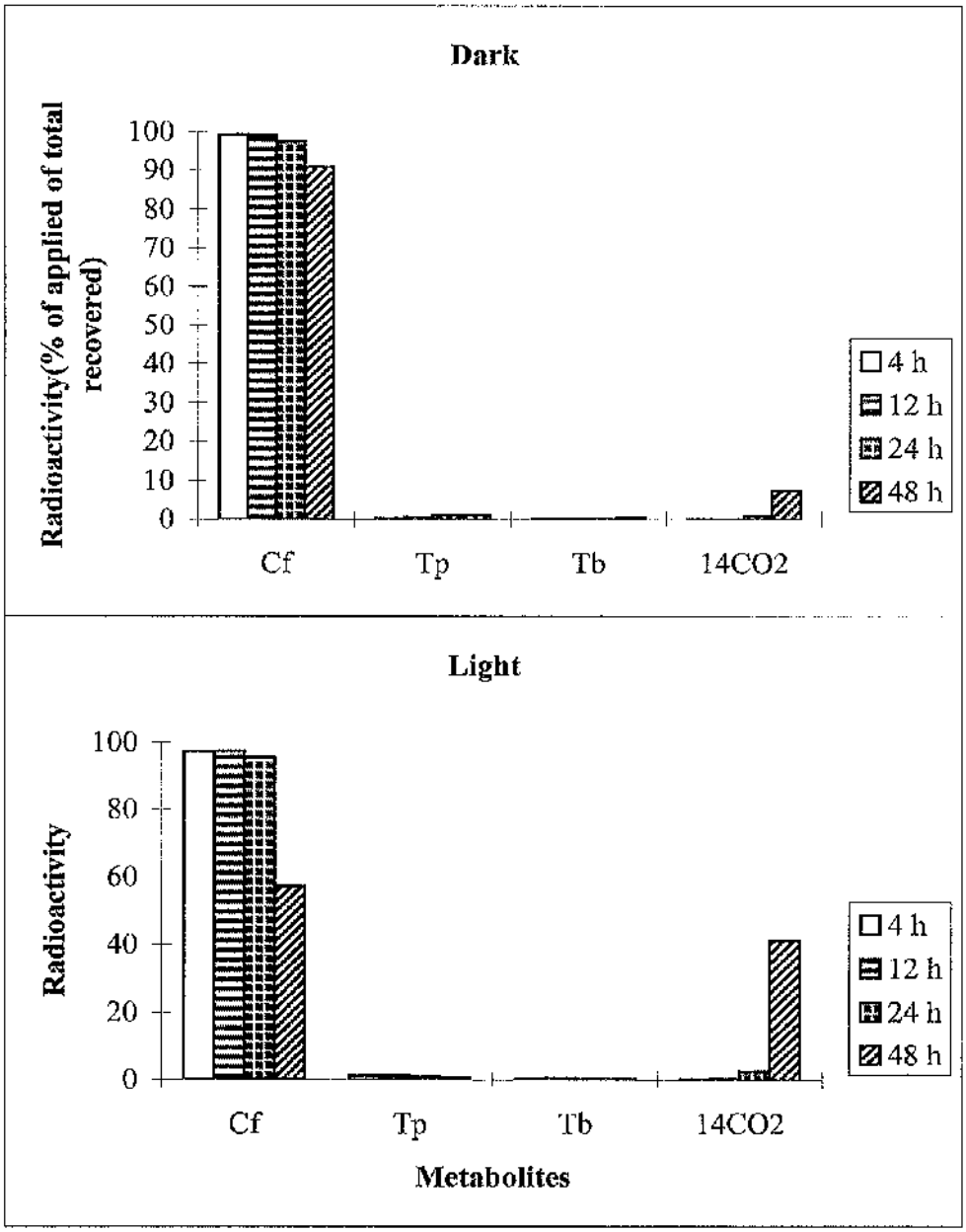


Fig. 3.4.1. Summary of the catabolism of [8-^{14}C]caffeine (1 μ Ci) by 100 mg of the first leaf of Iranian tea seedlings after incubation in light and darkness for 4, 12, 24 and 48 h. Data expressed as a percentage of recovered radioactivity. Cf, caffeine; Tp, theophylline; Tb, theobromine.

3.5. The effects of phytohormones on the metabolism of purine nucleotides and caffeine by tea leaves

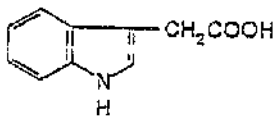
Unlike purine alkaloids, such as caffeine, phytohormones are present in plant tissues in only trace amounts, typically at the 10-50 ng g^{-1} level. Phytohormones, including abscisic acid (ABA), gibberellins, such as gibberellic acid (GA_3), the auxin, indole-3-acetic acid (IAA) and the cytokinin, zeatin (**Fig. 3.5.1**), co-ordinate growth, development and responses to environmental stimuli. They act by altering the expression of genes, changing the properties of membrances and by both inducing the synthesis of new enzymes and modulating the activity of existing enzymes. The response of cells to these actions can redirect metabolism and alter development (Clarke 1996). It was therefore of interest to investigate the effect of phytohormones on the metabolism of purine nucleotides and purine alkaloids. To this end, 100 mg samples of the first leaf of tea seedlings were incubated in the presence and absence of 10^{-6} M ABA, GA_3, IAA and zeatin. The leaves were pretreated with phytohormone for 4 h prior to the addition of either 0.5 μCi [8-^{14}C]adenine or [8-^{14}C]caffeine and incubation in either light or darkness. Samples were collected after 12 h and 24 h and radiolabelled components in methanolic extracts and the nucleic acid fraction analysed as described in Section 2.4.

3.5.1. Effect of phytohormones on the metabolism of [8-^{14}C]adenine

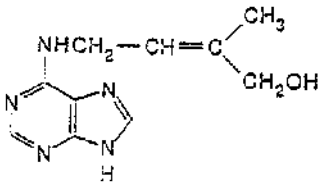
Data obtained following the incubation of [8-^{14}C]adenine with the first leaves of Iranian tea seedlings in the presence and absence of 10^{-6} M ABA, GA_3, IAA and zeatin in light and dark are presented in **Tables 3.5.1** and **3.5.2.** In control incubations in darkness 37 ± 1.0% and 38 ± 2.0% of the recovered radioactivity was associated with caffeine after 12

and 24 h respectively. The equivalent figures for theobromine were 8.3 ± 0.5% and 2.2 ± 0.1% while $^{14}CO_2$ evolution represented 12.1 ± 0.1% and 28.8 ± 0.5% of the recovered radioactivity. The phytohormones did not have a particularly marked effect on adenine metabolism although there is trend towards a slightly lower level of caffeine accumulation with no obvious pattern to the extent of $^{14}CO_2$ release (**Table 3.5.1**).

