CLASSIFICATION OF AN *OCIMUM* GERMPLASM COLLECTION (NCRPIS, AMES) AND INVESTIGATION OF ANTIFUNGAL ACTIVITY

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A thesis submitted to the Institute of Biomedical and Life Sciences of
The University of Glasgow
for the degree of
Doctor of Philosophy

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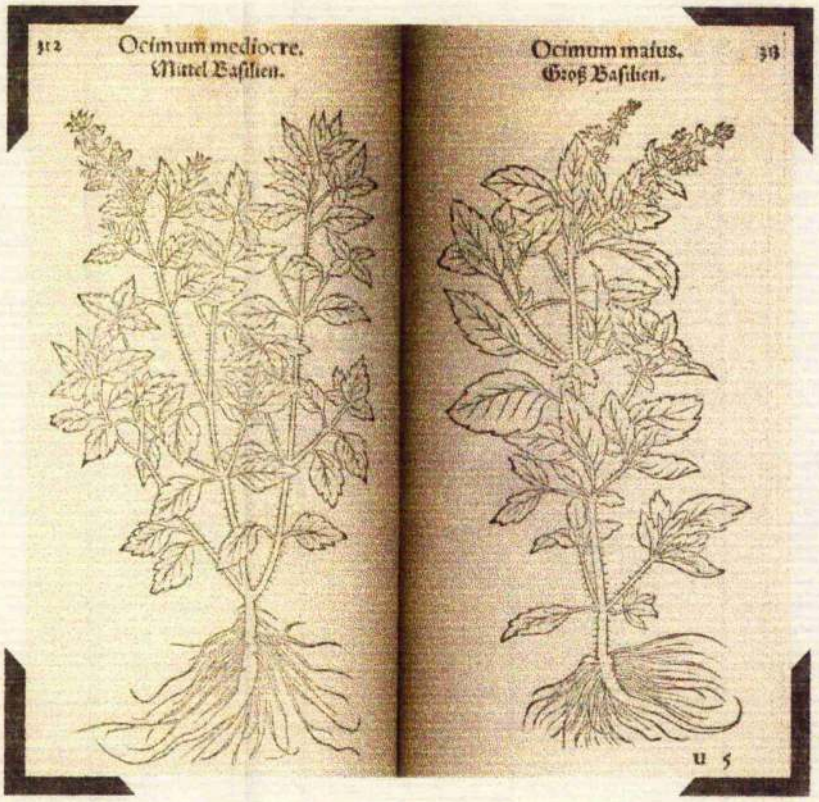
Auchincruive Estate, Ayr Campus
December 2003



Fuch's Botanical (1545)

Leonhard Fuch classified three species of *Ocimum* in his botanical in 1545: *O. minutum* (small leaves), *O. mediocre* (mid-sized leaves) and *O. maius* (large leaves).

ABSTRACT

The genus *Ocimum* belongs to the family Labiatae and is distributed worldwide, growing in the warmer parts of both hemispheres. It is an important genus with many medicinal, veterinary, pesticidal and culinary uses. The taxonomic classification of *Ocimum* is difficult and confusing. Accordingly, a joint effort began in 1999 between the PI Station at Ames, Iowa and SAC (Scottish Agricultural College) Auchincruive, Ayr to classify the *Ocimum* germplasm collection using classical morphological comparison coupled with chemotaxonomy (GC and GC-MS). Overall, fifty species of *O. basilicum*, thirteen *O. americanum*, seven *O. tenuiflorum*, three *O. gratissimum* and a single accession of *O. selloi* were characterised. Novel chemical profiles were discovered in the collection including *O. americanum* species containing up to 71% fenchone, 47% camphor and 30% limonene and a caryophyllene chemotype of *O. tenuiflorum*. Considering components ≥5%, limonene was restricted to accessions of *O. americanum*. Also, on examination of components ≥5%, several appeared to be species specific within this collection.

High oil yields (up to 5%) were found in *O. americanum* species from Zambia. These yields are exceptional for plants of the genus *Ocimum*. Plants of *O. basilicum* were found to differ in phenotype while producing a similar chemical profile. Plants grown in both Iowa and the west of Scotland produced oil of almost identical quality and quantity. In contrast, *O. americanum* plants were phenotypically alike but produced an array of essential oil profiles. In addition, variation between essential oils from leaves and flowers of *O. tenuiflorum* was also found.

Antifungal testing of selected *O. basilicum* oils and individual components demonstrated activity *in vitro* with concentrations as low as 2ppm, with methyl chavicol and linalol giving the largest reductions in fungal growth *in vitro*. In glasshouse experiments, good control of *Botrytis fabae* and *Uromyces viciae-fabae* infection of broad bean was also achieved with foliar application of selected *Ocimum* essential oils and individual chemical components. *O. basilicum* essential oils and individual components on polyamine biosynthesis, catabolism and excretion were investigated. As an initial approach to determining the mode of action of the essential oil of *Ocimum basilicum*, it was decided to study effects on fungal polyamine metabolism. Although test compounds did not significantly deplete intracellular levels of the major polyamines spermine, spermidine, putrecine and cadaverine, definite effects were seen on the activity of key biosynthetic and catabolic enzymes.

This work provides, for the first time, a detailed chemotype analysis of the genus *Ocimum* and shows that the whole essential oil and some of its individual components possess fungicidal activity. With increasing interest in the use of plant essential oils as crop protection agents, the essential oil of *Ocimum* may provide a useful additional means of controlling plant pathogens.

ACKNOWLEDGEMENTS

I am grateful for the award of the W J Thomson scholarship and the provision of facilities with which to carry out my investigations.

I am thankful to my supervisor Dr Katja Svoboda for the help, support and encouragement she has given me during my years at Auchincruive.

I am grateful to Prof Dale Walters who helped make this project possible and provided guidance and invaluable advice along the way.

I am also grateful to Dr Mark Widrlechner and Kathy Reitsma from NCRPIS, Ames for allowing me to analyse their International *Ocimum* Germplasm Collection and for all their help throughout the project.

The help of Prof Yoshinori Asakawa and assistant Miss Nishiki in the identification of essential oil components was greatly appreciated, as was that of John Black for providing basil oils and Andrew Syred for microscopic analysis.

I would like to thank the staff of the Plant Biology Department who helped me during my time there and Jim, Tommy and Jan at the gardens for all their help. I would like to extend my thanks to the many other staff at Auchincruive and elsewhere who gave me help and advice throughout my degree and PhD.

Last but by no means least I would like to express my profound appreciation to my husband Scott, my daughters Stacey and Nicola and my parents for their support and unending patience and understanding.

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LIST OF ABBREVIATIONS

A	amp
ADC	arginine decarboxylase
AdoMet	adenosylmethionine
AdoMetDC	adenosylmethionine decarboxylase
am	ante meridiem
APC	aminopropylcadaverine
BCPC	British Crop Protection Council
BGCI	Botanic Gardens and Conservation International
BSA	bovine serum albumin
c.	circa
°C	degrees Celsius
CABI	CAB International
CAM	crassulacean acid metabolism
cat.no.	catalogue number
CBD	Convension on biological diversity
CHA	cyclohexylamine
Ci	Curie
cDNA	complementary deoxyribose nucleic acid
CDP-ME	cytidine-diphospho-methylerythritol
cm	centimetre
cm^2	centimetre squared
Co.	Company
$^{14}CO_2$	radiolabelled carbon dioxide
CTP	cytidine triphosphate
cv	cultivar
d	day
DAHP	deoxy-arabino-heptulosonate phosphate
DAO	diamine oxidase
DFMO	difluoromethylornithine
DMAPP	dimethylallyl diphosphate
DNA	deoxyribose nucleic acid
DOXP	deoxy-xylulose-phosphate
dpm	disintigrations per minute
DTT	dithiothreitol
EC	European Community
EDTA	ethylenediaminetetra-acetic acid
EPSP	enolpyruvylshikimate phosphate
ER	endoplasmic reticulum
et al	and others
eV	electron voltage
FAO	Food and Agriculture Organiation
FPP	farnesyl diphosphate
FS	Forest Services
FW	fresh weight
g	gramme
GABA	gamma amino butyric acid

GC	Gas chromatography
GC-MS	Gas chromatography-mass spectromertry
GLC	Gas liquid chromatography
GP	glyceraldehyde-phosphate
GRIN	Germplasm Resource Information Network
GPP	geranyl diphosphate
h	hour
HCL	Hydrochloric acid
HMBDP	hydroxy-methylbut-enyl diphosphate
HMG-CoA	hydroxy-methylglutaryl CoA
in vitro	in an artificial environment (in glass)
in vivo	in the living organism
IPP	isopentenyl diphosphate
IRAD	L'institut de Recherche contre les Cancers de l'Appareli Digestif
IRRI	International Rice Research Institute
IVNo.	Inventory number
IVP	Inventory place
IVS	Inventory species
KH_2PO_4	potassium dihydro orthophosphate
km	kilometre
km^2	kilometre squared
KOH	potassium hydroxide
l	litre
$l\text{-}^{14}C$	radiolabelled carbon
LD_{50}	lethal dose to 50% of a population
LDC	lysine decarboxylase
Ltd	limited
M	molar
m	metre
m^2	metre squared
MECDP	methyl-erythritol cyclodiphosphate
MEP	methyl-erythritol-phosphate
$MFMOCH_3$	monofluoromethyl-dehydro-ornithine methyl ester
mg	milligram
MGBG	methylgloxal bis guanylhydrazone
$MgSO_4$	magnesium sulphate
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
mmol	millimole
mol	mole
MVA	mevalonic acid
NADPH	nicotinamide adenine dinucleotide phosphate
$NaNO_3$	sodium nitrate
NCGRP	National Centre for Genetic Preservation
NCRPIS	North Central Region Plant Introduction Station
NGRL	National Germplasm Resource Laboratory

NGRP	National Germplasm Research Programme
NMR	nuclear magnetic resonance
No.	number
NPGS	National Plant Germplasm System
NPK	Nitrogen Potassium Phosphate
NRCS	National Resource Conservation Service
NRE	National Resources and Environment
NSSL	National Seed Storage Laboratory
ODC	Ornithine decarboxylase
p	page
pp	pages
PAO	polyamine oxidase
PEP	phosphoenolpyruvate
pers.comm.	personal communication
PETL	Plant Exploration and Taxonomy Laboratory
PI	Plant Introduction
pm	post meridiem
post-	after
ppm	parts per million
pre-	before
psi	pounds per square inch
RH	relative humidity
RIA	radio immunosorbent assay
RNA	ribose nucleic acid
rpm	revolutions per minute
s	seconds
SAC	Scottish Agricultural College
SBML	Systematic Botany and Mycology Laboratory
sp.	species (singular)
spp.	species (plural)
SSAT	spermidine/spermine acetyl transferase
t	tonne
TLC	thin tissue chromatoraphy
Tris	trizma base
t/yr	tonne pr year
UCI	University of California, Irvine
UCSD	University of California, San Diego
UK	United Kingdom
US	United States
USDA	United States Department of Agriculture
UV	Ultraviolet
v/v	(volume/volume)
v/w	(volume/weight)
w/w	(weight/weight)
YACS	yeast artificial chromosomes
>	greater than
<	less tan
≤	less than or equal to
≥	greater than or equal to

α	alpha
β	beta
δ	delta
γ	gamma
m	milli
n	nano
μ	micro
=	equals
%	percent
™ or ®	registered trademark

Eucalyptol is used to represent 1,8-cineole throughout the text
Citral is used where both neral and geranial isomers are present

1 LITERATURE REVIEW

PART A: CLASSIFICATION AND PHYTOCHEMICAL INVESTIGATION OF *OCIMUM* SPECIES

1.1 THE IMPORTANCE OF BIODIVERSITY

Evolutionary biologists, taxonomists and conservationists have been declaring a biodiversity crisis for many years. However, a general lack of understanding of what this actually means and the consequences have resulted in their cries essentially falling on deaf ears. Biological diversity is the variation among all living things on earth, including the genetic variability below the species level which can be seen as chemical, morphological and physiological differences and ultimately DNA sequence variation. The term also encompasses the variation in habitat and environment with different ecosystems supporting unique combinations of living organisms interacting with each other and the soil, water and air which is around them. Although often distinctly different and separate, the actions of organisms in one ecological niche can affect others in all parts of the world (Secretariat of the CBD, 2001).

Including plants, animals and microorganisms, around 1.75 million species have been identified and estimates of the total number in existence range from 3 to 100 million (Secretariat of the CBD, 2001). This is the result of over 3.5 billion years of evolution including natural occurrences such as atmospheric changes, species interaction, hybridisation and speciation. In addition, mutation, adaptation and ultimately natural selection along with human artificial selection have led to the massive array of genetic material contained in living organisms today (Postlethwait & Hopson, 1992). This resulting biological diversity supplies the raw materials for our agriculture, horticulture,

pharmaceutical, medicinal and fuel industries, not to mention the fundamentals such as clean air, water, food, clothing, shelter and waste treatment, most of which we take for granted (UNDPI, 1997; BGCI, 2003). It allows plants to adapt and us to breed or engineer them to be resistant to pests and diseases. It enables us to grow plants in new environments e.g. with different temperature, water availability or salinity. We can develop plants to be higher yielding, more nutritious, last longer or simply look nicer.

Although events such as forest fires, floods, volcanic eruption, winds and earthquakes can result in a great number of species losses, the threats to biodiversity cannot solely be attributed to natural events. Ever increasingly human activities are having a profound detrimental effect on our natural resources. The predominant cause of extinctions being habitat destruction especially forests, wetlands and reefs associated with deforestation, pollution and other human developments (UNDPI, 1997; Secretariat of the CBD, 2001; Kirchner, 2002). It is predicted that 34,000 plant and 5,200 animal species face extinction. Thirty percent of breeds of our main farm animals are likely to die out along with one in eight birds. Seventy-five percent of crop varieties have been lost since 1900 and according to the FAO (Food and Agriculture Organisation) a further 50,000 will disappear each year. As the world's population obtains 90% of its energy from 20 food crops, with 50% coming from only four (rice, maize, wheat and potatoes) (UNDPI, 1997), measures must be taken to avert a global food crisis.

In addition, 45% of the world's forests were wiped out last century, with Madagascar alone losing 96% (Kirchner, 2002). With around a quarter of medicinal plants found in rainforests, we do not only stand to lose medicines we already rely on but destroy a wealth of undiscovered potential. Other threatened areas include coral reefs and coastal

mangroves with many lost altogether and others in a vulnerable state. Overall, extinctions are occurring at 100 to 1000 times higher than background rates (Johnson, 2002) and unless there are dramatic changes this trend will continue. Many countries are dealing with this problem by collecting and maintaining germplasm. There are around 1,600 botanic gardens worldwide holding tens of thousands of species (BGCI, 2003). The largest facility of this type is run by the United States Department of Agriculture (USDA).

1.2 THE ROLE OF THE USDA: PAST AND PRESENT

In 1862, President Abraham Lincoln founded the United States Department of Agriculture (USDA). At that time, the Department provided information on obtaining good seed and cultivation methods for the 90% of the people who were farmers. Today, although a mere 2% of American people belong to farming communities, the USDA provides a service to every American, many overseas partners and through conservation efforts benefits all around the world. The USDA's mission is to provide safe, affordable, nutritious and accessible food. A huge effort is also made to provide affordable housing with clean running water, electricity, telecommunications and nowadays, medical links and distance learning opportunities to encourage the development of strong communities able to trade in world markets. The department prides itself on being the largest exporter of agriculture products and the largest provider of assistance to developing countries. Education, technical assistance and food aid are given which in return can open up new markets for American people.

The USDA is also a world leader in research. In addition to increasing efficiency and quality of crops and livestock and improving human and animal health, many products

are developed using energy and other non-food crops. The department also endeavours to increase water use efficiency and reduce the use of chemical pesticides as part of their extensive conservation programme (USDA, 2002).

1.2.1 Conservation in the USDA

The main division of the USDA which deals with conservation is the Natural Resources and Environment (NRE) mission area; the goal of which is to ensure the health of the land through sustainable management. Such work is carried out by two agencies; the Forest Services (FS) and the Natural Resource Conservation Service (NRCS). The FS maintain species diversity, ecological productivity and overall health and promote the rural economic development of over 191 million acres of national forest and grassland and 472 million acres of private forests. These areas not only supply the raw materials for industry but provide recreation opportunities and possess a wealth of invaluable genetic material. The FS also model fire, insect, disease and drought cycles in a bid to understand their involvement in shaping ecosystems (NRE, 2002).

The NRCS is involved in 3,000 conservation sites across America. The agency works with farmers and other landowners, communities, governments, professional societies, environmental groups and volunteers. By monitoring levels of natural resources, pollution and soil erosion and promoting cultural practices such as terracing and contour farming, a great reduction in soil erosion from wind and water has been achieved. This also reduces water pollution and thus improves drinking water quality and fish and wildlife habitats (NRE, 2002).

However despite all efforts and not dismissing the great many considerable achievements, 'no one country or even continent has all the genetic resources necessary to sustain crops at the level that is needed today. Conditions and needs continue to change and collecting genetic diversity is how you have the resources to deal with them' (Stoner, 1998).

1.2.2 The National Plant Germplasm System (NPGS)

In 1898, the US department of agriculture predicted the country would be facing famine by 1931. This prompted the establishment of the plant introduction programme. Mark Carleton was sent on the department's first official plant exploration to Russia. One of the results of this trip was the introduction of new durum and hard red wheat varieties to the US. The durum wheat made tastier pasta and the hard red made better bread. In addition their drought tolerance allowed wheat to be grown on the Great Plains and the Northwest, hence in five years wheat production in the US soared from 60,000 to 20 million bushels per year. This was the first of many successful expeditions of this kind and even today most of the United States' fruit, vegetable and grain crops are those originally brought from Asia, Europe or South America. Although the US has native crops such as strawberries, pecans and tepary beans, only one of the world's top 20 major crops originated in North America; the sunflower and ironically it was a Russian researcher who used the germplasm to breed the varieties which started the sunflower oil industry (Kaplan, 1998). Today, the National Germplasm Resource Laboratory (NGRL) at Beltsville facilitates such plant explorations and assists curators at the germplasm maintenance sites (Stoner, 1998).

The base collection of the NPGS is held in the National Seed Storage Laboratory (NSSL) at the National Centre for Genetic Resources Preservation (NCGRP), Fort Collins, Colorado. With over 450,000 accessions representing more than 10,000 species, the facility is the largest genebank in the world (Becker, 1998). One of the objectives of the centre is the longterm preservation of the entire US germplasm collection. This provides a back up measure in the event of any losses from the active collections. The NSSL also preserve collections for other countries. Germplasm is stored for the International Rice Research Institute (IRRI) of the Philippines, who are concerned that a typhoon or other disaster could wipe out their valuable genetic resources (Kaplan, 1998). The centre at Fort Collins also conducts research programmes to investigate seed viability and develop new and improved technology for the longterm preservation of all forms of plant germplasm (Becker, 1998).

Since 1898, the USDA has considered its collections of genetic resources to be the common heritage of mankind. Its mission was then and still is to acquire, characterise, evaluate, document, preserve and distribute all forms of germplasm important to food and agriculture. To endorse this the department has always maintained a policy of unrestricted exchange, free of charge to researchers around the world. This policy was reaffirmed by Congress in 1990 with the establishment of the National Germplasm Resource Programme (NGRP) (Stoner, 1998).

1.2.3 The Germplasm Resource Information Network (GRIN)

GRIN is a computer database maintained by the NSSL. Original data on the network came from the nomenclature files of the former Plant Exploration and Taxonomy Laboratory (PETL) which dates back to 1898. The taxonomic nomenclature of the

450,000 plus accessions managed within the NPGS are included. These are mostly plants of economic importance including food, fibre, timber and medicinal. Also included are noxious weeds and endangered plants. Names are assigned in accordance with the international rules of botanical nomenclature by taxonomists at the Systematic Botany and Mycology Laboratory (SBML) using available literature and through consultations with specialists around the world. Since 1994, this database has been freely accessible to all via the internet (Wiersema & León, 1999).

1.2.4 The North Central Region Plant Introduction Station (NCRPIS)

From the base collection at the NSSL, germplasm is distributed to the four active Plant Introduction Stations at Ames (Iowa), Geneva (New York), Griffin (Georgia) and Pullman (Washington); established in 1948 to represent the main agricultural environments of the US (Kaplan, 1998). The NCRPIS at Ames houses vast collections of corn, sunflower and a wide diversity of fruit and vegetables. In addition, the station holds collections of speciality crops such as mustard, exotic vegetables and several herbs and aromatic plants (Widrlechner, 1989; Lyons-Johnson, 1998). One of the most important collections, which provides a valuable source of germplasm but has lacked a comprehensive, published characterisation is the collection of *Ocimum* (Morales & Simon, 1996, 1997).

Germplasm is acquired predominantly from foreign and domestic plant explorations and by exchange with other genebanks and collections. Each sample is evaluated to determine whether or not it represents new germplasm for the collections. If so a Plant Introduction (PI) number is allocated and the information accompanying a sample is logged onto the GRIN database.

Different techniques are used by scientists at the plant introduction stations and throughout the world to characterise unknown accessions. These include morphological description, GC and GC-MS analyses and isozyme and DNA comparison. Yield trials and disease, abiotic and biotic stress resistance testing are also carried out. Desirable traits found have been used in many scientific studies and crop improvement and genetic enhancement programmes at NCRPIS.

The controlled pollination programme at NCRPIS is one of the most extensive in the country with many crops pollinated by hand and others by honey bees, flies or other pollinating insects contained in field cages. Seeds produced are stored in temperature (4°C) and humidity (25% RH) controlled rooms (NCRPIS, 2002).

Agriculture sustains virtually all human life on earth. The agriculture systems of the US and the NCR in particular depend predominantly on crops brought into the country years ago. Environmental changes including global warming and new cultivation methods such as reduced tillage, mean crops must be produced which can withstand exposure to new pathogens and adapt and grow in new environmental conditions. Overall, the ability of the NCR to produce high yielding food and non-food crops, to reduce chemical inputs and soil and water contamination and depletion, depends upon its germplasm collection. Not only does the station support its own region but with around 15,000 samples per year distributed to researchers worldwide, it makes a significant contribution to human welfare as a whole (Lyons-Johnson, 1998).

1.3 THE FAMILY LABIATAE

1.3.1 Taxonomy and distribution of the Labiatae

The Labiatae (Lamiaceae), the mint family, one of the largest and most distinctive groups of angiosperms, is also one of the most highly evolved of all plant families (Cantino, 1992a; Hedge, 1992). Paleobotanical and archeobotanical findings of nutlets, seeds and fruit and ancient writings of the Labiatae have been reported as far back as 6000 BC (Nuñez & de Castro, 1992), but circumstantial evidence suggests the family have been in existence since the Cretaceous period; c.100 million years ago (Hedge, 2002 pers. comm.). The family consists of over 250 genera and almost 7000 species (Wagstaff *et al.*, 1995) which are widely distributed throughout warmer climates and the tropics, with a high percentage of isolated genera (Hedge, 1992).

Due to the great diversity, classification of the family has always been difficult and since the classifications of Bentham and Briquet in the 19th century (Paton, 1992), many revisions have been suggested including Erdtamn (1945), El-Gazzar & Watson (1970) and Cantino *et al.* (1992). In addition to studies using classical anatomical, morphological and phytochemical comparison, many recent works have involved cladistic analysis using chloroplast DNA base sequence information (Wagstaff *et al.*, 1995; Paton *et al.*, 1999).

1.3.2 General morphology of the Labiatae

Plants of the Labiatae are usually herbaceous and shrubby but can be woody or climbing. They are helophytic, mesophytic or xerophytic. Stems are quadrangular, with decussate leaves which are opposite or whorrled. Stipules are absent. Flowers are hermaphrodite, mostly zygomorphic but rarely actinomorphic. They are bilabiate and

axillary or whorlled. Calyx is persistant with five united sepals which are often bilabiate. Corolla is sympetalous, tubular and bilabiate. There are two or four declinate stamens which are one or two celled. Anthers are divergent and open lengthwise. Ovaries are superior with two deeply lobed carpels with an ovule in each carpel. Style are gynobasic and stigma mostly bifid. Fruits have four nutlets which are free or paired. Seeds can be endospermic or non-endospermic, usually with straight embryos. In essential oil producing types, glandular trichomes are found on most plant parts. Photosynthetic system is C3 or CAM (Hutchinson, 1959; Watson & Dallwitz, 2000).

1.3.3 Outline of production and uses of the Labiatae

Some 40% of Labiates produce aromatic essential oils (Lawrence, 1992). Many tropical species are rich in these oils but so far have not been commercially exploited (Harley & Reynolds, 1992). Annual hecterage of Labiate production worldwide is estimated at over 500,000 hectares and oil production per year is almost 15,000 t (Lawrence, 1992). The list of uses in both past and present times for plants of the Labiatae is endless. Mints are one of the two major sources of culinary herbs (Richardson,1992). Most well known include basil, thyme, sage and oregano. In a study of medicinal plants used by North American Indian cultures, the Labiatae ranked third in ethnobotanical importance and first in the areas of neurology and pulmonary medicine with essential oils reported to be the key to their medicinal properties. Basil specifically is reported to be one of the most important plants in popular medicine (Heinrich, 1992).

Production of essential oils by plants is believed to be predominantly a defence mechanism against pests and diseases. Many studies carried out to date have investigated the antibacterial, antifungal and insecticidal properties of the Labiates and

shown them to have potential in the agriculture, horticulture and human healthcare markets. Also, essential oils produced by plants of the Labiatae have been noted as possible taxonomic markers, with oil-rich and oil-poor species corresponding with Erdtman's two subfamilies; Nepetoideae and Lamioideae (Cole, 1992).

1.3.4 The genus *Ocimum*

1.3.4a Taxonomy of *Ocimum*

As noted by Hedge (1992), classification of the Labiatae above genus level is very foggy. However, it is widely recognised by workers studying the genus *Ocimum* that classification below this level is equally difficult (Simon *et al.*, 1990; Grayer *et al.*, 1996; Paton & Putievsky, 1996). Above genus level, *Ocimum* is placed in the tribe *Ocimae* (subtribe *Ocimimae*) of the subfamily *Nepatoideae* (Cantino *et al.*, 1992). Many infrageneric classifications have been recorded, possibly the first being that by Fuch in 1545 who reported 3 species. Paton (1992) delimited the genus to 30 species, but previous reports can be found which include up to 176 species (Sobti & Pushpangaden, 1978; Martins *et al.*, 1997; Khan *et al.* 2002).

The infrageneric classification described by Paton (1992) includes sections *Ocimum* (with subsections; *Ocimum* and *Gratissima*), *Heirocymum* and *Gymnocymum* previously described by Bentham (Paton, 1992). However, in contrast to Bentham, Paton removed section *Hemizygia* and recognised subsection *Hiantia* as a separate genus; *Becium*. He also suggested that *Heirocymum* was not a natural group. A parsimony analysis by Paton *et al.* (1999) substantiated these groupings with the exception of section *Hierocymum* who's members were distributed throughout different clades as previously suspected. In a study of pollen morphology by Harley *et al.* (1992), section *Heiocymum* and

subsections *Foliosa* and *Nudicaulia*, previously reported by Briquet were retained (Paton, 1992).

The great disparity in the classification of *Ocimum* is due to the diverse morphology found within the genus. This is the result of interspecific hybridisation and the occurrence of polyploidy (Simon *et al.*, 1990), although Sobti & Pushpangadan (1978) report cross species hybridisation to be rare. The taxonomical challenge becomes no less at the intraspecific level, with possibly the most well known, *Ocimum basilicum* renound for its highly polymorphic character and freely cross-pollinating nature (Gulati, 1979; Grayer *et al*, 1996; Paton & Putievsky, 1996). Hence, to date, more than 60 varieties and cultivars of *O. basilicum* have been identified (Simon *et al.*, 1984). In addition to differences in morphological character, these plants also produce an array of essential oils, seed oils and other biologically active compounds.

1.3.4b Distribution of *Ocimum*

Even after thoroughly searching the literature, it is difficult to find a definitive origin for the genus *Ocimum*. Many articles refer to it as originating in India. This is probably due to the fact that *O. sanctum* or Holy basil known in India as Tulsi has been worshipped there for centuries. *O. sanctum* and other basil species are cultivated in India and are widely used in indigenous medicine. Although a great deal of literature can be found which associates basil with India, more and more evidence is being uncovered which suggests species to be native to Africa, tropical America and Asia, indicating multiple centres of origin (Harley *et al.*, 1992; Hedge, 1992; Paton *et al.*, 1999; Simon *et al.*, 1999).

The genus is essentially tropical. Plants can be found worldwide, distributed throughout the warmer parts of both hemispheres from sea-level to 1830m with the greatest number of species in the tropical rainforests of Africa, South America and Asia (Sobti & Pushpangadan, 1978). Figure 1 by Harley *et al.* (1992) shows a possible worldwide distribution of the genus. It is even difficult to define the present day distribution of cultivated basil. In addition to unknown and wrongly classified species, much production, especially in the developing world, is for local use and thus not recorded in official trade figures. Lawrence (1992) estimated total world production of *Ocimum* oils per annum as; *O. gratisimum* - 55t, *O. basilicum* - 42t and *O. americanum* - 100kg. The USA is by far the largest importer, with figures for 1986-1988 rising from 1400t to 1800t per year. Europe is the second largest, with imports ranging from 5t/yr (Switzerland) to 200-350t/yr (Germany, UK & France). Italy probably produces the largest quantity for the domestic market (5000t/yr) followed by France (2000t/yr) (Putievsky & Galambosi, 1999). The same authors calculated that around 1700ha of basil is grown for oil production, ~2200 ha is produced for the dried herb market and 10000-15000 ha is grown for domestic consumption. Thus overall the estimated worldwide cultivation area is around 50000 ha.

1.3.4c Morphology of *Ocimum*

Plants of *Ocimum* are annual with a taproot or suffrutices with a rhizome. They can be woody shrubs or softer. Leaves are petiolate or sessile, often serrated at the edges (Plate 1). Some of the plants growing in arid regions have deciduous leaves which produce a scar when they fall. The inflorescence is typically a thyrse supporting 1-3 (usually 3) opposite flowered cymes. Cymes are subtended by bracts which sometimes

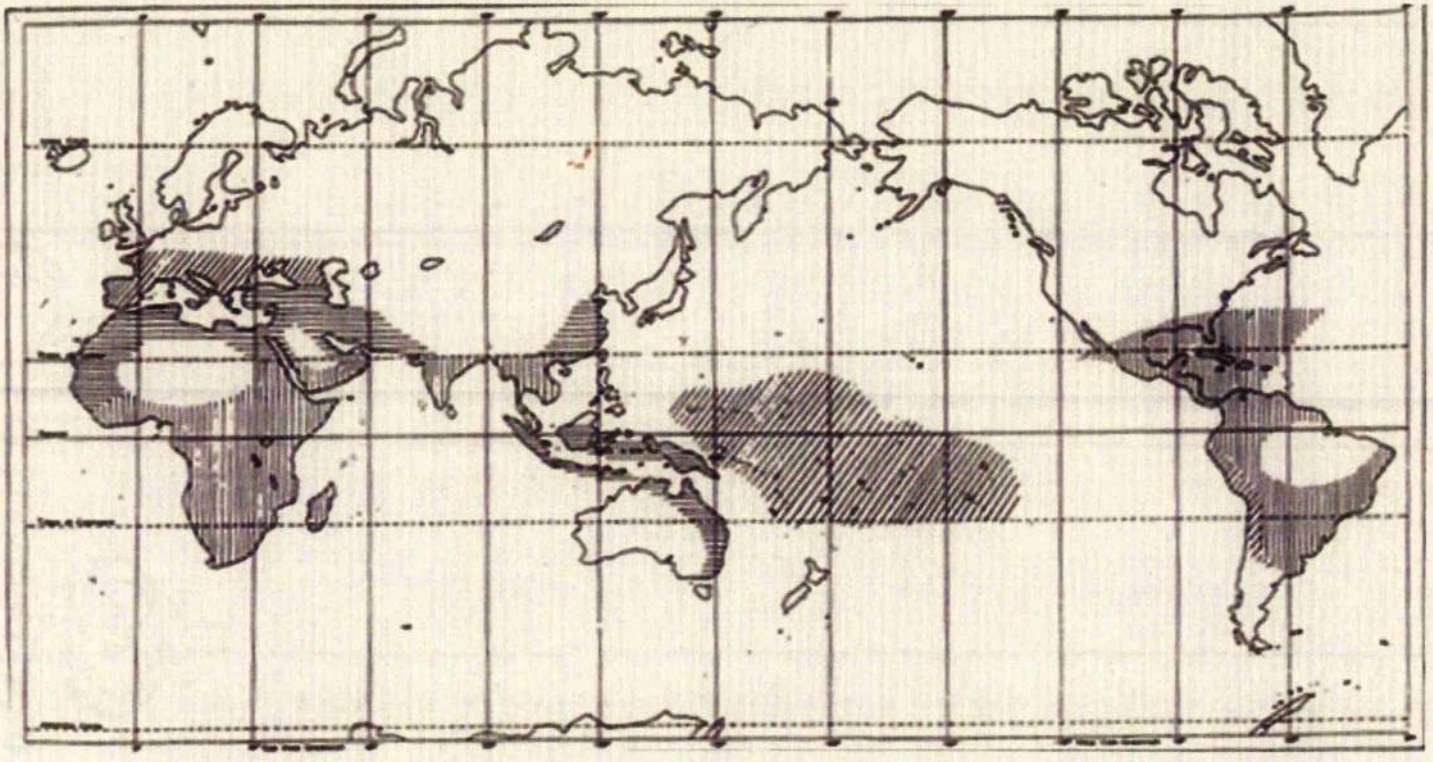


Figure 1 Worldwide distribution of *Ocimum*. Vertical shading - probable natural populations, horizontal shading - probably naturalised from cultivation but maybe natural and diagonal shading - cultivated, often naturalised populations (after Harley *et al.*, 1992)

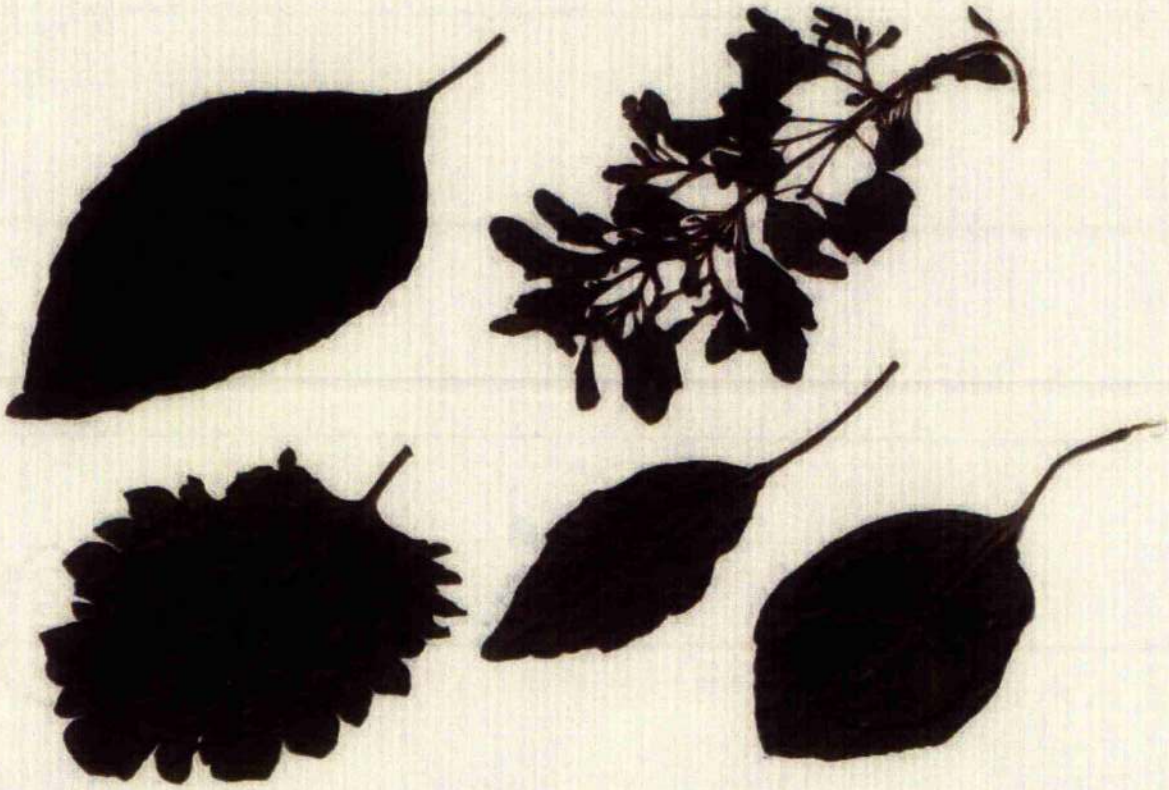


Plate 1 Variation in leaf morphology of *O. basilicum*

abscisc, leaving a scar which can develop into a bowl shaped structure which acts as a nectary. The cyme branches are fused or unfused to the stem. The calyx is tubular or funnel shaped and is usually straight or slightly curved and occasionally bent over at a right angle. It is 5-lobed with a single lobed upper lip and a 4-lobed lower lip. The upper lip is decurrent on the tube, forming inconspicuous or prominent wings. In some species the upper lip becomes membranous and swollen and the entire calyx is dispersed as opposed to the nutlets only. The throat of the calyx is open and either glabrous or bearded with a ring of hairs below the mouth. The corolla is usually straight, rarely curved downward and can be gibbous. The tube normally dilates towards the mouth but can be parallel walled. The posterior lip is 4-lobed. These can be equal or 2 median lobes can exceed 2 lateral ones. The anterior lip is entire, usually horizontal and occasionally deflexed.

The androecium consists of 4 stamens; 2 posterior and 2 anterior. The former is attached near the base of the corolla or at the midpoint, occasionally this is uncertain. The anterior are attached near the mouth. Posterior stamens can be straight or bent and have an inconspicuous, conspicuous or absent appendage. Anthers usually have 2 equal (occasionallly unequal) locules which are divergent or parallel.

The ovary of all species is in 4 parts; each part being a single seeded nutlet or mericarp. Nutlets are spherical or elliptic and are usually glabrous and rarely pubescent. They produce varying amounts of mucilage when wet. The style is gynobasic with bifid apex and subulate lobes. The disc of most species has 4 equal lobes (Paton *et al.*, 1999). Table 1 lists some distinguishing morphological features of species in the germplasm

	Ocimum spp.			
Characteristics	*O. basilicum*	*O. americanum*	*O. gratissimum*	*O. tenuiflorum*
Habit	20-60cm/herb	15-70cm/herb	60-250cm/herb	up to 1m/herb or woody herb
Leaf margin	entire-shallowly serrate	entire-serrate	serrate	serrate
Petiole	2-40mm	3-20mm	5-30mm	7-15mm
Fruiting calyx	6mm/throat open	4-5mm/throat open	6mm	3mm/throat open
Corolla	5-8mm pink/white/creamy yellow	4-6mm white/pale mauve	3-5mm greeny/dull white/pale yellow	3mm pink/white
Stamen ext	2-4mm	1-3mm/outgrowth near base	1mm	
Ovaries	glab	glab	glab	
Nutlets	black 1.5-2mm/ovoid mucilageous when wet	black 1.5mm/ovoid mucilageous when wet	brown 1.5mm+/-spherical little mucilage when wet	brown 1mm+/-ovoid little mucilage when wet

Table 1 Distinguishing morphological features of *Ocimum* spp. in NCRPIS germplasm collection

collection from NCRPIS, Ames. See also diagnostic key diagrams by Paton (1992) and Albuquirque & Andrade (1998) in Appendix 1.

1.4 VARIOUS USES OF *OCIMUM* SPECIES

1.4.1 Culinary

All parts of the basil plant, it's essential oils, oleoresins and seeds are widely utilised in the food and drink industries. The use of essential oils is now largely replacing that of dried leaves (Simon *et al*, 1984). There is a growing trend towards using herbs and spices in cooking in the UK. This is partly due to increased tourism and partly to our desire to eat healthier. Herbs and spices confer several health benefits in addition to making low fat dishes tastier (Mäkinen & Pääkkönen, 1999).

Possibly the most familiar use of basil is in Italian cooking. It is the perfect complement to tomatoes, either in a fresh side salad, in sauces and pasta dishes or as a pizza topping or garnish. It is also widely used in French, Greek and Mexican cuisine (Putievsky & Galambosi, 1999). Most meats, seafood and eggs are enhanced by basil and it is commonly added to soups and stews. Lemon types are especially enjoyable with fish. In addition to tomatoes basil enriches other vegetables including mushrooms, sweet peppers, aubergines and broccoli. This versatile herb also goes well with fruit, especially baked apple. It is often added to baked goods and breakfast cereals. Sweet dishes, confectionery, snack foods and chewing gum are flavoured with basil. Savoury butters, oils and vinegars are an excellent way to incorporate the attractive flavours of basil into almost any dish. In the near East the seeds are eaten alone or added to bread dough (Small, 1997). It's superior character can be appreciated in the French liqueur Chartreuse (Leung & Foster, 1996) and many other beers and spirits (Mäkinen & Pääkkönen, 1999). Tonic teas are made from infusions of leaves and cooling beverages made from leaves and seeds are enjoyed in places such as Barbados. Speciality products include basil lime marmalade, lemon pesto and basil cream, yoghurt and mustard.