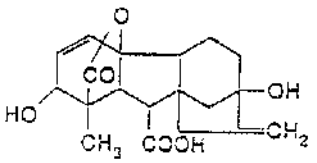
In light 13.8 ± 0.4% and 24.6 ± 0.4% of the recovered radioactivity was incorporated into caffeine after incubations of 12 and 24 h, respectively, with values of 6.9 ± 0.9% and 2.8 ± 0.4% for theobromine and 35.1 ± 1.1% and 45.5 ± 2.3% for $^{14}CO_2$. In the 12 h incubations, the presence of ABA, GA_3, IAA and to a lesser degree zeatin, resulted in slight increase in the incorporation of label into theobromine and caffeine, in some instances with lower, and others with higher, $^{14}CO_2$ output. After 24 h, however, this trend appeared to be reversed and there was a small reduction in the level of caffeine accumulation in the presence of all four phytohormones (**Table 3.5.2**). Thus, the data obtained demonstrate that ABA, GA_3, IAA and zeatin do not have any substantial effects on either adenine metabolism or purine alkaloid biosynthesis in the first leaf of Iranian tea seedlings.



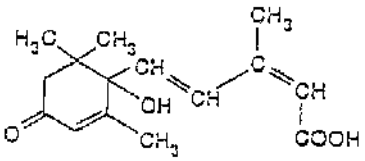
Indole-3-acetic acid



Zeatin.



Gibberllic acid



Abscisic acid

Fig. 3.5.1. Structure of phytohormones. Indole-3-acetic acid, Gibberllic acid, Abscisic acid and Zeatin.

Table. 3.5.1. Summary of the metabolism of [8-^{14}C]adenine (0.5μ Ci) by the 100 mg fresh weight of the first leaf of Iranian tea seedlings after incubation for 12 and 24 h in darkness in the presence and absence of 10^{-6} M ABA, GA_3, IAA and zeatin.

Metabolites	Control		ABA		GA_3		IAA		Zeatin	
	12h	24h	12h	24h	12h	24h	12h	24h	12h	24h
ATP+ADP	5.1 ± 1.1	4.5 ± 0.9	5.6 ± 1.5	5.4 ± 1.4	9.8 ± 0.5	7.1 ± 0.1	6.7 ± 0.7	4.2±0.5	5.3±1.3	4.5±0.8
AMP	1.7 ± 1.1	1.3 ± 0.3	2.4 ± 0.3	1.4 ± 0	2.5 ± 0.5	1.6 ± 0.4	1.7 ± 0.2	1.2±0.4	1.6±0.3	1.3±0.4
All+Aln	0.4 ± 0.1	0.8 ± 0.4	0.6 ± 0.2	0.5 ± 0.1	0.4 ± 0.1	0.7 ± 0.1	0.5 ± 0.1	0.5±0.3	0.2±0.1	0.5±0.3
Ad	0.3 ± 0	0.2 ± 0.1	0.4 ± 0.1	0.3 ± 0	0.4 ± 0.1	0.3 ± 0.2	0.3 ± 0.2	0.2±0.1	0.4±0.1	0.3±0.1
Tb	8.3 ± 0.5	2.2 ± 0	6.4 ± 0.2	2.3 ± 0.1	7.6 ± 0.3	2.7 ± 0.2	4.9 ± 0.3	2.6±0.9	6.4±1	1.8±0.1
Tp	0.5 ± 0.1	0.3 ± 0.1	0.3 ± 0	0.2 ± 0.1	0.6 ± 0.1	0.2 ± 0.2	0.2 ± 0.1	0.2±0	0.3±0.2	0.4±0.2
Cf	37 ± 1	38 ± 2	27.8 ± 0.8	34.9 ± 0.2	29.7 ± 1	31.8 ± 0.5	25.4 ± 3.3	32.2±0.9	32.5±0.6	26.9±0.9
NA	34.4 ± 0.3	24.1 ± 2.2	39.6 ± 0.9	34.7 ± 2.1	32 ± 0.6	26 ± 1.6	33±0.8	27.1±0.4	30.9±0.3	30.6±0.6
$^{14}CO_2$	12.1 ± 0.1	28.8 ± 0.5	17 ± 0.1	20.3 ± 0.7	17 ± 2.7	29.2 ± 1	27.3±2.4	32.1±0.8	22.8±1.2	33.9±0.9
recovered radioactivity	412 ± 16	454 ± 20	487 ± 14	553 ± 2	365 ± 4	480 ± 16	357 ± 17	336 ± 9	350 ± 2	424 ± 16

Levels of residual [8-^{14}C]adenine (Ad) and radiolabelled metabolites ATP, ADP, AMP, allantoin (All), allantoic acid (Aln), theobrimine (Tb), theophylline (Tp), caffeine (Cf), nucleic acid fraction (NA) and $^{14}CO_2$ expressed as a percentage of the total recovered radioactivity ± standard error (n = 2). Recovered radioactivity expressed as kdpm ± standard error (n = 2).

Table. 3.5.2. Summary of the metabolism of [8-^{14}C]adenine (0.5μCi) by the 100 mg fresh weight of the first leaf of Iranian tea seedlings after incubation for 12 and 24 h in light in the presence and absence of 10^{-6} M ABA, GA_3, IAA and zeatin.

Metabolites	Control		ABA		GA_3		IAA		Zeatin	
	12h	24h	12h	24h	12h	24h	12h	24h	12h	24h
ATP+ADP	2.6 ± 0.6	2.1 ± 0.9	5.2 ± 0.1	3 ± 0.8	7.2 ± 0.2	1.2 ± 0.3	6.3 ± 0.3	1.5 ± 0.5	3.8 ± 0.7	2.2 ± 0.5
AMP	1.9 ± 0.3	1.0 ± 0.1	2.5 ± 0.2	1.8 ± 0.1	2.9 ± 0.1	1.2 ± 0.2	3.3 ± 0.4	1.1 ± 0.1	2.3 ± 0.2	1.5 ± 0.1
All+Aln	0.5 ± 0.2	0.3 ± 0.1	1.1 ± 0.2	0.3 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.9 ± 0.3	0.3 ± 0	0.6 ± 0.1	0.3 ± 0
Ad	0.1 ± 0.1	0.03 ± 0	0.2 ± 0.1	0.02 ± 0	0.12 ± 0.1	0.1 ± 0.1	0.2 ± 0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.03
Tb	6.9 ± 0.9	2.8 ± 0.4	8.8 ± 0.1	5.0 ± 1.8	10.8 ± 0.1	3.0 ± 0.7	10.3 ± 0.9	3.1 ± 0.4	8.6 ± 0.6	3.4 ± 0.6
Tp	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.3 ± 0	0.6 ± 0	0.1 ± 0.1	0.6 ± 0.1	0.2 ± 0.1	0.5 ± 0.1	0.2 ± 0.1
Cf	13.8 ± 0.4	24.6 ± 0.4	16.8 ± 1.5	20.9 ± 0.3	20.2 ± 1.7	18.2 ± 0.8	19.5 ± 1	16.4 ± 0.1	13.8 ± 0.7	18.3 ± 0.4
NA	39.7 ± 0.1	21.8 ± 2.2	30.3 ± 0.3	37.9 ± 5.4	36.0 ± 0.7	24.1 ± 0.1	32.3 ± 0.1	20.5 ± 0.7	29.6 ± 0.7	25.6 ± 0.2
$^{14}CO_2$	35.1 ± 1.1	45.5 ± 2.3	34.7 ± 1.1	31.1 ± 4.4	21.6 ± 0.6	51.8 ± 1.2	26.7 ± 1.7	57 ± 0.2	41.0 ± 1.6	48.6 ± 0.8
recovered radioactivity	506 ±16	730 ± 83	615 ± 5	383 ± 33	612 ± 49	425 ± 36	612 ± 50	682 ± 19	469 ± 2	718 ± 27

Levels of residual [8-^{14}C]adenine (Ad) and radiolabelled metabolites ATP, ADP, AMP, allantoin (All), allantoic acid (Aln), theobrimine (Tb), theophylline (Tp), caffeine (Cf), nucleic acid fraction (NA) and $^{14}CO_2$ expressed as a percentage of the total recovered radioactivity ± standard error (n = 2). Recovered radioactivity expressed as kdpm ± standard error (n = 2).

3.5.2. Effect of phytohormones on the catabolism of [8-^{14}C]caffeine

The effects of phytohormones on the metabolism of [8-^{14}C]caffeine by the first leaf of Darjeeling tea seedlings incubated in light and darkness are presented in **Table 3.5.3** and **3.5.4**. In darkness, phytohormone treatment resulted in a more rapid rate of decline in [^{14}C]caffeine accompanied by an increased evolution of $^{14}CO_2$. This effect was apparent after 12 h and was further enhanced future after a 24 h incubation (**Table 3.5.3**). ABA and IAA were especially effective in increasing the rate of caffeine catabolism in darkness. In the light, a weaker promotion of [^{14}C]caffeine catabolism was observed, but only after 24 h, in the presence of zeatin and to a lesser extent ABA and GA_3 (**Table 3.5.4**). The data provide no indications as to how the promotory effects of the phytohormones on caffeine degradation are mediated. This is a topic that requires more detailed investigation.

Table. 3.5.3. Summary of the metabolism of [8-^{14}C]caffeine (1 μCi) by 100 mg fresh weight of the first leaf of Darjeeling tea seedlings after incubation for 12 and 24 h in darkness in the presence and absence of 10^{-6} M ABA, GA_3, IAA and zeatin.

Metabolites	Control		ABA		GA_3		IAA		Zeatin	
	12h	24h	12h	24h	12h	24h	12h	24h	12h	24h
Xanthine	0.8 ± 0.5	0.5 ± 0.1	0.2 ± 0.1	0.1 ± 0	0.5 ± 0.2	0.1 ± 0	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.2
Theobromine	1.8 ± 0.2	0.5 ± 0	0.6 ± 0.2	0.2 ± 0.1	1.2 ± 0.2	0.5 ± 0.2	0.5 ± 0.4	0.2 ± 0	1.0 ± 0.1	0.2 ± 0
Theophylline	0.3 ± 0.1	0.4 ± 0	0.2 ± 0.1	0.1 ± 0	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.2	0.2 ± 0.1	0.6 ± 0.5	0.2 ± 0.1
Caffeine	96 ± 0.6	92.5 ± 0.3	87.5 ± 0.9	24.4 ± 0.8	83.1 ± 1.4	63.8 ± 0.2	70.9 ± 0.9	32.5 ± 1.2	68.6 ± 1.2	49.7 ± 1.4
$^{14}CO_2$	1.3 ± 0.1	6.3 ± 0.2	11.6 ± 1.1	75.3 ± 0.9	15 ± 0.9	35.4 ± 0.5	28.2 ± 0.3	67 ± 1	29.7 ± 0.6	49.7 ± 1.2
recovered radioactivity	1626 ± 25	1837 ± 18	1622 ± 26	2111 ± 10	1536 ± 73	1696 ± 40	1451 ± 14	1806 ± 21	1560 ± 20	1700 ± 4

Levels of residual [8-^{14}C]caffeine and metabbolites expressed as a percentage of the total recovered radioactivity ± standard error (n = 2). Recovered radioactivity expressed as kdpm ± standard error (n = 2).

Table. 3.5.4. Summary of the metabolism of [8-^{14}C]caffeine (1 μCi) by 100 mg fresh weight of the first leaf of Darjeeling tea seedlings after incubation for 12 and 24 h in light in the presence and absence of 10^{-6} M ABA, GA_3, IAA and zeatin.

Metabolites	Cotrol		ABA		GA_3		IAA		Zeatin	
	12h	24h	12h	24h	12h	24h	12h	24h	12h	24h
Xanthine	0.8 ± 0.05	0.1 ± 0.04	0.5 ± 0.3	0.1 ± 0.03	0.4 ± 0.1	0.3 ± 0.1	0.8 ± 0.5	0.1 ± 0.04	0.6 ± 0.3	0.5 ± 0
Theobromine	1.3 ± 0.2	0.6 ± 0.2	1 ± 0.4	0.5 ± 0.4	0.5 ± 0.1	1 ± 0.2	0.8 ± 0.6	0.5 ± 0.3	1.4 ± 0.5	1.8 ± 0.7
Theophylline	1 ± 0.4	0.7 ± 0.5	0.5 ± 0.3	0.2 ± 0.1	0.2 ± 0.2	0.2 ± 0	0.6 ± 0.3	0.2 ± 0.1	0.7±0.4	0.2 ± 0.1
Caffeine	95.6 ± 0.8	95.2 ± 0.7	94.7 ± 1	85.6 ± 0.4	96.3±0.1	83.4 ± 0.9	97.8 ± 1.2	91.2 ± 0.2	97.4 ± 1.1	54.9 ± 3.8
$^{14}CO_2$	1.5 ± 0.3	3.6 ± 0.2	3.4 ± 0.1	13.8 ± 0.8	2.3 ± 0.2	15.2 ± 1.1	0.2 ± 0	8.1 ± 0.1	0.1 ± 0	43.3 ± 2.7
recovered radioactivity	1453 ± 71	1812 ± 27	1477 ± 22	1627 ± 54	1416 ± 41	1498 ± 28	1558 ± 49	1676 ± 64	1490 ± 63	1619 ± 29

Levels of residual [8-14C]caffeine and metabbolites expressed as a percentage of the total recovered radioactivity ± standard error (n = 2). Recovered radioactivity expressed as kdpm ± standard error (n = 2).