Overall, basil can enhance the colour and flavour of a wide array of food and drinks. In addition, basil essential oils, like many others, have long since been known to reduce microbial contamination and thus extend the shelf life of food (Deans & Ritchie, 1987; Lachowicz *et al.*, 1998; Basilico & Basilico, 1999). Basil has also been found to produce an abundance of anthocyanins which could provide an inexpensive source of stable red pigment for the food industry (Phippen & Simon, 1998).

1.4.2 Medicinal

Although no basil species are noted in the British or American Pharmacopoeias or the Chinese Materia Medica, sweet basil was reinstated in the French Pharmacopoeia in 1989 (Bruneton, 1999). Medicinal uses of these species have so far been essentially restricted to indigenous systems of medicine, where knowledge is often passed on through word of mouth. However, a growing body of scientific evidence substantiating claims of the biological properties of these plants is likely to expand their use in medicine to the West.

Basil is traditionally administered orally in the treatment of gastrointestinal disorders such as indigestion, bloating, flatulence, colic and other undefined stomachaches. Members of the mint family are already recognised as having these properties and are widely used in western medicine. Basil alleviates urinary tract infections. Kidney complaints can be greatly relieved as can diarrhoea and constipation. Intestinal parasites and worms are eliminated by ingestion of the plant and possibly due mainly to anti-inflammatory effects, preparations are prescribed for respiratory complaints. In Iran, seeds are used for various chest and lung conditions. Elsewhere crushed, dried leaves are sniffed or vapours inhaled to alleviate congestion. Treatments are also widely used to

relieve symptoms of cold, flu, coughs and catarrh. In the Comoros the plant is used as an expectorant to give relief from painful ear infections and in Papua New Guinea the leaves are chewed to relieve toothache. Antimicrobial, antiinflamatory and cooling properties, cleanse, soothe and aid wound healing. In Benin, baths infused with basil are used to calm hypertension, headaches and other nervous disorders (Nuñez & deCastro, 1992; Small, 1997).

1.4.3 Veterinary

Although not widely reported in the literature, basil preparations are administered in some cases to animals. *Ocimum basilicum* is used as an expectorant. *O. micranthum* is given along with *Chenopodium ambrosioides* to treat stomach pain and colic and a basil paste is used to massage the udders (Ketzis, 2000).

1.4.4 Pesticidal

After the advent of synthetic chemicals, the use of natural products in crop protection and medicine was greatly reduced. However, increased awareness of the deleterious effects of many of these chemicals on human health and the environment has led to demand for a return to natural alternatives. In addition to medicinal applications, the biological properties of *Ocimum* species such as antifungal, insecticidal and aphicidal afford them great potential in the areas of agriculture and horticulture. Many investigations of such attributes have given scientific back up to applications employed by indigenous systems. *O. sanctum* extracts have been shown to give good control of phytopathogenic fungi; *Pyricularia oryzae*, *Cochliobolus miyabeanus* and *Rhizoctonia solani* (Tewari & Najak, 1991). Khanna *et al.* (1991) demonstrated *O. gratissimum*

essential oil to inhibit fungal, bacterial and viral plant pathogens and Rai *et al.* (1999) found *O. basilicum* to be effective against several *Fusarium* species.

Basil is reported to repel flies and mosquitos and studies have shown nematocidal and allelopathic activity. Also juvocimene 1 and 2 found in basil are potent juvenile hormone mimics (Leung & Foster, 1996). A comprehensive review of the bioactivity of basil and its essential oils is given by Holm (1999).

1.4.5 Other uses

Sweet basil is classically used in the perfumery industry. The chemical content of the oils is very important. Different chemical components command different prices on the world market (Putievsky & Galambosi, 1999). Methyl chavicol is used in the manufacture of fine fragrances, while linalol type oils are usually constituents of lower value perfumes (Bruneton, 1999).

Basil oils are widely used in soaps, shampoos and other hair products. In some parts crushed leaves are used to dress and scent the hair and are believed to retard the greying process (Nuñez & deCastro, 1992). Many toothpastes and mouthwashes also contain basil (Holm, 1999). Seed oils which predominantly contain linoleic, linolenic, oleic and palmitic acids are commonly used in the cosmetics industry (Putievsky & Galambosi, 1999).

Many different types of basil are highly ornamental. They are used in bouquets and flower arrangements and in plantings (Foster, 1975; Simon & Reiss-Bubenhiem, 1988). Powdered basil is used as snuff and burning basil oil not only creates a pleasant aroma,

but inhalation of vapours is said to sharpen the mind and increase concentration. The latter was verified in a study carried out at SAC, Auchincruive (Svoboda *et al.*, 2002). One of the oldest uses is possibly in religious ceremonies including marriage. In India, basil is referred to as tulsi. It is considered sacred and has been worshipped and utilised there for centuries.

1.5 BIOCHEMISTRY OF ESSENTIAL OILS

1.5.1 Glandular trichomes in the family Labiatae as the site of essential oil production

The essential oils of Labiatae species are produced in external secretory structures called glandular trichomes. Most of these plants, including those in the genus *Ocimum*, have both peltate glands and capitate hairs (Hardman, 1972; Werker *et al.*, 1993; Gang *et al.*, 2001). Glandular trichomes have a stalk consisting of one or more cells. Attached to each stalk is a gland head, which in the Labiatae differ in cell number, from the single celled capitate hairs of *Ocimum basilicum* to the 18 celled peltate gland heads of *Mentha fruticosa*. In most species of Labiatae examined, head cells of the peltate glands consist of four middle cells surrounded by a number of cells forming two distinct circles. *Ocimum* peltate glands have typically only four cells. Capitate hairs can have variable head structures as in *Salvia* species or be of one type like *Coridothymus capitatus*. A unicellular stalk and head is most common (Werker *et al.*, 1993). Not all essential oil producing plants have glandular trichomes, the oils are often produced in internal structures such as the secretory cells of cinnamon (Lamiaceae) bark and secretory sacs in citrus (Rutaceae) rind. Umbeliferae such as fennel have secretory canals throughout, poppy (Papaveraceae) have laticifers and pine species (Pinaceae) have secretory ducts in their needles (Hardman, 1972; Fahn, 1979, 1987; Werker *et al.*, 1994).

1.5.1a Initiation, distribution and basic morphological characteristics of glandular trichomes

In developmental studies Hanlidou *et al.* (1991) found glandular trichomes to be formed prior to stomata. In some plants trichomes occur during the normal course of development while some have been found to be produced in response to stimuli. The following evolutionary trends for the different types has been suggested. Internal

secretory cells ⇒ internal secretory ducts and cavities ⇒ external glandular trichomes. It is thought that secretory tissue which develops in response to stimuli preceded sectretory tissue formed during the normal course of development and that non-glandular trichomes evolved into glandular trichomes (Fahn, 1987).

Epidermal cells differentiate into the characteristic glandular structures. These are usually short-stalked (occasionally long) and can be slightly sunken into the epidermal tissue, often on the underside of the leaf which provides protection (Hardman, 1972; Hanlidou *et al.*, 1991). Plate 2 shows a peltate glandular trichome of *Ocimum basilicum*. Turner *et al.* (2000b) noted prolonged gland initiation and largest number of glands on abaxial basal and middle zones of *Mentha piperita* (mint) and found new and mature glands occuring together within leaf zones. Werker *et al.* (1993) also made these observations in *Mentha*. The number of glandular trichomes per leaf is genetically determined and although the number rises as the leaf gets larger, the density of trichomes decrease with increasing leaf area. Qualitative and quantitative changes in volatile oil occur with leaf age and location and stage of trichome development (Maffei *et al.*, 1989; Werker *et al.*, 1993). Work referred to by Gough (1995) suggests that change in leaf orientation and UV light could be responsible for these variations.

The walls of the peltate glands are cutinised and a cuticle proper and cuticle layer cover the gland head, between which a subcuticular space develops and essential oil accumulates (Fahn, 1979). Plate 3 illustrates the raised cuticle of a peltate gland of *Ocimum basilicum*. Essential oil does not accumulate in the subcuticular space of the capitate hairs to the same extent as it does in the peltate glands. When the cuticle of the peltate gland ruptures, a large amount of essential oil is released very quickly, whereas

Plate 2 Scanning electron micrograph of peltate glandular trichome on leaf surface of *Ocimum basilicum* (seed source: Richters)

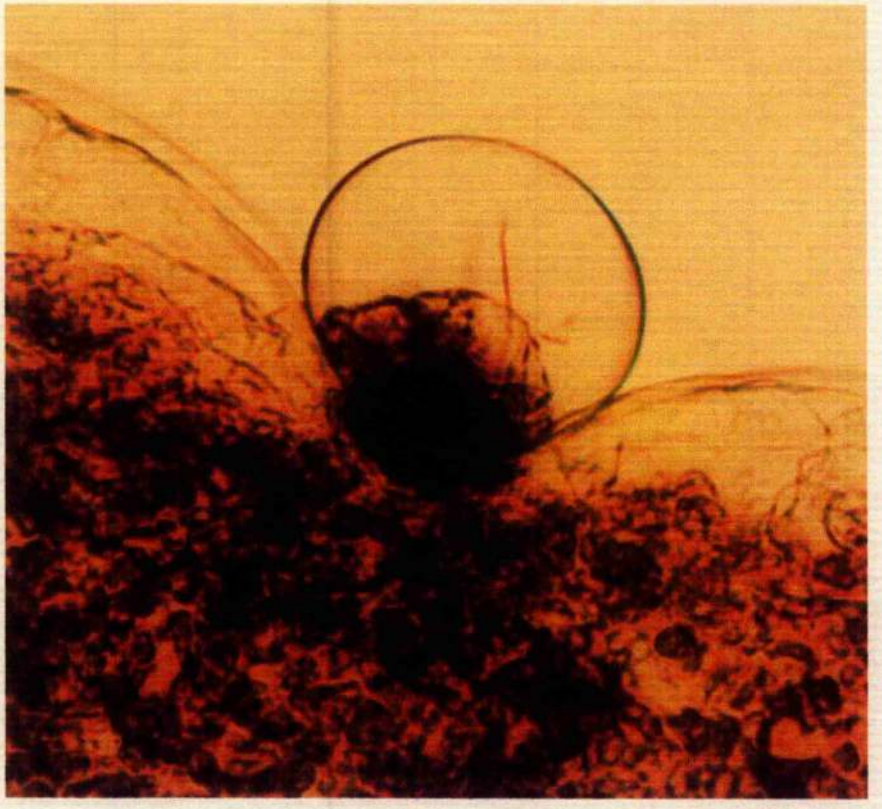


Plate 3 Photomicrograph of glandular trichome on upper leaf surface of *Ocimum basilicum* (seed source: Richters)

capitate glands continuously release small amounts of oil, hence the absence of the extended dome. The Labiatae have both types of glands providing a dual protection system (Werker *et al.*, 1993). Illustrations of a wide range of secretory structures including glandular trichomes can be found in Svoboda & Svoboda (2001).

1.5.1b Ultrastructural changes in glandular cells

Until the late 1980s, the lack of techniques to isolate cells from glandular trichomes hampered biosynthetic studies on terpenes. The surface abrasion technique developed by Gershenzon made progress possible (Gershenzon, 1987, 1992). Several workers believed terpene synthesis occured in the glands (Schnepf, 1974; Fahn, 1979; Sevinate-Pinto & Antunes, 1991) and supporting evidence of this soon accumulated. Gershenzon found that the activity of geranyl pyrophosphate phosphohydrolase, limonene synthase and limonene hydroxylase was localised to the glands in *Mentha* spp. (Gershenson, 1987). McKaskill *et al.* (1992) found isolated gland cells of peppermint to be non-specifically permeable and when provided with the necessary precursors and substrate the cells proved capable of *de novo* synthesis of monoterpenes. Yamaura *et al.* (1991) studied thymol and γ-terpineol and using quantitative analysis of essential oils and tracer experiments, determined that essential oil production took place in the glandular trichomes of thyme. Duke & Paul (1993) determined that the plastids were highly involved in terpene biosynthesis in the glands of annual wormwood. They also found that glandular chloroplasts contained lower levels of starch than mesophyll chloroplasts. The discovery in 1993 by Rohmer *et al.* of the deoxy-xylulose phopshate (DOXP) pathway and suspected influx/efflux of metabolites across the plastid membrane during terpene synthesis substantiated these findings and explained the many anomalies found by workers carrying out tracer experiments in the study of terpene biosynthesis. Detailed

descriptions of biosynthetic pathways involved in essential oil production can be found in Section 1.5.2.

1.5.1c Microscopic evidence of ultrastructural transformations

Ultrastructural changes during oil production in glandular secretory cells, such as the appearance of highly developed leukoplasts containing fibrous bundles were noted as far back as the 1960s, as were extensive endoplasmic reticulum (ER), abundant vacuoles, and mitochondria (Loomis, 1967; Schnepf, 1974; Fahn, 1979). Endopolyploidy and binucleate cells have also been observed in glandular trichomes (Corsi & Pagni, 1990). From such observations it was suggested that plastids and ER at least were involved in essential oil biosynthesis and transport.

More recently, using cryofixation and electron microscopy, Turner *et al.* (2000a) illustrated and discussed in depth, the ultrastructural transformations in gland head and stalk cells of *Mentha piperita*. The former supported the idea that leukoplasts and ER played major roles in essential oil biosynthesis and transport. Changes observed in stalk cells were believed to be in relation to carbon substrate supply. Bourett *et al.* (1994) reported 'budding' of the plasmalemma during essential oil production of *Nepeta racemosa*. Abundant mitochondria were seen close to this activity, suggesting that they provided energy for this export of oil which is against a concentration gradient.

1.5.1d Immunochemical and molecular studies of essential oil biosynthesis

Microscopic investigations, enzyme assays and tracer experiments using radiolabelled substrates have provided a vast amount of information on the structure and biochemistry of glandular trichomes. However, a comprehensive biosynthetic map of the glandular

trichomes has not yet been constructed. Immunochemical and functional genomics approaches now taken by many workers have overcome problems experienced in classical biochemical studies and have proved to be powerful tools in the identification of biosynthetic enzymes and subcellular compartmentalisation of steps in the pathways of essential oil production. Turner *et al.* (1999) confirmed from studies using polyclonal antibodies that the initial steps of monoterpene biosynthesis took place in the leucoplasts of *Mentha* gland cells. Antibodies were raised to limonene synthase in the leucoplasts of peppermint peltate gland secretory cells during the period of essential oil production while labelling was absent from stalk, basal and mesophyll cell plastids. A small amount of label was detected in the peltate secretory cell leucoplasts during pre- and post-secretory stages and in capitate glands. From DNA sequence information, limonene synthase was found to bear an amino terminal plastid targetting (transit) peptide. However, the fact that limonene hydroxylase possesses an amino terminal membrane insertion sequence suggests subsequent steps in the pathway are carried out in one or more other subcellular locations.

Lange *et al.* (1999) constructed a cDNA library of the mint peltate gland. As expected there were no genes for the mevalonate pathway but several involved in the deoxy-xylulose pathway and further terpenoid synthesis were identified. Lange *et al.* (1998) characterised the first enzyme of the pathway as deoxy-xylulose phosphate synthase. Lange & Croteau (1999a, b) described both deoxy-xylulose phosphate reductoisomerase and isopentenyl monophosphate kinase and Ramos-Valdivia *et al.* (1997) characterised IPP isomerase.

Burke *et al.* (1999) cloned geranyl diphosphate synthase cDNAs and found that coexpression of both a large subunit and a small subunit were required to produce functional geranyl diphosphate, thus confirming the enzyme's heterodimeric nature. Other enzymes found later in terpene biosynthesis include: 1,8 cineole synthase, menthofuran synthase, isopiperitenone reductase, pulegone reductase, sabinene synthase, bornyl diphosphate synthase and menthol acetyltransferase (Wise *et al.*, 1998; Lange *et al.*, 1999).

Gang *et al.* (2001) concluded from studies using electron microscopy, enzyme assays and molecular analysis that peltate glands also appeared to be the major and possibly only site of phenylpropene storage and biosynthesis in basil leaves. In this work 10 - 60 fold increases in specific activity of key phenylpropene pathway enzymes were found. In addition an EST database was constructed from the peltate glands of their EMX-1 (methyl chavicol type basil) line and from this several enzymes directly involved in phenylpropene biosynthesis were identified.

1.5.2 Biosynthesis of volatile oils

The proportions of chemical components in essential oils of *Ocimum* species vary. Some oils of *O. basilicum* have 90%+ of one compound (e.g. methyl chavicol) whereas oils of *O. tenuiflorum* tend to be mixtures in which no single component dominates. Constituents of the *Ocimum* oils fall into two main groups; the monoterpene and sesquiterpene hydrocarbons and the aromatic phenylpropenes. In the late 1950s Bloch and Lynen established that the mevalonic acid pathway led to the production of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) in animals and yeast. These units are the building blocks of many compounds including more than

35,000 known terpenoids (Zenk, 2002). The synthesis of monoterpenes and sesquiterpenes was postulated by Wallach in the late 19th century and later substantiated in the mid 20th century by Ruzicka (Banthorpe, 1995). The 'biogenetic isoprene rule' dictates that each group of terpenes arise from the head-to-tail condensation of a specific number of isoprene units, often followed by one or more classic reactions such as cyclisation and rearrangement. The reactive hemiterpenes, IPP and DMAPP are the pyrophosphate esters of dimethylallyl alcohol and methyl butenol respectively.

Although the isoprene rule still holds true, recent studies have established that there are two separate pathways leading to the production of IPP and it's isomer DMAPP. The new pathway was first discovered in eubacteria by Rohmer *et al.* 1993, in algae by Schwender *et al.* (1996) and in higher plants by Schwarz (1994). Initial findings included the preferential incorporation of mevalonic acid (MVA) into cytosolic sterols rather than plastidic isoprenoids. In contrast, photosynthetically fixed $^{14}CO_2$ was rapidly incorporated into plastidic isoprenoids. Mevalonin, a highly specific inhibitor of hydroxy-methylglutaryl CoA (HMG-CoA) reductase, blocked accumulation of cytosolic sterols and ubiquinone but not plastidic phytol, carotenoids and plastoquinone. Also isolated plastids were found to be unable to synthesise IPP from MVA (Lichtenthaler 1997a, b). The realisation that in organisms including higher plants, the DOXP pathway was localised in the plastids and IPP production in the cytosol was via the MVA pathway, suggested that the DOXP pathway was primitive. The pathway is believed to have been imported into eukaryotic cells by endosymbiotic cyanobacteria-like progenitors of the present chloroplasts of higher plants and algae; the latter seemingly losing the MVA pathway (Lichtenthaler, 1999; Schwender *et al.*, 2001; Gao *et al.*, 2002) The existence or extent of exchange between the two pathways is still uncertain.

Workers have found some evidence of the transfer of IPP, GPP and FPP (Disch *et al.*, 1998; Rohmer, 1999). Figure 2 illustrates the proposed compartmentalisation of the two pathways.

The DOXP pathway has been found to exist in many pathogenic bacteria including, *Mycobacterium tuberculosis* (tuberculosis), *Treponema pallidum* (syphilis), *Helicobacter pylori* (gastric ulcer), *Vibrio cholerae* and *Pseudomonas aeruginosa* (wound infection) and the protozoan causal agent of malaria; *Plasmodium falciparum.* As it appears not to be present in humans and other mammals, inhibitors of the pathway are potential herbicides and antibiotic and antimalarial drugs. The herbicide fosmidomycin has already been shown to inhibit the DOXP pathway and in fact suppress the growth of multi-drug resistant *P. falciparum* and cure malaria infected mice (Lichtenthaler *et al.*, 2000; Zeidler *et al.*, 2000). The synthesis of the less common phenylpropanoids occurs via the shikimic acid pathway which is also known to function in the chloroplasts of higher plants.

1.5.2a Mevalonate pathway

The mevalonate pathway is so called because of it's central intermediate, mevalonic acid (MVA). The initial step in the pathway is the Claisen condensation by an acetyltransferase (EC 2.3.1.9) of two molecules of acetic acid thioesters. A subsequent aldol condensation of the resulting acetoacetate with a molecule of acetyl coenzyme A (CoA) is catalysed by the enzyme HMG-CoA synthase (EC 4.1.3.5) to form a molecule of HMG-CoA. This is then reduced to MVA by the NADPH-dependant HMG-CoA reductase (EC 1.1.1.34). The conversion of HMG-CoA to MVA is irreversible and hence rate-limiting (Herbert, 1981; Dewick, 1997).

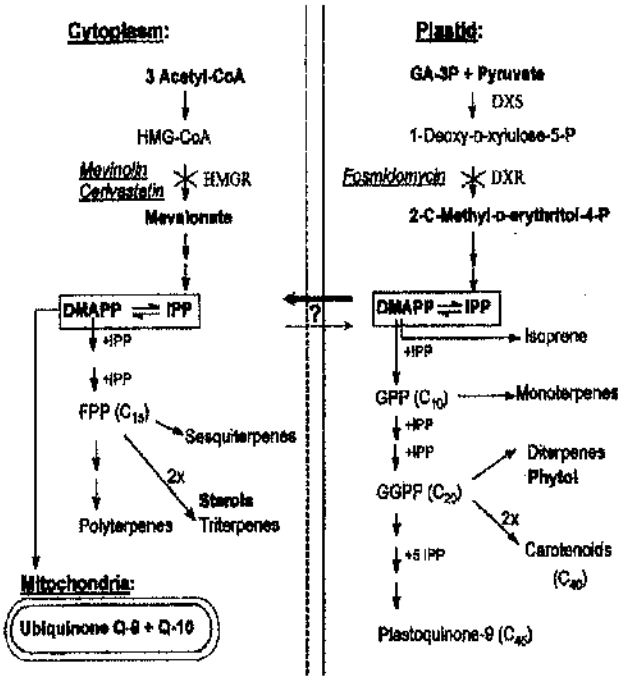


Figure 2 Proposed compartmentalisation of the two pathways of IPP and isoprenoid biosynthesis in higher plants, red algae and chrysophytes (after Lichtenthaler, 2000)

Mevalonic acid is transformed into the reactive hemiterpenoids. The phosphotransferase, mevalonate kinase (EC 2.7.1.36) catalyses the conversion of mevalonate to phosphomevalonate. This is followed by a second phosphorylation by phosphomevalonate kinase (EC 2.7.4.2) resulting in the formation of diphosphomevalonate, which is subsequently decarboxylated/dehydrated by the enzyme diphosphomevalonate decarboxylase (EC 4.1.1.33) to form IPP. Finally, the transformation of IPP into the highly reactive DMAPP is catalysed by IPP isomerase (EC 5.3.3.2). The reaction is reversible but tends to favour DMAPP (Figure 3) (Dewick, 1997).

The coupling of one molecule of IPP to a molecule of DMAPP induced by dimethylallyltransferase (EC 2.5.1.1) forms geranyl diphosphate (GPP) which is the ten carbon precursor of monoterpenes. Further additions of IPP onto allylic prenyl pyrophosphate units form the precursors of higher classes of terpenes; sesquiterpenes (15C), diterpenes (20C), sesterterpenes (25C) and so on (Figure 4) (McGarvey & Croteau, 1995; Bruneton, 1999).

Monoterpenes and sesquiterpenes have a rich diversity of acyclic, monocyclic and bicyclic structures which can become oxygenated, forming functionalised molecules such as alcohols, aldehydes, ketones, esters, ethers, phenols and oxides, examples of which are shown in Figure 5 (see also Appendix 2). There are around 1000 known monoterpenes and more than 7000 sesquiterpenes (Bohlmann *et al.*, 1998). Unoxygenated monoterpene hydrocarbons usually account for very little of the essential oil constituents in *Ocimum* species. However many oxygenated monoterpenes are major components (Hiltunen & Holm, 1999). Structural variation within sesquiterpenes

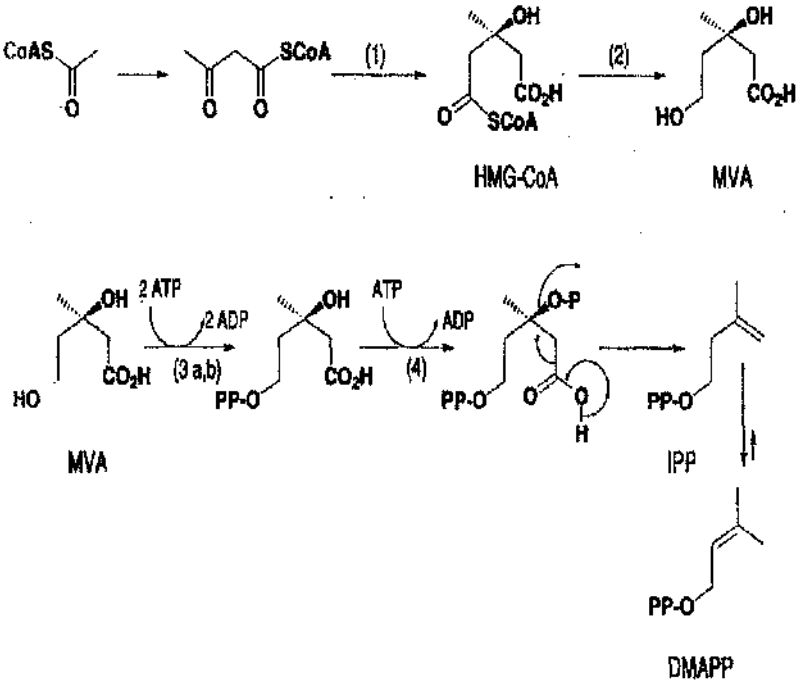


Figure 3 The mevalonate pathway (after Bruneton, 1999)

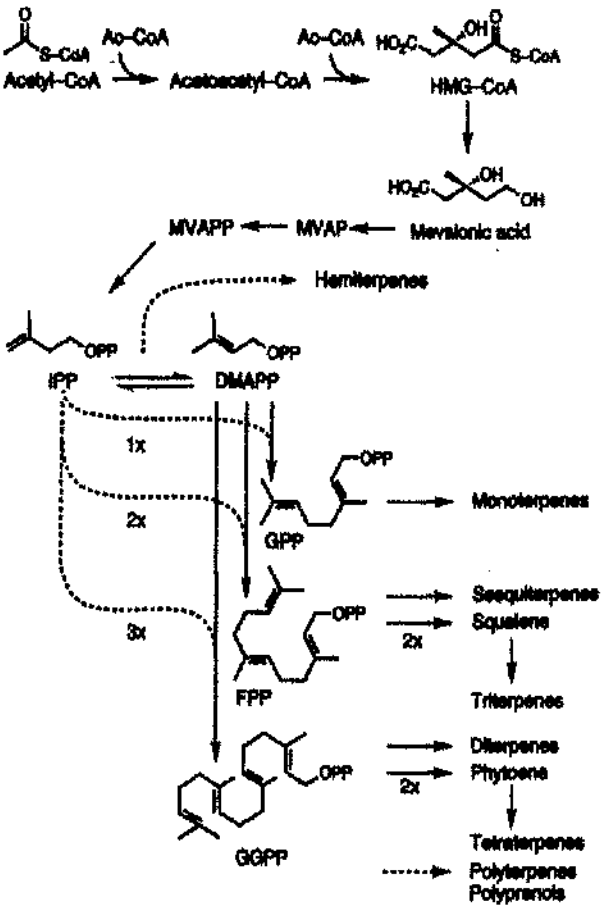
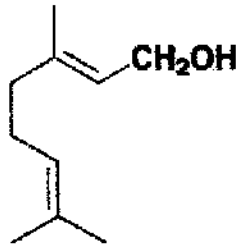
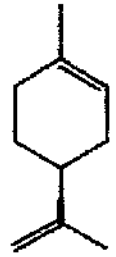


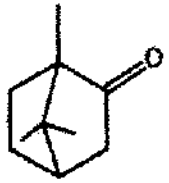
Figure 4 Outline of terpenoid skeleton biosynthesis
(after McGarvey & Croteau, 1995)



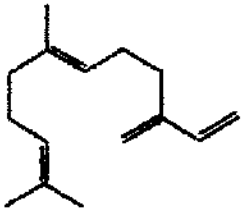
geraniol



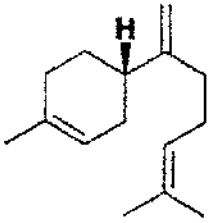
limonene



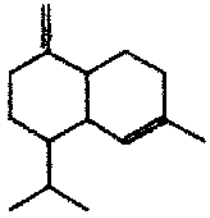
camphor



farnesene



β-bisabolene



γ-cadinene

Figure 5 Acyclic and cyclic mono- and sesquiterpenes found in *Ocimum* spp. (after Bruneton, 1999; Hiltunen & Holm, 1999)

is greater, due to the increased chain length and second double bond of the precursor molecule, farnesyl diphosphate (FPP). With some exceptions sesquiterpenes do not usually account for a great proportion of *Ocimum* essential oils (Hiltunen & Holm, 1999).

1.5.2b Shikimate pathway

In contrast to monoterpenes and sesquiterpenes, relatively few phenylpropenes have been identified (< 50) (Hay & Waterman, 1993). Phenylpropenes are synthesised via the shikimate pathway and although occurring in lesser quantities than monoterpenes, when present they are more potent. The first step in the pathway is the coupling of phosphoenolpyruvate (PEP) from glycolysis with D-erythrose 4-phosphate from the pentose phosphate cycle to form deoxy-arabino-heptulosonate phosphate (DAHP) by an aldol condensation reaction. This is catalysed by the enzyme DAHP synthase (EC 2.5.1.54). The elimination of the phosphate group, followed by a second aldol condensation initiated by dehydroquinate synthase, cyclises DAHP to dehydroquinate (HDQ). Dehydration of DHQ to dehydroshikimate by dehydroquinate dehydratase (EC 4.2.1.10) followed by a subsequent reduction of dehydroshikimate by shikimate dehydrogenase (EC 1.1.1.25) forms the central intermediate, shikimic acid.

The phosphotransferase shikimate kinase (EC 2.7.1.71) modifies shikimic acid to phosphoshikimate. The unusual condensation of the latter with a second PEP molecule by enolpyruvylshikimate phosphate (EPSP) synthase (EC 2.5.1.19) forms EPSP. A trans 1,4 conjugate elimination of phosphoric acid by chorismate synthase (EC 4.2.3.5) then converts EPSP to chorismic acid.

Amination of chorismic acid leads via anthranilate acid to tryptofan, the precursor of many alkaloids and microbial antibiotics. Chorismic acid also leads to the formation of the aromatic amino acids; phenylalanine and tyrosine. This involves firstly a unique Claisen-type pericyclic rearrangement promoting the production of prephenate. This is catalysed by chorismate mutase (EC 5.4.99.5). Dehydrogenation by prephenate dehydrogenase (EC 1.3.1.12) and dehydration by prephenate dehydratase (EC 4.2.1.51) generate hydroxyphenylpyruvate and phenylpyruvate respectively. Finally transamination reactions catalysed by tyrosine transaminase (EC 2.6.1.5) and aromatic amino-acid transaminase (EC 2.6.1.57) produce tyrosine and phenylalanine. Phenylalanine monoxygenase (EC 1.14.16.1) also converts phenylalanine to tyrosine (Figure 6) (Bruneton, 1999; Gang *et al.*, 2001, 2002a, b).

Phenylalanine is further transformed into cinnamic acid and ultimately to phenylpropenes such as methyl chavicol and eugenol. Until recently little was known about the biosynthesis of these compounds. Figure 7 illustrates intermediate and putative intermediate steps in the biosynthesis of methyl chavicol and methyl eugenol proposed by Gang *et al.* (2001, 2002a, b).

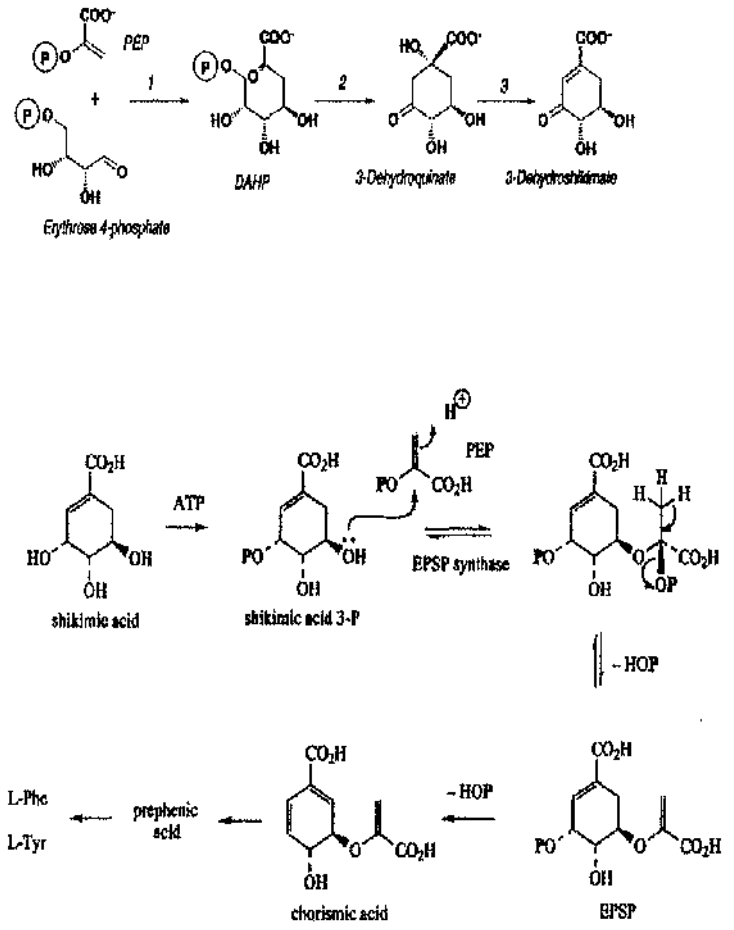


Figure 6 The shikimate pathway (after Bruneton, 1999)

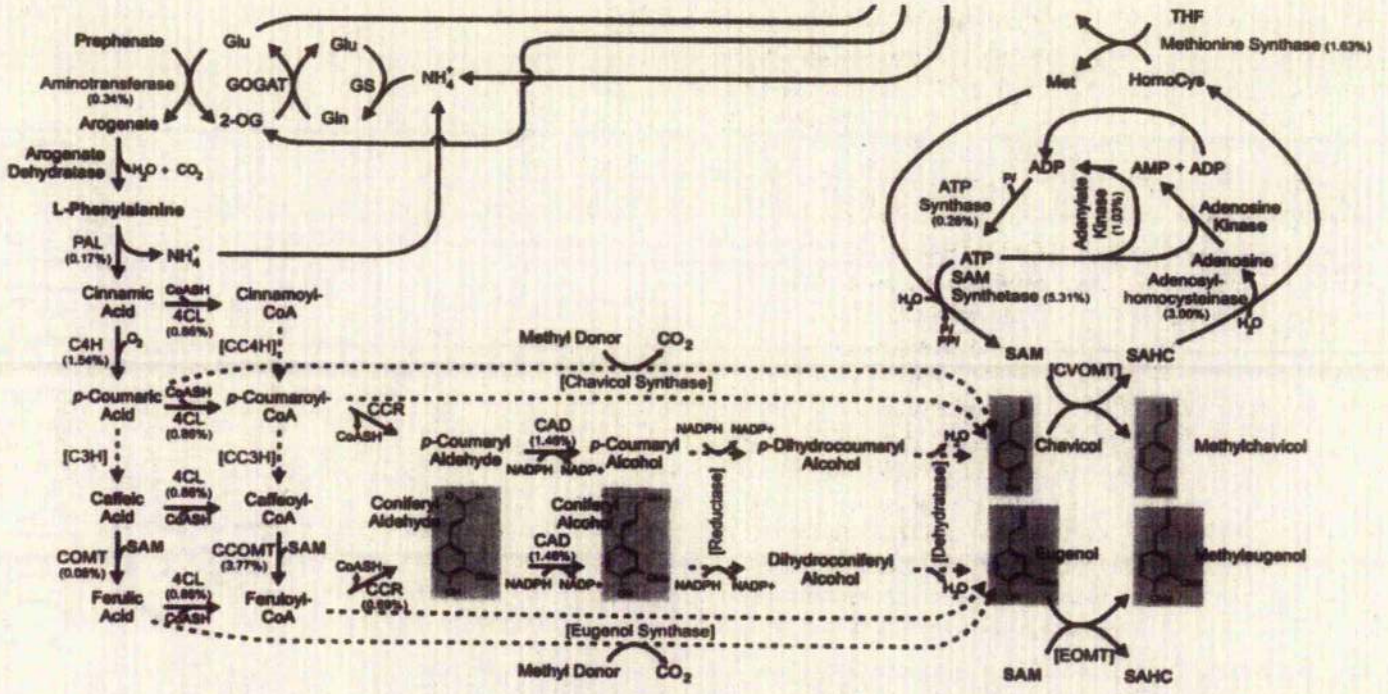


Figure 7 Proposed biosynthetic pathway to phenylpropenes in basil: C4H, cinnamate - 4 - hydroxylase; C3H, ρ-coumarate 3-hydroxylase; CC3H, ρ-coumarate-CoA 3-hydroxylase; CCR, cinnamyl-CoA reductase and CAD, cinnamyl alcohol dehydrogenase. Dotted lines indicate hypothetical reactions; hypothetical intermediates are boxed. The phenylpropanoid pathway involving methylations of CoA esters (as opposed to the free acids) is shown in brackets (after Gang *et al.*, 2001)

1.5.2c Deoxy-xylulose-phosphate pathway

The first step in this novel pathway involves a transketolate-type reaction in which a C2 moiety derived from pyruvate is transferred to D-glyceraldehyde-3-phosphate (GP) to form 1-deoxy-D-xylulose-5-phosphate (DOXP). This step is catalysed by the enzyme DOXP synthase (EC 4.1.3.37). DOXP reductoisomerase (EC 1.1.1.267) then converts DOXP to 2C-methyl-D-erythritol 4-phosphate (MEP). This is followed by transformation to 4-(cytidine 5-diphospho)-2-C-methylerythritol (CDP-ME) by reaction with cytidine triphosphate (CTP). This is catalysed by MEP cytidylyltransferase phospho-CDP-ME. Subsequent cyclisation of this product by methyl-D-erythritol cyclodiphosphate (MECDP) synthase (EC 4.6.1.12) leads to the formation of 2C-methyl-D-erythritol 2,4-cyclodiphosphate (MECDP). The latter intermediate is transferred into IPP and DMAPP via (E)-4-hydroxy-3-methylbut-2-enyl diphosphate (HMBDP) (Gao *et al.*, 2002). This plastidic pathway is illustrated in Figure 8.

1.5.3 Chemotaxonomy of *Ocimum* species

The disparity in taxonomical classification of *Ocimum* has been discussed. Due to this recognised confusion, basil essential oils have traditionally been characterised by their chemical composition and region of production. In earlier works, the oils were usually classified into four or five categories (Table 2). Probably the most well known oil is the European, Mediterranean or Sweet basil type (sometimes subdivided into French and American). It is distilled in Europe and the US from *Ocimum basilicum* and contains *d*-linalol and methyl chavicol as major components. Its linalol odour is described as being sweet, aromatic, anise-like and floral. This oil has the finest odour and is of the highest quality and hence is of great commercial value.

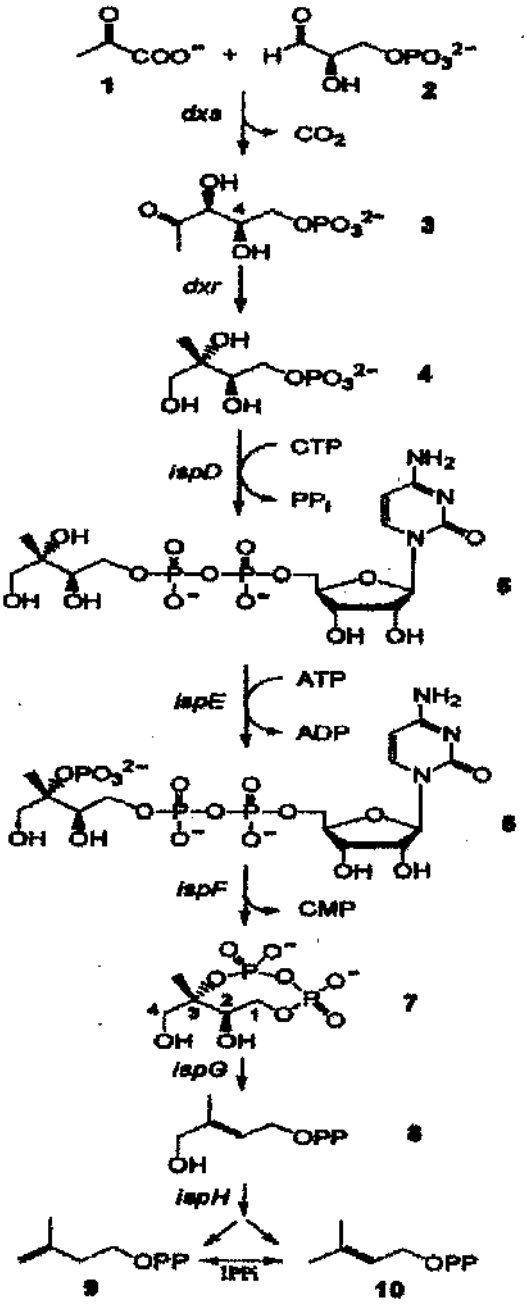


Figure 8 Deoxyxylulose phosphate pathway for the biosynthesis of terpenoids: 1, pyruvic acid; 2, D-glyceraldehyde 3 - phosphate; 3, 1-deoxy-D-xylulose 5-phosphate; 4, 2C-methyl-D-erythritol 4-phosphate; 5, 4-diphosphocytidyl-2C-methyl- D - erythritol; 6, 4-diphosphocytidy l -2C-methy l -D-erythritol 2 -phosphate; 7, 2C-methyl-D-erythritol 2,4-cyclodiphosphate; 8, (E) -4-hydroxy-3-methylbut-2-enyldiphosphate; 9, isopentenyl diphosphate; 10, dimethylallyl diphosphate (Zenk, 2003)

Type	Components	Region	Tentative spp.
European/Mediterranean	methyl chavicol/linalol	Europe/US/Mediterranean	*O. basilicum*
Reunion/Exotic/African/ Comoro	methyl chavicol/camphor	Reunion/Comoros/ Madagascar/Africa/Seychelles	unknown
Bulgarian	methyl cinnamate	Bulgaria/Sicily/Egypt/ Haiti/E. Indies/Indonesia/W.Africa	*O.canum*
Java	eugenol	Java/Seychelles/Samoa/ USSR/S.Pacific Isles	*O.gratissimum*
Egyptian	methyl chavicol/linalol	Egypt	unknown

Table 2 Traditional classification system for *Ocimum* essential oil

A second type is also commercially important but is inferior in quality to the European oil. This is due to the presence of camphor which creates a harshness. This oil is usually referred to as Reunion, Exotic, Camphor and occasionally Comoro type. It is produced in the Reunion Islands, Comoro Islands, Malagasy Republic, Africa, Seychelles, in the Reunion Islands, Comoro Islands, Malagasy Republic, Africa, Seychelles, Thailand and Vietnam. The main constituents of this oil are methyl chavicol and camphor but it is unclear from which species of *Ocimum* the oil is produced. It's odor is described as warm, spicy, camphoraceous and anise-like.