CHAPTER 4

CONCLUSION

4. Conclusions

Caffeine and smaller amounts of theobromine were detected by HPLC in aqueous extracts of the shoot of Iranian tea seedlings. Some samples that were analysed also contained trace levels of a compound which co-chromatographed with paraxanthine, one of the less frequently detected purine alkaloids. The highest concentrations of caffeine (>30 mg g^{-1} fresh weight) were present in the apical bud and first leaf and the concentrations of both caffeine and theobromine declined progressively as the age of the leaves increased. Cotyledons and roots had a very low purine alkaloid content. Analysis of Iranian tea seedlings from May 1993 to March 1994, during which period they increased in age from 5.5 to 15 months, demonstrated fluctuations in the concentrations of caffeine and theobromine with highest amounts present in summer months and the lowest in winter in November and January. While this could be due to the increasing age of the plants, it is more likely to be due to seasonal factors. A parallel investigation of the purine alkaloid content of Darjeeling tea seedlings produced very similar data to the study with Iranian tea seedlings, with the exception that paraxanthine was not detected in any samples of Darjeeling tea. A number of earlier, and less detailed, studies have provided indications of the existence of a purine alkaloid gradient within tea seedlings and Fujimori et al. (1991) have demonstrated seasonal variations in the levels of the three *N*-methyltransferase activities involved in caffeine biosynthesis (see **Fig. 1.3**) in desalted preparations from young tea leaves. There are no reports in the literature on long distance transport of caffeine within either tea or coffee plants which implies that high levels of caffeine in young leaves and the peak concentrations in mid-summer indicate that caffeine biosynthesis activity is highest in the summer months and

that the biosynthetic capacity of young leaves greatly exceeds that of older, more basal, leaf tissues

The study by Kato et al. (1997) on the NMT activities involved in the conversion of xanthosine to caffeine in cell-free preparations from young leaves of *Camellia sinensis*, is of interest in view of the detection of paraxanthine in some samples of Iranian tea (**Table 3.1.3 - 3.1.5**). In substrate specificity studies with partially purified NMT, Kato et al. (1997) showed that 7-methylxanthine, formed from xanthosine via 7-methylxanthosine is metabolised to theobromine at a ten-fold faster rate than its conversion to paraxanthine. The *N*-1 methylation of theobromine yielding caffeine is much slower than the *N*-3 methylation that converts paraxanthine to caffeine. However, because of the preferred conversion of 7-methylxanthine to theobromine, the major route to caffeine most probably proceeds via xanthosine → 7-methylxanthosine → 7-methylxanthine → theobromine → caffeine pathway (see **Fig. 1.5**). The alternative 7-methylxanthine → paraxanthine → caffeine is likely to function as one of a number of minor pathways that also contribute to the production of caffeine (Kato et al. 1997). However, the appearance of paraxanthine in shoots of Iranian tea harvested in September and November 1993 and January 1994, in concentrations higher than those of theobromine (**Tables 3.1.3 - 3.1.5**), raises the possibility that at certain times of the year the role of paraxanthine in caffeine biosynthesis in tea may be more important than is currently realised. Had time permitted, this possibility would have been investigated by comparing the metabolic fate of methylxanthines fed to cell-free preparations obtained from young Iranian tea leaves harvested in mid-summer and mid-winter in which endogenous paraxanthine was, respectively, absent and present.

Metabolism studies demonstrated that [8-^{14}C]adenine served as an effective substrate for [^{14}C]caffeine production and that there were large differences in the metabolism of adenine by young mature and aged leaves of Iranian tea. The young, first, second and third leaves were much more active sites of caffeine biosynthesis than mature and aged leaves. For instance, in [8–^{14}C]adenine incubations with the first leaf, 48.4 ± 0.9% of the recovered radioactivity was associated with [^{14}C]caffeine after 24 h, while with mature and aged leaves there was a <2% incorporation. Thus, the apical to basal endogenous caffeine gradient in the shoot of tea seedlings is mirrored by a decline in the capacity for caffeine biosynthesis as the leaves age.

In studies with [8-^{14}C]adenine and first and second leaves of Iranian tea in light and darkness, there was a much more extensive accumulation of [^{14}C]caffeine in darkness than in light which became evident after a 12 h incubation. The first leaves of Iranian tea seedlings also catabolised to [8–^{14}C]caffeine more rapidly in light than in darkness but this effect did not become apparent until 48 h after the beginning of the incubation period. The lower rate of incorporation of [8-^{14}C]adenine into caffeine in light is, therefore, not a consequence, at least initially, of a light-induced increase in the rate of catabolism of caffeine. These findings are in keeping with both the report of Konoshi et al (1972) who demonstrated that the caffeine content of tea shoots is higher in darkness than in light and the work of Ogutuga and Northcote (1970a) showing that tea callus accumulates more caffeine in light than in darkness. Exactly what steps in the pathway between adenine and caffeine are affected by light remains to be determined. The high levels of $^{14}CO_2$ produced when leaves are incubated in the presence of [8-^{14}C]adenine (see Tables 3.2.6 and 3.2.7), implies that in light the purine catabolism pathway may be more active and thereby limit the amount of substrate available for

purine alkaloid biosynthesis. It is, however, possible that light may also down-regulate some of the steps in the caffeine biosynthesis pathway (see **Fig. 1.3**).

Pulse-chase experiments were carried out with [8-^{14}C]adenine and [8-^{14}C]guanine. The kinetics of the metabolism were similar with an initial accumulation of label in theobromine declining as the level of [^{14}C]caffeine increased. This supports the widely held view that theobromine is the immediate precursor of caffeine and is not in keeping with the proposals of Nazzario and Lovatt (1993a,b) that caffeine is synthesized from xanthine and that theobromine, produced by two separate de novo and salvage pathways, is not converted to caffeine. [8-^{14}C]Adenine was also converted to guanine which was incorporated into nucleic acids. The amount of [^{14}C]caffeine produced from [8-^{14}C]guanine appeared to be slightly less than the levels produced in parallel incubations with [8-^{14}C]adenine.