The third type of oil reported is less important commercially. This is the Bulgarian or methyl cinnamate type. It is distilled in Bulgaria, Sicily, Egypt, Haiti, East Indies, Indonesia, West Africa, Guatamala and Pakistan. It is reported to be produced from *Ocimum canum* but due to its lack of prominence on the world market little information is available on its characteristics.

The fourth oil is the Java or Russian type which is produced in Java, the former USSR, Seychelles, Samoa, North Africa and the South Pacific Islands. It is said to be distilled from *O. gratissimum* and has a high eugenol content. Lawrence (1992) reported that Russian basil, *O. gratissimum* L. (including Clocimum) accounted for the greatest proportion (55 tonnes) of the world annual production of basil essential oil. An Egyptian basil has also been reported which has a higher methyl chavicol and lower linalol content to the European oil. This oil is also produced commercially (Lawrence, 1976-1994; Gulati, 1979; Pruthi, 1980; Farrell, 1985; Simon *et al.*, 1990, 1999; Reineccius, 1994; Burdock, 1996; Leung & Foster, 1996).

More recent scientific studies have identified several new chemotypes within *Ocimum*, such as citral, eucalyptol, thymol and fenchone (Table 3). An excellent review of the essential oils of the genus is given by Hiltunen & Holm (1999).

The majority of components in basil oils are monoterpene and sesquiterpene hydrocarbons and aromatic phenylpropenes. Structures and descriptions of the major components found in basil oils are shown in Appendix 2.

Enzyme reference numbers stated in this section were obtained from the NC-IUBMB (1992) Enzyme Nomenclature Database.

Chemotype	Species	Author
Methyl chavicol	*bas, sell*	1,2,4,5,8,9,10,11,14,17,19,20,21,22,25,28,30,31,34,48,51,58,60
Linalol	*bas, can, grat*	2,4,5,6,7,13,15,17,23,25,26,27,29,32,33,35,44,45,48,54,55,59
Linalol/methyl chavicol	*bas*	7,9,27,33,45,48,58
Eugenol	*bas, grat, urtic, trich, can, sanc*	7,8,36,39,40,42,43,44,50,55,57
Thymol	*grat*	37,54,55,61,62
1,8-cineole	*grat, can*	50,54
1,8-cineole/eugenol	*grat*	50
Methyl chavicol/methyl eugenol	*bas*	8,48
Methyl eugenol	*bas, sell, urtic, sanc*	8,25,44,51,55
Methyl cinnamate	*bas*	12
Methyl cinnamate/linalol	*bas*	16,29
β-caryophyllene	*bas*	24
Geranial	*amer,* x *cit*	44,48
Fenchone	*can*	44
β–bisabolene	*sanc*	44
Linalol/eugenol	*bas*	48
Geranial/neral	x *cit*	48
Eugenol/cis β-ocimene	*urtic*	55
Camphor	*can*	56

Table 3 Chemotypes of *Ocimum* previously reported in the literature. *bas=O. basilicum, can=O. canum*, x *cit=O.*x citriodorum, *grat=O. gratissimum, sanc=O. sanctum, sell=O. selloi, trich=O. trichodon*, urtic=O. urticifolium. Numbers are emboldened in square brackets at the end of each corresponding reference in the reference section

PART B: INVESTIGATION OF ANTIFUNGAL ACTIVITIES OF *OCIMUM BASILICUM* ESSENTIAL OILS

The importance of preserving germplasm has been discussed and the need for a satifactory classification system has also been stressed. To utilise the potential of medicinal plants, especially in western medicine and crop protection, scientific testing must be carried out to ensure optimum efficacy and safety. In the present work, in order to study the antifungal activity of *Ocimum* essential oils and individual chemical components, it was necessary to choose a model test system. Various models have been used for this type of work. One such model utilises *Vicia faba* L. (broadbean) infected with *Botrytis fabae* Sardiña (broad bean chocolate spot) or *Uromyces viciae-fabae* (Pers.) Schroet (broad bean rust) (Havis *et al*, 1994; Mackintosh & Walters, 1998; Letessier *et al*, 2001). It was decided, therefore, to use this system to study the antifungal activity of the essential oil from *Ocimum*.

1.6 HOST PLANT – PATHOGEN SYSTEM

1.6.1 *Vicia faba* L.

Possibly one of the oldest cultivated, the broad bean, is an economically important crop plant worldwide. Within Europe, the UK is by far the largest producer (Knott, 1997), accounting for almost half of EU annual production. Widely used in organic farming, it is predominantly a high protein animal feed but is also produced for human consumption, especially in the middle East (Duke, 1983; Bulson *et al.*, 1997). The disappearance of farm horse power, lack of suitable varieties and serious disease outbreaks led to the 2240000 ha produced in the UK in 1873 being greatly reduced. However, due to the EC subsidy scheme initiated in 1978, much work began with the

aim of producing higher quality varieties and cultivars such as, low tannin, higher yielding, early maturing, shorter stemmed and disease resistant, in order to increase production especially for niche markets (Knott, 1997).

1.6.2 *Uromyces viciae-fabae* (Pers.) Schroet

Uromyces viciae-fabae is the casual agent of leaf, stem and pod rust of broad bean (Thomas & Sweet, 1990). Rusts caused by Basidiomycetes of the order Uredinales are among the most destructive plant diseases (Agrios, 1997). *U. viciae-fabae* predominantly infects broad beans but can also induce disease in peas, vetch and lentils (Lapwood *et al.*, 1984). Early infection is seen as defoliation of the plant but the disease normally takes hold late in the growing season, manifesting itself as small randomly distributed red-brown pustules, classically surrounded by haloes of chlorotic tissue (Parry, 1990).

The pathogen survives overwinter in the form of uredospores or resting mycelium on living plant material such as volunteer (self-sown) beans, crop debris or autumn sown crops. Infection is spread by windblown spores and is exacerbated by conditions of relatively high temperature and leaf wetness and soils deficient in potassium. Warm days with dew at night encourage spore germination (Parry, 1990; Thomas & Sweet, 1990).

U. viciae-fabae was first reported in Scotland in 1924 (Foister, 1961) and has a worldwide disrtibution. Severe attacks have been reported in the past in Europe, Australia, Canada, N & E Africa, W Asia and the Middle East (Rashid & Bernier, 1986; Sache & Zadoks, 1994). In general, the disease is not severe, partly due to its lateness of infection, but 30% losses have been reported in artificially infected trials (Parry, 1990).

Control measures for the disease include cultural methods which involve the removal of volunteers and crop debris. Chemical control includes foliar applied fungicides containing active ingredients such as fenpropomorph, cyproconazol and chlorothalonil (Buczacki *et al.*, 1981; Parry, 1990; CABI & BCPC, 2001).

1.6.3 *Botrytis fabae* Sardiña

Botrytis fabae was first noted on broad bean in Scotland in 1924 (Foister, 1961). Originally ascribed to *B. lathyri* then *B. cinerea*, it is now known to be caused by the two mitosporic fungi *B. fabae* and *B. cinerea* (Parry, 1990). *Botrytis* diseases are probably the most common and most widely distributed diseases of vegetables, ornamentals, fruits and field crops throughout the world (Agrios, 1997). There are two stages of the disease. An early 'non-aggressive' phase, recognised by small red-brown spots peppered over the leaves and stems and a later 'aggressive' phase or 'blight' during which the discrete spots enlarge and coalesce to form larger spots which in severe cases can cover the plant (Parry, 1990; Thomas & Sweet, 1990). Aggressive chocolate spot can cause up to 50% crop loss or even complete crop failure (Bretag, 1995). Early infection can be caused by either *B. fabae* or *B. cinerea*, but later infection is generally found to be due to the former only.

Environmental conditions affect the severity of the disease. Infection is favoured by damp, humid weather (15-20°C; 100% R.H. optimal) and wind plays a major role in the spread of the disease (Harrison, 1983, 1984; Fitt *et al.*, 1985). The pathogens survive overwinter either on crop debris, volunteer beans or autumn sown crops or as sclerotia in the soil. Initial infection occurs by windblown conidia or infected seed. Waterlogging,

high plant density, plant stress, acidic and/or mineral deficient soils can exacerbate the disease.

Control measures include cultural methods to reduce aggressive infection, such as planting at optimal density to reduce humidity in the crop and avoiding oversheltered or low lying areas to avoid waterlogging and frost. Improving drainage and use of optimal fertiliser rates can also help by reducing plant stress. Chemical control is widely used primarily in the form of foliar applied fungicides but also seed treatments. Recommended active ingrediants for foliar applications include benomyl, chlorothalonil, cyproconazole, carbendazim and carbendazim. Seed treatments containing captan, thiabendazole, chlorothalonil and thiram are also available (Parry, 1990; CABI & BCPC, 2001).

1.7 THE POLYAMINES

In addition to simply determining whether or not a medicinal plant possesses biological activity, it would be beneficial to elucidate the mode of action of such biocidal agents. This knowledge would not only aid in obtaining optimum efficacy and safety, but would provide the templates from which additional new products could be designed. Polyamines are essential cellular metabolites and are key components in chromosome structure. Evidence from previous scientific studies suggests that polyamines are a likely target for the biologically active components of essential oils (Carnesecchi *et al*, 2001). Further, since work in the Department of Plant Biology at SAC highlighted polyamine metabolism as a potentially useful target for fungicide development, it was decided to study the effect of the essential oil of *Ocimum* on polyamine metabolism in *B. fabae*.

1.7.1 Polyamines: a brief history

The term 'Polyamines' refers to a group of compounds which are found in almost every living cell and are inherent to survival, adaptation and evolution. The polyamines are synthesised via enzyme controlled biochemical pathways and contain multiples of amine groups within their structures. Monoamines command a field of research of their own but curiously diamines are encompassed in the term polyamines by workers in this field. In their natural free form the polyamines are aliphatic polycations (Davis, 1990). This design provides a superior binding capability to that of point-charged cations like Mg^{2+}, especially to polyanions such as DNA molecules (Tabor & Tabor, 1984).

The Dutch scientist, Anton van Leeuwenhoek, considered the greatest early microscopist, is also credited with the first discovery of a polyamine. He reported to the Royal Society of London in 1678, the crystalisation of a mysterious substance, now

known to be the phosphate salt of spermine. Although later observed by other scientists, spermine phosphate was only identified as such in 1878 by Schreimer.

During the late 1880s, Brieger isolated two diamines from putrifying animal corpses and bacteria and aptly named them putrescine and cadaverine. These were characterised soon after by Ladenburg and von Udránszky and Baumann respectively (Smith, 1982; Cohen, 1998). During the following 40 years knowledge of the origins, distribution and functions of these amines accumulated and as isolation techniques were improved, derivatives of the three compounds were observed.

Almost two and a half centuries after Leeuwenhoek's discovery, two teams headed by, Dudley and Wrede described the isolation, composition, structure and synthesis of the tetramine spermine. This was followed by the discovery and definition of the triamine spermidine one year later by Dudley and co-workers (Smith, 1982). During the next 40 years research in this field remained fragmentary, although enzymes involved in the biosynthesis and degradation of polyamines, along with many new classes and derivatives of the four main polyamines: spermine, spermidine, cadaverine and putrescine were found.

Around 1970, growth in research on polyamines accelerated with activity centred in Japan, Europe and the Americas. Work by this time was being carried out on a wide range of biological materials and with the development of instrumentation and assays such as GC (Gas Chromatography), GC-MS (Gas Chromatography-Mass Spectrometry) RIA (Radio Immunoassay) and NMR (Nuclear Magnetic Resonance), the list of natural free polyamines expanded substantially to that shown in Table 4.

Trivial name	Chemical Abbreviation	Chemical Name	Structure
Diamines			
Putrescine	4	1,4-Diaminobutane	$H_2N(CH_2)_4NH_2$
Cadaverine	5	1,5-Diaminopentane	$H_2N(CH_2)_5NH_2$
Diaminopropane	3	1,3-Diaminopropane	$H_2N(CH_2)_3NH_2$
2-Hydroxyputrescine	—	*S*(+) 1,4-Diaminobutane-2-ol	$H_2NCH_2CHOHCH_2CH_2NH_2$
Triamines			
Spermidine	3,4	1,8-Diamino-4-azaoctane	$H_2N(CH_2)_3NH(CH_2)_4NH_2$
sym-Homospermidine	4,4	1,9-Diamino-5-azanonane	$H_2N(CH_2)_4NH(CH_2)_4NH_2$
Aminopropylcadaverine	3,5	1,9-Diamino-4-azanonane	$H_2N(CH_2)_3NH(CH_2)_5NH_2$
Norspermidine (caldine)	3,3	1,7-Diamino-4-azaheptane	$H_2N(CH_2)_3NH(CH_2)_3NH_2$
7-Hydroxyspermidine	—	1,8-Diamino-4-azaoctane-6-ol	$H_2N(CH_2)_3NHCH_2CHOHCH_2CH_2NH_2$
Tetramines			
Spermine	3,4,3	1,12-Diamino-4,9-diazadodecane	$H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$
Thermine (norspermine)	3,3,3	1,11-Diamino-4,8-diazaundecane	$H_2N(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH_2$
Thermospermine	3,3,4	1,12-Diamino-4,8-diazadodecane	$H_2N(CH_2)_3NH(CH_2)_3NH(CH_2)_4NH_2$
Canavalmine	4,3,4	1,13-Diamino-5,9-diazatridecane	$H_2N(CH_2)_4NH(CH_2)_3NH(CH_2)_4NH_2$
Aminopropylhomospermidine	3,4,4	1,13-Diamino-4,9-diazatridecane	$H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_4NH_2$
N,*N*^{1}-bis(3-Aminopropyl)-cadaverine	3,5,3	1,13-Diamino-4,10-diazatridecane	$H_2N(CH_2)_3NH(CH_2)_5NH(CH_2)_3NH_2$
Aminopentylnorspermidine	5,3,3	1,13-Diamino-6,10-diazatridecane	$H_2N(CH_2)_5NH(CH_2)_3NH(CH_2)_3NH_2$
Aminobutylhomospermidine	4,4,4	1,14-Diamino-5,10-diazatetradecane	$H_2N(CH_2)_4NH(CH_2)_4NH(CH_2)_4NH_2$
N^1-Aminopropylnorspermidine	3(3)3	*tris*(3-Aminopropyl)amine	$H_2N(CH_2)_3N(CH_2)_3NH_2$ $\vert$ $(CH_2)_3NH_2$
N^1-Aminopropylspermidine	3(3)4	*N*^{1}-(3-Aminopropyl)-1,8-diamino-4-azaoctane	$H_2N(CH_2)_3N(CH_2)_4NH_2$ $\vert$ $(CH_2)_3NH_2$
Pentamines			
Caldopentamine	3,3,3,3	1,15-Diamino-4,8,12-tri-azapentadecane	$H_2N(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH_2$
Homocaldopentamine	3,3,3,4	1,16-Diamino-4,8,12-triazahexadecane	$H_2N(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH(CH_2)_4NH_2$
N^1-bis(Aminopropyl)norspermidine	3(3)(3)3	*tetrakis*(3-Aminopropyl)ammonium	$H_2N(CH_2)_3$ $(CH_2)_3NH_2$ $\diagdown$ $\diagup$ N^+ $\diagup$ $\diagdown$ $H_2N(CH_2)_3$ $(CH_2)_3NH_2$
Thermopentamine	3,3,4,3	1,16-Diamino-4,8,13-tri-azahexadecane	$H_2N(CH_2)_3NH(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$
N^1-bis(Aminopropyl)spermidine	3(3)(3)4	*N*^{1}-*tris*(Aminopropyl)-ammoniobutylamine	$H_2N(CH_2)_3$ $(CH_2)_3NH_2$ $\diagdown$ $\diagup$ N^+ $\diagup$ $\diagdown$ $H_2N(CH_2)_3$ $(CH_2)_4NH_2$
Hexamines			
Caldohexamine	3,3,3,3,3	1,19-Diamino-4,8,12,16-tetraza-ane	$H_2N(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH$-$(CH_2)_3NH_2$
Homothermohexamine	3,3,4,3,3	1,20,Diamino-4,8,13,17-tetraza-ane	$H_2N(CH_2)_3NH(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH$-$(CH_2)_3NH_2$
Thermohexamine	3,3,3,4,3	1,20-Diamino-4,8,12,17-tetraza-ane	$H_2N(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH(CH_2)_4NH$-$(CH_2)_3NH_2$
Homocaldohexamine	3,3,3,3,4	1,20-Diamino-4,8,12,16-tetraza-ane	$H_2N(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH$-$(CH_2)_4NH_2$

Table 4 Natural free polyamines (Cohen, 1998)

1.7.2 Distribution of polyamines

A vast amount of research has shown that, with some exceptions, the diamine putrescine, the triamine spermidine and the tetramine spermine have a universal distribution throughout species of the five kingdoms. Polyamine titre, however, varies both inter- and intraspecifically and localisation within the individual has been found. For example, only 1-2% of putrescine, spermidine and spermine in spinach leaf cells is reported to be in the chloroplast and only 0.5-2% of the same three polyamines have been found in the mitochondria of *Helianthus tuberosus* (Jerusalem artichoke) tubers. In rat liver cells, a greater proportion of polyamines has been found in the nucleus than in the cytoplasm and in humans, the bulk of the total spermine content is reported to be contained in the male prostate gland. Polyamines have also been found in blood cells rather than plasma (Cohen, 1998).

The less common diamine cadaverine, has a more sporadic distribution. Not only is its overall occurrence less widespread, its presence within organisms is not consistent. Kuznetsov *et al.* (2002) reported the accumulation and translocation of cadaverine in *Mesembryanthemum crystallinum* L. (common ice plant) after heat shock treatment. After treatment with the ornithine decarboxylase inhibitor, DFMO (difluoromethylornithine), Foster & Walters (1990) found an increase in cadaverine in *Pyrenophora avenae* Ito & Kuribay, the causal agent of leaf-stripe in oats. Also, the accumulation of cadaverine and the conversion of cadaverine to spermidine analogues in DFMO treated tumour cells was noted by Alhonen-Hongisto & Jänne (1980).

An increase in cadaverine and its higher derivative aminopropylcadaverine (APC) was found in cultured animal cells, *Neurospora crassa* (bread mould) and bacteria when

deficient in the main polyamines (Dion & Cohen,1972; Paulus *et al.*, 1982). Zarb & Walters (1994) observed an increase in cadaverine and derivatives in ectomycorrhizal fungi following polyamine depletion, while Foster & Walters (1993) found that the putresecine analogue, keto-putresciene induced an increase in cadaverine and that cadaverine could support growth in the absence of spermidine. This supported previous work in which Alhonen-Hongisto & Jänne (1980) and Paulus *et al.* (1982) reported cadaverine and aminopropylcadaverine to be compensating polyamines.

Post-1970, improved extraction methods and increased interest in organisms living in extreme environments such as hot springs led to the discovery of unusual polyamine profiles. Examples of these are given in Section 1.7.6, along with their putative functions and potential use in evolutionary studies and classification.

1.7.3 Biosynthesis of polyamines

It was originally thought that animals and fungi had a single route leading to the production of putrescine, while plants and bacteria had two (Zarb & Walters, 1993). Evidence now suggests that this distinction is not so clear cut. Arginine decarboxylase (ADC; EC 4.1.1.19), thought to be lost from fungi and animals when the three kingdoms (plant, animal and fungi) diverged (Malmberg, 2002), has been found to exist in fungi including *Verticillium dahliae* (wilt), *Fusarium oxysporum* (wilt), *Helminthosporium* (leaf spot) and *Ceratocystis* spp. (wilt) (Khan and Minocha, 1989a) and the mycorrhizal fungus *Laccaria proxima* (Zarb & Walters, 1994). Polyamines can also be taken up by cells and freed from conjugated, bound and compartmentalised pools. For the purpose of this review, the universal route will be classed as the ornithine decarboxylase (ODC: EC

4.1.1.17) pathway and the second route, most commonly found in plants and bacteria, will be classed as the arginine decarboxylase (ADC: EC 4.1.1.19) pathway (Figure 9).

In the ODC pathway, the production of the diamine putrescine involves decarboxylation of the amino acid ornithine by the enzyme ODC. The second route to putrescine requires decarboxylation of the amino acid arginine by the enzyme ADC to form the intermediate agmatine followed by conversion to putrescine in bacteria and fungi by the enzyme agmatinase (EC 3.5.3.11) or agmatine deiminase (EC 3.5.3.12). This is a two step process in plants with agmatine converted first to N-carbamoylputrescine by agmatine iminohydrolase (EC 3.5.3.12) followed by conversion of this compound to putrescine by N-carbamoylputrescine amidohydrolase (EC 3.5.1.53). Arginine is also directly converted to ornithine by the enzyme arginase and this can lead to complications in assaying ADC activity in plants (Smith, 1985).

In order to convert putrescine to spermidine, the addition of an aminopropyl group is required. The decarboxylation of S-adenosylmethionine by the enzyme S-adenosylmethionine decarboxylase (AdometDC; EC 4.1.1.50) provides the aminopropyl moiety, which is then attached to putrescine in a reaction catalysed by the aminopropyl transferase spermidine synthase (EC 2.5.1.16) to form the triamine spermidine. The subsequent addition of a second aminopropyl group catalysed by spermine synthase (EC 2.5.1.22), converts spermidine to the tetramine spermine, thus spermidine and spermine are essentially higher derivatives of putrescine (Smith, 1982; Tabor & Tabor, 1984; Davis, 1990).

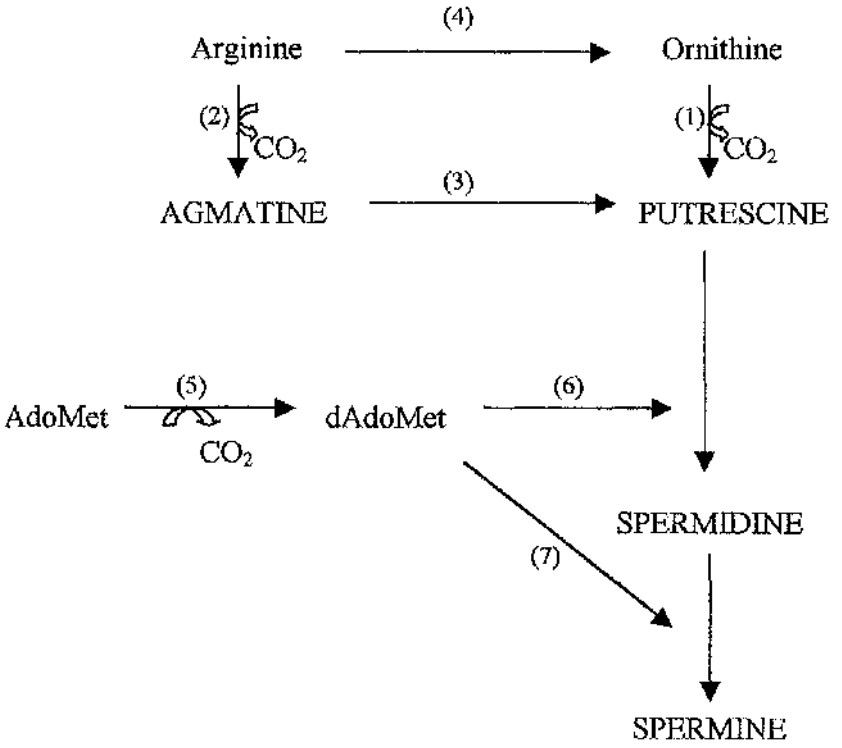


Figure 9 The biosynthetic pathway of major fungal polyamines. (1) ornithine decarboxylase (2) arginine decarboxylase (3) agmatinase or agmatine deaminase (4) arginase (5) S-adenosylmethionine decarboxylase (6) spermidine synthase (7) spermine synthase.

Cadaverine is synthesised by decarboxylation of the amino acid lysine. In plants this is carried out by the enzyme lysine decarboxylase (LDC; EC 4.1.1.18). It has been reported, however, that cadaverine is also formed via ODC in animal cells and *N. crassa*, albeit inefficiently (Paulus *et al.*, 1982).

1.7.4 Regulation of cellular levels of polyamines

1.7.4a Control by major enzymes of biosynthesis

As indicated earlier, there are four main steps in the major polyamine biosynthesis pathway in all organisms. These are irreversibly catalysed by the enzymes ODC, AdoMetDC, spermidine synthase and spermine synthase. Initially, putrescine is produced via ODC. This enzyme is highly regulated and rate-limiting to the pathway (Foster & Walters, 1990). It is regulated by substrate supply and enzyme activity and enzyme concentration is most likely controlled by the rate of protein translation and enzyme turnover; the latter being very high (Tabor & Tabor, 1984). This enzyme is also reported to be subject to feedback control by the products of polyamine biosynthesis; putrescine, spermidine and spermine, with an increase in polyamines reported to reduce ODC activity and *vice versa* (Pegg, 1988). In addition, the inhibitory protein, antizyme, induced by polyamines in animal and some plant cells, has been found to reduce ODC activity by forming a complex with the enzyme (Tabor & Tabor, 1984).

The second major enzyme in the pathway is AdoMetDC which is also highly regulated and rate-limiting. This enzyme is directly activated by putrescine in mammals and fungi, by magnesium in *E-coli* but by neither compound in plants (Cohen, 1998). An increase in spermidine and spermine, downregulates AdoMet and conversely a decrease in these products has been found to induce upregulation (Tabor & Tabor, 1984; Pegg, 1988).

In contrast to ODC and AdoMetDC, the aminopropyl transferases spermidine synthase and spermine synthase are not highly regulated or rate-limiting and do not have a high turnover rate. Spermidine synthase is said to be subject to feedback control. Flux through the two synthases depends on the availability of the substrate dAdoMet, the amount of the enzymes and their amine substrates (Tabor & Tabor, 1984).

Although evidence exists to suggest these enzymes are subject to feedback control and it is well known that other cellular enzymes are controlled by this mechanism, many authors agree that feedback is not a control mechanism, in fungi at least. The fact that many polyamines are available to cells other than those in the intracellular free pool, means that feedback control by polyamines would be extremely difficult. It is most likely for this reason that the more complex controls of adjusting protein translation rate and enzyme turnover rate have evolved to help control cellular levels of polyamines (Davis, 1990).

1.7.4b Catabolism of polyamines

Like many other things which are essential to life, in excess, polyamines (especially spermine) are damaging to living cells. In addition to controlling biosynthesis and in order to combat the toxic effects of greatly elevated levels of polyamines, cells have developed sophisticated catabolism systems. To date two pathways for polyamine catabolism are known to exist in fungi: retroconversion (Figure 10) and degradation. The former involves acetylation of the tetramine spermine by the enzyme spermine acetyltransferase (SSAT) to form N^1-acetyl spermine, which is then cleaved by polyamine oxidase (PAO; EC 1.5.3.11) to release the triamine spermidine. Spermidine can also be converted in a similar way. In this case, SSAT catalyses the addition of an

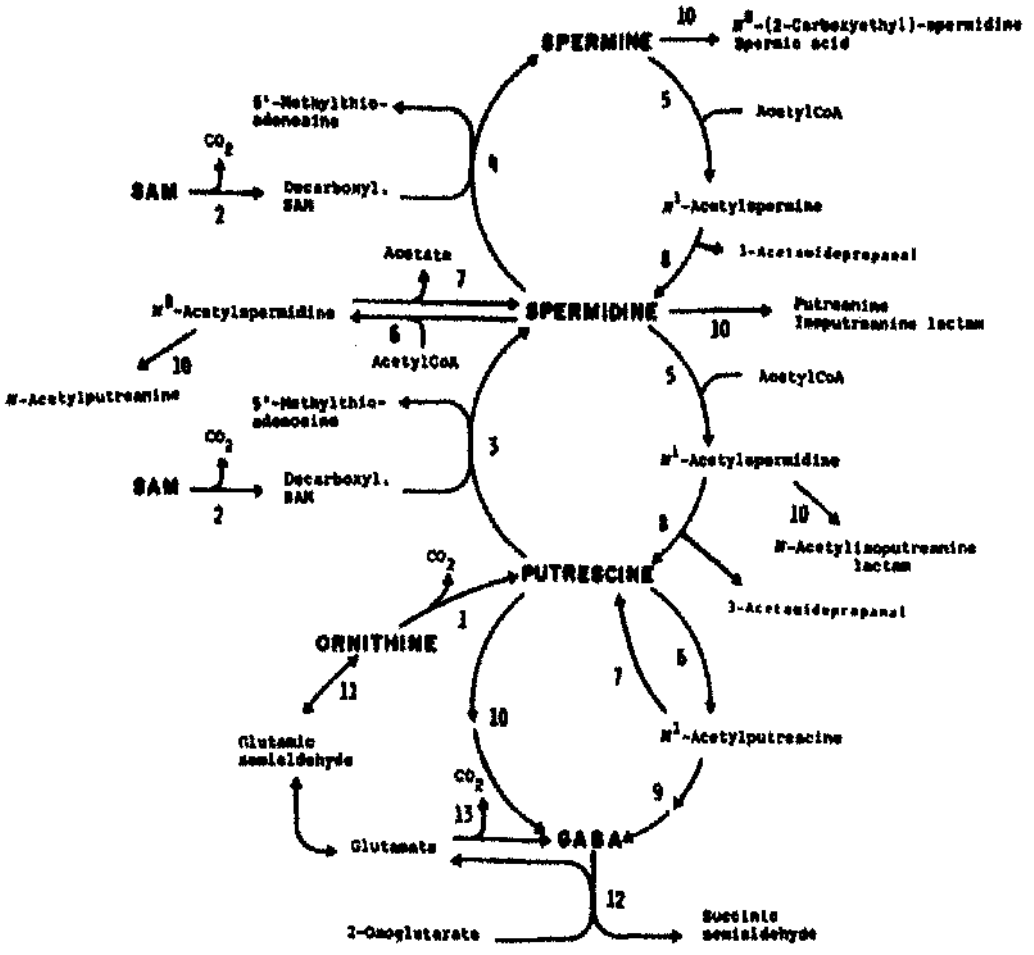


Figure 10 The interconversion pathway in vertebrates
(after Cohen, 1998)

acetyl group from acetyl CoA to spermidine to form its N^1-acetyl derivative, which is in turn reduced to the diamine putrescine by PAO. Putrescine can also be acetylated then further transformed to GABA (γ-aminobutyric acid) (Cohen, 1998). An illustration and description of the fungal polyamine degradation pathway can be found in Walters (2002).

1.7.4c Other regulatory mechanisms

Other mechanisms which alter the cellular pool of free polyamines include, non-specific electrostatic attraction and specific binding to nucleic acids, conjugation with e.g. acetyl groups, alkaloids, phenolics and proteins, sequestration into intracellular organelles such as the vacuole and influx and efflux across the cell membrane (Davis, 1990; Cohen, 1998).

1.7.5 Functions of polyamines

Work with inhibitors of polyamine biosynthesis and experiments using mutants have uncovered many functions of polyamines which are essential to the growth and development of living organisms. In addition, polyamine profiling has generated ideas of their roles in adaptation and evolution. The essentiality of certain amino acids was realised early in the 20th century, but it was not until 1948 that Herbst and Snell described the absolute requirement of polyamines for growth. They demonstrated the necessity of exogenous putrescine or equivalent in growth of *Hemophilus parainfluenzae* (Cohen, 1998). This prompted investigations into the role of polyamines in growth and development.

1.7.5a Associations of polyamines with nucleic acids

The suspicion of an interaction between polyamines and nucleic acids began when polyamines were found to transfer along with T-even (*E-coli*) phage DNA. Correlations were found between polyamines and nucleic acids. Abundance and biosynthesis of RNA (mainly ribosomal) paralleled that of spermidine in mammalian liver cells, increased spermine and spermidine levels coincided with increased DNA and RNA during chick embryo development and polyamines have been found to affect the rate of DNA synthesis in *E-coli*. Inhibition of cell proliferation was demonstrated in Ehrlich ascites tumour cells by polyamine depletion induced using the ODC inhibitor difluoromethylornithine (DFMO). Polyamines are also reported to play an important role in RNA transcription (Smith, 1982). Overall it seems clear that polyamines exert a stabilising and/or regulating effect on DNA and RNA molecules, although the exact mechanisms have yet to be elucidated.

Many models of polyamine-nucleic acid complexes have been postulated. Liquori *et al.* (1967) proposed spermine and spermidine binding to neighbouring phosphates with aminopropane groups on one strand of DNA, then crossing the minor groove with the putrescine moiety (Figure 11a, b). In contrast, other workers proposed nonspecific models. In general workers using X-ray techniques have shown polyamines (in crystal form) to bind in a specific manner to varying but well defined sites on DNA, and DNA-polyamine interactions (in solution) examined using NMR and other techniques usually demonstrate a non-specific electrostatic attraction conforming to the polyelectrolyte theory (Lyubartsev & Nordenskiöld, 1997). Studies on RNA-polyamine binding revealed the existence of H-bonding and that secondary amines had an important role in

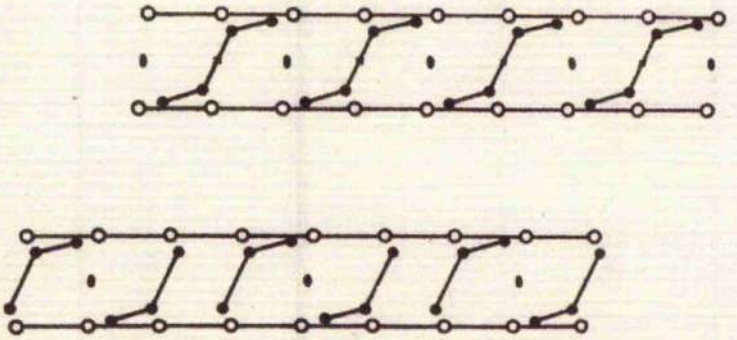


Figure 11a Schematic model showing spermidine and spermine molecules in the narrow grooves of DNA (Liquori *et al.*, 1967)

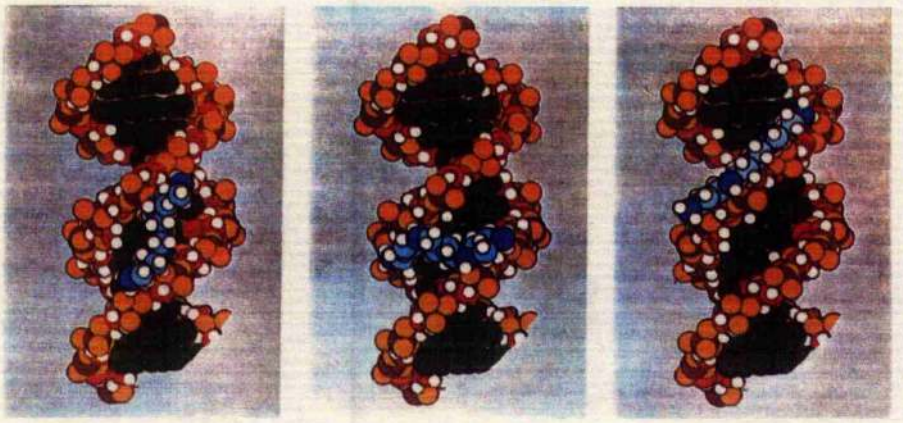


Figure 11b Probable configurations of the spermine-DNA complex (Suwalsky *et al.*, 1969)

such complexes. Overall, it appears that non-specific and specific interactions between polyamines and nucleic acids exist.

1.7.5b Changes in polyamines during development

Polyamines have been shown to affect rates of nucleic acid synthesis and in many cases to stimulate protein synthesis in extracts of prokaryote and eukaryote cells. In addition, qualitative and quantitative changes in polyamines have been recorded during specific developmental changes, as described below.

1.7.5c Polyamines and fungal development

Elevated levels of polyamines are associated with morphogenesis in *Mucor racemosus* (Inderlied *et al.*, 1980), while in *Sclerotium rolfsii* mycelial growth and sclerotium formation are associated with an increase in polyamine biosynthesis (Shapira *et al.*, 1989). Increases of over 100-fold occurred in the activity of ODC and AdoMetDC during the emergence of the germ-tube and before DNA doubling in *Aspergillus nidulans*. Within 16 hours, a sharp fall in activity of both enzymes was observed (Stevens *et al.* (1976). Polyamine biosynthesis is also associated with the dimorphic transition from yeast to mycelium in *Mucor rouxii* and *M. bacilliformis* (Calvo-Mendez *et al.*, 1987).

1.7.5d Polyamines and mammalian development

ODC and AdoMetDC activities increase during foetal development in mammals, hence embryonic rat tissue was found to be higher in spermidine content than tissues of older animals, while both spermine and spermidine were found to decline with age in the rat brain. A fifty fold increase in levels of spermine and spermidine were observed during

chick embryo development, followed by an 80% drop over the subsequent 18 day period (Cohen, 1998).

1.7.5e Polyamines and plant development

Polyamines have been found to be associated with rhizogenesis in tobacco and application of polyamines is reported to stimulate cell division and tissue growth in dormant *Helianthus* tubers (Cohen, 1998). Increased ODC has been reported during tobacco ovary development (Slocum & Galston, 1985) and polyamine metabolism was found to be related to fruit set and growth in apple (Biasi *et al.*, 1991). Polyamines can induce embryogenesis in carrot (Galston & Sawhney, 1990) and affect pollen tube development, pollen germination and pollen tube length in lily flowers (Rajam, 1989).

1.7.5f Involvement of polyamines in counteracting stress

Elevated putrescine has been found to be involved in the counteraction of many stresses (Galston & Sawhney, 1990). Polyamines have been shown to reduce the effect of temperature on nucleic acids. Tabor (1961) demonstrated that spermine increased the Tm (melting temperature) of transforming DNA in the thermophilic bacterium *Bacillus subtilis*. A partially purified T2 (*E-coli*) phage DNA, labile to freezing and thawing, was able to retain its activity in the presence of spermidine and diamines (Cohen, 1998). Similarly, phage DNA readily damaged by the shearing effect of stirring, remained intact in the presence of spermine. Spermine has been shown to preserve membranes, stabilise enzymes and protect other fragile structures such as cell walls, protoplasts and mitochondria (Cohen, 1998). Galston & Sawhney (1990) found ADC specifically to be increased during stress conditions such as anaerobiosis, potassium defficiency, water stress and the presence of atmospheric pollutants.

1.7.6 Polyamines and taxonomy

Studies of polyamine metabolism and their functions have provided us with information on their general distribution and an insight into their roles in adaptation to a range of stresses. The discovery of higher polyamines in organisms living in extreme environments prompted wider polyamine profiling and the investigation of polyamines as potential taxonomic markers.

In the early 70s, scientists began to investigate how thermophilic organisms managed to grow and multiply in such high temperatures. It was first discovered that polyamines increased the melting temperature of nucleic acids (Tabor, 1961; Mandel, 1962). Further work revealed that they stabilised protein synthesising machinery. *T. thermophilus* was found to be unable to efficiently form polypeptides at 65°C in the absence of spermine in particular (Ohno-Iwashita *et al.*, 1975). The new tetramines, norspermine (named thermine) and diamino-4,8-diazadodecane (also called thermospermine) were discovered in this organism, along with new pentamines and hexamines with tertiary and quaternary structures (Oshima, 1975, 1979). Thermine and the triamine norspermidine (or caldine), were also found in the archaebacterium, *Caldariella acidophla* (Cohen, 1998).

More extreme Gram positive thermophiles of the genus *Saccarococus* were reported to contain tertiary and quaternary branched tetra- and pentamines and the extreme thermophilic *Thermatoga* species which exist at temperatures around 78°C, were found to produce the long-chain polyamines caldopentamine, caldohexamine and caldoheptamine (Zellner & Kneifel, 1993). In the same work, increasing temperature (up to 85 °C) was found to result in both an increase in total amount of long-chain polyamines and the chain length strongly suggesting adaptation to increased

temperature. Systematic studies utilising nucleate and protein sequences placed organisms such as the sulphur-metabolising thermoacidophilic Archaebacteria *Sulfolobus acidocaldarius* among the earliest prokaryotes. The abundance of higher polyamines found in these organisms support the suggestion of an ancient role in the survival of genetic material and also the hypothesis that life on earth began in volcanic thermal springs.

The specific polyamine profiles of other organisms also suggest essentiality and functions conferring evolutionary advantages. For example, 2-hydroxyputrescine was found to be contained uniquely in all members of the β-class of the proteobacteria. These organisms contain putrescine as their major polyamine and also produce very low levels of spermidine (Busse & Auling, 1988). A study was carried out on two groups of carbon monoxide utilising organisms designated pseudomonads. Hydroxypolyamines were found to be produced by one group only, which led to questions of their place within the Proteobacteria (Hamana & Matsuzaki, 1990a). Examination of twelve species of sulphur-oxidising eubacteria classified as *Thiobacillus* revealed five distinct types. Only one group consisting of four species contained hydroxypolyamines. *Aeromonas* and *Vibrionaceae* both contain diaminopropane but can be distinguished by the fact that only the latter contains norspermidine (Hamana & Matsuzaki, 1990b).

As the polyamine profiles of more and more organisms are examined, a greater number of functions should be revealed. Even with the information currently available, the polyamines appear to have potential as markers in systematic studies. A review by Hamana and Matsuzaki (1992) discusses the use of polyamines in microbial systematics.

1.7.7 Inhibition of polyamine biosynthesis: applications

1.7.7a Cancer treatment

The use of inhibitors of polyamine biosynthesis extended during the late 60s when the finding of unusual expansion and decay of ODC in actively growing mammalian tissue sparked interest in the field of medicine, especially in the use of such inhibitors as chemotherapeutic agents in the treatment of cancer (Galston & Sawhney, 1990). Compounds in use at this time, however, were mainly reversible competitive inhibitors. These included the polyamines themselves, analogues of substrates or products of enzyme catalysed reactions and antagonists of enzyme cofactors. These proved to have limited success *in vivo* and this prompted the design and synthesis of the 'suicide inhibitors' which react directly and irreversibly with the enzymes themselves (Walters & Mackintosh, 1997). One of the first and possibly most studied to date is the ODC inhibitor DFMO (difluoromethylornithine), developed by Merrell-Dow Pharmaceuticals. This inhibitor was not without drawbacks, its passive uptake meant high doses were required to have an effect and the rapid turnover of ODC allowed cells to make a quick recovery after treatment. However, its low mammalian toxicity retained interest in its development, as a potential drug for human use (Cohen, 1998).

A vast amount of work was done using inhibitors to deplete cellular levels of polyamines. However, cells have many compensating measures against depletion of individual substrates or enzymes such as upregulation, production of compensatory enzymes or polyamines, increased uptake, reduced excretion and release of polyamines from bound and sequestered pools. In light of this, new multifaceted approaches to depleting cellular polyamine levels were explored. Success was achieved against childhood leukemia using the AdoMetDC inhibitor MGBG (methylgloxal bis

guanylhydrazone) after pre-treatment with DFMO, with all patients in the study experiencing remission (Siimes *et al.*, 1981). Promising results have also been obtained in treating cancers with other combinations, for example, DFMO administered with interferon or bleomycin. It has been suggested that the DFMO destabilises the nucleic acids on which the drugs act (Cohen, 1998). More recent studies taking place at the University of California, Irvine have looked at the use of DFMO as a chemopreventative measure against colon cancer. Positive results have led the team to carry out further investigations with DFMO alone and in combination with other anti-cancer drugs (UCI, 1998).

On the whole, however, successes have been limited. One of the possible reasons why DFMO has failed to completely halt the growth of cancerous tumours is that cells can gather polyamines from extracellular sources such as food and intestinal bacteria. This knowledge has been taken into account by scientists at the University of California San Diego School of Medicine and Lund University, Sweden. The team is now investigating a two pronged approach whereby DFMO which stops polyamine biosynthesis in the cell is used in conjunction with a heparin sulphate inhibitor to stop the cells from gathering polyamines from the extracellular matrix. This study is in progress and initial results look promising (UCSD, 2001).

1.7.7b Inhibition of fungal growth and development

Building on previous observations, Galston & Sawhney (1990) demonstrated that depletion of polyamines by DFMO induced ODC inhibition, led to a reduction in growth of phytopathogenic fungi which was reversible by addition of exogenous polyamines. The knowledge that plants possess two routes to polyamine production and thus the

ability to compensate for ODC inhibition, while most fungi appeared to possess only the one route, led them to investigate the potential of DFMO as a fungicidal agent.

Several workers carried out *in vitro* studies using the ornithine analogue DFMO and also reported reduced fungal growth, reversible with application of exogenous polyamines (Barker *et al.*, 1993; Biondi *et al.*, 1993; Bharti & Rajam, 1996). Initial *in vivo* experiments by the Galston group on rust infection of *Phaseolus vulgaris* (field bean) found that infection decreased with application of increasing concentrations of DFMO. In addition, translocation of the compound was reported in these plants (Galston & Sawhney, 1990). Walters (1986) demonstrated successful inhibition of the rust pathogen, *Uromyces viciae-fabae* (Pers.) Schroet (rust) on *Vicia faba* L. (broad bean) by application of DFMO. Walters also found the compound to be systemic and nonphytotoxic. Positive results were then obtained *in vivo* following DFMO treatment of a range of important crop pathogens.

West & Walters (1988) investigated the effects of a range of ODC inhibitors on *Erysiphe graminis* (powdery mildew) infection of *Hordeum vulgare* L. (barley). Inhibition was observed after treatment with all compounds with greatest control achieved with a DFMO-MGBG combination. In this study, post-inoculation treatments were found to be more effective than pre-inoculation treatments. The combination of DFMO and spermidine was found to give good control of *Botrytis cinerea* (grey mould) of *Senecio* sp. and tomato. Both components were effective individually to a lesser extent (Elad,1991).

Later work involved the examination of polyamine analogues. Investigations of such compounds stemmed from initial studies carried out by Foster and Walters (1993) on two putrescine analogues keto-putrescine and N-acetylputrescine. The latter was shown to be most effective, inhibiting growth of *Pyrenophora avenae* (oat stripe), *Pyricularia oryzae* (rice blast) and *Phytophthora infestans* (potato blight) *in vitro* and providing *in vivo* control of a range of crop pathogens *Erysiphe graminis, Uromyces viciae-fabae, Botrytis fabae* (broad bean chocolate spot), *Puccinia hordei* (barley brown rust), *Phytophthora infestans* and *Podosphaera leucotricha* (apple powdery mildew).

A succession of experiments was carried out in which putrescine analogues, E-1,4-diaminobut-2-ene (E-BED) and E-N,N,N',N'-tetraethyl-1,4-diaminobut-2-ene (E-TED) were tested for antifungal activity *in vitro* and in the glasshouse. Both compounds exhibited considerable antifungal activity against economically important crop pathogens *Erysiphe graminis* f.sp. *hordei* (barley powdery mildew), *U. viciae-fabae*, *B. fabae*, *P. infestans*, *P. leucotricha* and *P. avenae*. E-BED was also effective against *B. cinerea* and *P. oryzae*. In these studies, post-inoculation treatments were found most effective (Havis *et al.*, 1994a, b). In the field, E-BED gave good early season control of *E. graminis*, with results comparable to a commercial standard. In addition, increased plant height, dry weight and grain weight was reported after treatment with this compound (Havis, 1993).

The efficacy of the spermidine analogue, norspermidine and the spermine synthase inhibitor cyclohexylamine (CHA) as antifungal agents was also investigated. Norspermidine was shown to possess protective, eradicant and systemic activity when tested against the crop pathogens *U. viciae-fabae*, *B. fabae*, *E. graminis* f.sp. *hordei*, *P.*

leucotricha and *P. infestans* (Mackintosh, 1997). Both compounds applied pre-inoculation were found to reduce barley powdery mildew by up to 77% and both gave results comparable to a commercial fungicide in field trials (Mackintosh & Walters, 1998).

1.7.8 Polyamines: a look to the future

Polyamines themselves, polyamine analogues and other compounds able to inhibit polyamine biosynthesis have potential as novel fungicidal agents (Rajam & Galston, 1985; Rajam *et al.*, 1985; Walters, 1986; West & Walters, 1988; Foster & Walters, 1990, 1993; Zarb & Walters, 1993, 1994; Khan & Minocha, 1989b; Elad, 1991; Havis *et al.*, 1994a, b; Havis *et al.*, 1997; Walters & Mackintosh, 1997; Mackintosh *et al.*, 1997; Mackintosh & Walters, 1998). Polyamine inhibitors have also shown potential as chemotherapeutic agents in the treatment of cancerous tumours (UCI, 1998; Carnesecchi *et al.*, 2001; UCSD, 2001). Other possible applications include the use of spermine to aid re-isolation of DNA from agarose gels and improve plant gene amplification by PCR and pre-incubation with spermine derivatives to increase transformation efficiency in YACS (yeast artificial chromosomes). Also, antimicrobial and antioxidant properties of polyamines provide further opportunities for future investigation (Cohen, 1998).

Enzyme reference numbers stated in this section were obtained from the NC-IUBMB (1992) Enzyme Nomenclature Database.

2 OBJECTIVES

As our natural resources, including rainforests containing many medicinal plants are being destroyed at a phenomenal rate, preserving germplasm is of utmost importance. The *Ocimum* collection held at NCRPIS has already been used in several research and crop improvement programmes and in addition to other reported properties is highly likely to possess a wealth of undiscovered potential. However, simply collecting germplasm is not enough. A satisfactory classification system must be in place to allow the plants to be investigated and utilised. An important part of this project is to carry out a chemotaxonomic study of the plants in the *Ocimum* germplasm collection held at the NCRPIS. The results of this work will be coupled with a morphological analysis being carried out by Dr Mark Widrlechner, a taxonomist at the PI station in Ames. This will ultimately provide a comprehensive classification of the collection.

An extensive literature search for components and oil profiles previously reported to be present in *Ocimum* species will be used to identify novel chemical components and chemotypes within the collection. This again may highlight potential for development of new products or cultivation of new types of basil. In addition to making a sound classification of all plants in the collection, an attempt will be made to identify connections between chemical composition, geographical location and species of *Ocimum* in order to broaden our overall knowledge and understanding of the genus.

This project will also investigate the utility of chemical components from *Ocimum* as fungicidal agents. While classical chemical fungicides are in widespread use within

Western Europe, there are several reasons for looking to alternative crop protection treatments. For example, the occurrence and severity of plant pathogens evolves swiftly with changes in cropping methods. In addition, EU policies direct research towards environmentally benign methods for plant protection. With fewer new chemical fungicides as a result of escalating development costs and legislative compliance, alternative environmentally benign approaches are sought. Since *Ocimum* plants have been widely reported as having a great number of biological properties, their antifungal properties will be investigated.

If the *Ocimum* essential oils and/or components are seen to possess antifungal activity, an attempt will be made to elucidate their mode of action. Although several reports of biological activity of essential oils can be found, little if any information is known of their mechanism of action.

The objectives of this project were to:

1. Provide a detailed chemotype analysis of the *Ocimum* collection held at NCRPIS;
2. Examine the antifungal and fungicidal effects of the essential oil of *Ocimum*, looking at both the whole oil and individual components of the oil;
3. Study the mode of action of the essential oil of *Ocimum* against selected plant pathogenic fungi, concentrating on effects on polyamine metabolism.

3 MATERIALS AND METHODS

3.1 CULTIVATION OF PLANTS

3.1.1 Cultivation of plants at the North Central Region Plant Introduction Station, Ames

On 30th June 1999, seeds of 88 accessions from the National Plant Germplasm System *Ocimum* spp. collection were started in a glasshouse at the NCRPIS in Ames, Iowa. Two hundred seeds of each accession were sown without pre-treatment into 32-cell containers. Each cell measured 3.8cm^2 x 12.7cm and contained 150ml of growing medium consisting of commercial potting mix, local soil and perlite in the ratio of 6:3:1 (v/v).

The seedlings were thinned to one plant per cell and fed weekly with a commercial liquid fertiliser (NPK 20:10:20) throughout the growing season. Plants of 82 of the accessions were harvested for analysis between late July and December. The stems were cut just above soil level at early flowering stage. The plant material was placed in large, fine mesh, polyester bags and dried in a forced-air drier at 29-32°C. When the samples were crisp to the touch (after 48-72 h), the mesh bags were placed into plastic bags and these were tied shut. They were boxed for shipment to Scotland by Express Mail and delivered in approximately 3 d.

The taxonomic identities of the accessions were confirmed just before the plants were harvested. This was done by comparing the plants' morphological characteristics to the diagnostic keys and taxonomic descriptions published by Paton (1992) and Albuquerque and Andrade (1998) (Appendix 1).

3.1.2 Cultivation of plants at SAC, Auchincruive

Cultivation of plants from 3 accessions of *Ocimum* (*O. basilicum*, PI172996, *O. basilicum*, PI207498 & *O. tenuiflorum*, PI414201) was carried out at SAC, Auchincruive (plant passport information can be found in Appendix 13a). One hundred seeds of each type were sown on 16th May, 2000, in a peat-based growing medium (Scotts UK Professional, Ipswich, UK), in module trays with one seed per module. Seeds were planted approximately 5mm below the soil surface. Module trays were placed in the large section of the first glasshouse on entering Diamond Field. Plants were watered daily or when soil showed signs of drying. Germination took place between 7 and 10 days. Plants were transferred on 15th June to 7.6cm diameter pots with the same compost and on 5th & 6th July were planted into the ground inside the unheated glasshouse. The growing medium inside the glasshouse was a mix of local soil (fine sandy loam) and a peat-based potting compost (9:3 v/v). One kilogram of an NPK (20:10:20) fertiliser was applied to the soil one day prior to planting (at 20g/m^2) and black polythene sheeting was used to suppress weed growth. No supplementary lighting or pesticide treatment was used during this part of the project.

3.2 DISTILLATION OF PLANT MATERIAL

3.2.1 Distillation of plants cultivated at NCRPIS

On arrival at Auchincruive, the dried plant material was catalogued and transferred to brown paper bags. The bags were placed in a drying oven (30°C) overnight prior to distillation to allow the leaves to be removed from the stems with minimum disturbance to the oil glands. Samples were weighed, then hydrodistilled for 2 hours using British Pharmacopoeia distillation apparatus and procedure (British Standards, 1985) (Appendix

3). The oil obtained was measured and transferred to glass vials. The vials were stored in a dark fridge at 0-5°C until required for further analysis.

Oil yield was calculated as percentage volume per weight:

e.g. 1ml oil / 50g plant material x 100 = 2% (v/w)

3.2.2 Distillation of plants cultivated at SAC

Samples of fresh material of *O. tenuiflorum* PI414201 were harvested and separated into batches of leaves, flowers and whole plants. Separate distillations were carried out and Gas Chromatography traces obtained for each oil. The 100 *O. basilicum* PI172996, 100 *O. basilicum* PI207498 and remaining *O. tenuiflorum* were harvested, dried at room temperature, hydrodistilled and oil yields were calculated as described above in Section 3.2.1.

3.3 GAS LIQUID CHROMATOGRAPHY (GLC) OF ESSENTIAL OILS

GLC was used to determine the chemical compositions of the essential oils. This was carried out on a PU4550 gas chromatograph connected to an SP4400 integrator. The following operating conditions were used: Carbowax (polyethylene glycol) 20m column; (25m x 0.25mm diameter; film thickness, 0.25μm); carrier gas, nitrogen, adjusted to a linear flow of 30ml/min; injector and detector temperatures, 250°C; oven temperature was programmed, 50-200°C, at 5°C/min and held isothermal for 10 min; sample size, 0.2μl; split, 1:100. The main components were identified by comparing their retention times on the column to those of known standards.

3.4 GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS) OF ESSENTIAL OILS

GC-MS analyses were carried out by Anu Thompson at Oceanography Labs, Liverpool University using an HP5890 gas chromatogram fitted with a split-splitless injector. The injector temperature was set at 230°C. The oven temperature was programmed as follows: 50°C isothermal for 5 min, increased 5°C min^{-1} to 200°C; isothermal at 200°C for 5 mins; increased 10°C min^{-1} to 230°C, isothermal at 230°C for 5 min. A fused silica capillary column (DB-WAX 30m, 0.25mm id, 0.25μm film thickness) was introduced directly into the electron-impact source of a VG-TS250 magnetic sector mass spectrometer. Helium was used as a carrier gas. Typical operating conditions were: electron voltage, 70eV, emission current, 300μA; photo-multiplier, 300V and source temperature, 230°C. The instrument was operated in full acquisition mode and cycled from 50-650 Daltons every second. Data were collected on a VAX-3100 workstation and were processed using VG-OPUS software. The mass spectra were compared with those stored in the mass-spectrometer database using NIST library.

3.5 ACQUISITION OF FUNGAL PATHOGENS

Botrytis cinerea Pers. and *Botrytis fabae* Sardiña were both obtained from Dr J G Harrison, SCRI, Dundee.

Uromyces viciae-fabae (Pers.) Schroet was obtained from Professor P G Ayres, Lancaster University.

3.6 *IN VITRO* EXPERIMENTAL ASSESSMENT OF ANTIFUNGAL ACTIVITIES OF SELECTED *OCIMUM BASILICUM* ESSENTIAL OILS AND INDIVIDUAL COMPONENTS AGAINST *BOTRYTIS FABAE*

3.6.1 Effects of two different *O. basilicum* essential oils tested against *B. fabae* grown on solid media

In vitro bioassays were carried out to investigate the antifungal activity of two selected *O. basilicum* essential oils; methyl chavicol chemotype Sweet basil oil from the Comoros (oil 1) and linalol chemotype basil oil from Egypt (oil 2). The major chemical components of the two oils are shown in Table 5. The oils were supplied by Mr John Black (Aroma Trading Limited, New Rookery Farm, Silverstone, NN12 8UP, England) and were of the highest quality. The two oils were chosen as they are both from common types of basil oil and are commercially produced and therefore freely available.

Oil	Component	% of whole oil
Oil 1 (Methyl chavicol chemotype	Methyl chavicol	76.1
from the Comoros)	Linalol	18.6
Oil 2 (Linalol chemotype	Linalol	53.0
from Egypt)	Eugenol	12.4
	Eucalyptol	7.7
	Caryophyllene	5.0

Table 5 Percentage of major chemical components in *Ocimum* essential oils used in antifungal experiments

Antifungal effects of the two oils were investigated by measuring radial mycelial growth of the test pathogen *Botrytis fabae* growing on autoclaved (15 min at 121°C and 103kPa) solid medium X (Last & Hamley, 1956) (Appendix 4). This involved placing a 7mm diameter plug of *B. fabae* (and agar) from a 4-day-old stock plate onto a new Petri dish containing the same media amended with a quantity of the test compound. Test compounds were added using a sterile pipette when the media was cool but still molten (40-50°C).

A preliminary experiment was set up to establish the technique and range of concentrations suitable for the two oils. Six concentrations ranging from 0-4000ppm were incorporated into sterile media. Four replicates were set up for each concentration and plates were incubated in the dark at 18-20°C. Three radial measurements of mycelial growth were taken each day for 4 days. From the results of this experiment, it was decided that ranges of 0-1000ppm and 0-300ppm were suitable for oil 1 and oil 2 respectively.

3.6.2 Effects of five major components of basil oil tested individually against *B. fabae* grown on solid media

Five individual components: methyl chavicol, linalol, eugenol, eucalyptol and caryophyllene were purchased from Sigma Aldrich (Table 6). From a preliminary experiment, a concentration range was chosen to look at the effects of the compounds on radial mycelial growth of *B. fabae*: 0-1000ppm for methyl chavicol, 0-600ppm for linalol, 0-200ppm for eugenol, 0-800ppm for eucalyptol and 0-800ppm for caryophyllene (Section 3.6.1).

Component	Sigma Reference No.
Methyl chavicol	A 2,920-8
Linalol	L 260-2
Eugenol	E 5504
Eucalyptol	C 8144
Caryophyllene	C 9653

Table 6 Individual chemical components used in antifungal experiments

3.6.3 Effects of two different combinations of chemical components of basil oil tested against *B. fabae* grown on solid media

Two combinations of the major chemical components of the two basil oils (Table 5) were made up to assess how much of the antifungal activity of the oils was attributed to the main components and thus the importance of the minor and trace components of the oils when used as novel fungicides. Major components were mixed in the same proportions as the corresponding oils and tested at the same concentrations; combination 1 corresponding to oil 1 and thus made up [80:20] and tested at 0-1000ppm and combination 2 corresponding to oil 2 and made up [10:68:6.5:15.5] and tested at 0-300ppm.

3.6.4 Growth curve of *B. fabae* in liquid culture

A growth curve was constructed for *B. fabae* in order to determine the timing of its exponential growth phase. Conical flasks containing 100ml of liquid medium X (Last & Hamley, 1956) were inoculated with 7mm diameter plugs of *B. fabae* taken from 4-day-old stock plates. Flasks were placed in a Gallenkamp orbital incubator set at 90 rpm and

18-20°C. Fungal material from three flasks was harvested every 12 hours for 108 hours. The biomass was harvested using a fine mesh sieve, centrifuged (for 15 min at 20000g and 4°C using 50ml round bottom tubes in a JA30.50 rotor) and weighed. The fresh weight of fungal material was plotted as a function of time.

3.6.5 Effects of two different *O. basilicum* essential oils tested against *B. fabae* in liquid culture

Four replicate flasks were prepared for each concentration of the test compound containing sterile media, a concentration of essential oil and a 7mm diameter plug of fungus (cut into 6 sections) taken from a 4-day-old stock plate. All flasks were then placed in the dark in a Gallenkamp orbital incubator set at 18-20°C and 90 rpm. After 3 days, fungal material was harvested using a fine mesh sieve, rinsed with distilled water, centrifuged (for 15 min at 20000g and 4°C) and weighed. The fungal material was then placed in polythene bags, labelled and stored at -20°C for use in further experiments. This technique was an adaption from Deans *et al.* (1994) and Gundidza *et al.* (1993).

A preliminary experiment was carried out to establish a suitable range of concentrations at which to test oils 1 and 2 (Table 5) in liquid media. From this, it was decided that ranges of 0-30ppm and 0-50ppm were suitable for oils 1 and 2 respectively.

3.6.6 Effects of three major components of basil oil tested individually against *B. fabae* in liquid culture

From an initial experiment the following ranges were chosen to test the three major components against *B. fabae* in liquid media; 0-30ppm for methyl chavicol, 0-120ppm for linalol and 0-30ppm for eugenol. The oils and individual components were tested at

different concentrations in order to inhibit but not kill the pathogen so that fungal material could be harvested for further experimentation.

3.7 GLASSHOUSE EXPERIMENTS

3.7.1 Establishment of plants and their inoculation

A glasshouse experiment was carried out at Mansionfield Experimental Unit to test two basil oils (Table 5) and three individual chemical compounds (Table 6) found in varying concentrations in these and other basil oils against *Uromyces viciae-fabae* (broad bean rust). Broad bean seeds, cv. Bunyard's Exhibition, were obtained from Unwins Seed Co. (Cambridge, UK). The best seeds were chosen on the basis of size, colour and plumpness. Seeds were pre-soaked and those which failed to imbibe water were discarded. On 15th January, 2002, seeds were sown in 10.2 cm diameter pots containing Levington™M3 compost (Scotts UK Professional, Ipswich, UK). Experimental plants were inoculated with the rust pathogen by painting a spore suspension (25mg/100ml) onto the leaves. The two basil oils; methyl chavicol type and linalol type and three individual chemicals; methyl chavicol, linalol and eugenol were tested at two concentrations and at four temporal stages (Section 3.7.2). Plants were covered with polythene bags for 48 hours post-inoculation to increase humidity. Plants were placed in trays and irrigated from below throughout the experiment.

Glasshouse temperature throughout the experiment ranged from an average minimum of 8 °C to an average maximum of 23 °C. The average maximum from first treatment till assessment was 28°C. Daylight was supplemented from 7am to 10pm using high pressure mercury flourescent lamps (Philips HLRG 400W) placed 1.2m apart and 1m above the surface of the plant pots. Plants were watered twice daily or when the soil

showed signs of drying and no treatments other than experimental compounds were applied to the plants during the experiment.

3.7.2 Application of essential oils and their individual components

Treatments were applied at 1000 & 5000ppm. This was the effective concentration from Petri-dish experiments and five times that for the two whole oils, methyl chavicol and linalol. These concentrations were approximately 10 and 50 times the *in vitro* effective concentrations for eugenol. The first two leaflets were sprayed to run-off (using a hand held spray bottle which produced a fine mist) with each test compound and the first two pairs of leaflets were inoculated. Treatments were made up as follows:

1000ppm = 50µl of test compound in 50 ml distilled H_2O + 0.001% (v/v) *Tween 20

5000ppm = 250µl of test compound in 50 ml distilled H_2O + 0.001% (v/v) *Tween 20

*Tween 20 was used as an adjuvant for the test compound.

The four treatments were applied 3 days and 3 hours pre-inoculation and 3 hours and 3 days post-inoculation. The first treatments were carried out on Friday 7th March, 2002 and plants were assessed 15 days post-inoculation by determining the percentage leaf area infected using a standard area diagram.

The experiment was repeated as described above to test the same compounds against *Botrytis fabae* (broad bean chocolate spot). Seeds were planted on 3rd April, 2002, treatments were carried out between 26th April and 2nd May, 2002 and plants were assessed 5 days post-inoculation on 4th May, 2002 using a standard area diagram. The

experimental plants were inoculated with the chocolate spot pathogen by painting a spore suspension ($4x10^5$ conidia ml^{-1}) onto the leaves. Glasshouse temperature throughout the experiment ranged from an average minimum of 9°C to an average maximum of 31°C. The average maximum from first treatment till assessment was 34°C.

3.7.3 Experimental design

Five replicate plants per treatment were included and five control plants which were inoculated but not treated. Treatments were listed and numbered from 1 to 200 (5 test compounds x 2 concentrations x 5 replicates x 4 timings) and a random number (from 1 to 200) was allocated to each one corresponding to a plant on the experimental plan to achieve a complete randomised experimental design.

3.8 MICROSCOPIC EXAMINATION OF BOTRYTIS FABAE SARDIÑA (broad bean chocolate spot)

Due to the effects on growth habit observed in Petri dish experiments where *B. fabae* was treated with methyl chavicol and linalol type basil oils, further investigation was carried out by Andrew Syred (Microscopix Laboratory, Wales) using light and scanning electron microscopy. Liquid culture experiments were set up as described in Section 3.6.5 and samples were sent to Microscopix laboratory in Wales for examination.

3.9 DETERMINATION OF THE EFFECTS OF TWO *OCIMUM BASILICUM* ESSENTIAL OILS AND THREE MAJOR COMPONENTS ON POLYAMINE BIOSYNTHESIS, CATABOLISM AND EXCRETION IN *BOTRYTIS FABAE*

As described in Sections 3.6.5 and 3.6.6, fungal material was grown in liquid culture amended with varying concentrations of two *Ocimum* essential oils and three individual major components of the oils. After harvesting, the fungal material was immediately

placed in a freezer at -20°C until required for polyamine and enzyme determination assays.

3.9.1 ODC activity

In order to measure ODC activity in the fungal tissue, a crude enzyme extract was prepared by grinding 0.5g of fungal tissue in a pre-chilled mortar and pestle containing 1.75ml of buffer [0.04M KH2 PO_4, 0.04M KOH, 2mM 1,4-dithiothreitol (DTT), 1mM magnesium chloride, 0.1mM ethylenediaminetetra-acetic acid (EDTA), 0.1mM pyridoxal-5-phosphate - pH adjusted to 7.6]. Extracts were placed in glass test tubes and sonicated using a Soniprep 150 for 10 cycles of 10 s on/20 s off. Test tubes were kept on ice during sonication. Suspensions were then centrifuged for 15 min at 24000g and 0°C. The supernatant (cytosolic fraction) was dialysed in 30 volumes of buffer overnight at 4°C, using 16mm diameter dialysis sacks with a molecular weight cut off of 12000 (Sigma diagnostics cat. no. 250-11). ODC activity was assayed by measuring the $^{14}CO_2$ released after incubation with [1-^{14}C]ornithine - specific activity: 52mCi/mmol (Amersham Pharmacia Biotech UK Ltd). A reaction mixture was made up of 0.3ml reaction medium [50mM Tris/HCL, 0.05mM L-ornithine monohydrochloride, 0.031mM pyridoxal-5-phosphate - pH adjusted to 8.0], 0.1ml enzyme extract and 2.5µl (0.125µCi) of L-[1-^{14}C] ornithine hydrochloride. The reaction was carried out in glass test tubes. Rubber stoppers with 35mm long, 22 gauge needles inserted axially holding 6mm diameter filter papers impregnated with 2M KOH. Test tubes were placed in a water bath at 37°C for 30 minutes, after which the reaction was terminated with 0.2ml of 6% perchloric acid (v/v). Test tubes were incubated for a further 30 minutes then filter papers were removed and placed in 10ml of Emulsifier Safe scintillant (Packard Bioscience) and left to stand overnight. $^{14}CO_2$ released during the reaction and trapped

by the KOH was measured using a Packard Tri-Carb 1900 TR liquid scintillation counter. A Bradford protein determination assay (Bio-Rad Laboratories Ltd.) was carried out, with BSA (bovine serum albumin) as a standard, on the original cytosolic fraction to enable radioactivity counts in disintegrations per minute to be converted and expressed as pmol $^{14}CO_2$ (mg protein)$^{-1}$ hour^{-1}. The Bradford assay was carried out according to the manufacturer's instructions which are shown in Appendix 5.

3.9.2 AdoMetDC activity

A crude enzyme extract was prepared by grinding, sonication and centrifugation as described in section 3.9.1, after which 0.753g of ammonium sulphate was dissolved in the cytosolic fraction. Suspensions were then centrifuged at 24000g for 20 min at 0^{O}C. The pellet obtained was resuspended in 1.75ml of buffer and dialysed as described in section 3.9.1. AdoMet activity was assayed by measuring $^{14}CO_2$ released after incubation with S-adenosyl-L-[*carboxyl*-^{14}C]methionine - specific activity: 60mCi/mmol (Amersham Pharmacia Biotech UK Ltd). A reaction mixture was made up of 0.3ml of reaction medium [0.1M sodium phosphate, 0.2mM S-adenosyl-L-methionine, 1.0mM putrescine - pH adjusted to 7.4], 0.1ml of enzyme extract and 0.1µl S-adenosyl-L-[*carboxyl*-^{14}C] methionine. Subsequent steps in the assay were carried out as described in section 3.9.1.

3.9.3 Incorporation of radiolabelled ornithine into polyamines

Due to the fact that the substrate for spermidine synthase; decarboxylated S-adenosyl-L-methionine is not commercially available, an indirect measure of the activity of this enzyme was taken. This was done by allowing radiolabelled ornithine to be incorporated

into polyamines. The amount of radioactivity measured in the polyamines gives an indirect assessment of spermidine synthase.

An ODC assay as described in section 3.9.1 was carried out. After the final incubation in the water bath, 100µl of each sample was placed in a clean glass test tube. Radioactivity incorporated into polyamines was measured in dpm's (disintegrations per minute) in the scintillation counter. In order to do this, polyamines were separated using thin layer chromatography (TLC). A fluorescent label was also attached so that samples could be identified and scraped from TLC plates. Saturated sodium carbonate in distilled water (200µl) and 400µl dansyl chloride in acetone (30mg per ml) were added to each 100µl aliquot. Care was taken to avoid the dansyl chloride solution being exposed to light throughout the experiment. Markers were made up in the same way using 100µl of distilled water containing all polyamines to be measured in place of the 100µl sample. Reaction mixtures were incubated in a Gallenkamp benchtop oven at 60°C for 25 min, then 100µl of L-proline in distilled water (100mg per ml) was added and samples were incubated for a further 10 min at room temperature to stop the reaction. Dansylated polyamines were then extracted by adding 500µl of toluene and vortexing for 20 seconds. Aliquots from the toluene layer of each sample and markers (25µl) were spotted onto silica-gel TLC plates (Whatman LK6D) which had been activated in an oven at 110°F for 90 minutes. Plates were placed in pre-prepared TLC tanks containing chloroform:triethylene (12:1) (v/v). When separated, polyamines were identified using ultraviolet light. Spots were scraped from the plates into 10ml of Emulsifier-safe scintillant (Packard Bioscience) and radioactivity measured using a Packard Tri-Carb

1900 liquid scintillation counter. Values obtained as disintegrations per minute were expressed as dpm [mg protein]$^{-1}$.

3.9.4 Determination of free polyamines in fungal tissue

Extracts were prepared by grinding 0.5g of fungal tissue in a pre-chilled mortar and pestle using 1ml of 10% perchloric acid. The suspensions were centrifuged at 24000g for 25 minutes at 0°C. Volumes (100µl) of the supernatant were placed into clean glass test tubes and dansylated, then separated on TLC plates as described in Section 3.9.3. Spots were scraped off the plates into 20ml scintillation vials containing 4 ml of ethyl acetate. Standard curves were generated by making a dilution series (0.01µg/ml - 100µg/ml) in 10% perchloric acid, dansylating and separating as described in Section 3.9.3. Fluorescence was measured using a Perkin-Elmer LS5 luminescence spectrometer at excitation 365ηm and emission 506ηm. Fluorescence readings were converted and expressed as µmol g^{-1} FW and plotted against concentration (Appendix 6).

3.9.5 Determination of free polyamines in surrounding growth media

To determine whether polyamines were being excreted by the fungus into the surrounding media, the procedure for measuring free polyamines in fungal tissue was followed using samples of media in which the fungal pathogen had been growing during the inhibition experiment. Media was stirred well then 100µl samples were placed into clean glass test tubes. A 200µl volume of perchloric acid was added and subsequent steps in the free polyamine extraction protocol were followed. Spots were scraped off the plates into 20ml scintillation vials containing 4ml of ethyl acetate. Fluorescence was

measured using a Perkin-Elmer LS5 luminescence spectrometer at excitation 365ηm and emission 506ηm. Fluorescence readings were converted and expressed as μmol g^{-1} FW.

3.9.6 DAO activity

To measure diamine oxidase activity, 0.6g of fungal tissue was ground in a pre-chilled mortar and pestle with 4ml of buffer [100mM potassium phosphate, 2mM 1,4-dithiothreitol - pH adjusted to 8.0]. The crude enzyme extract was centrifuged at 20000g for 20 minutes at 4°C. A 0.5ml volume of the supernatant was placed in a clean glass test tube to which 0.5ml of reaction medium [100mM potassium phosphate, 1.0 mM putrescine, 30μg catalase] and 0.15μCi of [1,4-^{14}C] putrescine dihydrochloride - specific activity: 114mCi/mmol (Amersham Biotech UK Ltd) was added. Test tubes were placed in a water bath at 37°C for 30 min. The reaction was then stopped by adding 1ml of 4M KOH and shaking. Product was extracted by adding 2ml of toluene and vortexing for 10 seconds and letting stand for 30 min. An aliquot (1ml) was taken from the toluene layer, then transferred to a 20ml scintillation vial containing 10ml of Emulsifier safe scintillant (Packard Biosscience). Radioactivity was measured using a Packard Tri-Carb 1900TR liquid scintillation counter. A Bradford protein determination assay was carried out, with BSA as a standard, on the original extract to enable radioactvity counts in disintegrations per minute to be converted and expressed as pmol product [mg protein]$^{-1}$ hour^{-1}.

3.9.7 PAO activity

Polyamine oxidase activity was determined as described for diamine oxidase in section 3.9.6 with the following modifications. The PAO reaction medium contained [100mM potassium phosphate, 1mM spermidine and 30μg catalase - pH adjusted to 8.0] and the

radiolabel used was [^{14}C] spermidine trichloride. The grinding buffer was the same as described for DAO in Section 3.9.6.

3.10 STATISTICAL ANALYSES

Data values shown throughout this research are means of the number of replicates per treatment used in each experiment (indicated in each individual data set).

The reliability of each mean value representing the population mean is indicated by the standard error of the mean. These values are illustrated by plus and minus ($\pm$) error bars above and below data points on each graph.

A student's *t*-test was used to test the significance of the difference between a treatment and control mean (Sokal & Rohlf,, 1981). The variances in the populations from which the two samples were taken were assummd to be equal. Significant differences between a treatment and control mean are denoted as *($P \leq 0.05$), **($P \leq 0.01$) or ***($P \leq 0.001$).

To allow standard curves to be constructed in order to convert fluorimetric values (365/506nm) into concentration of individual polyamines (μg/ml), a dilution series was made and absorbance measured. The best straight-line fit between the two variables was determines using simple regression analysis and expressed as an equation based on -

$$y = mx + c$$

y = Absorbance (365/605nm)

m = gradient of the straight line

x = polyamine concentration (μg/ml)

c = y-axis intercept

4 RESULTS

4.1 CHARACTERISATION OF NCRPIS GERMPLASM COLLECTION

Accessions of the *Ocimum* germplasm collection were grown at the Plant Introduction Station at Ames and morphologically characterised by Dr M P Widrlechner using identification keys compiled by Paton (1992) and Albuquerque & Andrade (1998) (Appendix 1). On the basis of morphological characters, each accession was preliminarily classified and named under the corresponding species. This list was retained for comparison with chemical data (Table 8).

4.2 CULTIVATION OF PLANTS AT SAC, AUCHINCRUIVE

Three accessions of the collection (Section 3.1.2) were successfully cultivated in the ground within a glasshouse at Diamondfield Experimental Unit. This allowed basic observation of germination and morphological stages of development and a comparison of oil profiles with the same accessions grown in Ames. All plants were harvested at early flowering stage, hydrodistilled and analysed by GC and GC-MS. Plants of *Ocimum basilicum* accessions PI207498 and PI172996 both produced oils containing 93% methyl chavicol (Table 7) compared to 88% and 91%, respectively when grown in Iowa. Leaves, flowers and whole plants of *O. tenuiflorum* accession PI414201 were distilled separately to determine if variation existed between oil profiles within the plant. Differences were observed between the plant parts; flowers produced an oil consisting mainly of eucalyptol, β-bisabolene and an unknown fraction, while oil obtained from the leaves contained mainly methyl chavicol, eugenol and eucalyptol. It was also noted that fresh whole plant material distilled from plants grown at Auchincruive contained all five main components in essentially equal proportions (although methyl chavicol was

			% of each identified components in total oil				
Inventory number	**Oil yield (% v/w)**	**plant part**	**euc**	**methyl chav**	**β-bisab**	**?**	**eugenol**
414201q	0.1	flowers	37	5	20	15	6
414201q	0.1	leaves	12	18	-	6	40
414201q	0.1	whole plant (fresh)	16	8	18	14	22
414201q	0.3	whole plant (dried)	13	20	19	12	5

Table 7 Chemical composition of *O. tenuiflorum* from NCRPIS germplasm collection, grown at SAC, Auchincruive

euc = eucalyptol

β-bisab = β-bisabolene

methyl chav = methyl chavicol

? = unknown

q = US Maryland

slightly lower than the rest), while the dried whole plant material sent from Iowa contained a substantially higher amount of methyl chavicol and lower amount of eugenol.

4.3 ESSENTIAL OIL YIELD OF PLANTS IN THE NCRPIS *OCIMUM* COLLECTION

Oil yields from plants in the collection were calculated as percent volume per weight (%v/w). These ranged from 0.3 to 5.0%. The highest yielding species was *O. americanum*. A large number of *O. basilicum* accessions was examined and these were found to have oil yields between 0.5 and 2.9 %. Oil yields of the full collection and those accessions grown at SAC are shown in Tables 7 and 8.

4.4 GAS LIQUID CHROMATOGRAPHY OF ESSENTIAL OILS

Essential oils of the accessions in the germplasm collection were analysed by GC. Oil profiles are shown in Table 8 and Appendix 10.

4.4.1 Accessions of *Ocimum basilicum*

Final results were recorded for fifty accessions of *O. basilicum* from thirteen different geographical locations. Throughout the study, it became obvious that the correct labelling and documentation of the history of each individual accession was important, both for plant classification and greater awareness of the overall distribution of the genus. In addition, it was noted that common names do not distinguish between different chemotypes.