The effects of ABA, GA_3, IAA and zeatin on [8-^{14}C]adenine metabolism by the first leaf of Darjeeling tea seedlings were investigated in light and darkness. Overall, the phytohormones had relatively little effect on either adenine metabolism in general or, more specifically, the conversion of adenine to theobromine and caffeine. Incubations with [8-^{14}C]caffeine showed that in darkness the phytohormones, most notably ABA and IAA, increased the rate of catabolism of caffeine after 12 h. In light the effect was less marked with caffeine degradation being enhanced by zeatin, and to a lesser degree ABA and GA_3, but only after an incubation period of 24 h. Exactly, how these growth regulators exert their effect remains to be determined. As yet it is unclear whether they activate specific steps in the caffeine degradation pathway, such as the conversion of caffeine to theophylline, which is the rate limiting step in purine alkaloid catabolism

(Ashihara et al. 1997) or whether their influence is an indirect consequence of their hormonal effects on plant growth and development.

REFERENCES

Anderson, A. & Gibbs, M. (1962). The biosynthesis of caffeine in the coffee plant. *J. Biol. Chem. 237*, 1941-1944.

Anderson, J. D. (1979). Purine nucleotide metabolism of germinating soyabean embryonic axes. *Plant Physiol. 63*, 100-104.

Ashihara, H. (1993). Purine metabolism and the biosynthesis of caffeine in mate' leaves. *Phytochemistry 33*, 1427-1430.

Ashihara, H. & Fujimori, N. (1993). Adenine metabolism and biosynthesis of theobromine and caffeine in developing leaves of coffee plants. *Proceedings of the 15th International Colloquim on Coffee,* Montpellier, ASIC, Paris, pp. 767-769.

Ashihara, H., Fujimori, N., Suzuki, T., & Waller, G. R. (1991a). Purine alkaloids (caffeine and theobromine) in flowers of tea and other *Camellia* plants. *Proceedings of the International Symposium on Tea Science,* Shizuoka, Japan, pp. 180-184.

Ashihara, H., Gillies, F. M. & Crozier, A. (1997). Metabolism of caffeine and related purine alkaloids in leaves of tea (*Camellia sinensis*). *Plant Cell Physiol. 38*, 413-419.

Ashihara, H. & Kubota, H. (1986). Patterns of adenine metabolism and caffeine biosynthesis in different parts of tea seedlings. *Physiol. Plantarum. 68*, 275-281.

Ashihara, H. & Kubota, H. (1987). Biosynthesis of purine alakaloids in *Camellia* plants. *Plant Cell Physiol. 28*, 535-539.

Ashihara, H., Kubota, H. & Suzuki, T. (1991b). Biosynthesis of caffeine and theobromine in different parts of tea seedlings. *Proceedings of the International Symposium on Tea Science,* Shisuoka, Japan, pp. 175-179.

Ashihara, H., Li, X. N. & Ukaji, T. (1988). Effect of inorganic phosphate on the biosynthesis of purine and pyrimidine nucleotides in suspension-cultured cells of *Catharanthus roseus*. *Ann. Bot.* ***61***, 225-232.

Ashihara, H., Mitsui, K., Yabuki, N. & Anygaard, P. (1991c). Adenosine metabolism and growth of adenosine-requiring mutant cells of *Datura innoxia*. *Int. J. Purine and Pyrimidine Res.* ***2***, 129-135.

Ashihara, H., Monteiro, A. M., Gillies, F. M. & Crozier, A. (1995a). Biosynthetic pathways of caffeine in *Coffea arabica* leaves. *Proceedings of the 16 th International Colloquim on Coffee,* Kyoto, ASIC, Paris, pp. 589-598.

Ashihara, H., Monteiro, A. M., Moritz, T., Gillies, F. M. & Crozier, A. (1996a). Catabolism of caffeine and related purine alkaloids in leaves of *Coffea arabica* L. *Planta* ***198***, 334-339.

Ashihara, H., Monterio, A. M., Gillies, F. M. & Crozier, A. (1996b). Biosynthesis of caffeine in leaves of coffee. *Plant Physiol.* ***111***, 747-753.

Ashihara, H., Shimizu, H., Takeda, Y., Suzuki, T., Gillies, F. M. & Crozier, A. (1995b). Caffeine metabolism in high and low caffeine containing cultivars of Camellia sinensis. *Z. Naturforsch.* ***50C***, 602-607.

Atkins, C. A., Rainbird, R. & Pate, J. S. (1980). Evidence for a purine pathway of ureide synthesis in N_2-fixing nodules of copea [*Vigna unguiculata* (L.) Walp.]. *Z. Pflanzenphysiol. Bd. S.* ***97***, 249-260.

Banerjee, B. (1992). Botanical classification of tea. In: K. C. Willson & M. N. Clifford (Ed.), *Tea cultivation to consumption*. Chapman and Hall, London pp. 25-50.

Barankinewicz, J. & Paszkowski, J. (1980). Purine metabolism in mesophyll protoplasts of tobaco (*Nicotina tabacum*) leaves. *Biochem. J.* ***186***, 343-350.

Baumann, T. W. & Frischknecht, P. M. (1988a). Caffeine; Production by plant [*Coffea* Sp.]. In: Y. P. S. Bajaj (Ed.), *Biotechnology in Agriculture and*

Forestry. Vol. 4, Medical and Aromatic Plants I. Springer-Verlag, Heidelberg, pp. 264-281.

Baumann, T. W. & Frischknecht, P. M. (1988b). Purines, *Cell culture and Somatic Cell Genetics of Plants*. Vol. 5, Academic Press, London, pp. 403-417.

Baumann, T. W. & Gabriel, H. (1984). Metabolism and extraction of caffeine during germination of *Coffea arabica* L. *Plant and Cell Physiol. 25*, 1431-1436.

Baumann, T. W., Looser, E. D. & Wanner, H. (1978). 7-Methylxanthosine an intermediate in caffeine biosynthesis. *Phytochemistry 17*, 2077-2082.

Buchanann, J. M., Sonne, J. C. & Delluva, A. M. (1948). Biological precursors of uric acid, II. The role of lactate, glycine, and carbon dioxide as precursors of the carbon chain and nitrogen atom 7 of uric acid. *J. Biol. Chem. 173*, 81-98.

Chou, C. H. & Waller, G. R. (1980). Isolation and identification by mass spectrometry of phytotoxins in *Coffea arabica*. *Bot. Bull. Academia Sinica. 21*, 25-34.

Clarke, A. (Ed.). (1996). Control systems in plants (Fourth ed.). Benjamin/Cumming, Menlo Park, California.

Cloughley, J. B. (1982). Factors influencing the caffeine content of black tea: Part 1- The effect of field variables. *Food Chem. 9*, 269-276.

Coddington, A. (1985a). Guanosine. In: H. U. Bergmeyer (Ed.), *Methods of enzymatic analysis*. Vol. 7, Purines, Pyrimidines, Nucleosides. VCH, Weinheim, pp. 117-121.

Coddington, A. (1985b). Inosine. In: H. U. Bergmeyer (Ed.), *Methods of enzymatic analysis*. Vol. 7, Purines, Pyrimidines, Nucleosides. VCH, Weinheim, pp. 121-125.

Cordell, G. A. (1981). Introduction to alkaloids a biogenetic approach. John Wiley and Sons, New York, pp. 952-959.

Crozier, A., Monteiro, A. M., Moritz, T., Gillies, F. M. & Ashihara, H. (1995). Purine alkaloid catabolism pathways in *Coffea arabica* leaves. *Proceedings of the 16 th International Colloquim on Coffee,* Kyoto, ASIC, Paris, pp. 606-615.

Eden, T. (1976). *Tea.* (Third ed.). Longman London.

Finger, A., Kuhr, S. & Engelhardt, U. H. (1992). Chromatography of tea constituents. *J. Chromatogr.* ***624***, 293-315.

Food Agriculture Organization (F. A. O) (1994). Production year book. Vol. 48, pp. 174.

Frischknecht, P. M., Baumann, T. W. & Wanner, H. (1977a). Caffeine production in tissue cultures of *Coffea arabica. Proceedings of the 8 th International Colloquim on Coffee,* ASIC, Paris, pp. 139-142.

Frischknecht, P. M., Baumann, T. W. & Wanner, H. (1977b). Tissue culture of *Coffea arabica* growth and caffeine formation. *Planta Medica*, ***31***, 344-350.

Frischknecht, P. M., Eller, B. M. & Baumann, T. W. (1982). Purine alkaloid formation and CO_2 gas exchange in dependence of development and of enviromental factors in leaves of *Coffea arabica* L. *Planta* ***156***, 295-301.

Frischknecht, P. M., Ulmer-Dufek, J. & Baumann, T. W. (1986). Purine alkaloid formation in buds and developing leaflets of *Coffea arabica*: Expression of an optimal defence strategy? *Phytochemistry* ***25***, 613-616.

Fujimori, N. & Ashihara, H. (1990). Adenine metabolism and the synthesis of purine alkaloids in flowers of *Camellia. Phytochemistry* ***29***, 3513-3515.

Fujimori, N. & Ashihara, H. (1993). Biosynthesis of caffeine in flower buds of *Camellia sinensis. Ann. Bot. 71*, 279-284.

Fujimori, N. & Ashihara, H. (1994). Biosynthesis of theobromine and caffeine in developing leaves of *Coffea arabica. Phytochemistry* ***36***, 1359-1361.

Fujimori, N., Suzuki, T. & Ashihara, H. (1991). Seasonal variations in biosynthetic capacity for the synthesis of caffeine in tea leaves. *Phytochemistry* ***30***, 2245-2248.

Garrett, R. H. & Grisham, C. M. (1995). *Biochemistry*. Saunders College Publishing, Fort Worth.

Harler, C. R. (1963). *Tea manufacture*. Oxford University Press London.

Henderson, J. F. & Paterson, A. R. P. (1973). *An introduction to nucleotide metabolism*. Academic Press London.

Hirose, F. & Ashihara, H. (1984a). Changes in the activity of enzymes involved in purine salvage and nucleic acid degradation during the growth of *Catharanthus roseus* cells in suspension culture. *Physiol.Plant.* ***60***, 532-538.

Hirose, F. & Ashihara, H. (1984b). Fine control of purine nucleotide biosynthesis in intact cells of *Catharanthus roseus*. *J. Plant Physiol.* ***116***, 417-423.

Inoue, T. & Adachi, F. (1962). Studies on biogenesis of tea components. *Chem. Pharm. Bull.* ***10***, 1212-1214.

Janick, P. (1986). *Horticultural Science*. (Fourth ed.). W.H.Freeman, New York.

Jones, J. W. & Robins, R. K. (1963). Methylation studies of certain naturally occurring purine nucleosides. *J. Am. Chem. Soc.* ***85***, 193-201.

Kalberer, P. (1965). Breakdown of caffeine in the leaves of *Coffea arabica* L. *Nature*, No. ***4971***, 597-598.

Kato, M. (1989). *Camellia sinensis* L. (tea): In: Y. P. S. Bajaj (ed), In Vitro regeneration, *Biotechnology in Agriculture and Forestry*. Vol. 7, Medical and Aromatic Plants. Springer-Verlag, Heidelberg, pp. 82-98.

Kato, M., Kanehara, T., Shimizu, H.,Suzuki, T., Gillies, F. M., Crozier, A. & Ashihara, H. (1996). Caffeine biosynthesis in young leaves of *Camellia sinensis*: in vitro studies on *N*-methyltransferase activity involved in the conversion of xanthosine to caffeine. *Physiol. Plantarum **98***, 629-636.

Kihlman, B. A. (1977). *Caffeine and chromosomes*: Elsevier Scientific Publishing Company, Amsterdam, New York, Oxford.

Konishi, S., Ozasa, M. & Takahashi, E. (1972). Metabolic conversion of *N*-methyl carbon of glutamylmethylamide to caffeine in tea plants. *Plant and Cell Physiol. **13***, 365-375.

Lehninger, A. L. (1982). *Biochemistry*. Worth Publishers Inc. New York.

Levenberg, B., Hartman, S. C. & Buchanan, J. M. (1956). Biosynthesis of the purines (Further studies in vitro on the metabolic origin of nitrogen atoms 1 and 3 of the purine ring. *J. Biol. Chem. **220***, 379-390.

Looser, E., Baumann, T. W. & Wanner, H. (1974). The biosynthesis of caffeine in the coffee plant. *Phytochemistry **13***, 2515-2518.

Mazzafera, P., Crozier, A. & Magalhaes, A. C. (1991). Caffeine metabolism in *Coffea arabica* and other species of coffee. *Phytochemistry **30***, 3913-3916.