Inventory No. *	Oil yield (% v/w)	% of each identified component in total oil: Linalol	Geraniol	Geranyl acetate	Methyl eugenol	Citral	Methyl chavicol	Eugenol	Eucalyptol	Camphor	Beta-pinene	Fenchone	Limonene	Methyl cinnamate	Anethole	Beta-bisabolene	unknown	Elemene	unknown	Beta-Caryophyllene	Germacrene D	Ocimene	Copaene	Caryophyllene oxide	Asarone
297498a	2.3	...	...	...	5.8	...	87.7	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
211589a	1.6	42.1	...	...	36.9	...	...	...	5.1	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
197442d	2.9	77.4	5.2	...	...	...	...	...	5.3	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2658870e	1.6	57.2	...	...	6.3	...	...	13.4	6.0	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
5812966f	1.7	64.3	...	...	...	...	5.3	7.4	5.8	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
1903066h	0.7	8.9	...	...	4.2	...	56.9	4.6	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2531578i	1.8	...	...	...	...	48.7	38.6	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2963905h	1.3	7.2	...	...	4.9	...	66.1	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2963916	1.6	...	...	...	...	39.4	49.4	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
24983i	1.5	47.9	...	...	...	...	...	13.2	8.2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
3584635	1.8	70.9	10.2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
3584635	0.6	20.8	25.7	9.1	...	...	...	8.1	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
3584675	1.5	58.7	9.9	...	...	...	5.5	...	7.2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
3584685	1.3	15.9	...	...	...	...	57.7	...	10.3	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
3584695	1.3	36.8	7.9	...	...	...	26.3	...	9.9	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
3584705	0.7	8.9	11.0	...	...	...	39.9	...	16.4	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
3584715	1.2	44.9	5.8	...	...	...	...	...	10.5	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
3584725	1.3	69.6	6.4	...	...	...	8.1	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
3686935	1.2	47.7	...	...	...	...	14.3	5.7	12.1	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
3686975	1.3	20.0	...	...	...	...	65.3	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
3686985	2.0	68.3	...	...	...	...	4.2	...	10.1	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
3686995	1.1	38.6	...	...	...	...	...	...	26.0	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
3794125	1.3	49.8	4.6	...	...	...	21.2	...	5.0	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
3794135	1.0	71.4	7.2	4.2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
3794145	2.3	58.8	13.6	...	...	...	...	...	4.0	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
1705795	1.3	45.4	...	...	9.6	...	...	6.3	6.0	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
1705795	0.5	25.8	...	...	...	...	...	62.3	5.0	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
1705815	1.4	46.4	...	...	15.9	...	...	10.8	4.4	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
1726965	2.5	...	...	...	...	...	91.1	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
1729975	1.8	...	...	...	...	...	91.8	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
1729985	1.8	...	...	...	...	...	90.9	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
1737465	0.9	51.0	...	...	4.8	...	12.3	3.6	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
1742855	0.7	20.7	...	...	5.7	...	51.1	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
1757505	2.1	78.2	8.8	...	...	...	...	...	4.0	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
1766665	0.8	30.0	...	...	37.4	...	...	6.2	5.8	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
1822465	1.0	39.9	...	...	16.0	...	...	...	4.6	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
1938695	1.0	43.1	...	...	20.3	...	...	...	10.7	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
4741955	0.8	37.9	...	...	...	...	5.4	11.7	13.6	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
4141945	1.0	31.9	...	...	...	...	32.9	...	8.0	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
4141955	1.7	22.2	...	...	...	...	54.9	...	4.8	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
4141965	1.3	35.9	...	...	...	...	38.7	6.2	5.5	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
4141975	0.8	30.5	...	...	8.0	...	...	13.9	16.7	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
4141985	1.7	11.2	...	...	...	...	65.2	...	4.8	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
4141995	1.7	20.9	...	...	...	...	55.5	...	8.2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
4142005	1.7	8.5	...	...	...	...	69.0	...	4.6	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
7772r	...	31.2	...	...	...	48.9	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
6421s	0.8	48.8	...	...	...	...	...	16.8	6.0	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
3584645	1.4	68.4	...	...	...	...	8.0	...	9.4	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
3584665	1.1	43.7	20.7	...	5.6	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
24444g	2.8	...	...	...	...	84.0	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
5009425	solid	...	...	...	...	...	...	...	...	46.8	...	...	13.3	...	...	...	...	...	...	...	...	...	...	...	...	...
5009435	2.2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
5009445	3.7	...	...	...	...	...	...	...	...	...	...	70.4	7.3	...	...	...	...	...	...	...	...	...	...	...	...	...
5009455	2.8	...	...	...	...	...	...	...	...	...	...	70.5	6.7	...	...	...	...	...	...	...	...	...	...	...	...	...
5009475	3.9	...	...	...	...	...	...	...	...	...	...	68.6	7.3	...	...	...	...	...	...	...	...	...	...	...	...	...
5009485	3.4	...	...	...	...	...	...	...	51.8	...	7.1	...	16.3	...	...	...	...	...	...	...	...	...	...	...	...	...
5009495	4.7	...	...	...	...	...	...	...	49.1	...	6.1	...	15.5	...	...	...	...	...	...	...	...	...	...	...	...	...
5009505	dropped	...	...	...	...	...	...	...	46.9	...	7.1	...	19.5	...	...	...	...	...	...	...	...	...	...	...	...	...
5009515	4.0	...	...	...	...	...	...	...	27.1	...	...	...	6.9	47.5	...	...	...	...	...	...	...	...	...	...	...	...
5009935	3.8	...	...	...	...	...	...	...	44.4	...	7.3	...	30.0	...	...	...	...	...	...	...	...	...	...	...	...	...
5009545	3.9	...	...	...	...	...	...	...	46.3	...	6.4	...	20.6	...	...	...	...	...	...	...	...	...	...	...	...	...
16796	2.3	...	...	...	...	...	31.0	9.2	11.4	12.2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
23155b	1.3	...	...	...	13.4	...	...	13.9	...	...	...	...	...	...	...	...	...	...	5.7	15.4	41.9	...	...	...	...	...
23154c	0.9	...	...	...	...	...	...	5.9	15.8	...	...	...	...	...	15.8	22.3	11.3	...	...	...	...	...	...	...	...	...
2887795	1.8	...	...	...	...	...	...	33.0	...	...	...	...	...	...	...	5.0	...	26.5	...	...	8.2	...	...	...	...	...
23157c	0.8	...	...	...	...	...	...	8.2	17.6	...	...	...	...	...	17.4	16.3	10.8	...	...	...	...	...	...	...	...	...
4142015	0.8	...	...	...	...	...	20.0	5.4	15.2	...	...	...	...	...	...	19.4	12.2	...	...	...	...	...	...	...	...	...
4142045	0.7	...	...	...	...	...	13.8	12.4	18.9	...	...	...	...	...	...	15.4	7.1	...	...	...	...	...	...	...	...	...
4142055	1.2	...	...	...	...	...	10.1	36.2	14.9	...	...	...	...	...	...	8.9	5.4	...	...	...	...	...	...	...	...	...
7833	0.5	...	...	...	...	...	4.3	48.8	...	...	...	...	...	...	...	...	...	...	...	5.0	...	6.9	...	...	10.8	...
24449m	0.3	...	...	...	...	...	...	11.7	...	...	...	...	...	...	...	...	...	...	...	...	...	...	5.2	27.8	...	...
5009525	4.3	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	9.3	7.8	...	7.5	23.0	...	...
2113695	...	7.0	...	...	64.0	...	6.3	...	2.7	...	...	...	...	...	...	...	...	...	...	...	...	...	...	7.3	...	...

Table 8 Chemical components in accessions of NCRPIS germplasm collection

* Origin

a = Afghanistan; b = Cuba; c = Denmark; d = Ethiopia; e = Greece; f = Hungary; g = India; h = Iran; i = Italy; j = Macedonia; k = Maldives; l = Sri Lanka; m = Tanzania; n = Turkey; o = Uruguay; p = US California; q = US Maryland; r = US New Mexico; s = US Pennsylvania; t = Yugoslavia; u = Zambia

Table 8 shows the relative proportions of seven components (>5%) found in the *O. basilicum* group: eucalyptol (5.0-26.0%), linalol (7.2-77.4%), eugenol (5.0-52.3), citral (39.4-48.9%), methyl eugenol (5.0-37.4%), methyl chavicol (5.0-91.8%) and geraniol (5.0-25.7%). Linalol occured most frequently, followed by eucalyptol then methyl chavicol. Eugenol, methyl eugenol and geraniol were present in one third to one quarter of samples. Plants containing highest amounts of linalol were mainly from C & SE Europe and the Middle East, although the accession with highest linalol content was from Ethiopia. Plants with highest methyl chavicol were mainly from the Middle East. Linalol and methyl chavicol appeared to have an inverse relationship. Samples with highest amounts of linalol also contained geraniol but had little methyl chavicol. In contrast, oils with highest methyl chavicol little or no linalol. Linalol/citral and methyl chavicol/citral types had no other major components.

Eucalyptol, eugenol and geraniol were always accompanied by linalol. Highest methyl chavicol samples did not contain these three components. Samples with a higher methyl eugenol content had no methyl chavicol. With one exception, samples containing geraniol had no eugenol or methyl eugenol. Geraniol and geranyl acetate were mainly found in samples from Macedonia. In contrast, no methyl eugenol and little eugenol was present in the Macedonian plants. Only three accessions contained citral but in these cases it accounted for almost half the oil. Overall, four out of the seven main components were oxygenated monoterpenes (eucalyptol, linalol, citral and geraniol) and three were phenylpropenes (methyl chavicol, eugenol and methyl eugenol).

Taking a chemotype component as ≥30% (denoted in bold), seven chemotypes were noted: methyl chavicol, linalol, eugenol, methyl chavicol/linalol, methyl chavicol/citral,

linalol/citral and linalol/methyl eugenol. No sesquiterpenes were found in any of these oils in quantities ≥ 5%, although cadenine at least was present at around 4% in two accessions.

4.4.2 Accessions of *Ocimum americanum*

Results for the *O. americanum* spp. included thirteen accessions, eleven of which are from Zambia (Table 8). Analysis of the essential oils from these samples produced seven distinct groups with seven main components: camphor (47%), fenchone (70-71%), eucalyptol (34-52%), methyl cinnamate (48%), methyl chavicol (31%), limonene (7-30%) and citral (84%). Each of the seven main components constituted a different chemotype. All eleven accessions from Zambia contained limonene. *O. americanum* was the only species to contain this component. *O. americanum* was also the only species to contain fenchone, methyl cinnamate and a lesser component, β-pinene, which always occurred along with eucalyptol and limonene. Fenchone and eucalytol chemotypes had the lowest amount of limonene, whilst camphor and eucalyptol/β-pinene had higher amounts. Overall, *O. americanum* species consisted of oxygenated monoterpene chemotypes (camphor, fenchone, eucalyptol and citral), an oxygenated monoterpene/monoterpene hydrocarbon chemotype (eucalyptol/limonene) and phenylpropene chemotypes (methyl chavicol and methyl cinnamate). Again, sesquiterpenes did not constitute the major components of the oils.

4.4.3 Accessions of *Ocimum tenuiflorum*

Seven different accessions of *O. tenuiflorum* were in the collection. Three samples produced distinct chemotypes (caryophyllene and eugenol x2) and the four other

samples produced oils made up of components no greater than 22% (Table 8). The essential oil from sample A23155 contained over 40% of the sesquiterpene caryophyllene and lesser amounts of phenylpropenes eugenol and methyl eugenol, a small amount of the sesquiterpene elemene and over 15% of an unknown fraction. Plants of accession PI288779 produced oil containing 33% of the phenylpropene eugenol along with a substantial amount of the sesquiterpene elemene (26.5%) and lesser amounts of sesquiterpenes caryophyllene and β-bisabolene. Essential oil of the third chemotype PI414205 consisted of 36.7% eugenol (phenylpropene) along with eucalyptol (oxygenated monoterpene), methyl chavicol (phenylpropene), β-bisabolene (sesquiterpene hydrocarbon) and an unknown fraction.

Accessions PI414201 and PI414204 produced similar oils, neither of which contained components over 20% and consisted of methyl chavicol, β-bisabolene, eucalyptol, eugenol and an unknown fraction. This species produced the widest range of major essential oil components and in contrast to *O. basilicum* and *O. americanum*, the oils contained substantial amounts of sesquiterpenes. All accessions contained eugenol and all except the Cuban samples produced β-bisabolene. Most also contained eucalyptol and an unknown fraction. The two eugenol chemotypes from Maryland, US and India had low β-bisabolene and other accessions with low eugenol had higher β-bisabolene. Also, quantities of the unknown fraction increased with higher β-bisabolene.

4.4.4 Accessions of *Ocimum gratissimum*

The collection contained three accessions of *O. gratissimum*, one of which (A7833) produced essential oil with a main component ≥30% (Table 8). The oil consisted of

40.8% eugenol plus lesser amounts of caryophyllene, caryophyllene oxide, ocimene (E and Z) and methyl chavicol. Essential oil from accession A24448 contained 27.8% caryophyllene and small amounts of eugenol and copaene. The largest component of the third *O. gratissimum* accession PI500952 was again caryophyllene (23.0%) and minor components included caryophyllene, germacrene, copaene and linalol. A7833 was of the variety *macrophylla* and A24448 and PI500952 were both var. *gratissimum*, this distinction was reflected in the essential oil profiles. As with *O. tenuiflorum*, main chemical components were either phenylpropene or sesquiterpene (oxygenated) and additional constituents included sesquiterpene hydrocarbons, phenylpropenes, monoterpene hydrocarbons and oxygenated monoterpenes.

4.4.5 Accessions of *Ocimum selloi*

A single accession of *O. selloi* was grown at SAC from seed provided by NCRPIS in order to observe the morphological features of the species as little information could be obtained from the literature. Visually these plants were completely different from any other *Ocimum* species. Plants grew very low to the ground in a rosette-like fashion. Leaves were dark green in colour and were very thick and waxy with an abundance of leaf hairs. When the leaves were rubbed, unlike most other plants in the genus, the essential oil did not smell pleasant. GC-MS results indicated the *O. selloi* to be a methyl eugenol chemotype with small amounts of linalol, methyl chavicol (or anethole), caryophyllene and asarone (Table 8).

4.5 GAS CHROMATOGRAPHY-MASS SPECTROMETRY OF ESSENTIAL OILS

Twenty-eight samples representing species, chemical and geographical variation within the collection were sent to Liverpool University and analysed by GC-MS (Appendix 8). Two samples containing a main component constituting 70% of the oil from *O. americanum* species were sent along with 5 other oils (Appendix 9) to the laboratory of Professor Asakawa in Tokushima, Japan where they were verified by GLC, GC-MS, IR and NMR. Tables were compiled from all analyses, coupled with the morphological characterisation and a final classification of the germplasm collection was made. A summary table listing chemical components >30% and chemical components >20% can be found in Appendix 10

4.6 *IN VITRO* ANTIFUNGAL BIOASSAYS

4.6.1 Effects of methyl chavicol type basil oil on *B. fabae* grown on solid media

Experiments were carried out as described in Section 3.6.1. Visually, mycelium appeared less dense on treated plates in comparison to controls. In addition to the sparse, stringy appearance, overall growth was affected (Plate 4). As can be seen in Figure 12, radial growth of mycelia declined with increasing concentrations of the test compound. All concentrations tested in this experiment resulted in significant reductions ($P \leq 0.001$) of mycelial growth at all assessment times in comparison to controls. Greatest reductions were observed with treatments of 1000ppm on d 2, 3 and 4.

4.6.2 Effects of linalol type basil oil on *B. fabae* grown on solid media

In contrast to samples treated with the methyl chavicol type oil, mycelial growth was visually more dense with a defined periphery. Significant reductions in growth were

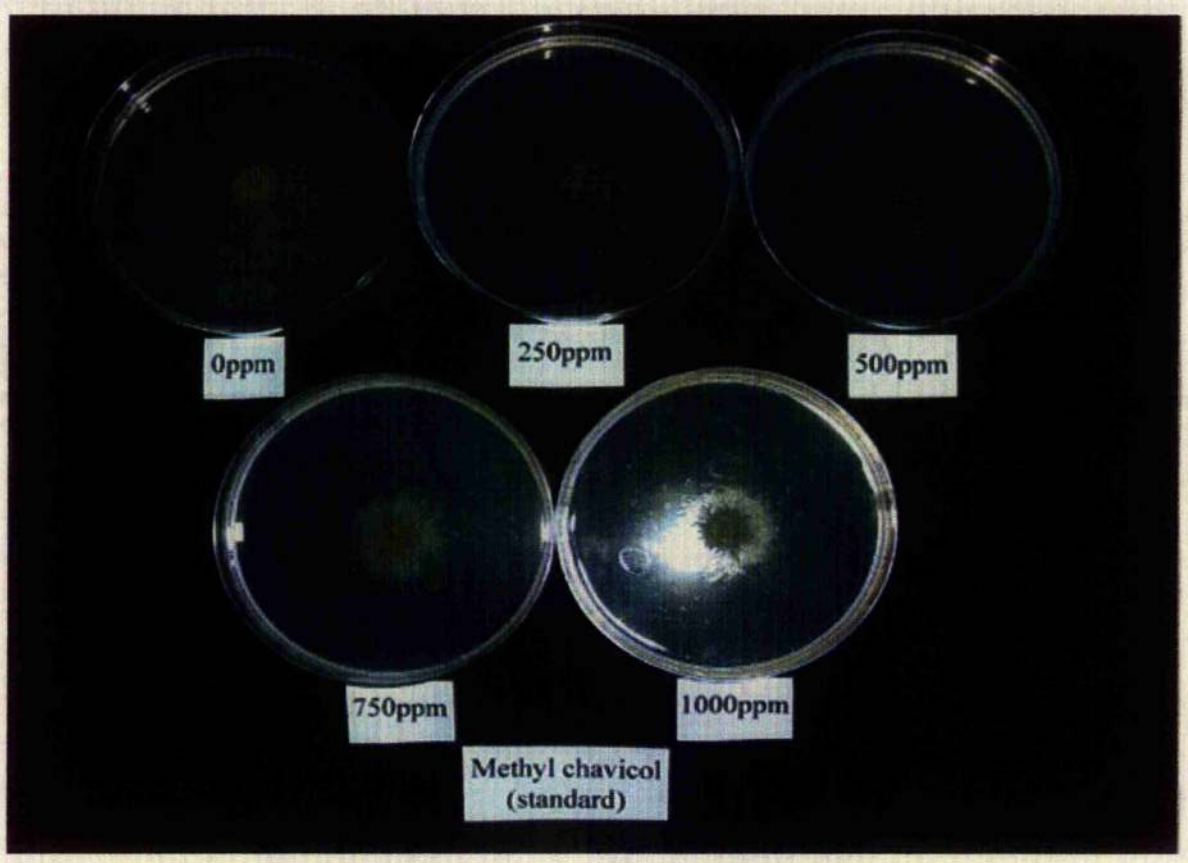


Plate 4 Effects of different concentrations of methyl chavicol type basil oil on *Botrytis fabae* grown on solid media

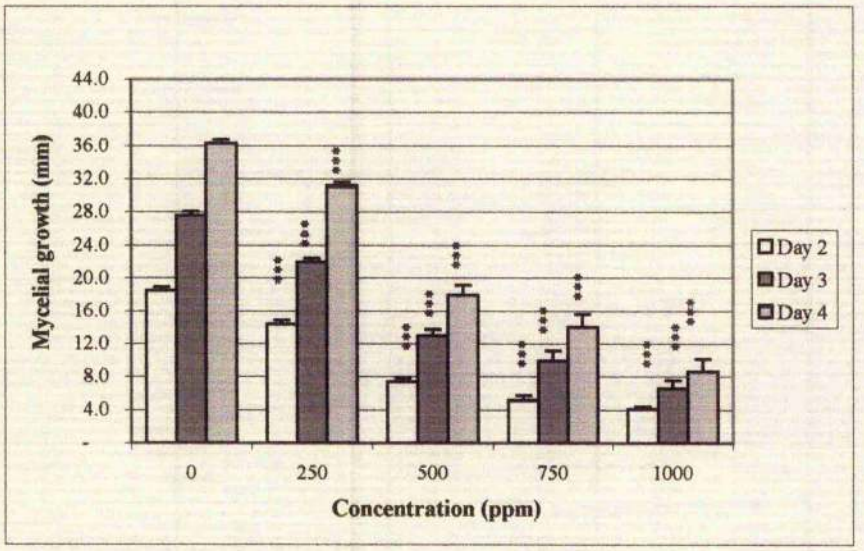


Figure 12 Effects of various concentrations of methyl chavicol type basil oil (0-1000ppm) on mycelial growth of *Botrytis fabae* grown on solid media. Values are the means of 3 radial measurements of 4 replicates 2, 3 and 4 days after inoculation. Error bars indicate standard error of the mean. Significant differences from controls calculated using the student's *t*-test are shown as $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$

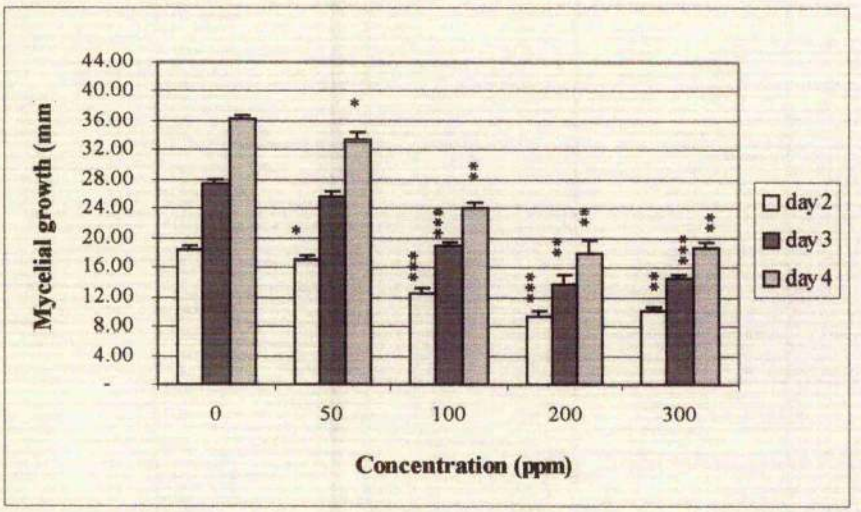


Figure 13 Effects of various concentrations of linalol type basil oil (0-300ppm) on mycelial growth of *Botrytis fabae* grown on solid media. Values are the means of 3 radial measurements of 4 replicates 2, 3 and 4 days after inoculation. Error bars indicate standard error of the mean. Significant differences from controls calculated using the student's *t*-test are shown as $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$

observed with all concentrations of the test oil at all assessment times apart from 50ppm on d 3 (Figure 13). Treatment with linalol type basil oil led to significant reduction in growth 2 and 4 d after inoculation of plates ($P \leq 0.05$). All other concentrations and assessment times gave significant decreases in fungal growth at the $P \leq 0.001$ level.

4.6.3 Effects of five individual components of basil oil on *B. fabae* grown on solid media

4.6.3a Methyl chavicol

Methyl chavicol, which makes up 76.1% of oil 1 (Table 5), was tested against *B. fabae* as described in Sections 3.6.1 and 3.6.2, at the same concentrations as the whole oil. Figure 14 shows that significant reductions in growth were observed at all concentrations, with growth decreasing as concentration was increased. Greatest control was again observed at 1000ppm on d 2, 3 and 4.

4.6.3b Linalol

Linalol makes up 18.6% of oil 1 and 53% of oil 2. All concentrations at all assessment times caused significant decreases in fungal growth (Figure 15). Exposure of *B. fabae* to 300ppm linalol led to a greater reduction in fungal growth than that achieved with the whole oil.

4.6.3c Eugenol

Eugenol is the second largest component of the linalol type oil but only makes up 12.4%. All concentrations of the oil at all assessment times were shown to reduce fungal growth significantly compared to controls (Figure 16). No growth was observed after 4 d

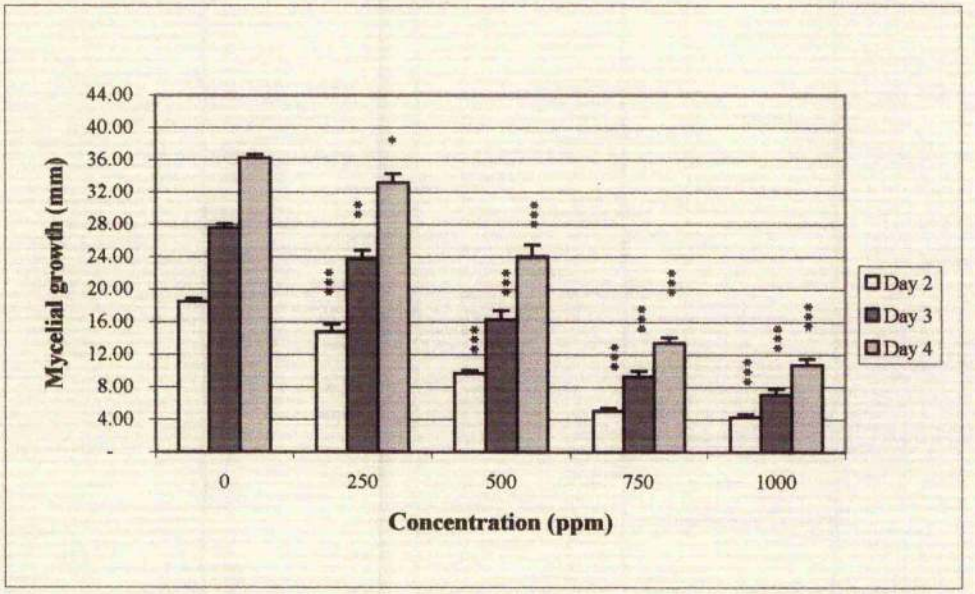


Figure 14 Effects of various concentrations of methyl chavicol (0-1000ppm) on mycelial growth of *Botrytis fabae* grown on solid media. Values are the means of 3 radial measurements of 4 replicates 2, 3 and 4 days after inoculation. Error bars indicate standard error of the mean. Significant differences from controls calculated using the student's *t*-test are shown as * $P \leq 0.05$; ** $P \leq 0.01$; *** $P < 0.001$

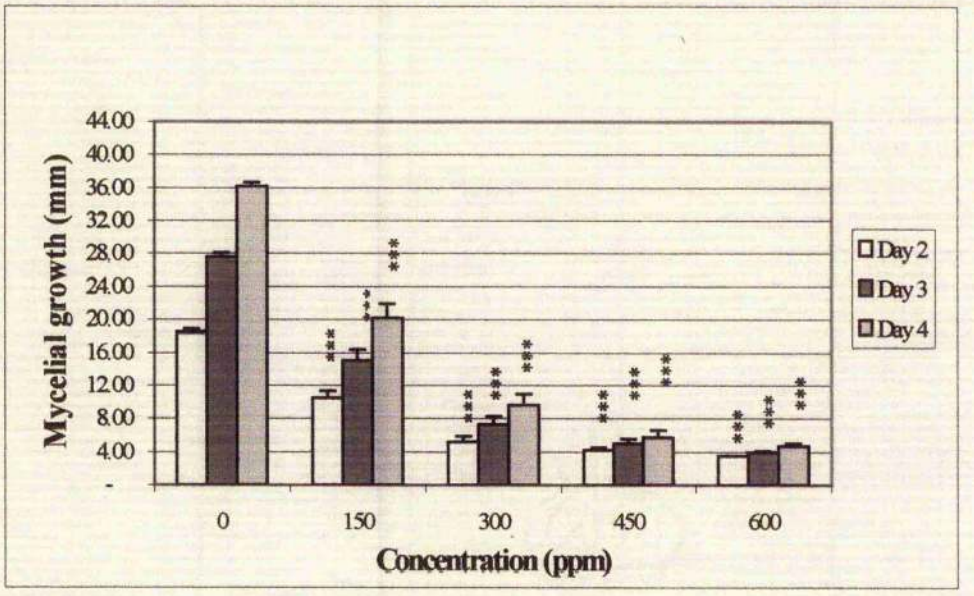


Figure 15 Effects of various concentrations of linalol (0-600ppm) on mycelial growth of *Botrytis fabae* grown on solid media. Values are the means of 3 radial measurements of 4 replicates 2, 3 and 4 days after inoculation. Error bars indicate standard error of the mean. Significant differences from controls calculated using the student's *t*-test are shown as * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

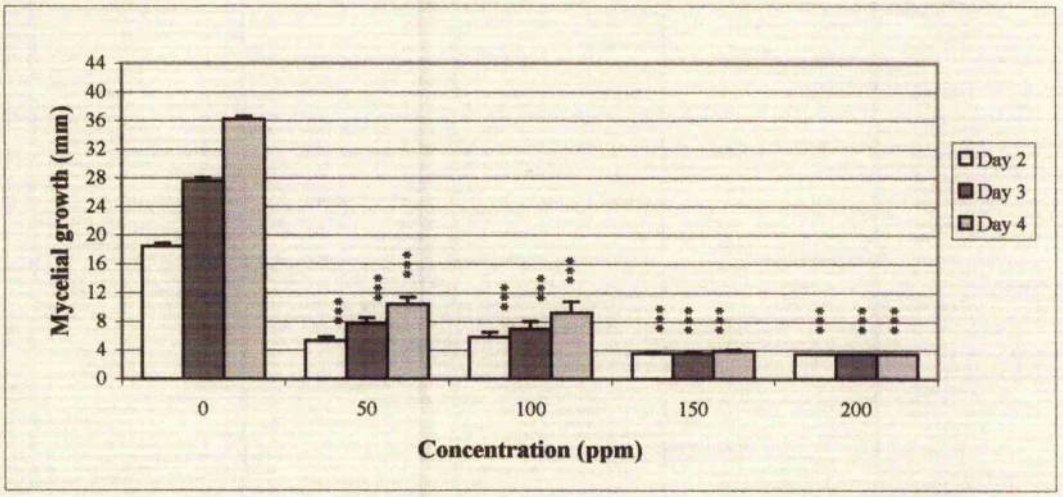


Figure 16 Effects of various concentrations of eugenol (0-200ppm) on mycelial growth of *Botrytis fabae* grown on solid media. Values are the means of 3 radial measurements of 4 replicates 2, 3 and 4 days after inoculation. Error bars indicate standard error of the mean. Significant differences from controls calculated using the student's *t*-test are shown as $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$

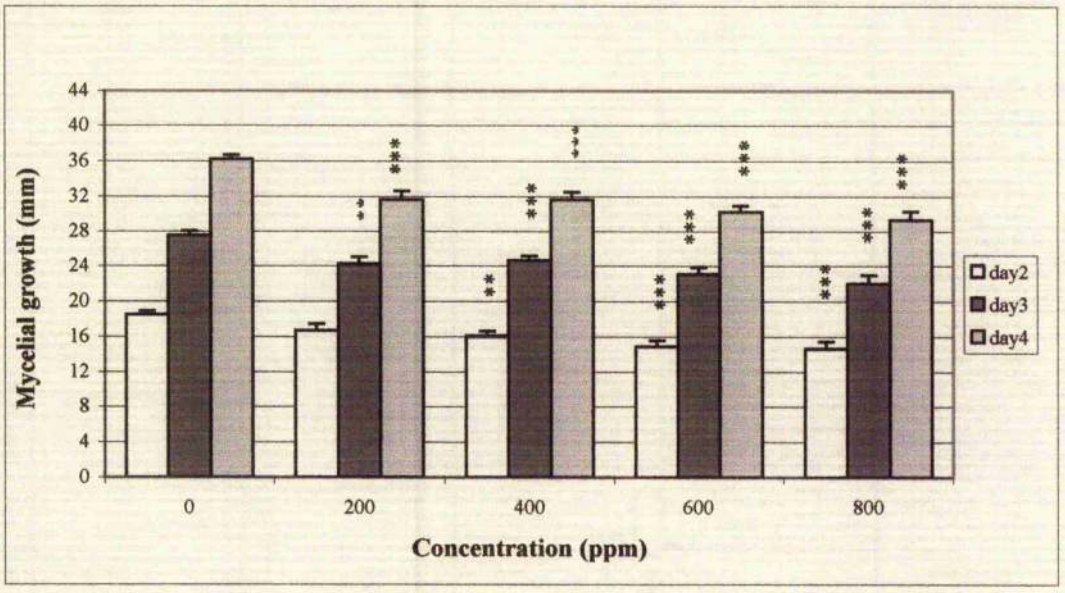


Figure 17 Effects of various concentrations of eucalyptol (0-800ppm) on mycelial growth of *Botrytis fabae* grown on solid media. Values are the means of 3 radial measurements of 4 replicates 2, 3 and 4 days after inoculation. Error bars indicate standard error of the mean. Significant differences from controls calculated using the student's *t*-test are shown as $* P \leq 0.05$; $** P \leq 0.01$; $*** P < 0.001$

exposure to 150 and 200 ppm of the test compound. Minimal fungal growth was noted after 4 d with the lowest concentration, 50ppm.

4.6.3d Eucalyptol

Eucalyptol constitutes 7.7% of the linalol type oil. Figure 17 shows that all concentrations at all timings with the exception of 200ppm on d 2 gave significant reduction in fungal growth ($P \leq 0.001$). However, growth inhibition did not increase greatly from 200 to 800ppm.

4.6.3e Caryophyllene

Carryophyllene makes up 5% of oil 2 and although it did not reduce growth significantly at 200 and 400ppm (Figure 18), treatment with 600 and 800ppm caryophyllene caused significant decreases in growth.

4.6.4 Effects of combination 1 on *B. fabae* grown on solid media

The major components of the methyl chavicol type basil oil were mixed together in proportions similar to those of the whole oil [80:20]. This combination was tested as described in Sections 3.6.1 and 3.6.3 at the same concentrations as the methyl chavicol type oil and methyl chavicol single component (250-1000ppm). As with the whole oil, significant reductions in mycelial growth were observed with all concentrations tested at all assessment times, with inhibition increasing with concentration (Figure 19).

4.6.5 Effects of combination 2 on *B. fabae* grown on solid media

The main components of the linalol type basil oil were mixed together in proportions similar to those of the whole oil [10:68:6.5:15.5]. As can be seen from Figure 20, there

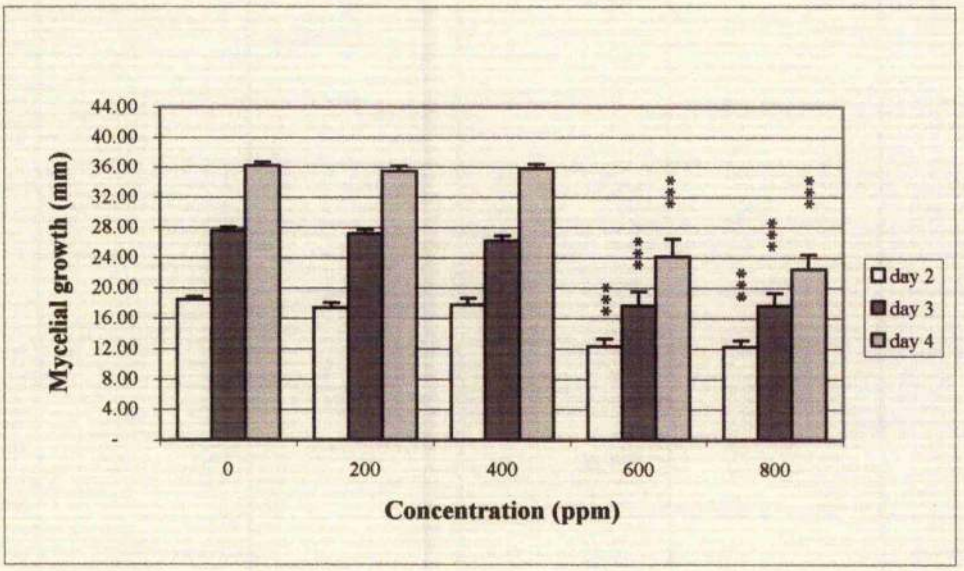


Figure 18 Effects of various concentrations of caryophyllene (0-800ppm) on mycelial growth of *Botrytis fabae* grown on solid media. Values are the means of 3 radial measurements of 4 replicates 2, 3 and 4 days after inoculation. Error bars indicate standard error of the mean. Significant differences from controls calculated using the student's *t*-test are shown as $*P< 0.05$; $**P< 0.01$; $***P< 0.001$

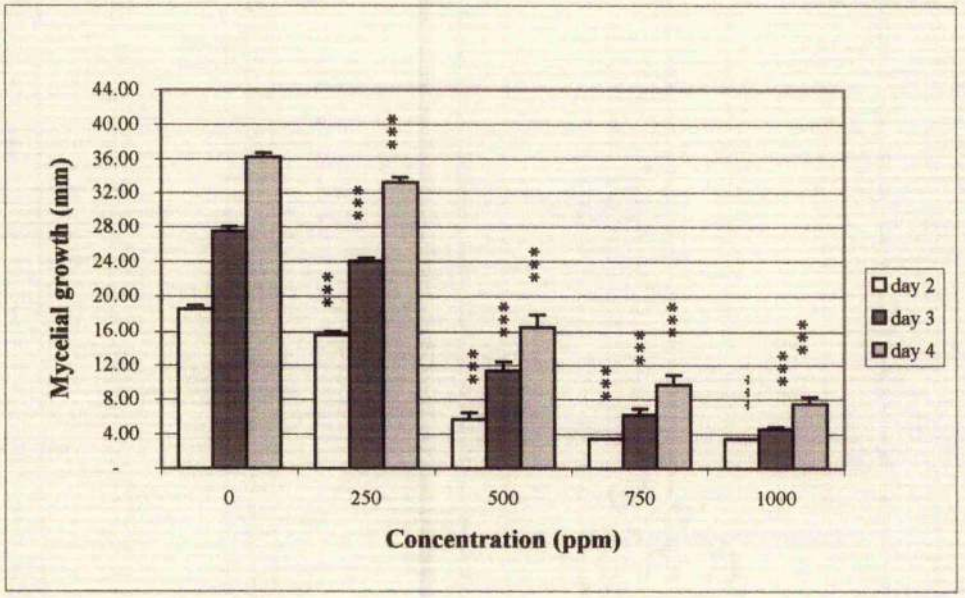


Figure 19 Effects of various concentrations of combination 1 (0-1000ppm) on mycelial growth of *Botrytis fabae* grown on solid media. Values are the means of 3 radial measurements of 4 replicates 2, 3 and 4 days after inoculation. Error bars indicate standard error of the mean. Significant differences from controls calculated using the student's *t*-test are shown as $*P\leq 0.05$; $**P\leq 0.01$; $***P\leq 0.001$

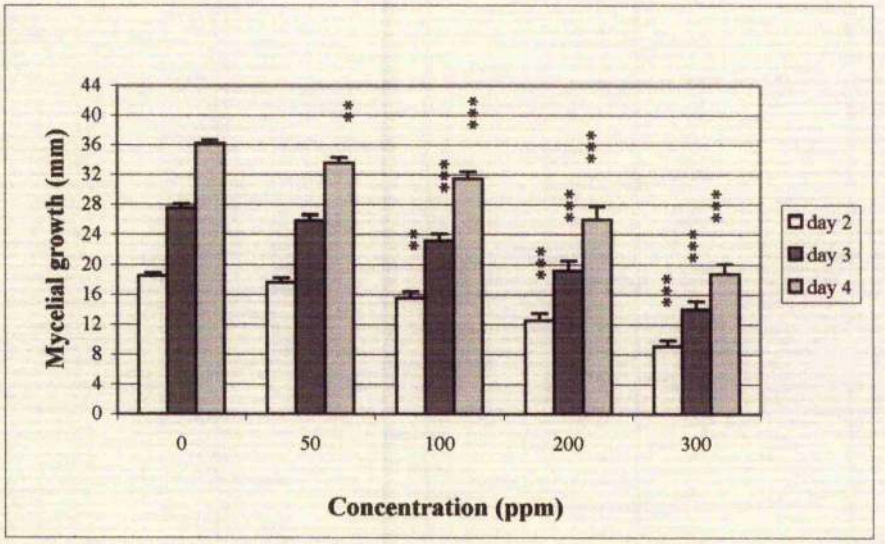


Figure 20 Effects of various concentrations of eugenol (0-300ppm) on mycelial growth of *Botrytis fabae* grown on solid media. Values are the means of 3 radial measurements of 4 replicates 2, 3 and 4 days after inoculation. Error bars indicate standard error of the mean. Significant differences from controls calculated using the student's *t*-test are shown as * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

were no significant reductions in growth with treatments of 50ppm on d 2 and 3 but a significant reduction was observed on d 4. Exposure to 100ppm showed significant reductions 2, 3 and 4 d following inoculation. Concentrations of 200 and 300ppm showed significant decreases in growth at all assessment times.

4.6.6 Growth curve of *B. fabae* in liquid culture

The growth of *B. fabae* in liquid culture was observed and a growth curve constructed. *B. fabae* is a relatively fast growing fungus with exponential growth taking place between 60 and 90 hours. Fungal material for experiments was harvested during the period of most rapid growth which, as can be seen from Figure 21, was 72 to 84 h.

4.6.7 Effects of methyl chavicol type basil oil and methyl chavicol on *B. fabae* in liquid culture

Experiments were carried out as described in sections 3.6.5 and 3.6.6. As can be seen in Figures 22 and 23, reductions in mycelial growth were observed with concentrations of both test compounds down to 2ppm. Treatment with 2-15ppm methyl chavicol oil led to significant reductions in fungal growth, while treatment with 20ppm and above caused complete inhibition of mycelial growth. Treatments of 2, 5 and 10ppm methyl chavicol resulted in significant inhibition of fungal growth, while no growth was observed in the flasks following treatments of 15ppm and above.