Mazzafera, P., Crozier, A. & Sandberg, G. (1994). Studies on the metabolic control of caffeine turnover in developing endosperms and leaves of *Coffea arabica* and *Coffea dewevrei*. *J. Agric. Food Chem. **42***, 1423-1427.

Nagata, T. (1986). Differences in caffeine, flavanols and amino acids contents in leaves of cultivated species and hybrids in the genus *Camellia*. *JARQ **19***, 276-280.

Nagata, T. (1991). Differences in tea components in the genus *Camellia*. *Proceedings of the International Symposium on Tea Science,* Shzuoka, Japan, pp. 100-104.

Nagata, T. & Sakai, S. (1984). Differences in caffeine, flavanols and amino acids contents in leaves of cultivated species of *Camellia*. *Japan. J. Breed. 34*, 459-467.

Nagata, T. & Sakai, S. (1985a). Purine base pattern of *Camellia irrawadiensis*. *Phytochemistry 24*, 2271-2272.

Nagata, T. & Sakai, S. (1985b). Caffeine, Flavanol and aminoacid contents in leaves of hybrids of the section Dubiae in the genus *Camellia*. *Japan. J. Breed. 35*, 1-8.

Nathanson, J. (1984). Caffeine and related methylxanthines: Possible naturally occurring pesticides. *Science 226*, 184-187.

Nazario, G. M. & Lovatt, C. J. (1993a). Purine metabolism and alkaloid biosynthesis in leaves of *Coffea arabica* L. *Proceedings of the 15 th International Colloquim on Coffee,* Montpellier, ASIC, Paris, pp 155-162.

Nazario, G. M. & Lovatt, C. J. (1993b). Regulation of purine metabolism in intact leaves of *Coffea arabica*. *Plant Physiol. 103*, 1195-1201.

Nazario, G. M. & Lovatt, C. J. (1993c). Separate de novo and salvage purine pools are involved in the biosynthesis of theobromine but not caffeine in leaves of *Coffea arabica* L. *Plant Physiol. 103*, 1203-1210.

Negishi, O., Ozawa, T. & Imagawa, H. (1985a). Methylation of xanthosine by tea-leaf extracts and caffeine biosynthesis. *Agric. Biol. Chem. 49*, 887-890.

Negishi, O., Ozawa, T. & Imagawa, H. (1988). *N*-Methyl nucleosidase from tea leaves. *Agric. Biol. Chem. 52*, 169-175.

Negishi, O., Ozawa, T. & Imazawa, H. (1985b). Conversion of xanthosine into caffeine in tea plants. *Agric. Biol. Chem. 49*, 251-253.

Negishi, O., Ozawa, T. & Imazawa, H. (1985c). The role of xanthosine in the biosynthesis of caffeine in coffee plants. *Agric. Biol. Chem. 49*, 2221-2222.

Negishi, O., Ozawa, T. & Imazawa, H. (1991). Biosynthesis of caffeine in tea plant. *Proceedings of the International Syposium on Tea Science,* Shizuoka, Japan, pp. 105-109.

Negishi, O., Ozawa, T. & Imazawa, H. (1992). Biosynthesis of caffeine from purine nucleotides in tea plant. *Biosci. Biotech. Biochem. 56*, 499-503.

Negishi, O., Ozawa, T. & Imazawa, H. (1994). Guanosine deaminase and guanine deaminase from tea leaves. *Biosci. Biotech. Biochem. 58*, 1277-1281.

Neuenschwander, B. & Baumann, T. W. (1991). Purine alkaloid formation during somatic embryo development of *Coffea arabica* L. *Proceedings of the 14 th International Colloquim on Coffee,* San Francisco, ASIC, Paris, pp. 595-600.

Nobusawa, E. & Ashihara, H. (1983). Purine metabolism in cotyledons and embryonic axes of Black gram (*Phaseolus nungo* L.) seedlings. *Int. J. Biochem. 15*, 1059-1065.

Ogutuga, D. B. A. & Northcote, D. H. (1970a). Caffeine formation in tea callus tissue. *J. Expt. Bot. 21*, 258-273.

Ogutuga, D. B. A. & Northcote, D. H. (1970b). Biosynthesis of caffeine in tea callus tissue. *Biochem. J. 117*, 715-720.

Poulton, J. E. (1981). Transmethylation and demethylation reactions in the metabolism of secondary plant products. In: P. K. Stumpf and E. E Conn (Eds), *The Biochemistry of Plants.* Vol. 7, Secondary plant products. Academic Press, London, pp. 667-723.

proiser, E. & Serenkov, G. P. (1963). On caffeine biosynthesis in tea leaves. *Biokhimia 28*, 857-861.

Roberts, M. F. & Waller, G. R. (1979). *N*-methyltransferase and 7-methyl-*N*-nucleoside hydrolase activity in *Coffea arabica* and The biosynthesis of caffeine. *Phytochemistry 18*, 451-455.

Ross, C. W. (1981). Biosynthesis of nucleotides In: P. K. Stumpf and E. E. Conn (Eds), *The Biochemistry of Plants*. Vol. 6, Proteins and Nucleic acids. Academic Press, London, pp. 169-205.

Sandberg, G., Crozier, A. & Emstsen, A. (1987). Indole-3-acetic acid and related compounds. In: L. Rivier, A. Crozier (Eds), Principles and Practice of Plant Hormone Analysis, Vol. 2., Academic Press, London, pp. 169-301.

Schubert, K. & Boland, M. J. (1990). The ureides. In: B. J. Miflin & P. J. Lea (Eds), *The Biochemistry of Plants*. Vol. 16, Intermediary Nitrogen Metabolism. Academic Press, London, pp. 197-282.

Schultess, B. H. & Baumann, T. W. (1995a). Stimulation of caffeine biosynthesis in suspension-cultured coffee cells and the in situ existence of 7-methylxanthosine. *Phytochemistry **38***, 1381-1386.

Schultess, B. H., Wyss, G. S. & Baumann, T. W. (1991). The effect of ethephon and adenine on purine alkaloid synthesis in coffee cell suspension cultures. *Proceedings of the 14 th International Colloquim on Coffee,* San Francisco, ASIC, Paris, pp. 601-607.

Schulthess, B. H. & Baumann, T. W. (1995b). Are xanthosine and 7-methylxanthosine caffeine precursors? *Phytochemistry **39***, 1363-1370.

Schulthess, B. H., Morath, P. & Baumann, T. W. (1996). Caffeine biosynthesis starts with the metabolically channelled formation of 7-methyl-XMP a new hypothesis. *Phytochemistry 41*, 169-175.