4.6.8 Effects of linalol type basil oil and linalol on *B. fabae* in liquid culture

Figures 24 and 25 show that significant reductions in mycelial growth were observed with treatments of 30-50ppm linalol whole oil and virtually no growth was found in

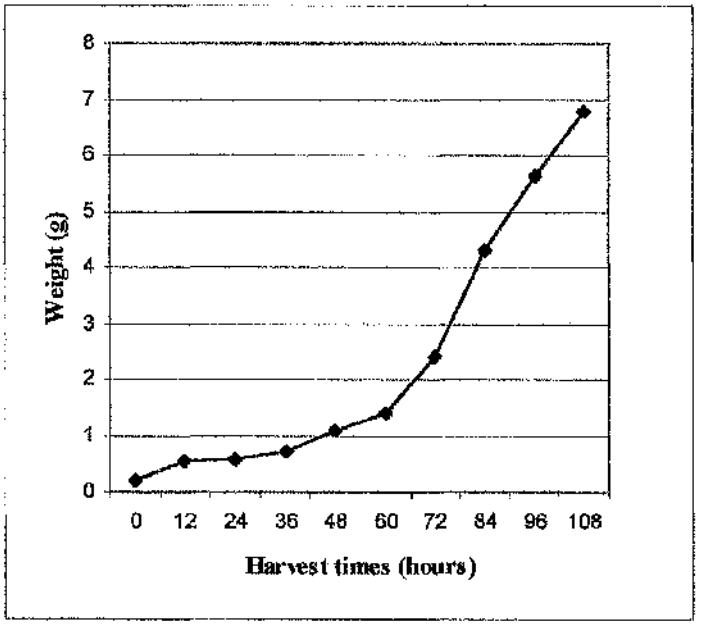


Figure 21 Growth curve of *Botrytis fabae* in liquid culture

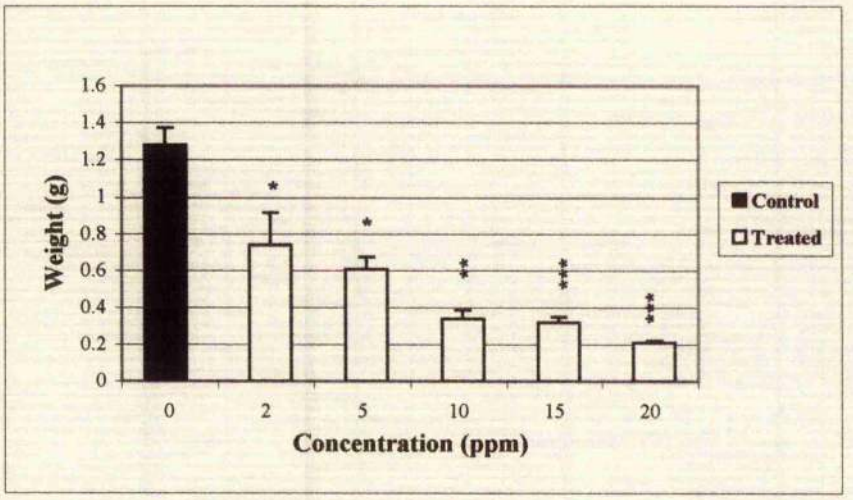


Figure 22 Effects of various concentrations of methyl chavicol type basil oil (0-20ppm) on mycelial growth of *Botrytis fabae* in liquid culture. Values are the means of 4 replicates. Error bars indicate standard error of the mean. Significant differences from controls calculated using the student's *t*-test are shown as $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$

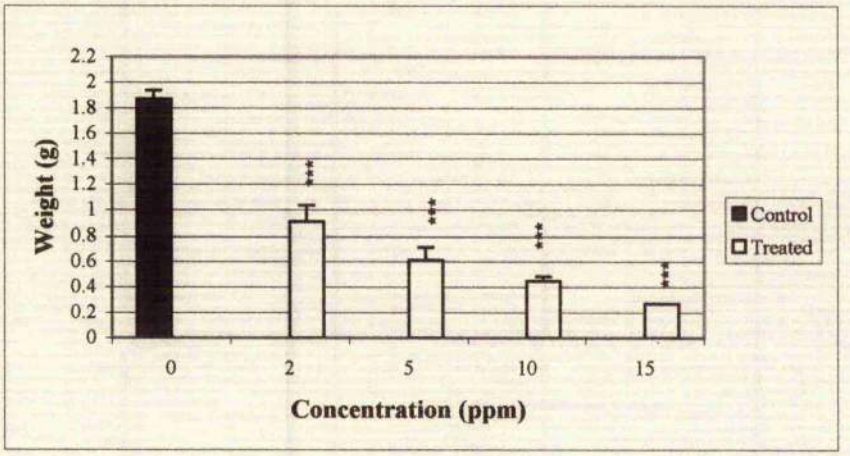


Figure 23 Effects of various concentrations of methyl chavicol (0-15ppm) on mycelial growth of *Botrytis fabae* in liquid culture. Values are the means of 4 replicates. Error bars indicate standard error of the mean. Significant differences from controls calculated using the student's *t*-test are shown as $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$

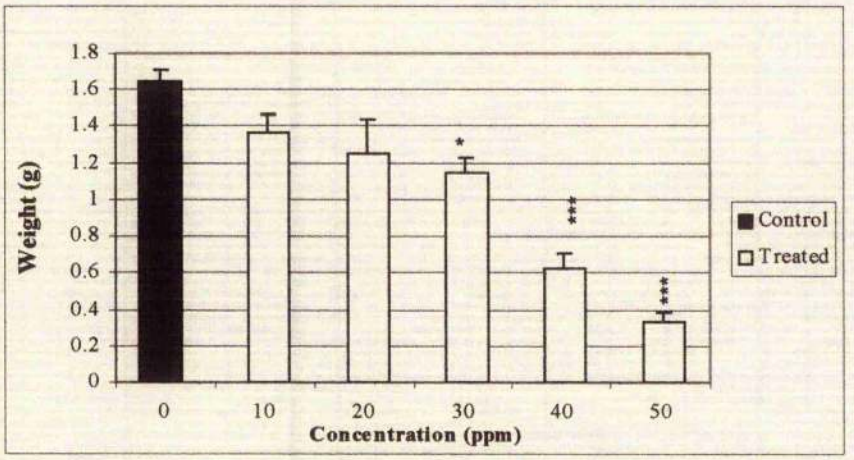


Figure 24 Effects of various concentrations of linalol type basil oil (0-50ppm) on mycelial growth of *Botrytis fabae* in liquid cullture. Values are the means of 4 replicates. Error bars indicate standard error of the mean. Significant differences from controls calculated using the student's *t*-test are shown as $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$

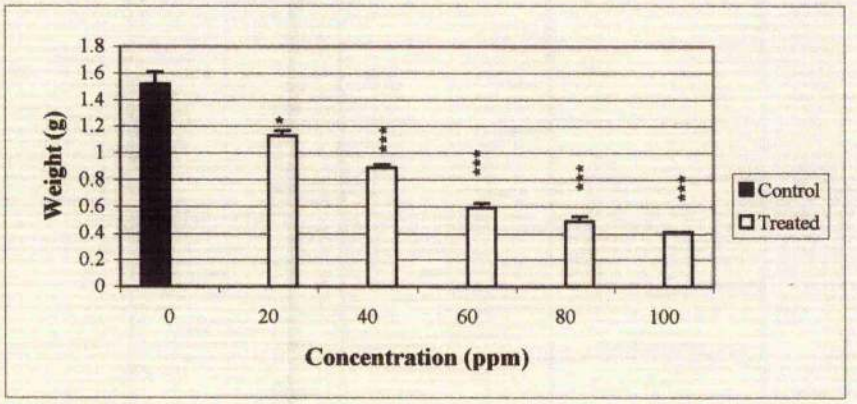


Figure 25 Effects of various concentrations of linalol (0-100ppm) on mycelial growth o5 *Botrytis fabae* in liquid culture. Values are the means of 4 replicates. Error bars indicate standard error of the mean. Significant differences from controls calculated using the student's *t*-test are shown as $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$

flasks containing 50ppm of the oil. Significant reductions in mycelial growth were found following treatment with linalol at concentrations of 20ppm and higher.

4.6.9 Effects of eugenol on *B. fabae* in liquid culture

As can be seen in Figure 26, eugenol caused significant decreases in growth when applied at 10ppm and above with very little growth detected in flasks containing 30ppm.

4.7 GLASSHOUSE EXPERIMENTS

Antifungal activity of two basil oils (methyl chavicol and linalol types) and three individual major components (methyl chavicol, linalol and eugenol) against *B. fabae* and *Uromyces viciae-fabae* was investigated *in vivo* in two experiments carried out at Mansionfield Experimental Unit as described in Section 3.7.

4.7.1 Effects of methyl chavicol type basil oil and methyl chavicol on *B. fabae* infection of broad beans

Both methyl chavicol whole oil and methyl chavicol reduced infection significantly in comparison to controls (Figures 27 & 28). With the exception of methyl chavicol applied 3 d pre-inoculation, applications of 5000ppm reduced infection to a greater extent than treatment with 1000ppm. Greatest control was achieved with treatments applied 3 h post-inoculation. Methyl chavicol whole oil and methyl chavicol applied at 5000ppm completely inhibited growth of the pathogen and 1000ppm reduced infection by an average of 96% and 95%, respectively.

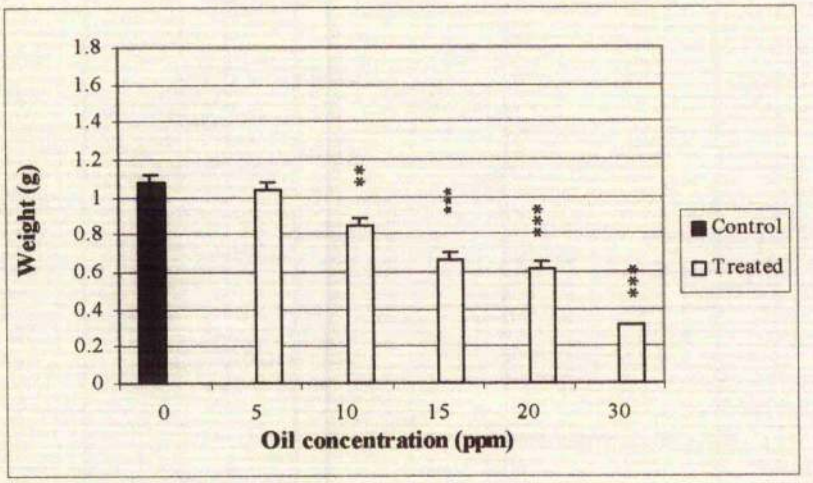


Figure 26 Effects of various concentrations of eugenol (0-30ppm) on mycelial growth of *Botrytis fabae* in liquid culture. Values are the means of 4 replicates. Error bars indicate standard error of the mean. Significant differences from controls calculated using the student's *t*-test are shown as $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$

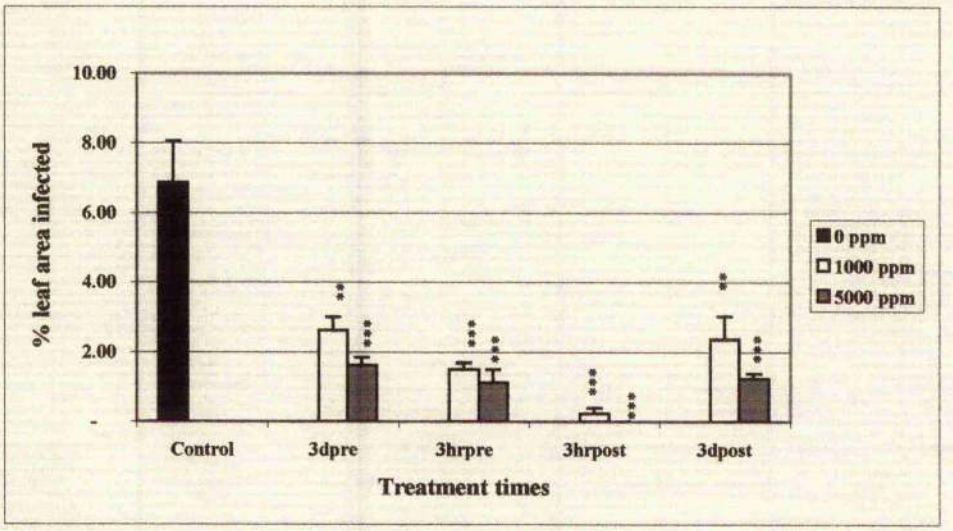


Figure 27 Effects of methyl chavicol type basil oil (1000 & 5000ppm) on *Botrytis fabae* infection of broad beans. Values are the means of 2 leaflets on 4 replicate plants. Error bars indicate standard error of the mean. Significant differences from controls calculated using the student's *t*-test are shown as $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$

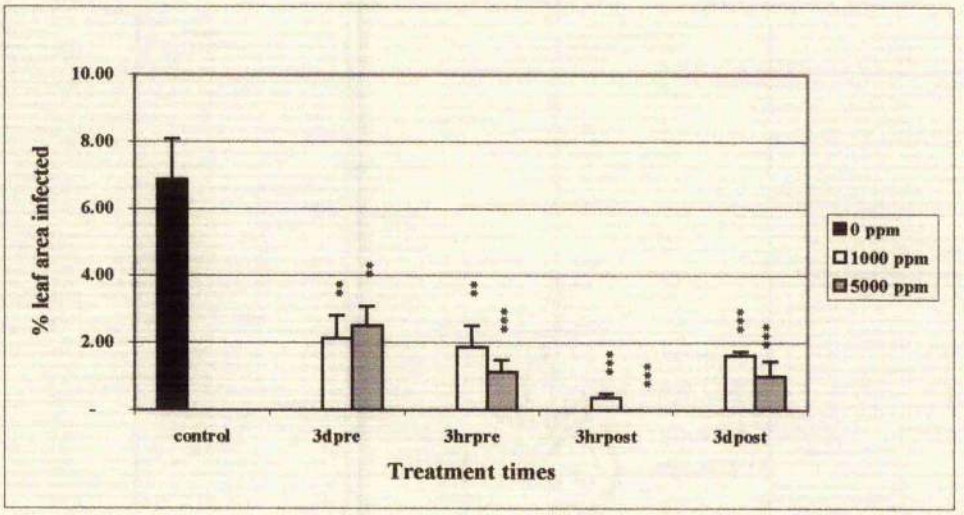


Figure 28 Effects of methyl chavicol (1000 & 5000ppm) on *Botrytis fabae* infection of broad beans. Values are the means of 2 leaflets on 4 replicate plants. Error bars indicate standard error of the mean. Significant differences from controls calculated using the student's *t*-test are shown as $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$

4.7.2 Effects of linalol type basil oil and linalol on *B. fabae* infection of broad beans

Figures 29 and 30 show that both compounds significantly reduced infection when applied both pre- and post-inoculation. Most effective applications were again 3 h post-inoculation. Linalol oil applied at a concentration of 5000ppm gave complete control of the pathogen. Linalol on its own applied at 5000ppm reduced the infection average by 98%.

4.7.3 Effects of eugenol on *B. fabae* infection of broad beans

Eugenol at 1000 and 5000ppm significantly reduced infection at all timings (Figure 31), with 3 h post-inoculation being the most effective treatment. In this case 1000ppm gave complete control and 5000ppm reduced the mean infection by 98%.

4.7.4 Effects of methyl chavicol oil and methyl chavicol on *U. viciae-fabae* infection of broad beans

When applied 3 d pre-inoculation, only methyl chavicol at a concentration of 5000ppm had a significant effect on the pathogen (Figures 32 & 33). Good control was achieved with treatments applied 3 h pre- and 3 d post-inoculation. Complete inhibition was observed with all treatments applied 3 h post-inoculation.

4.7.5 Effects of linalol oil and linalol on *U. viciae-fabae* infection of broad beans

Figures 34 and 35 show that only linalol whole oil applied at a concentration of 5000ppm reduced infection 3 d pre-inoculation. Good control was achieved with all treatments applied 3 h pre- and 3 d post-inoculation. Again, no rust pustules were found on leaves treated 3 h post-inoculation.

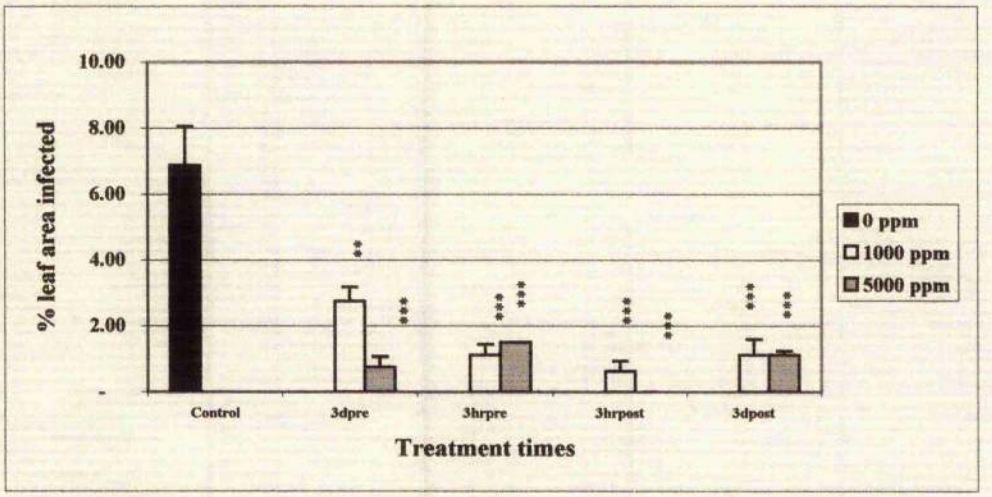


Figure 29 Effects of linalol type basil oil (1000 & 5000ppm) on *Botrytis fabae* infection of broad beans. Values are the means of 2 leaflets on 4 replicate plants. Error bars indicate standard error of the mean. Significant differences from controls calculated using the student's *t*-test are shown as $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$

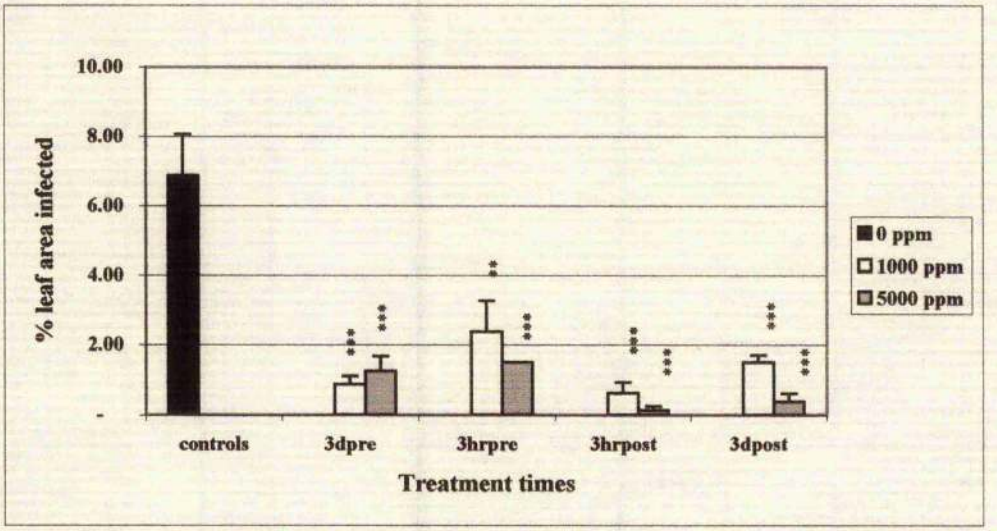


Figure 30 Effects of linalol (1000 & 5000ppm) on *Botrytis fabae* infection of broad beans. Values are the means of 2 leaflets on 4 replicate plants. Error bars indicate standard error of the mean. Significant differences from controls calculated using the student's *t*-test are shown as $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$

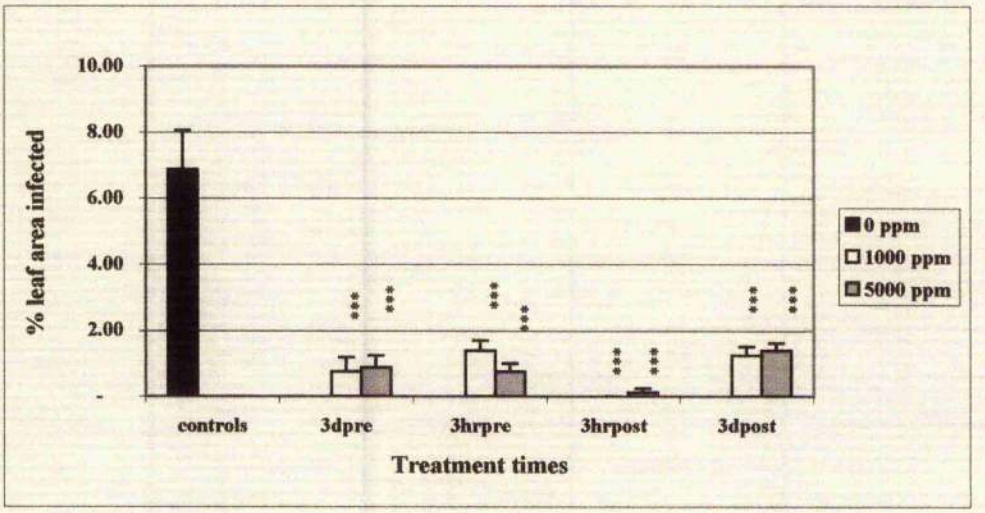


Figure 31 Effects of eugenol (1000 & 5000ppm) on *Botrytis fabae* infection of broad beans. Values are the means of 2 leaflets on 4 replicate plants. Error bars indicate standard error of the mean. Significant differences from controls calculated using the student's *t*-test are shown as $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$

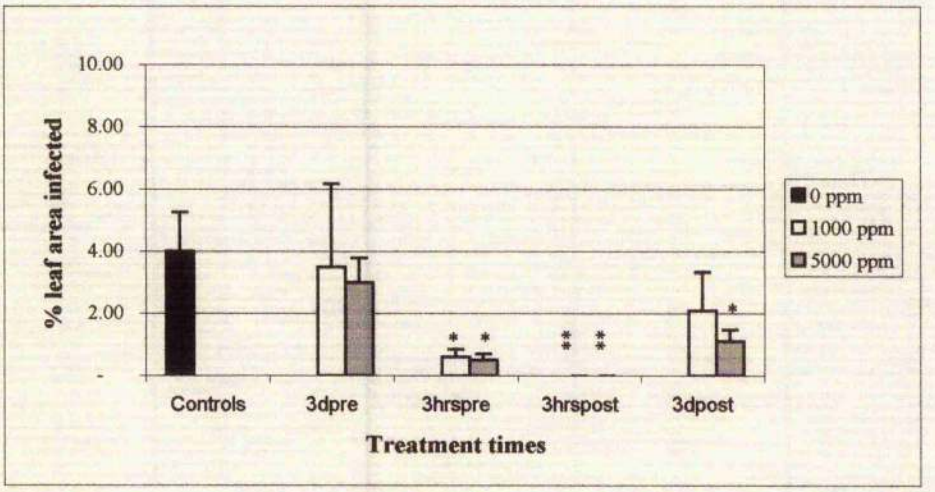


Figure 32 Effects of methyl chavicol type basil oil (1000 & 5000ppm) on *Uromyces viciae-fabae* infection of broad beans. Values are the means of 2 leaflets on 4 replicate plants. Error bars indicate standard error of the mean. Significant differences from controls calculated using the student's *t*-test are shown as $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$

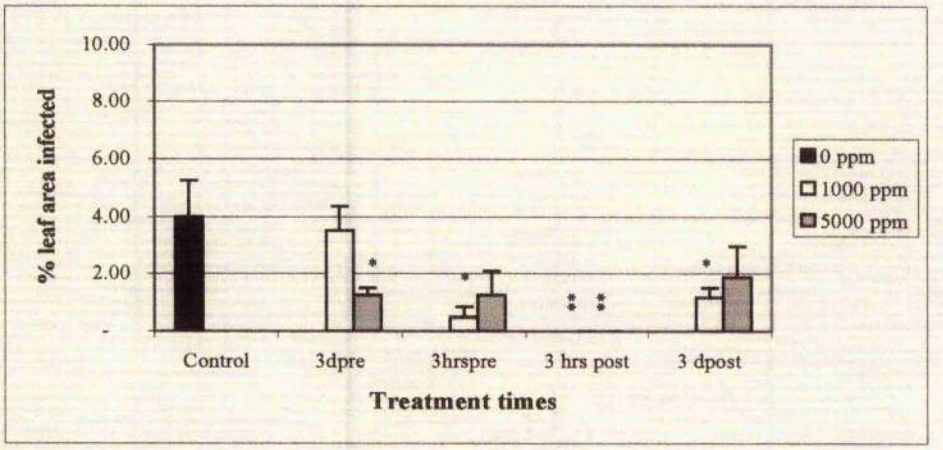


Figure 33 Effects of methyl chavicol (1000 & 5000ppm) on *Uromyces viciae-fabae* infection of broad beans. Values are the means of 2 leaflets on 4 replicate plants. Error bars indicate standard error of the mean. Significant differences from controls calculated using the student's *t*-test are shown as $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$

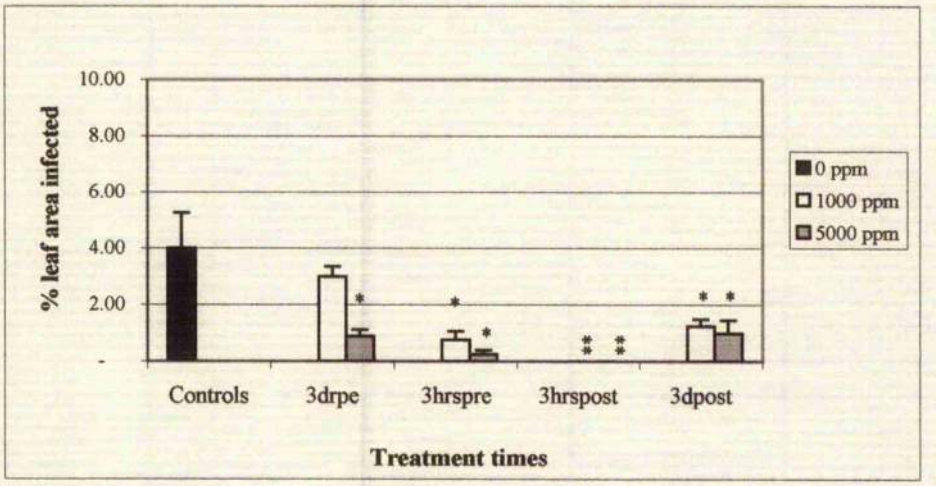


Figure 34 Effects of linalol type basil oil (1000 & 5000ppm) on *Uromyces viciae-fabae* infection of broad beans. Values are the means of 2 leaflets on 4 replicate plants. Error bars indicate standard error of the mean. Significant differences from controls calculated using the student's *t*-test are shown as $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$

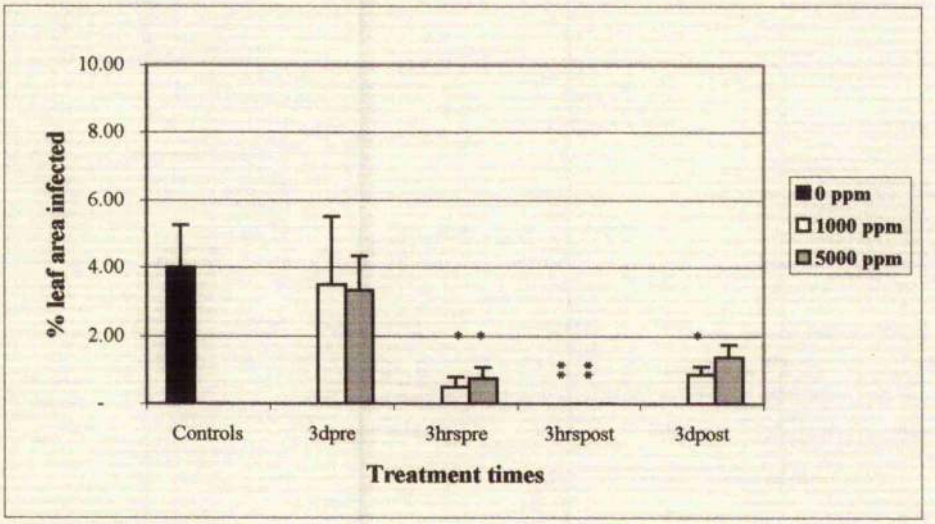


Figure 35 Effects of linalol (1000 & 5000ppm) on *Uromyces viciae-fabae* infection of broad beans. Values are the means of 2 leaflets on 4 replicate plants. Error bars indicate standard error of the mean. Significant differences from controls calculated using the student's *t*-test are shown as $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$

4.7.6 Effects of eugenol on *U. viciae-fabae* infection of broad beans

Like all other treatments applied 3 h post-inoculation, eugenol gave complete control of the rust pathogen (Figure 36). Treatments applied 3 h pre-, 3 d pre- and 3 d post-inoculation showed significant inhibition of infection.

4.8 MICROSCOPIC EXAMINATION OF *BOTRYTIS FABAE* SARDIÑA (broad bean chocolate spot)

Photomicrographs and scanning electron micrographs were taken of *B. fabae* grown in liquid culture amended with either methyl chavicol type or linalol type basil oil. As can be seen from Plate 5a,b, c & d no distinct physical differences were noted.

4.9 EFFECTS OF SELECTED *OCIMUM BASILICUM* ESSENTIAL OILS AND THREE INDIVIDUAL COMPONENTS ON POLYAMINE BIOSYNTHESIS, CATABOLISM AND EXCRETION IN *BOTRYTIS FABAE*

Material harvested from liquid bioassays (Sections 3.6.5 and 3.6.6) was used to investigate the effects of five test compounds; methyl chavicol and linalol type basil oils, methyl chavicol, linalol and eugenol on polyamine biosynthesis, catabolism and excretion in the broad bean pathogen, *B. fabae* (Section 3.9). Results are shown for flux of radiolabelled ornithine into polyamines, activities of the biosynthetic enzymes ODC and AdoMetDC and activities of the breakdown enzymes PAO and DAO. Free polyamine content of the fungal cells in the presence of all test compounds and excretion of polyamines into the surrounding medium after treatment with eugenol are also given.

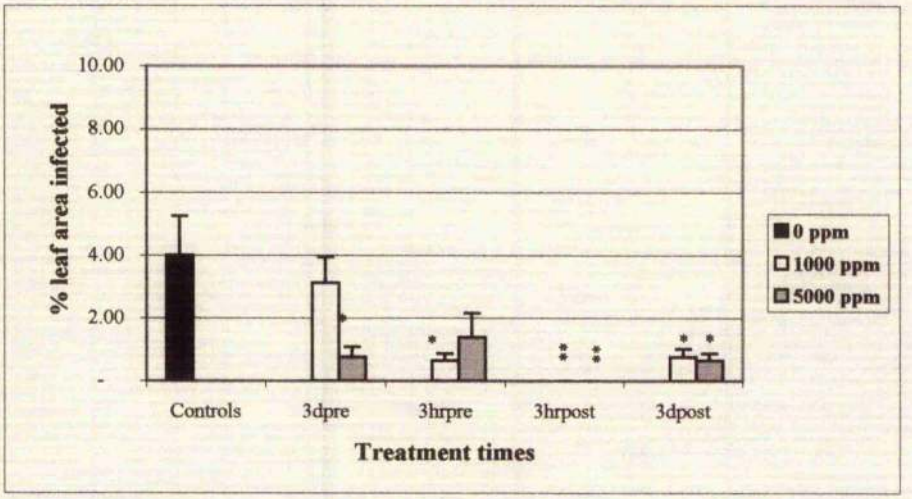


Figure 36 Effects of eugenol (1000 & 5000ppm) on *Uromyces viciae-fabae* infection of broad beans. Values are the means of 2 leaflets on 4 replicate plants. Error bars indicate standard error of the mean. Significant differences from controls calculated using the student's *t*-test are shown as $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$

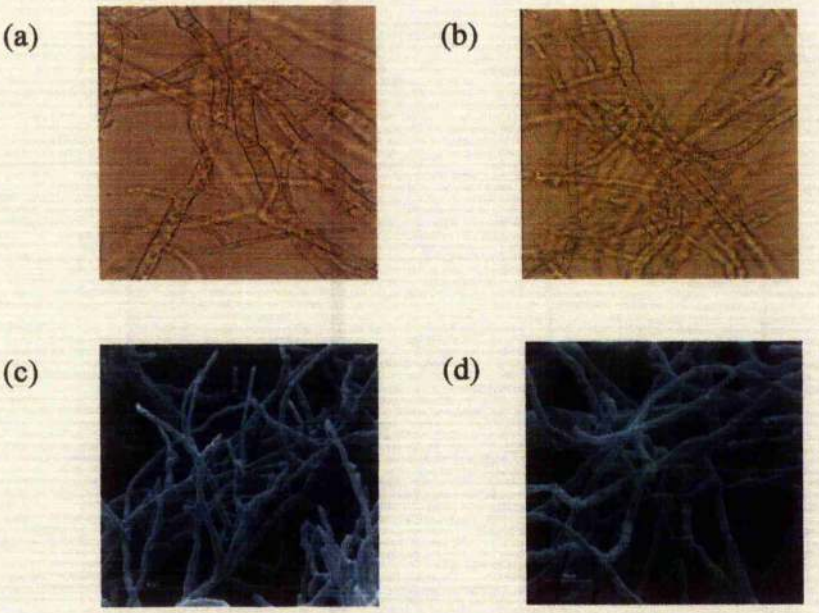


Plate 5 (a) Photomicrograph of *Botrytis fabae* control (field width 0.29mm) (b) Photomicrograph of *Botrytis fabae* treated with 10ppm methyl chavicol (field width 0.29mm) (c) Scanning electron micrograph of *Botrytis fabae* control (bar = 40 μm) (d) Scanning electron micrograph of *Botrytis fabae* treated with 20ppm linalol (bar = 20μm)

4.9.1 Effects of methyl chavicol type basil oil on polyamine biosynthesis and catabolism

There was no significant difference in the incorporation of radiolabelled ornithine into the four main polyamines in treated samples compared to controls and no difference was found in activity of the first enzyme of the biosynthetic pathway, ODC (Table 9). However, a significant increase ($P \leq 0.01$) was observed in AdoMetDC activity in treated samples. Significant increases ($P \leq 0.001$) were also noted for both catabolic enzymes, PAO and DAO. Nonetheless, no differences were found in the levels of the four polyamines isolated in their free state from the fungal cell extracts.

4.9.2 Effects of methyl chavicol on polyamine biosynthesis and catabolism

Again, no differences were found in the formation of the four polyamines from radiolabelled ornithine (Table 10). When synthesis was investigated, there was no difference in ODC activity but in contrast to the whole oil, a significant reduction in AdoMetDC activity was observed. Effects on the catabolic enzymes were also different from the whole oil. No difference was found in DAO activity and a small but significant increase was noted in PAO activity. No differences were observed in levels of free polyamines extracted from the fungal tissue.

4.9.3 Effects of linalol type basil oil on polyamine biosynthesis and catabolism

No differences were noted in flux of radiolabelled ornithine into the polyamines (Table 11). No difference was recorded in ODC activity although a small but significant decrease was seen in AdoMetDC activity. However, similar to the methyl chavicol type oil, a significant increase in DAO activity and in PAO activity was observed. No differences were found in free polyamine levels of the fungal tissue.

Effect of methyl chavicol type oil on activity of diamine and polyamine oxidase in *B. fabae*

	Enzyme activity (pmol product [mg protein]$^{-1}$hr^{-1}) ± SE			
	DAO		PAO	
Control	170 ± 15.7		57 ± 1.9	
Methyl chavicol whole oil	392 ± 14	***	121 ± 6.7	***

Effect of methyl chavicol type oil on activity of ornithine and S-adenosylmethionine decarboxylase in *B. fabae*

	Enzyme activity (pmol CO^2 [mg protein]$^{-1}$hr^{-1}) ± SE	
	ODC	AdoMetDC
Control	47 ± 5.0	5 ± 0.6
Methyl chavicol whole oil	58 ± 4.7	9 ± 0.5 **

Effect of methyl chavicol type oil on incorporation of radio-labelled ornithine into polyamines in *B. fabae*

	Radioactivity in polyamines (dpm [mgprotein]$^{-1}$)			
	Spermine	Spermidine	Cadaverine	Putrescine
Control	28 ± 3	48 ± 23.5	57 ± 22.3	45 ± 2.9
Methyl chavicol whole oil	43 ± 18.7	206 ± 114.6	17 ± 0	77 ± 28.4

Effect of methyl chavicol type oil on free polyamine concentration in *B. fabae*

	Free polyamine concentrations (μmol g^{-1} FW)			
	Spermine	Spermidine	Cadaverine	Putrescine
Control	1387 ± 76.9	1708 ± 100.2	158 ± 13.5	324 ± 16.2
Methyl chavicol whole oil	1420 ± 68.8	1689 ± 107.5	163 ± 16.1	238 ± 11.9

Table 9 Effects of methyl chavicol type basil oil on polyamine biosynthesis and catabolism. Values are the mean of four replicates ± standard error. Significant differences from controls calculated using the student's *t*-test are shown as *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$

Effect of methyl chavicol on activity of diamine and polyamine oxidase in *B. fabae*

	Enzyme activity (pmol product [mg protein]$^{-1}$hr^{-1}) ± SE	
	DAO	PAO
Control	403 ± 24.7	114 ± 3.9
Methyl chavicol standard	744 ± 176.4	135 ± 5.4 *

Effect of methyl chavicol on activity of ornithine and S-adenosylmethionine decarboxylase in *B. fabae*

	Enzyme activity (pmol CO^2 [mg protein]$^{-1}$hr^{-1}) ± SE	
	ODC	AdoMetDC
Control	37 ± 10.9	33 ± 3.5
Methyl chavicol standard	42 ± 7.8	8 ± 3.0 **

Effect of methyl chavicol on incorporation of radio-labelled ornithine into polyamines in *B. fabae*

	Radioactivity in polyamines (dpm [mg protein]$^{-1}$)			
	Spermine	Spermidine	Cadaverine	Putrescine
Control	132 ± 29.6	66 ± 27.9	92 ± 52.6	114 ± 8.1
Methyl chavicol standard	122 ± 12.2	103 ± 21.1	11 ± 0	292 ± 132.7

Effect of methyl chavicol on free polyamine concentration in *B. fabae*

	Free polyamine concentrations (μmol g^{-1} FW)			
	Spermine	Spermidine	Cadaverine	Putrescine
Control	1361 ± 46.8	1663 ± 96.4	293 ± 34.3	361 ± 36.9
Methyl chavicol standard	1852 ± 84.3	1710 ± 117.5	214 ± 10.1	222 ± 9.9

Table 10 Effects of methyl chavicol on polyamine biosynthesis and catabolism. Values are the mean of four replicates ± standard error. Significant differences from controls calculated using the student's *t*-test are shown as *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$

Effect of linalol type oil on activity of diamine and polyamine oxidase in *B. fabae*

	Enzyme activity (pmol product [mg protein]$^{-1}$hr^{-1}) ± SE			
	DAO		PAO	
Control	201 ± 8		83 ± 3.4	
Linalol whole oil	358 ± 21	***	107 ± 3.4	**

Effect of linalol type oil on activity of ornithine and S-adenosylmethionine decarboxylase in *B. fabae*

	Enzyme activity (pmol CO^2 [mg protein]$^{-1}$hr^{-1}) ± SE		
	ODC	AdoMetDC	
Control	64 ± 29.9	21 ± 4.0	
Linalol whole oil	61 ± 15.2	8 ± 3.1	*

Effect of linalol type oil on incorporation of radio-labelled ornithine into polyamines in *B. fabae*

	Radioactivity in polyamines (dpm [mg protein]$^{-1}$)			
	Spermine	Spermidine	Cadaverine	Putrescine
Control	96 ± 41	99 ± 23.2	66 ± 20.6	152 ± 28
Linalol whole oil	53 ± 8.4	60 ± 15.2	100 ± 10	216 ± 42.4

Effect of linalol type oil on free polyamine concentration in *B. fabae*

	Free polyamine concentrations (μmol g^{-1} FW)			
	Spermine	Spermidine	Cadaverine	Putrescine
Control	2050 ± 79.0	2291 ± 87.2	370 ± 14.3	555 ± 48.9
Linalol whole oil	1360 ± 104.9	1804 ± 102.6	257 ± 33.0	376 ± 41.0

Table 11 Effects of linalol type basil oil on polyamine biosynthesis and catabolism. Value are the mean of four replicates ± standard error. Significant differences from controls calculated using the student's *t*-test are shown as *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$

4.9.4 Effects of linalol on polyamine biosynthesis and catabolism

No difference was noted in the incorporation of radiolabelled ornithine into the four polyamines and neither was there any difference in ODC activity (Table 12). A small but significant increase was noted in AdoMetDC activity, but unlike the linalol whole oil, no difference was seen in DAO activity, although a significant difference was observed in PAO activity. Again, there were no differences in free polyamine levels of the fungal tissue.

4.9.5 Effects of eugenol on polyamine biosynthesis and catabolism

In the case of eugenol, a significant increase was found in the incorporation of radiolabelled ornithine into spermine and a small but significant decrease in flux of label into putrescine (Table 13). No difference at all was noted in ODC activity. However, a significant increase in AdoMetDC activity was observed. No difference was seen in DAO or PAO activity and free polyamine levels in fungal tissue did not vary significantly. Free polyamines were measured in the media surrounding samples treated with eugenol but no significant differences were found.