Sealy, J. (1958). A Revision of the Genus *Camellia*. *Royal Horticultural Society, London.*

Shimazaki, A. & Ashihara, H. (1982). Adenine and guanine salvage in suspension cultured cells of *Catharanthus roseus*. *Ann. Bot. **50***, 531-534.

Sonne, J. C., Buchananu, J. M. & Delluva, A. M. (1948). Biological precursors of uric acid I. The role of lactate, acetate, and formate in the synthesis of the ureide groups of uric acid. *J. Biol. Chem. 173*, 69-79.

Sonne, J. C., Lin, I. & Buchanan, J. M. (1956). Biosynthesis of the purines (precursors of the nitrogen atoms of the purine ring). *J. Biol. Chem. **220***, 369-378.

Suzuki, T. (1972). The participation of *S*-adenosylmethionine the biosynthesis of caffeine in the tea plant. *FEBS Lett. **24***, 18-20.

Suzuki, T., Ashihara, H. & Waller, G. R. (1991a). Distribution and metabolism of purine alkaloids in tea and other *Camellia* plants: A review. *Proceedings of the International Symposium on Tea Science,* Shizuoka, Japan, pp. 110-114.

Suzuki, T., Ashihara, H. & Waller, G. R. (1991b). Seasonal variations in the content and metabolism of caffeine in tea leaves. *Proceedings of the International Symposium on Tea Science,* Shisuoka, Japan, pp. 170-174.

Suzuki, T., Ashihara, H. & Waller, G. R. (1992). Purine and purine alkaloid metabolism in *Camellia* and *Coffea* plants. *Phytochemistry **31***, 2575-2584.

Suzuki, T. & Waller, G. R. (1982). Metabolism of caffeine in sterile callus tissue cultures of *Coffea arbica. Proceeding of the 5 th International Congress of Plant Tissue Culture,* pp.385-386.

Suzuki, T. & Takahashi, E. (1975a). Metabolism of xanthine and hypoxanthine in the tea plant (*Thea sinensis* L.). *Biochem. J. **146***, 79-85.

Suzuki, T. & Takahashi, E. (1975b). Biosynthesis of caffeine by tea leaf extracts (enzymatic formation of theobromine from 7-methylxanthine and of caffeine from the theobromine). *Biochem. J. **146***, 87-96.

Suzuki, T. & Takahashi, E. (1976a). Metabolism of methionine and biosynthesis of caffeine in the tea plant (*Camellia sinnensis* L.). *Biochem. J. **160***, 171-179.

Suzuki, T. & Takahashi, E. (1976b). Further investigation of the biosynthesis of caffeine in tea plants (*Camellia sinensis* L.). *Biochem. J. 160,* 181-184.

Suzuki, T. & Takahashi, E. (1976c). Caffeine biosynthesis in *Camellia sinensis*. *Phytochemistry 15,* 1235-1239.

Suzuki, T. & Takahashi, E. (1977). Biosynthesis of purine nucleotides and methylated puries in higher plants. *Drug Metabolism Reviews. 6,* 213-242.

Suzuki, T. & Waller, G. R. (1984a). Biodegradation of caffeine: Formation of theophylline and theobromine from caffeine in mature *Coffea arabica* fruits. *J. Sci. Food Agric. 35,* 66-70.

Suzuki, T. & Waller, G. R. (1984b). Biosynthesis and biodegradation of caffeine, theobromine and theophylline in *Coffea arabica* L. fruits. *J. Agric. Chem. 32,* 845-848.

Suzuki, T. & Waller, G. R. (1985a). Effects of light on the production and degradation of caffeine in *Camellia sinensis* L. seedlings. *Plant Cell Physiol. 26,* 765-768.

Suzuki, T. & Waller, G. R. (1985b). Purine alkaloids of the fruits of *Camellia sinensis* L. and *Coffea arabica* L. during fruit developinment. *Ann Bot. 56,* 537-542.

Suzuki, T. & Waller, G. R. (1985c). Total nitrogen and purine alkaloids in *Camellia sinensis* throughout the year. Is caffeine a nitrogen storage compound in tea in winter? *Proceedings of International Conference on Soil and Nutrition of Perennial Crops*. In: A. T. Bachik and E. Pushparajaha (eds), *Malasian Society of Soil Science*, Kuala Lumpur, pp. 259-267.

Suzuki, T. & Waller, G. R. (1986). Total nitrogen and purine alkaolids in the tea plant throughout the year. *J. Sci. Food Agric. 37,* 862-866.

Suzuki, T. & Waller, G. R. (1988). Metabolism and analysis of caffeine and other methylxanthines in coffee, tea, cola, guarana and cacao. In: H. F. Linskens & J.

F. Jackson (Eds), *Modern methods in plant analysis*. Vol. 8, Analysis of nonalcoholic beverages. Springer-Verlag, Heidelberg, pp. 184-220.

Takeo, T. (1992). Semi-fermented tea. In: K. C. Willson & M. N. Clifford (Eds), *Tea cultivation to consumption*. Chapman and Hall, London, pp. 413-457.

Thomas, R. & Schrader, L. E. (1981). Ureide metabolism in higher plants. *Phytochemistry 20*, 361-371.

Timson, J. (1977). Caffeine. *Mutation Research 47*, 1-52.

Ulbricht,T. L. V.(1964). Purines, pyrimidines and nucleotides and the chemistry of nucleic acids. In: R.Robinson (Ed), A course in organic chemistry. Vol. 25, Pergamon Press, New York, pp. 1.77.

Viani, R. (1991). The active constituents of tea; a brief review. *Proceedings of the International Symposium on Tea Science,* Shizuoka, Japan, pp. 1-5.

Waller, G. R., Ashihara, H. & Suzuki, T. (1993). Updated review of purine and purine alkaloid metabolism in *Coffea* and *Camellia* plants. *Proceedings of the 15 th International Colloquim on Coffee,* Montpellier, ASIC, Paris, pp. 141-154.

Waller, G. R., MacVean, C. D. & Suzuki, T. (1983). High production of caffeine and related enzyme activities in callus cultures of *Coffea arabica* L. *Plant Cell Reports 2*, 109-112.

Waller, G. R. & Suzuki, T. (1989). Caffeine metabolisim by *Coffea arabica* L. fruit. *Proceedings of the 13 th International Colloquim on Coffee,* ASIC Paris, 350-361.

Yabuki, N. & Ashihara, H. (1991). Catabolism of adenine nucleotides in suspension-cultured plant cells. *Biochim. Biophys. Acta. 1073*, 474-480.

GLASGOW UNIVERSITY LIBRARY