Effect of linalol on activity of diamine and polyamine oxidase in *B. fabae*

	Enzyme activity (pmol product [mg protein]$^{-1}$hr^{-1}) ± SE	
	DAO	PAO
Control	493 ± 56.4	192 ± 9.2
Linalol standard	485 ± 62.5	142 ± 1.3 **

Effect of linalol on activity of ornithine and S-adenosylmethionine decarboxylase in *B. fabae*

	Enzyme activity (pmol CO^2 [mg protein]$^{-1}$hr^{-1}) ± SE	
	ODC	AdoMetDC
Control	25 ± 6.7	13 ± 9.6
Linalol standard	17 ± 2.8	41 ± 5.3 *

Effect of linalol on incorporation of radio-labelled ornithine into polyamines in *B. fabae*

	Radioactivity in polyamines (dpm [mg protein]$^{-1}$)			
	Spermine	Spermidine	Cadaverine	Putrescine
Control	108 ± 28.3	65 ± 26.8	113 ± 32.3	72 ± 24.4
Linalol standard	89 ± 21.1	69 ± 31.3	67 ± 31	11 ± 6.7

Effect of linalol on free polyamine concentration in *B. fabae*

	Free polyamine concentrations (μmol g^{-1} FW)			
	Spermine	Spermidine	Cadaverine	Putrescine
Control	1880 ± 219.8	1851 ± 88.2	296 ± 13.9	407 ± 24.5
Linalol standard	867 ± 74.4	1309 ± 165.3	223 ± 39.8	114 ± 7.7

Table 12 Effects of linalol on polyamine biosynthesis and catabolism. Values are the mean of four replicates ± standard error. Significant differences from controls calculated using the stuednt's *t*-test are shown as *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$

Effect of eugenol on activity of diamine and polyamine oxidase in *B. fabae*

	Enzyme activity (pmol product [mg protein]$^{-1}$hr^{-1}) ± SE	
	DAO	PAO
Control	348 ± 65.6	92 ± 3.4
Eugenol standard	481 ± 127.8	105 ± 6.2

Effect of eugenol on activity of ornithine and S-adenosylmethionine decarboxylase in *B. fabae*

	Enzyme activity (pmol CO^2 [mg protein]$^{-1}$hr^{-1}) ± SE	
	ODC	AdoMetDC
Control	47 ± 8.4	14 ± 0.9
Eugenol standard	54 ± 9.9	73 ± 5.0 ***

Effect of eugenol on incorporation of radio-labelled ornithine into polyamines in *B. fabae*

	Radioactivity in polyamines (dpm [mg protein]$^{-1}$)			
	Spermine	Spermidine	Cadaverine	Putrescine
Control	48 ± 5.7	24 ± 8.5	42 ± 18.5	52 ± 10.4
Eugenol standard	314 ± 36.3 ***	186 ± 93.2	118 ± 29.8	17 ± 6.5 *

Effect of eugenol on free polyamine concentration in *B. fabae*

	Free polyamine concentrations (μmol g^{-1} FW)			
	Spermine	Spermidine	Cadaverine	Putrescine
Control	1993 ± 47.0	2300 ± 131.1	280 ± 29.9	629 ± 92.7
Eugenol standard	1602 ± 126.9	1858 ± 65.6	339 ± 32.3	747 ± 279.5

Effect of eugenol on polyamine excretion from *B. fabae*

	Free polyamine concentrations after 3 days (mg ml^{-1} buffer)			
	Spermine	Spermidine	Cadaverine	Putrescine
Control	129 ± 3.2	4 ± 0.7	9 ± 0.6	5 ± 0.7
Eugenol	98 ± 14.5	8 ± 0.6	9 ± 0.8	7 ± 0.8
Control + 200mg/ml Spermine	330 ± 7.2	8 ± 0.8	9 ± 0.8	4 ± 0.2
Eugenol+200mg/ml Spermine	341 ± 6.5	9 ± 0.2	9 ± 0.6	7 ± 0.2

Table 13 Effects of eugenol on polyamine biosynthesis and catabolism. Values are the mean of four replicates ± standard error. Significant differences from controls calculated using the student's *t*-test are shown as *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$

5 DISCUSSION

5.1 CHARACTERISATION OF NCRPIS GERMPLASM COLLECTION

A joint effort began in 1999 between the NCRPIS and SAC, Auchincruive to classify the accessions in the *Ocimum* germplasm collection held at the PI Station in Ames, using a combination of classical morphological comparison to diagnostic keys (Appendix 1) and chemotaxonomy, the latter including GLC, GC-MS, IR and NMR analyses (Sections 3.3, 3.4, 4.4 and 4.5). The *Ocimum* collection consisted predominantly of *O. basilicum*, a selection of *O. americanum* accessions, two small groups of *O. tenuiflorum* and *O. gratissimum* and a single accession of *O. selloi*.

5.2 CULTIVATION OF PLANTS AT SAC, AUCHINCRUIVE

Although essentially tropical, members of the genus *Ocimum* grew extremely well underglass at SAC in the west of Scotland. Morphological observation confirmed differences in phenotypes and similarities in chemotypes within the same species. For example *O. basilicum* (PI207498) from Afghanistan grew quite tall and had green leaves, while *O. basilicum* (PI172996) from Turkey was relatively short in stature and produced purple leaves. Nonetheless, both acquisitions had the same chemical composition (~90% methyl chavicol). These results were similar to those from Iowa. In addition to genetic factors, environmental conditions are known to influence oil yield and composition (Wijesekera, 1991; Piccaglia, 1998; Bruneton, 1999). However, it seems that in the case of this particular chemotype, these characteristics were not influenced.

In contrast, Table 7 shows the differences in chemical composition of essential oil from fresh and dried plants of *O. tenuiflorum* accession PI414201. Here, there were differences in the chemical composition of the leaves and flowers. It is important to consider these characteristics for each species individually, e.g. the oil composition of *Myrica gale* (bog murtle) does not differ in dry or fresh plant material, whilst *Dracocephalum moldavica* (dragonhead) has considerable changes in volatile components (Svoboda, 1998; Svoboda *et al.*, 1998).

5.3 ESSENTIAL OIL YIELD OF PLANTS IN THE NCRPIS GERMPLASM COLLECTION

Oil yields of plants in the collection were lowest for *O. gratissimum* with yields ranging from 0.3-0.5% (v/w). *O. tenuiflorum* produced yields between 0.7 and 1.8% (v/w), which is typical of *Ocimum* species (Simon *et al.*, 1990, 1999; Morales *et al.*, 1993). *O. basilicum* yields were slightly higher, from 0.5-2.9% and *O. americanum* yields were extremely high for plants of this genus at 2.3-5.0%. Oil yield for species of the genus *Ocimum* reported in the literature are classically low. In a previous study at SAC, yields of *O. basilicum* were 0.06-0.83% (Kyle, 1999) and Halva & Craker (1996) reported *O. basilicum* yields of 0.2-1.0% (v/w). However, Hiltunen (1999) reported higher yields for species of the genus: 0.5-1.5% for *O. basilicum*, 0.6% for *O. sanctum*, 0.38-0.65% for *O. canum* (*O. americanum*), 2.5-7.6% for *O. kilimanscharicum* and 0.5-1.4% for *O. gratissimum*.

Aromatic plants with extremely small oil yields (<0.01% v/w) are represented by such species as rose, melisa and jasmine. In contrast, yields of over 10% (v/w) are typical of plant species such as frankinsence and clove (Bruneton, 1999). Volatile oil yield is

genetically coded in particular plant species and although it can be influenced by environmental factors and ontogeny, substantial changes cannot be induced by external factors (Deans & Svoboda, 1988; Wijesekera, 1991; Collins *et al.*, 1994; Marotti *et al.*, 1994; Piccaglia, 1998; Bruneton, 1999). Genetic manipulation with its tools for changing gene expression could be the future solution for increased yield (Chappell, 1995; Bohlmann *et al.*, 1998; Lange *et al.*, 1999).

5.4 GAS LIQUID CHROMATOGRAPHY OF ESSENTIAL OILS

Chemotaxonomy is a widely recognised method in plant classification. It has been successfully used in the characterisation of many plant families (Deans & Svoboda, 1988; Svoboda *et al.*, 1990; Galambosi *et al.*, 1993; Piccaglia *et al.*, 1993; Collins *et al.*, 1994; Jackson & Hay, 1994; Marotti *et al.*, 1994; Svoboda *et al.*, 1995; Baratta *et al.*, 1998; Svoboda, 1999; Hogg *et al.*, 2001). In cases such as *Pimenta racemosa* (Miller) J. Moore var. *racemosa* (bay rum tree), which has three distinctly different chemotypes (Bruneton, 1999), GC or GC-MS can give an accurate characterisation. However, with complex genera such as *Ocimum* or *Satureja* (savoury), chemotaxonomy is a valuable tool but needs to be coupled with one or more other investigations such as morphological examination.

Such analyses of plant collections makes practical applications possible. For example, the development of new or improved flavour, fragrance and industrial products. Collections previously characterised from NCRPIS include those of *Agastache* (hyssop) (Charles *et al.*, 1991), *Anethum* (dill) (Charles *et al.*, 1995) and *Petroselinum* (parsley) (Simon & Quinn, 1998). Hopefully, by continuing to collect and analyse germplasm in this way it will be possible to discover new bioactive compounds. It will also enable the

improvement of quality and yield in crop plants and identify new food sources to compensate for the increasing population and reduction of available fertile land. In addition, it will help us to find and develop safe products to control the many pests and diseases which threaten our food supply.

5.4.1 Accessions of *Ocimum basilicum*

According to the literature, linalol is one of the most common chemical components found in essential oils of *O. basilicum*, *O. canum*, *O. micranthum* and *O. sanctum*. Linalol fractions up to 90% have been reported and the compound has previously been found to co-exist with methyl chavicol, eucalyptol, eugenol and geraniol (Hiltunen & Holm, 1999). Linalol chemotype seems to be an indigenous plant type in Turkey (Baser, 1999). Our study agrees with this literature, although no previous reports were found for plants from Macedonia. Twenty-six accessions of the NCRPIS collection were found to be linalol chemotypes. The known origins of samples and secondary identification names are listed in Appendix 10.

Eucalyptol was found to be present in *O. basilicum* accessions of the collection in differing amounts up to 26%. Eucalyptol has been found in *O. kilimanscharicum*, *O. keniense*, *O. gratissimum*, *O. canum*, *O. micranthum*, *O. sanctum* and *O. rubrum* from trace - 60%. It is usually low in *O. basilicum*, (<10%), thus making our findings unusual. One report has been made, however, of an *O. basilicum* eucalyptol chemotype Holm *et al.* (1989).

Citral has been reported in *O. canum*, *O. gratisimum*, *O. menthiflorum* and *O. pilosum*, however, little information can be found on *O. americanum* and *O. basilicum* citral

chemotypes. Simon *et al.* (1999) reported x *citriodorum* spp. to contain 24-61% linalol and 16-19% citral. They also reported x *citriodorum* containing 92% methyl chavicol. One of the linalol/citral types was designated 'Mrs Burns' which is usually classed as *O. basilicum*. Complete clarification of the origin of individual species and their hybrids can be achieved only by detailed genetic experiments or by DNA fingerprinting (Vieira *et al.*, 2001, 2003).

Although no sesquiterpenes ≥5% were found to be present in the oils of *O. basilicum* in this study, substantial amounts have been reported previously. Sesquiterpenes have also been found in oils of *O. canum*, *O. gratissimum* and *O. sanctum*. Small fractions have been found in *O. canum*, *O. trichodon* and *O. kiniense* (Hiltunen & Holm, 1999).

It is well known that varying amounts of methyl chavicol can be present in *O. basilicum* essential oils. In addition, eugenol and methyl eugenol have been recorded in *Ocimum basilicum* in various amounts (Hiltunen & Holm, 1999). Thus, our findings of these phenylpropenes in *O. basilicum* accessions were as expected.

O. basilicum plants were seen to vary morphologically, including those which produced similar essential oils e.g. PI207498 from Afghanistan and PI172996 from Turkey. Variation within *O. basilicum* specifically has been noted and Simon *et al.* (1990) reported that there could be more than 60 varieties. Examples of variation in leaf morphology can be seen in Plate 1. *O. basilicum* is one of the most commercially exploited species and is collected and cultivated worldwide. Hence, much of the variation is possibly due to breeding and free intercrossing.

5.4.2 Accessions of *Ocimum americanum*

These plants were very similar morphologically, which exemplifies the complications typically encountered in classification of the *Ocimum* genus. An example of detailed clarification of taxonomical position, using both GC and classical taxonomy, was the reclassification of *O. kilimandscharicum* in the collection. Plants of accession A1679 were initially classified as *O. kilimandscharicum*. However, after the essential oil analysis showed that the plant produced a relatively high amount of methyl chavicol accompanied by camphor, eucalyptol and eugenol, the accession was grown again to allow a reassessment of the morphological characteristics. Considering the GC and GC-MS results and a morphological comparison to the diagnostic key published by Kew (Paton, 1992), the accession was reclassified as *O. americanum* var. *pilosum*. With main component methyl chavicol and lesser component eugenol, plants of this accession constitute one of the seven distinct groups within the *O. americanum* collection (Section 4.4.2). The smaller compounds of this oil, camphor and eucalyptol, seem to be typical of the species.

The fact that limonene was present in quantities up to 30% is unusual as this component typically constitutes 0-1% of an oil (Hiltunen & Holm, 1999). These plants were collected from three different sources and although morphologically uniform they fell into many distinctly different chemical groups, in contrast to *O. basilicum* species which looked different but produced similar chemical profiles. Few reports of *O. americanum* could be found in the literature. Hiltunen & Holm (1999) cited methyl chavicol and citral chemotypes and Simon *et al.* (1999) reported finding linalol/eucalyptol and eucalyptol/ methyl chavicol/ β-bisabolene chemotypes. These findings are contrary to those from this work. Only one report was found in the literature of a basil with a major

component of fenchone. Lawrence (1988-1991) reported a fenchone chemotype of *O. canum*. The camphor chemotype of *O. americanum* found in this study also appears to be the first recorded.

5.4.3 Accessions of *Ocimum tenuiflorum*

According to the literature, four chemotypes of *O. sanctum* exist: citral, eugenol, methyl chavicol and chavibetonol (Hiltunen & Holm, 1999) *O. sanctum* is a synonyn of *O. tenuiflorum* and although this species is one of the most widely studied, especially in India, the caryophyllene chemotype found in this present work is again the first to be reported.

5.4.4 Accessions of *Ocimum gratissimum*

O. gratissimum is classicaly reported to produce a phenolic oil. It was originally thought the plants produced either eugenol or thymol. However, it was later found that both constituents were often present. In more recent studies several more chemotypes have been noted: eucalyptol (Silva *et al*, 1999), citral, methyl cinnamate (Ntezurubnza *et al*., 1987), linalol, geraniol, eugenol/sesquiterpene, ocimene, ocimene/methyl isoeugenol, eugenol/ocimene, eugenol/methyl eugenol and a sesquiterpene type (Yusuf *et al*., 1998). Which components constituted the sesquiterpene chemotypes and at which quantity a component determined a chemotype was not reported thus it is not possible to know if the accessions of *O. gratissimum* in this collection producing up to 28% caryophyllene oxide are novel. No thymol chemotypes were found in the NCRPIS collection.

5.4.5 Accession of *Ocimum selloi*

One report was found on this species by Martins *et al.* (1997) who analysed two accessions from a germplasm collection held at the University of Viçosa, Brazil. The accessions were different in both essential oil profile and morphological characteristics, accession A being a methyl chavicol chemotype and accession B methyl eugenol. Description and essential oil constituents of the latter were similar to those of the sample of *O. selloi* in this study.

5.5 GAS CHROMATOGRAPHY-MASS SPECTROMETRY OF ESSENTIAL OILS

Gas chromatography is a very effective tool in the analysis of individual components of essential oils. However, their identification is achieved only by comparison of peaks to standard compounds. For exact identification of major and minor peaks, it is necessary to verify their authenticity by GC-MS or NMR. Thirty samples which were analysed by these methods served as an extremely useful grid for resolution of the hundreds of GC traces which were generated during four years of investigation into *Ocimum* species.

5.6 CROP PROTECTION

Plant essential oils have been documented since ancient times for having biological activity. Many different oils are reported to have a range of properties including antioxidant, antibacterial, antifungal and insecticidal (Deans & Ritchie, 1987; Deans *et al.*, 1992; Gundidza *et al.*, 1993; Lima *et al.*, 1993; Piccaglia *et al.*, 1993; Deans *et al.*, 1994; Marotti *et al.*, 1994; Marinova & Yanishlieva, 1996; Baratta *et al.*, 1998; Holm, 1999; Rege *et al.*, 1999; Vasudevan *et al.*, 1999; Hogg *et al.*, 2001; Svoboda *et al.*, 2003). Athough utilised widely in flavour and fragrance and healthcare industries, the

potential of these oils as novel biocides in the agriculture and horticulture sectors is only just beginning to be realised.

5.6.1 Antifungal testing of selected *Ocimum basilicum* essential oils and components against *Botrytis fabae* grown on solid media

All test compounds in the present study had a significant effect on the mycelial growth of the chocolate spot pathogen; in the case of eugenol, down to 50ppm. Both combination 1 and 2 appeared to have similar effects to their corresponding oils methyl chavicol type and linalol type, respectively. Methyl chavicol results also mirrored those of the methyl chavicol whole oil. However, the lesser component of this oil, linalol showed even greater inhibitory effects than methyl chavicol when tested on its own.

The four main components of oil 2 (linalol, eugenol, eucalyptol and caryophyllene) mixed together to make combination 2 showed inhibition comparable to the whole oil. When tested individually eugenol and linalol had greater effects than the whole oil, while eucalyptol and caryophyllene demonstrated poorer inhibitory properties. So overall, the whole oil and combination of these components had an effect that was intermediary to the most and least effective components.

It has been reported that essential oil components are responsible for the biological activity of many plant extracts (Khanna *et al.*, 1991; Heinrich, 1992; Vasudevan *et al.*, 1999). This is not surprising as essential oils consist of compounds such as aldehydes, ketones, alcohols, lactones, esters, ethers and phenolic compounds. However, although activity may be due exclusively to these compounds, in some cases this may not always be the case. In addition, many findings show that activity can be attributed to the main

component (Sinha & Gulati, 1990), while others have found evidence to suggest this may not always be the case. Anise oil containing 44% (v/v) linalol and 27% (v/v) methyl chavicol was tested along with various linalol: methyl chavicol combinations. The antimicrobial activity of the whole oil proved superior to all combinations suggesting lesser components made an important contribution to the biological activity of that particular oil (Lachowicz *et al.*, 1998). Others have attributed activity to specific individual components such as linalol, methyl chavicol, eugenol, isoeugenol, methyl isoeuggenol, thymol, caryophyllene and eucalyptol (Lachowicz *et al.*, 1998; Holm 1999; Vasudevan *et al.*, 1999).

Whole oils may, however, confer other advantages such as a broader range of protection (Carleton *et al.*, 1992). Synergistic effects are known to exist. Also, less active or inactive components may enhance activity of other components by influencing availability, resorption or rate of reaction (Khanna *et al.*, 1991; Svoboda *et al.*, 2003) In addition, specificity including that due to MOA and defence capabilities of the target organism must also influence efficacy.

Although the majority of literature on the biological activity of essential oils appears to be in a medical context, several reports can be found on oils tested against food microflora and mycotoxins and phytopathogens. Freixa *et al.* (1998) found *O. micranthum* extract effective against *Micosporum* and *Trichophyton* spp. Nwosu & Okafor (1995) investigated the extracts of 10 medicinal plants collected in SE Nigeria for antifungal activity. *O. gratissimum* was effective against *Basidiobolus*. and *Trichophyton* spp. at a 1:10 dilution and inhibited *B. haptosporus* at a 1:40 dilution. The activity of the *O. gratissimum* extract compared favourably to conventional drugs used

for such infections. In a study by Professor Rai and co-workers, methyl cinnamate type basil essential oil was found to inhibit human and plant fungal pathogens of the genus *Fusarium* at dilutions down to 62.5 and 31.2 $\mu l ml^{-1}$ (Rai *et al.*, 1999). *O. basilicum* oil has also been found to inhibit *Fusarium avenecium* (Svoboda *et al.*, 2003).

5.6.2 Antifungal testing of selected *Ocimum basilicum* essential oils and components against *Botrytis fabae* grown in liquid culture

A second set of *in vitro* bioassays was carried out, this time assessing the antifungal effects of five test compounds (methyl chavicol oil, linalol oil, methyl chavicol, linalol and eugenol) against *B. fabae* grown in liquid culture. Effective concentrations were substantially lower in these experiments, probably due to the rotating flasks leading to increased contact between the test compound and the pathogen. No growth was detected after treatment with 20ppm methyl chavicol whole oil or methyl chavicol single component. Significant reductions in growth were observed with application of 40ppm linalol oil and substantial inhibition was seen after treatment of 40ppm linalol although to a lesser extent than with the linalol whole oil. Significant decreases in growth occurred after exposure to 10pp eugenol.

In order to investigate the effects of the oils and components on polyamine metabolism within the fungal pathogen, material had to be obtained from flasks containing treatments which inhibited but did not completely kill the pathogen, thus experiments were run with concentrations which allowed a reasonable harvest. Preliminary experiments were carried out to establish a suitable range of concentrations for each test compound, so unfortunately due to time constraints it was not possible to run further

experiments in order to establish exact MIC's or determine the nature of inhibition i.e. whether fungicidal or fungistatic.

Reports of this type of experiment include Zollo *et al.* (1998) who screened 13 Cameronian plants for antifungal activity against six human fungal pathogens. *O. gratissimum* was by far the most effective, especially against *Microsporum* and *Tricophyton* spp., with minimum inhibitory concentrations as low as 78ppm. *O. basilicum* was also very active, proving fungicidal to *T. rubrum* at 312ppm. In a search for natural food preservatives, Basilico & Basilico (1999) found methyl chavicol type basil essential oil able to inhibit *Aspergillus ochraceus* at 500 and 1000ppm and suppress production of ochratoxin A at 750 and 1000ppm. Lachowicz *et al.* (1998) examined essential oils from 5 varieties of *Ocimum basilicum* for inhibitory effects against a range of acid-tolerant food microflora. All five oils were effective against 29 out of 33 microbes with best effects found to be against the yeasts and moulds. Pure methyl chavicol exhibited a narrower spectrum of activity than the whole oils, inhibiting 16 out of the 33 strains. In contrast, pure linalol demonstrated the broadest range, inhibiting 30 out of 33.

5.7 GLASSHOUSE EXPERIMENTS

Glasshouse experiments demonstrated the basil oils and individual components to have significant inhibitory effects on both *B. fabae* and *U. v.-fabae* infection of broad beans. Again, the degree of inhibition by methyl chavicol type oil and methyl chavicol was similar and linalol alone controlled the disease to a greater extent than the linalol type whole oil. Overall, all applications significantly reduced infection in comparison to the controls. It would be valuable to carry out further experiments to determine the

longevity of these effects. Treatments at 3 h post inoculation completely stopped the disease from taking hold and most treatments whether applied at 1000ppm or 5000ppm significantly reduced infection. A second set of controls with treatments of water only would have determined whether the effect was partially due to the spraying. The reduction in infection, however, was certainly not completely due to spraying as 5000ppm was consistently more effective than 1000ppm thus it can be concluded that the test compounds were exerting most if not all the observed effect.

The reason for applying treatments at different timings was to attempt to define by what means the oils were inhibiting the pathogen. For example, if 3 d pre-inoculation treatments were especially effective, this could mean that the test compound(s) had protectant properties. If 3 h pre-inoculation treatments were particularly good at reducing infection, spore germination might have been prevented. 3 h post-inoculation treatments may also prevent germination but are more likely to be affecting later developmental processes such as appresorium formation, germ-tube penetration or even mycelial growth inside the leaf. The fact that all treatments at all times were so much less than the controls suggests that the compounds were having an effect in more than one way. This is not surprising in the case of the whole oils as they contain so many active constituents. However, it can be seen that the individual components appear to be equally effective. The individual chemicals tested in this experiment have been shown by other workers to be extremely potent when tested *in vitro* (Lachowicz *et al.*, 1998; Holm, 1999; Vasudevan *et al.*, 1999).

As mentioned previously, little work has been reported on the effects of essential oils on pests and diseases of crop plants, especially *in vivo* studies. Published work includes that

by Tewari & Nayak (1991) who found that an extract of *O. sanctum* was able to inhibit the three rice pathogens *Pyricularia oryzae* (rice blast), *Cochliobolus miyabeanus* (brown spot) and *Rhizoctonia solani* (scurf) both *in vitro* and on the host plant in glasshouse experiments. Best control was seen against *P. oryzae* with MICs of $4.9gl^{-1}$ *in vitro* and a mean of 2.4 compared to 8.5 for controls (and 1.8 for a commercial fungicide) on a disease scale of 0-9. Khanna *et al.* (1991) found that *O. gratissimum* exhibited greatest antifungal activity in their study of 10 plant essential oils. Complete inhibition of *Colletotrichum capsici* (anthractose) and *Sclerotium rolfsii* (stem rot) was achieved with treatments of 50-500ppm. *O. gratissimum* was also shown to inhibit Tobacco Mosaic Virus and human waterborne pathogens *Aeroomonas* and *E-coli*.

Experiments assessing the antifungal activity of hyssop oil against crop plant pathogens *P. avenae, P. oryzae* and *U.-viciae-fabae* was conducted previously at SAC, Auchincruive in which significant effects were observed *in vitro* but less consistant results were obtained *in vivo*. Nonetheless, hyssop oil was seen to reduce *U. viciae-fabae* infection of broad bean pre-and post-inoculation (Letessier *et al.*, 2001). The authors (and references within) concluded that this anomaly could have been due to diffusion of the volatile compounds away from the plants. Temperature readings as high as 34°C were recorded during glasshouse experiments in the present work, especially while treatments were being applied. It is, therefore, possible that much lower concentrations of these compounds would still reduce disease. With the exception of 5000ppm eugenol, treatments did not appear to cause phytotoxicity.

A suitable antifungal compound must be toxic to the pathogen but not to the host plant. Thus, *in vivo* testing is extremely important when searching for compounds to control

crop plant pathogens. In addition to highlighting phytotoxicity problems, such experiments can reveal compounds to have a different effect on the host plant from those found *in vitro*, due to inhibition taking place at specific stages of development such as spore germination or appresorium formation. This was found to be the case with some polyamine analogues (Havis *et al.*, 1994a, b). In the work discussed above by Letessier *et al.*, 2001, hyssop oil was found to inhibit germination of *B. fabae* and *U. viciae-fabae*.

Timescale experiments using microscopy and germination assays might define exactly which stage the compounds are acting on the pathogens. Overall, the fact that these oils have a low mammalian toxicity, are biodegradable, multifunctional, non-persistent in the environment and relatively cheap to produce make future developments an extremely attractive venture (Hay & Waterman, 1993).

5.8 EFFECTS OF TWO SELECTED *O. BASILICUM* ESSENTIAL OILS AND THREE INDIVIDUAL COMPONENTS ON POLYAMINE BIOSYNTHESIS, CATABOLISM AND EXCRETION IN *B. FABAE*

In this present work, it was found that application of selected *Ocimum* essential oils and individual components substantially inhibited growth of the fungal pathogens *B. fabae* and *U. viciae-fabae in vitro* and *in vivo*. Thus, in view of the suggestion that polyamines are a likely target for essential oils *in vivo* (Carnesecchi *et al*, 2001) further investigation was carried out to establish whether polyamine metabolism was a target for the antifungal activity.

5.8.1 Effects of methyl chavicol type basil oil and methyl chavicol on polyamine biosynthesis and catabolism

With treatments of methyl chavicol type oil, the data obtained for flux of label into spermidine was highly variable, so it is possible that there was an increase in spermidine due to the increase in AdoMetDC activity. As no increase in spermine was noted but PAO and DAO were very significantly increased, it is possible that spermidine was either being acetylated or bound to essential oil or other component(s) and subsequently broken down by the catabolic enzymes. After treatment with methyl chavicol alone, AdoMetDC activity was reduced and PAO slightly increased. Although the methyl chavicol oil was made up of 76% methyl chavicol and the antifungal activity of the oil and the single component appeared very similar, the effects on polyamine biosynthesis seemed to be quite different. It was, however, interesting to note that although methyl chavicol oil increased AdoMetDC activity and methyl chavicol decreased it, the resulting level of AdoMetDC in both cases was essentially equal. The increased PAO could again be due to breakdown of putrescine, acetylated polyamines or phenolic amide type compounds.

5.8.2 Effects of linalol type basil oil and linalol on polyamine biosynthesis and catabolism

The reason why PAO and DAO were increased so substantially after treatment with linalol type basil oil is not known, although the levels of the diamines could have been increased sufficiently to stimulate the catabolic enzymes. Linalol alone did not reduce AdoMetDC activity to the same extent as the other components. This suggests that it may have been another component(s) of the linalol oil or synergistic effects which caused the decrease in AdoMetDC activity after treatment with linalol whole oil . Although not statistically significant, there appears to be a reduction in free spermine

which could possibly account for the increase in PAO activity. However, although AdoMetDC activity was increased it was not accompanied by an increase in spermidine or spermine synthesis, suggesting that acetylation could have occurred.

5.8.3 Effects of eugenol on polyamine biosynthesis, catabolism and excretion

Eugenol caused the most dramatic changes in polyamine metabolism. AdoMetDC activity increased more than 5-fold and incorporation of radioabelled ornithine into spermine increased by more than 6-fold. Furthermore, no significant difference was seen in PAO and DAO activity. Data suggested a reduction in putrescine, but as ODC activity appeared unaffected this was probably due to the increased activity of AdoMetDC moving the radiolabel to spermine. One explanation for this could be that spermidine synthase was inhibited and thus the accumulation of AdoMet stimulated spermine synthesis. This was reported to be the case in chinese cabbage protoplasts treated with the spermidine synthase inhibitor cyclohexylamine (CHA) (Cohen, 1998).

5.8.4 Hypothetical explanation of complex relationships between polyamine metabolism and bioactive test compounds

Another explanation for the increased spermine should also be considered, that is the possibility that spermine was synthesised by a route other than that via putrescine and spermidine. Alternative pathways for the production of other polyamines have been suggested. Cataldi & Algranati (1986) found evidence of putrescine synthesis in *E-coli* via a pathway which does not involve ODC or ADC. Tait (1976) elucidated a pathway in which L-aspartic β-semialdehyde forms a Schiff base with putrescine. This is reduced to form carboxyspermidine which is in turn decarboxylated to spermidine. Walters & Cowley (1996) reported findings which indicated that APC and 3APC were formed in

some way other than by AdoMetDC and the aminopropyltransferases. Further investigation ruled out the L-aspartic β-semialdehyde route.

There is also the question of why spermine synthesis was increased greatly but free spermine levels did not appear to change. Work by Havis *et al.* (1996) found a 370% increase in spermine in *Phytophthora infestans* after exposure to E-TED. Further investigation revealed that the spermine was being displaced from intracellular binding sites and although mycelial growth was being inhibited, the pathogen was excreting much of the spermine in an attempt to control the size of the spermine pool. The data obtained in the present work eliminates elevated catabolic breakdown and excretion of spermine from the fungus into the surrounding media. However, several other possibilities remain. Polycations such as spermine readily bind to polyanionic molecules such as DNA and other negatively charged molecules or surfaces and although sequestrated free spermine would have been released during the experimental procedure, any other bound form of spermine whether cytosolic, compartmentalised or excreted would not have been detected. It is of course possible that the compounds retrieved from the TLC plate were not all purely spermine. A compound such as eugenol could have bound with spermine or derivatisation may have taken place and the resultant compound may still have been lifted by the carrier solvent and eluted in the same region as spermine. Such molecules may not stimulate catabolic enzymes. Polyamines can also be utilised in the formation of larger molecules including polyamine-protein complexes (Bagni & Tassoni, 2001).

Overall, in this present work, no statistically significant reductions were recorded in the free polyamine pools after treatment with the five test compounds. However, the

compounds did appear to influence polyamine metabolism and substantial (although not statistically significant) differences were observed in the data for cultures treated with linalol, suggesting that a larger scale investigation of this type would be valuable.

5.8.5 Findings from other investigations of polyamine inhibitors

When work began in the early 1980s on the development of crop protection agents using inhibitors of polyamine biosynthesis, two groups at the forefront of this work were the Galston group in the US and Walters and co-workers in Scotland. Rajam *et al.* (1985, 1986) demonstrated that DFMO, the irreversible suicide inhibitor of ODC, reduced *U. phaseoli* (rust) infection of *Phaseolus vulgaris,* and Walters (1986) found that DFMO controlled rust (*U. v.-fabae*) infection of *Vicia-faba* L. Both groups investigated these findings further. Rajam & Gaston (1985) studied the effects of DFMO and DFMA on four phytopathogenic fungi: *Botrytis sp., Botrytis cinerea, Rhizoctonia solani* and *Monilinia fruticola,* while the Walters group investigated the efficacy of a wide range of polyamine biosynthesis inhibitors including polyamine analogues keto-putrescine (Foster & Walters, 1993), (*E*)-1,4-diaminobut-2-ene (E-BED), (Havis *et al.*, 1994a), (*E*)-N,N,N'N'-tetraethyl-1,4-diaminobut-2-ene (E-TED) (Havis *et al.*, 1994b) and norspermidine (Mackintosh & Walters, 1998) and enzyme inhibitors such as MGBG, CHA (West & Walters, 1988; Foster & Walters, 1990; Mackintosh & Walters, 1998) and MFMOCH$_3$ (Zarb & Walters, 1993).

Foster & Walters (1990) found that MGBG alone and in combination with DFMO reduced mycelial growth of *Pyrenophora avenae* to a greater extent than DFMO alone. West & Walters (1988) found DFMO and MGBG individually and in combination to be powerful inhibitors of barley powdery mildew (*Erysiphe graminis*) infection *in vivo,*

while Mackintosh & Walters (1998) found the spermidine synthase inhibitor CHA and the spermidine analogue norspermidine comparable to commercial fungicides in a 2 year field trial against barley powdery mildew. Zarb & Walters (1993) reported that $MFMOCH_3$ reduced growth of the cocoa pathogen *Crinipellis perniciosa*. This compound was also found to inhibit barley powdery mildew infection (West & Walters, 1988). The polyamine analogue keto-putrescine was able to control a range of phytopathogenic fungi (Foster & Walters, 1993), as were E-BED and E-TED (Havis *et al.*, 1994a, b).

However, although many of the compounds investigated have exhibited a high degree of antifungal activity *in vitro*, reduced fungal infections on host plants and were shown to influence polyamine biosynthesis, in several cases it was concluded that perturbation of polyamine metabolism was unlikely to be their main mode of action (Havis *et al.*, 1994a, b; Mackintosh *et al.*, 1997). Although many analogues and inhibitors exert the desired effect, anomalies are often reported. For example, Zarb & Walters (1993) found putrescine and spermidine concentrations and AdoMetDC activity to be increased and spermine pools depleted in *C. perniciosa* after treatment with DFMO. In contrast, increases in ODC activity have been reported in cultured tumour cells after treatment with MGBG (Foster & Walters, 1990). The same authors also found that addition of exogenous polyamines along with inhibitors increased levels of inhibition. Also, many workers have observed that cadaverine can compensate for depletion of other polyamines (Alhonen-Hongisto & Janne, 1980; Foster & Walters, 1993). These cases demonstrate the complexity of the polyamine system, many parts of which are related to cellular homeostasis and fungal defence mechanisms which can control enzyme levels and alternative pathways at the level of transcription and translation.

Some inhibitors have been found to give good control against the phytopathogens when tested on the plant, but cause minimal disruption to polyamine biosynthesis when investigated *in vitro*. This is possibly because the antifungal effects occurr during specific stages of fungal development such as spore germination and appresorium formation (Havis *et al.*, 1994a, b) Therefore, both *in vitro* and *in vivo* experiments are important when investigating a novel compound for fungicidal activity.

5.8.6 Future possibilities of regulating polyamines in biological systems

As mentioned previously, claims of biological activity of many compounds are now the subject of scientific investigation, these include those of anticancer properties of essential oils. The oil of *Ocimum* species, especially that of *O. sanctum*, has been reported to inhibit tumour growth and scientific evidence is accumulating to support these claims (Vasudevan *et al.*, 1999). Polyamines are also known to be associated with the growth of cancerous tumours and are the focus of many cancer research projects. A team of researchers from L'Institut de Recherche contre les Cancers de l'Appareli Digestif (IRAD) in Strasbourg investigated the effects of geraniol on the growth of a human colon cancer cell line (Caco-2). They found that it reduced cell growth by 70% and at the same time inhibited DNA synthesis. A 50% decrease in ODC activity was observed which led to a 40% reduction of the intracellular pool of putrescine. Polyamine acetylation was also enhanced, thus indicating that the intracellular catabolism of polyamines was increased, although possibly due to feedback mechanisms, AdoMetDC activity was increased. The team concluded that polyamine metabolism was highly likely to be a target for the antiproliferative properties of geraniol. In the same report, the group made reference to other work in which monoterpenes have been found to exert antitumour activity. For example, limonene has been found to exert such activity against

mammary gland, lung, liver, stomach and skin cancers in rodents. Perillyl alcohol has shown chemoprotective effects against mammary gland, liver, pancreas and colon cancers in rodents and geraniol has previously been reported to be active against murine leukemia, hepatoma and melanoma (Carnesecchi *et al.*, 2001). In addition, paclitaxel, a diterpene from the yew tree has achieved a central role in the chemotherapy of malignant tumours and another diterpene, eleutherobin from coral has also shown potential as a cytostatic agent (Eisenreich *et al.*, 1998).

As evidence exists to show that essential oil components can inhibit both fungal development and tumour growth and that polyamine biosynthesis is likely to be a target for some terpenes and phenylpropenes at least, further investigation in this area should be persued. However, as seen in this present work and postulated previously, different components of these oils are likely to have different modes of action (Carleton *et al.*, 1992). Synergistic effects of essential oil components are likely and in addition, fungal compensatory mechanisms create further complications. For these reasons, possibly a more direct route to elucidating modes of action would be to observe differential gene expression in treated and control samples.

6 CONCLUDING DISCUSSION

In the present research, chemotype analysis was undertaken in order to provide information required to clarify the taxonomy of the genus *Ocimum*. GC and GC-MS coupled with classical morphological comparison has proved successful in the characterisation of this complex genus. The present work uncovered new chemical profiles including *O. americanum* spp. containing up to 71% fenchone, 47% camphor and 30% limonene and a caryophyllene chemotype of *O. tenuiflorum*. Considering components ≥5%, limonene was restricted to accessions of *O. americanum*. Oil yields of up to 5% were found in *O. americanum* species from Zambia which is unusual for plants of the genus *Ocimum*. Single component chemotypes and high yielding species have potential to be a source of flavour and fragrance compounds and provide germplasm for the improvement of existing lines and the development of new cultivars.

Many chemical profiles have been described and several essential oil components appear to be species specific within this collection at least. Also, relationships between the chemical components within species have been noted to provide additional information. Overall, these results can equip researchers with a new and useful reference for the identification of unknown *Ocimum* species. Due to the chemical and morphological complexity of the genus, no single analysis provided a definitive classification of an unknown species. However, building up a knowledge bank of morphological and chemical profiles and continuing to study intra- and inter-specific relationships can collectively aid classification. Phylogenetic and breeding studies will also aid our

understanding of the genus by providing an insight into the evolutionary patterns of species of the genus.

This research project has moved beyond the classification of *Ocimum* to examine the utility of selected essential oils and individual components. Accordingly, antifungal testing of selected *O. basilicum* oils and individual components demonstrated activity *in vitro* with concentrations as low as 2ppm. Good control of *B. fabae* and *U. viciae-fabae* infection of broad bean *in vivo* was also achieved. Further investigation of MICs and longevity of effects would be valuable, as would investigation of other oils and their components.

There has been renewed interest recently in the use of essential oils as crop protection agents and the work presented in this thesis shows that the essential oil of *O. basilicum* could be useful in controlling plant disease caused by certain pathogens. However, before the oil could be used in crop protection, thorough examination of the efficacy of the whole oil and its components in the field is necessary. It would be important to determine the effects of formulation, since this can influence significantly the persistence and activity of the chemical on the foliage. Such field studies should also include an analysis of the effects of the oil on crop growth and yield. Perhaps the most attractive aspect of using essential oils as crop protection agents is their favourable mammalian toxicity. Nevertheless, toxicity of the oil would need to be examined and LD_{50} values determined for the whole oil and its components. It is clear from the data presented in this thesis that the essential oil of *O. basilicum* does not exert its antifungal effects via polyamine metabolism. There are a very wide range of biochemical target sites which could be examined *in vivo* and *in vitro* to determine the mode of action of

chemical components derived from *Ocimum* species. Polyamine metabolism as assessed in this research exemplified one approach in this general area of research. In addition, an alternative strategy might be to take a molecular approach. For example, attempting to determine global gene expression using differential display. This would indicate genes which are up- or down-regulated following treatment, thus identifying avenues for further exploration.

In conclusion, this work has uncovered chemical profiles of *Ocimum* species never before reported in the literature. It has provided chemotypic and morphological descriptions of a large collection of *Ocimum* species which can serve as a useful reference to other workers attempting to classify unknown *Ocimum* species. Selected essential oils and individual chemical components of *Ocimum* have been shown to possess a high degree of antifungal activity shoing potential for development in the agriculture, horticulture, medicinal and pharmaceutical industries. Finally, although of tropical decent, these plants have been shown to grow well under glass in south west Scotland and in addition, exceptionally high yielding species have been shown to exist within the genus.

7 SUGGESTIONS FOR FUTURE WORK

Characterisation of more *Ocimum* species and varieties in order to gain a wider knowledge of the genus at present and it's phylogeny.

Assessment of the antifungal properties of more *Ocimum* essential oils and individual components; considering different combinations, minimum inhibitory concentrations and nature (fungicidal/fungistatic) and longevity of activity.

Assessment of antifungal activity of *Ocimum* essential oils and individual components against other pathogens including those which cause disease in humans.

Investigation into the mode of action of *Ocimum* oils and individual components.

Quantitative analysis of incorporation of substrate(s) into polyamines and investigate the possibility of an alternative pathway leading to spermine.

Toxicity testing and investigation of biodegradation of *Ocimum* essential oils and components.

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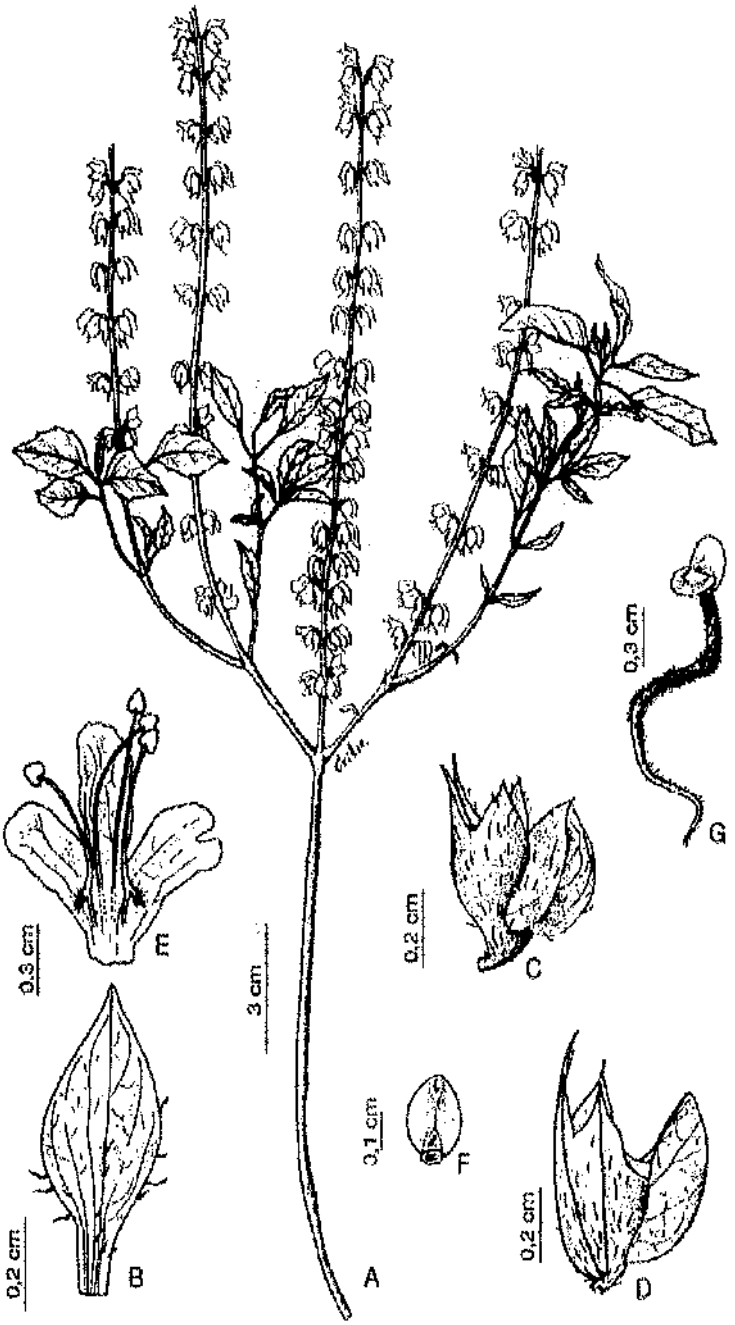
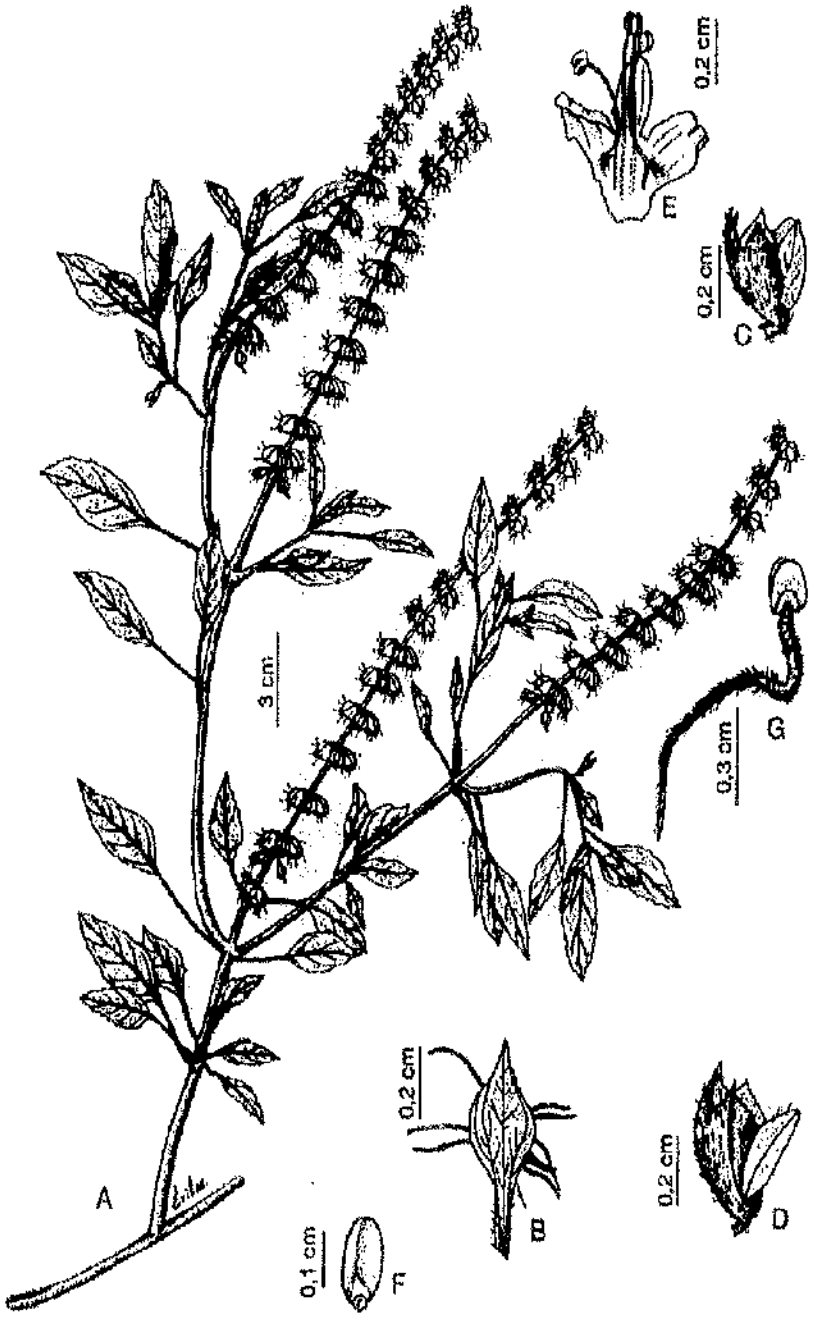
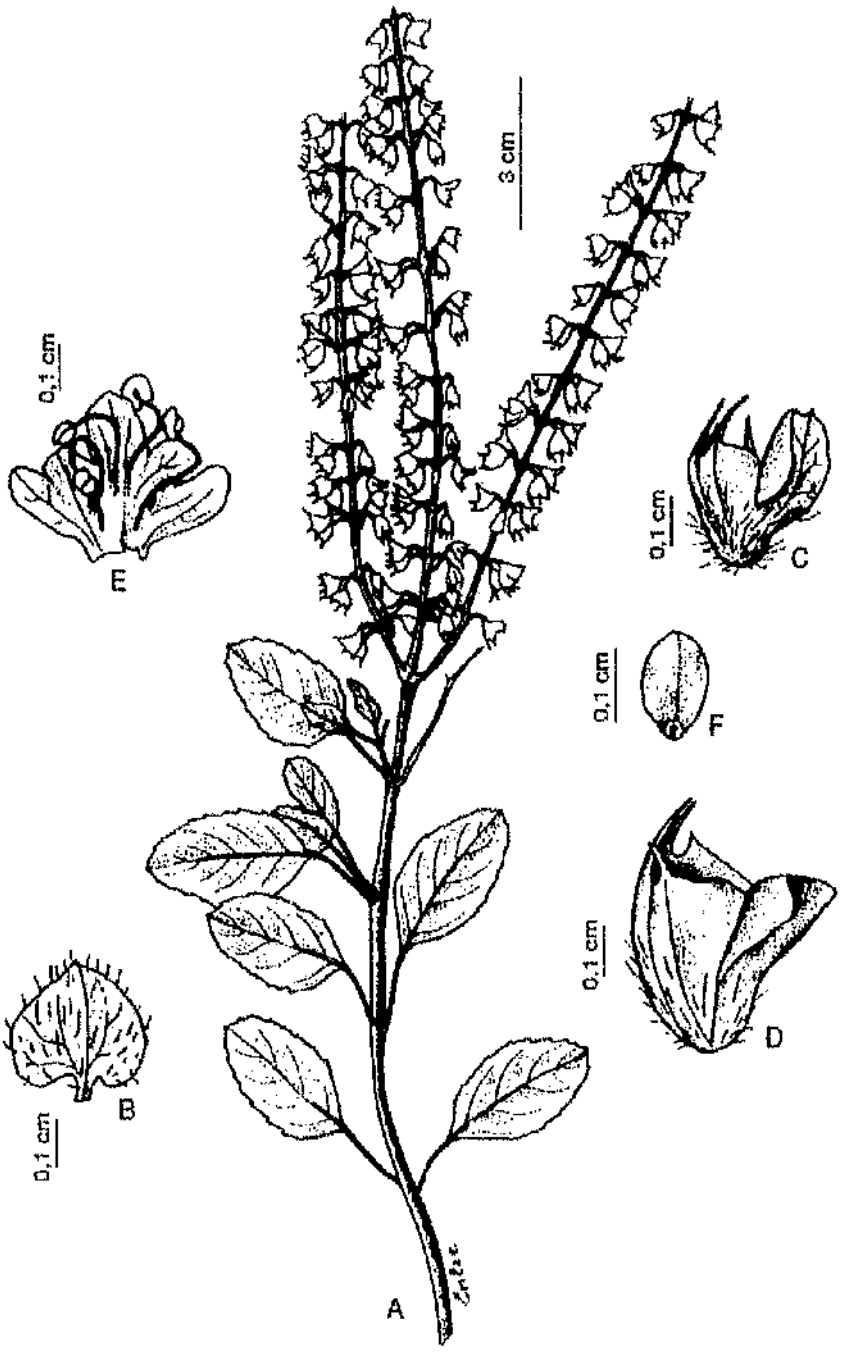


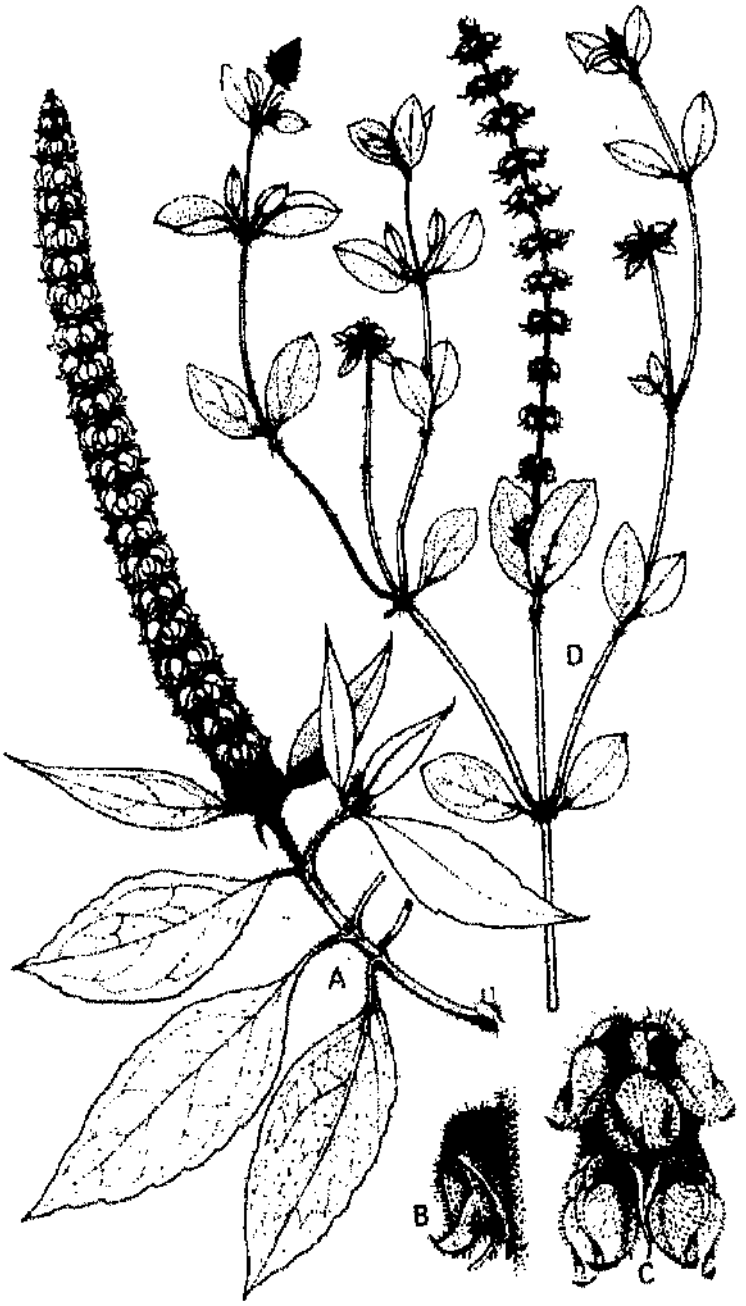
Appendix 1 Diagnostic key diagram for *Ocimum basilicum*: A, habit; B, bract; C, flowering calyx; D, fruiting calyx; E, longitudinal section of corolla; F, nutlet; G, seedling (after Albuquerque & Andrade, 1998)



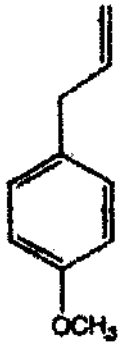
Appendix 1 (cont) Diagnostic key diagram for *Ocimum americanum*: A, habit; B, bract; C, flowering calyx; D, fruiting calyx; E, longitudinal section of corolla; F, nutlet; G, seedling (after Albuquerque & Andrade, (1998)



Appendix 1 (cont) Diagnostic key diagram for *Ocimum tenuiflorum* A, habit; B, bract; C, flowering calyx; D, fruiting calyx; E, longitudinal section of corolla; F, nutlet; G, seedling (after Albuquerque & Andrade, 1998)

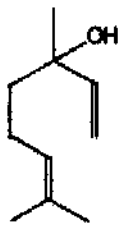


Appendix 1 (cont) Diagnostic key diagram for *Ocimum gratissimum* A, habit; B, fruiting calyx; C, part of mature inflorescence; D, *O. kenyense* (after Paton, 1992)



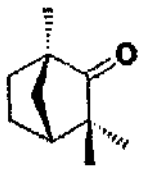
methyl chavicol

Major constituent of basil and tarragon (estragon) oils. Sweet, anisic, herbaceous odour. Widely used in the flavour industry and in the manufacture of fine perfumes.



linalol

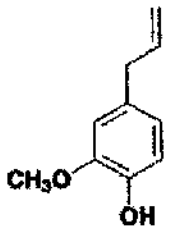
Major component of basil, rosewood, coriander and ho oils. Fresh, flowery odour; although can be spicy in coriander. Widely used in perfumery, soaps and detergents.



fenchone

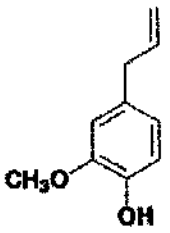
Usually isolated from Cedarleaf oil or produced synthetically. Warm-camphoraceous, powerful and diffusive, basically sweet odour. Warm, burning and bitter taste with a medicinal note. It is used to prepare artificial fennel oil and a few other essential oils and to perfume household products. In spite of its rather unpleasant taste it is used in various berry flavours and spice complexes and liqueurs.

Appendix 2 Components ≥ 20% found in essential oils of *Ocimum* spp. (Arctander, 1969; Burdock, 1996; Bruneton, 1999; Hiltunen & Holm, 1999)



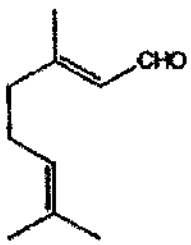
eugenol (R=H)

Major constituent of basil, clove, cinnamon leaf and pimento oils. Pungent, spicy odour. Warm and spicy flavour producing a burning sensation in the mouth. Extensively used in clove and carnation compositions. Also used in large amounts for oriental and spicy notes, rose bases, incense, certain Fougère and 'blue-grass' fragrances. It is a modifier in mint flavours, fruit complexes and rare flavours such as date. It is also a dental antiseptic.

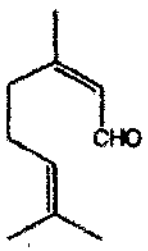


methyl eugenol (R=CH_3)

Warm, earthy, tenacious odour like ginger or tea. Used to create a warm background in perfumes. A modifier for spice flavours and its low volatility makes it ideal for baked goods.



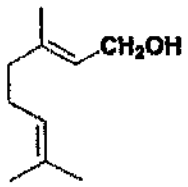
geranial



neral

Natural citral is nearly always a mixture of the two isomers; geranial and neral. Used widely as a powerful lemon flavour and fragrance. Can be concerted to geraniol and citronellol. Condensation with methylne groups produces pseudoionones; the starting material for the production of ionones and vitamins.

Appendix 2 cont

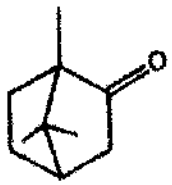


Found in nearly all terpene containing oils. A light, fresh, rose-like odour. Using different processes, can be converted to a range of compounds including cintronellal, citronellolcyclogeraniol, tetrahydrogeraniol, citral and geranyl esters.

geraniol

Fresh, light and sweet citrusy odour like orange peel oil. The taste is sweet, refreshing and citrus-like. Extensively used in perfumery and essential oil reconstruction as a refreshing top-note especially in large volume fragrances for household goods. It is used in many fragrances including floral jasmine and lavender, fruity fragrances and pine, woody or green odours. It is a modifier for lime, fruit and spice complexes. Obtained as a by product of orange juice production.

limonene



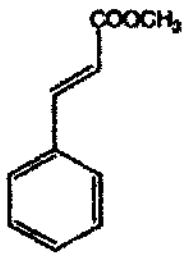
Warm-minty almost ethereal-diffusive odour of very low tenacity. Slightly bitter warm then cool taste. Occasionally used in perfumery, more often in artificial essential oils of the Lavender-Lavandin-Spike family. Used in certain types of masking odours in industry. Also important as a plasticizer.

camphor

Appendix 2 cont

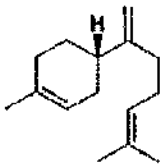
eucalyptol

One of the most widely distributed chemicals occurring in natural essential oils. Sweet, fresh odour and taste and cool mouthfeel. Used to improve odour in industrial products. Widely utilized in perfumery and flavouring, especially oral hygiene products.



methyl cinnamate

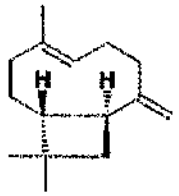
Powerful, tenacious, fruity-balsamic odour in extreme dilution more fruity, strawberry-like, less balsamic. Widely used in perfumes, usually at low concentrations. Also used in soaps and household products. Frequently used in flavour compositions such as cherry, strawberry, grape, peach, plum, butter, cream, vanilla and liqueur flavours.



β-bisabolene

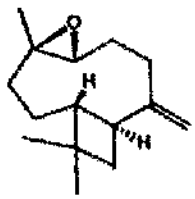
Pleasant, warm, sweet-spicy-balsamic odour. Has potential for use in perfumery and the reconstitution of essential oils especially of oriental, opopanac, chypre and novelty fragrance types.

Appendix 2 cont



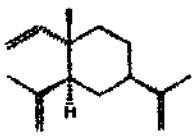
Woody-spicy, dry and tenacious odour. Dry-woody somewhat bitter taste. Used in perfumery. Generally produced from clove and cinnamon leaf and clove stem. Used as a spice flavour or as a fixative for more volatile spice flavourings.

caryophyllene



Woody, slightly ambergris-like odour. Can be prepared by treatment of β-caryophyllene with organic peracids.

caryophyllene oxide



elemene

Appendix 2 cont

1. Prepare sample. Take note of weight.
2. Rinse out flasks that have been steeping in 5% Decon 90, first with tap water then a further 3 times with distilled/deionised water.
3. Fill flask with sample and add distilled/deionised water.
4. Secure by fixing clamp around the neck of the flask.
5. Remove stopper and rinse out the distillation apparatus with tap water.
6. Attach distillation apparatus to flask and connect up the water supply for the condenser, to the tap. Turn on water, making sure the flow is moving in the right direction with a reasonable pressure.
7. Rinse out the gathering tube of the apparatus 3 times with distilled/deionised water and fill up tube with distilled/deionised water.
8. Switch on power to electromantle. Turn up to fill initially then turn down once oil begins to be distilled.
9. Leave for 2-3 hours until all the oil appears to have been distilled.
10. Measure the amount of oil.
11. Run off water from gathering tube and collect oil sample in labelled vial.
12. Rinse distillation apparatus under the tap. Replace stopper, refill with 5% Decon 90 and leave to steep.
13. Once cool, empty flask, refill with 5% Decon 90 and leave to steep for at least 3 hours.
14. Refill flask with distilled/deionised water ready for the next distillation.

Appendix 3 British Pharmacopoeia Distillation Apparatus Procedure (British Standards, 1985).

KH_2PO_4	1.52g
Mg SO_4	0.52g
$NaNO_3$	6.00g
Dextrose	10.00g
Peptone	2.00g
Casein hydrolysate	3.00g
Yeast extract	0.50g
Agar	20.00g

make up to 1 litre with sterile water

Appendix 4 Medium X (Last & Hamley, 1956)

Standard procedure for 20-140 μg protein; 200-1400μg/ml

1. Prepare and store reagents according to manufacturer's instructions.

2. Prepare samples by removing any particulate matter and/or treating any chemicals that may cause interference.

3. Prepare several dilutions of BSA (bovine serum albumin) protein standard containing approximately 0.2 -1.4 mg/ml.

4. Place 0.1ml of standards and appropriately diluted samples in clean, dry test tubes. Place 0.1ml of sample buffer in a test tube to use as a blank.

5. Add 5.0ml of diluted dye reagent.

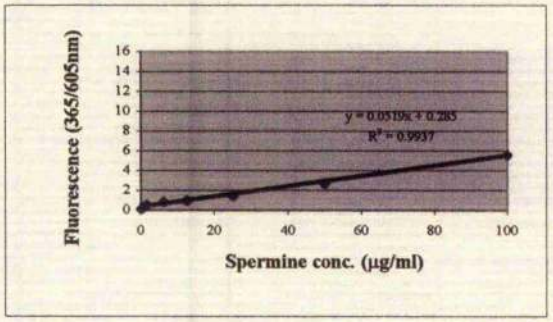
6. Vortex (try to avoid excess foaming); or mix several times by gentle inversion of the test tube.

7. Measure OD_{595} between 5 -60 minutes after preparation.

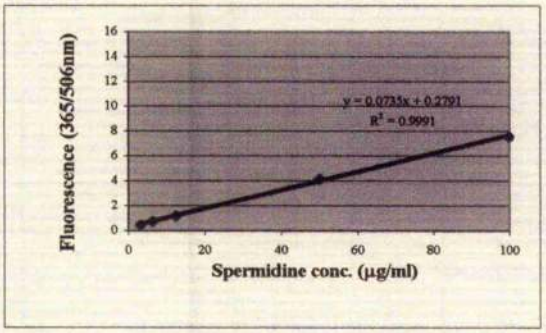
8. Plot OD_{595} versus concentration of standards. Read unknowns from the standard curve.

Appendix 5 The Bio-Rad Protein Assay based on the Bradford (1976) method

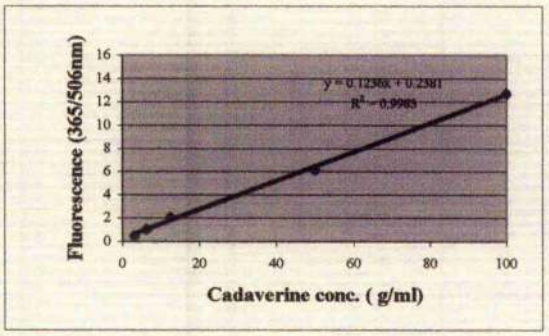
Spermine



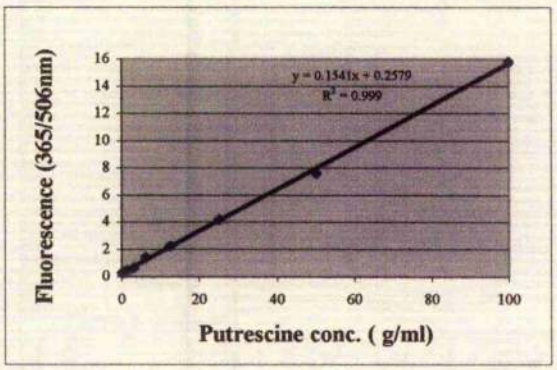
Spermidine



Cadaverine



Putrescine



Appendix 6 Standard curves for four major (dansylated) polyamines

IVP&No	Genus/Species/Variety	Source	Chemotype (30%)	Chemical group (20%)
A24444	*Ocimum americanum*	India	citral	citral
A1679	*Ocimum americanum*	US,Maryland	methyl chavicol	methyl chavicol
PI500942	*Ocimum americanum*	Zambia	camphor	camphor
PI500944	*Ocimum americanum*	Zambia	fenchone	fenchone
PI500945	*Ocimum americanum*	Zambia	fenchone	fenchone
PI500948	*Ocimum americanum*	Zambia	eucalyptol	eucalyptol
PI500951	*Ocimum americanum*	Zambia	eucalyptol>limonene	eucalyptol>limonene
PI500943	*Ocimum americanum pilosum*	Zambia	fenchone	fenchone
PI500947	*Ocimum americanum pilosum*	Zambia	eucalyptol	eucalyptol
PI500949	*Ocimum americanum pilosum*	Zambia	eucalyptol	eucalyptol
PI500950	*Ocimum americanum pilosum*	Zambia	methyl cinnamate	methyl cinnamate>eucalyptol
PI500953	*Ocimum americanum pilosum*	Zambia	eucalyptol	eucalyptol>limonene
PI500954	*Ocimum americanum pilosum*	Zambia	eucalyptol	eucalyptol
PI207498	*Ocimum basilicum*	Afghanistan Kabul	methyl chavicol	methyl chavicol
PI211586	*Ocimum basilicum*	Afghanistan,Kunduz	linalol>m.eugenol	linalol>m.eugenol
PI197442	*Ocimum basilicum*	Ethiopia	linalol	linalol
PI263870	*Ocimum basilicum*	Greece	linalol	linalol
PI531396	*Ocimum basilicum*	Hungary	linalol	linalol
PI190100	*Ocimum basilicum*	Iran	methyl chavicol	methyl chavicol
PI296390	*Ocimum basilicum*	Iran	methyl chavicol	methyl chavicol
PI296391	*Ocimum basilicum*	Iran	m.chavicol>citral	m.chavicol>citral

PI253157	*Ocimum basilicum*	Iran,Esfahan	citral>m.chavicol	citral>m.chavicol
A24983	*Ocimum basilicum*	Italy,Veneto	linalol	linalol
PI358463	*Ocimum basilicum*	Macedonia	linalol	linalol
PI358465	*Ocimum basilicum*	Macedonia		geraniol>linalol
PI358467	*Ocimum basilicum*	Macedonia	linalol	linalol
PI358468	*Ocimum basilicum*	Macedonia	methyl chavicol	methyl chavicol
PI358469	*Ocimum basilicum*	Macedonia	linalol	linalol>methyl chavicol
PI358470	*Ocimum basilicum*	Macedonia	methyl chavicol	methyl chavicol
PI358471	*Ocimum basilicum*	Macedonia	linalol	linalol
PI358472	*Ocimum basilicum*	Macedonia	linalol	linalol
PI368695	*Ocimum basilicum*	Macedonia	linalol	linalol
PI368697	*Ocimum basilicum*	Macedonia	methyl chavicol	methyl chavicol>linalol
PI368698	*Ocimum basilcum*	Macedonia	linalol	linalol
PI368699	*Ocimum basilicum*	Macedonia	linalol	linalol>eucalyptol
PI379412	*Ocimum basilicum*	Macedonia	linalol	linalol>methyl chavicol
PI379413	*Ocimum basilicum*	Macedonia	linalol	linalol
PI379414	*Ocimum basilicum*	Macedonia	linalol	linalol
PI170578	*Ocimum basilicum*	Turley,Aydin	linalol	linalol
PI170579	*Ocimum basilicum*	Turkey,Izmir	eugenol	eugenol>linalol
PI170581	*Ocimum basilicum*	Turkey,Canakkale	linalol	linalol
PI172996	*Ocimum basilicum*	Turkey,Kars	methyl chavicol	methyl chavicol
PI172997	*Ocimum basilicum*	Turkey,Kars	methyl chavicol	methyl chavicol

PI172998	*Ocimum basilicum*	Turkey,Van	methyl chavicol	methyl chavicol
PI173746	*Ocimum basilicum*	Turkey,Malatya	linalol	linalol
PI174285	*Ocimum basilicum*	Turkey,Elazig	methyl chavicol	methyl chavicol>linalol
PI175793	*Ocimum basilicum*	Turkey,Canakkale	linalol	linalol
PI176646	*Ocimum basilicum*	Turkey,Tokat	methyl eugenol>linalol	methyl eugenol>linalol
PI182246	*Ocimum basilicum*	Turkey,Maras	linalol	linalol
NSL15586	*Ocimum basilicum*	US,California	linalol	linalol>methyl eugenol
PI414193	*Ocimum basilicum*	US,Maryland	linalol	linalol
PI414194	*Ocimum basilicum*	US,Maryland	m.chavicol>linalol	m.chavicol>linalol
PI414195	*Ocimum basilicum*	US,Maryland	methyl chavicol	m.chavicol>linalol
PI414196	*Ocimum basilcum*	US,Maryland	linalol>m.chavicol	linalol>m.chavicol
PI414197	*Ocimum basilicum*	US,Maryland	linalol	linalol
PI414198	*Ocimum basilicum*	US,Maryland	methyl chavicol	methyl chavicol
PI414199	*Ocimum basilicum*	US,Maryland	methyl chavicol	methyl chavicol>linalol
PI414200	*Ocimum basilicum*	US,Maryland	methyl chavicol	methyl chavicol
A7772	*Ocimum basilicum*	US,New Mexico	citral>linalol	citral>linalol
NSL6421	*Ocimum basilicum*	US,Pensylvania	linalol	linalol
PI358464	*Ocimum basilicum*	Yugoslavia	linalol	linalol
PI358466	*Ocimum basilicum*	Yugoslavia	linalol	linalol>geraniol
A24448	*Ocimum gratissimum gratissimum*	Tanzania		caryophyllene oxide
PI500952	*Ocimum gratissimum gratissimum*	Zambia		caryophyllene oxide
A7833	*Ocimum gratissimum macrophylla*	Sri Lanka	eugenol	eugenol

A23155	*Ocimum tenuiflorum*	Cuba	caryophyllene	caryophyllene
A23154	*Ocimum tenuiflorum*	Denmark		B-bisabolene
PI288779	*Ocimum tenuiflorum*	India,Gujarat	eugenol	eugenol>elemene
A23157	*Ocimum tenuiflorum*	Maldives		
PI414201	*Ocimum tenuiflorum*	US,Maryland		methyl chavicol
PI414204	*Ocimum tenuiflorum*	US,Maryland		
PI414205	*Ocimum tenuiflorum*	US,Maryland	eugenol	eugenol

Appendix 7 Summary of chemotypes (components 30%) and chemical groups (components 20%) of germplasm accessions

IVP & No.	Species	Origin
A24983	*O. basilicum*	Italy
PI500951	*O. americanum*	Zambia
NSL15586	*O. basilicum*	California
PI531396	*O. basilicum*	Hungary
PI414205	*O. tenuiflorum*	Maryland
A7772	*O. basilicum*	New Mexico
PI500944	*O. americanum*	Zambia
PI288779	*O tenuiflorum*	India
NSL6421	*O. basilicum*	Pennsylvania
PI358469	*O. basilicum*	Macedonia
A23157	*O. tenuiflorum*	Maldives
PI500953	*O. americanum*	Zambia
PI358466	*O. basilicum*	Yugoslavia
A23154	*O. tenuiflorum*	Denmark
PI500950	*O. americanum*	Zambia
PI296391	*O. basilicum*	Iran
PI511865	*O. selloi*	Uruguay
PI500943	*O. americanum*	Zambia
PI263870	*O. basilicum*	Greece
A1679	*O. americanum*	Maryland
PI500942	*O. americanum*	Zambia
PI207498	*O. basilicum*	Afghanistan
PI500952	*O. gratissimum*	Zambia
PI414195	*O. basilicum*	Maryland
PI197442	*O. basilicum*	Ethiopia
A24448	*O. gratissimum*	Tanzania
A23155	*O. tenuiflorum*	Cuba
PI172997	*O. basilicum*	Turkey
A7833	*O. gratissimum*	Sri Lanka

Appendix 8 List of samples sent to Liverpool for GC-MS

IVP & No.	Species	Origin
Aroma Trading	*O. basilicum*	Comoros
Aroma Trading	*O. basilicum*	Eygptian
PI511865	*O. selloi*	Uruguay
PI500943	*O. americanum*	Zambia
PI500945	*O. americanum*	Zambia
PI414204	*O. tenuiflorum*	Maryland
PI414197	*O. basilicum*	Maryland

Appendix 9 List of samples sent to Japan for GC-MS

Plant Passport Information

Entry	IVP	IVNO	PVS	IVT	CINO	Genus	Species	Variety	Previous taxonomy	Improvement status	Secondary Identifier	Origin/Source
1	Ames	1679	89ncab01	SD	1	*Ocimum*	*canescens*	*pilosum*	*O. kilimandscharicum*	Unknown origin. Seeds donated via Plant Introduction Office, Beltsville, Maryland, USA.	PI 409558	US, Maryland
2	Ames	24484	98ncav01	SD	82	*Ocimum*	*canescens*			seeds donated by S M Damaan Area Afgarh, India via the National Centre for Agriculture Utilisation Research (NCAUR), Peoria, Illinois, USA	NG 62505	India
3	PI	500942	90ncai01	SD	62	*Ocimum*	*americanum*			Collected during 1981 Plant Expedition to Zambia	ZM 1453	Zambia
4	PI	500943	97ncai01	SD	63	*Ocimum*	*americanum*			Collected during 1981 Plant Expedition to Zambia	ZM 1503	Zambia
5	PI	500941	97ncai01	SD	64	*Ocimum*	*americanum*		*O. sp.*	Sesheke, Mongu District, Western Province, Zambia (1981 Plant Expedition)	ZM 1551	Zambia
6	PI	500945	90ncai01	SD	65	*Ocimum*	*americanum*			Collected during 1981 Plant Expedition to Zambia	Simukanka	Zambia
7	PI	500948	90ncai01	SD	68	*Ocimum*	*americanum*			Collected during 1981 Plant Expedition to Zambia	ZM 1906	Zambia
8	PI	500951	97ncai01	SD	71	*Ocimum*	*americanum*			Collected during 1981 Plant Expedition to Zambia	Cultivated	ZM 2282	Zambia
9	PI	500953	97ncai01	SD	73	*Ocimum*	*americanum*			Collected during 1981 Plant Expedition to Zambia	Wild	ZM 2523	Zambia
10	PI	500947	97ncai01	SD	67	*Ocimum*	*americanum*	*pilosum*		Collected during 1981 Plant Expedition to Zambia	Wild	ZM 1881	Zambia
11	PI	500949	97ncai01	SD	69	*Ocimum*	*americanum*	*pilosum*		Collected during 1981 Plant Expedition to Zambia	Cultivated	ZM 1974	Zambia
12	PI	500950	97ncai01	SD	70	*Ocimum*	*americanum*	*pilosum*		Collected during 1981 Plant Expedition to Zambia	Cultivated	ZM 2044	Zambia
13	PI	500954	97ncai01	SD	74	*Ocimum*	*americanum*	*pilosum*		Collected during 1981 Plant Expedition to Zambia	Wild	Lwimi	Zambia
14	NSL	6721	97nsfv51	SD	3	*Ocimum*	*basilicum*			W Atlee Burpee Co., Warminster, Pennsylvania, USA		Sweet Basil	US, Pennsylvania
15	Ames	7772	98ncal01	SD	4	*Ocimum*	*basilicum*			seed from Arizona		HB3/Mrs Bruce Lemon Basil	US, New Mexico
16	MSL	15386	97nsfb01	SD	6	*Ocimum*	*basilicum*			Ferry-Morse Seed Co., Mountain View, California, USA		Dark Opal	US, California
17	Ames	24983	99ncas01	SD	88	*Ocimum*	*basilicum*			A Bellandelli, ESASEB, Casalmaio, Veneto, Italy	Cultivar	Genovese	Italy, Veneto
18	PI	170579	97ncas01	SD	8	*Ocimum*	*basilicum*			Garden of Ali Kacuk Kiva, Bergama, Izmir, Turkey		2263	Turkey, Izmir
19	PI	170581	20ncai01	SD	9	*Ocimum*	*basilicum*			Ayvacik, Canakkale, Turkey		3061	Turkey, Canakkale
20	PI	172996	90ncai01	SD	10	*Ocimum*	*basilicum*			Seed dealer, Igdir, Kars, Turkey		Reyhan	Turkey, Kars
21	PI	172997	96ncai01	SD	11	*Ocimum*	*basilicum*			Seed dealer, Igdir, Kars, Turkey		Reyhan	Turkey, Kars
22	PI	172998	96ncai01	SD	12	*Ocimum*	*basilicum*			Seed dealer, Van, Van, Turkey		Reyhan	Turkey, Van
23	PI	173746	78ncai01	SD	13	*Ocimum*	*basilicum*			Soedanan, Malatya, Malatya, Turkey		Reyhan	Turkey, Malatya
24	PI	174284	89ncai01	SD	14	*Ocimum*	*basilicum*			Ercis, Van, Turkey		Zairun	Turkey, Van
25	PI	174285	97ncai01	SD	15	*Ocimum*	*basilicum*			Seed dealer, Elazig, Elazig, Turkey		Reyhan	Turkey, Elazig
26	PI	175793	97ncai01	SD	16	*Ocimum*	*basilicum*			Bahcedere, Canakkale, Turkey		Fesnegan	Turkey, Canakkale
27	PI	176646	89ncai01	SD	17	*Ocimum*	*basilicum*			Soedanan, Tokat, Tokat, Turkey		Reyhan	Turkey, Tokat
28	PI	182246	90ncai01	SD	18	*Ocimum*	*basilicum*			Elbistan, Maras, Turkey		Reyhan	Turkey, Maras
29	PI	190100	90ncai01	SD	19	*Ocimum*	*basilicum*			Seeds presented by Abdolla Ledi, Tehran, Iran		1	Iran
30	PI	197442	97ncai01	SD	20	*Ocimum*	*basilicum*			Diredawa, Ethiopia		16126	Ethiopia
31	PI	207498	90ncai01	SD	21	*Ocimum*	*basilicum*			from a market in Kabul, Afghanistan		12648	Afghanistan, Kabul
32	PI	211586	97ncai01	SD	22	*Ocimum*	*basilicum*		*Celosia*	Khanabad, Kondoz, Afghanistan		12831	Afghanistan
33	PI	253157	91ncab01	SD	24	*Ocimum*	*basilicum*			Esfahan bazaar, Esfahan, Iran		Rayhoen	Iran, Esfahan
34	PI	268870	90ncai01	SD	27	*Ocimum*	*basilicum*			Seeds presented by J Levanos, Texas, Virginia, USA			Greece
35	PI	296390	90ncai01	SD	29	*Ocimum*	*basilicum*			Seeds presented by th Office of Agriculture Attache, Tehran, Iran			Iran
36	PI	296391	76ncai01	SD	30	*Ocimum*	*basilicum*			Seeds presented by th Office of Agriculture Attache, Tehran, Iran			Iran
37	PI	358463	91ncai01	SD	31	*Ocimum*	*basilicum*			Donated by L Aladzajkov, Skopje University, Skopje, Macedonia		Obicen bosilek	Macedonia
38	PI	358464	97ncai01	SD	32	*Ocimum*	*basilicum*			Donated by L Aladzajkov, Skopje University, Skopje, Macedonia		Edrofiten	Yugoslavia
39	PI	358465	97ncai01	SD	33	*Ocimum*	*basilicum*			Donated by L Aladzajkov, Skopje University, Skopje, Macedonia		Srednofiten	Macedonia
40	PI	358466	91ncai01	SD	34	*Ocimum*	*basilicum*			Donated by L Aladzajkov, Skopje University, Skopje, Macedonia		Krosten	Yugoslavia
41	PI	358467	76ncai01	SD	35	*Ocimum*	*basilicum*			Donated by L Aladzajkov, Skopje University, Skopje, Macedonia		Domasen snen	Macedonia
42	PI	358468	76ncai01	SD	36	*Ocimum*	*basilicum*			Donated by L Aladzajkov, Skopje University, Skopje, Macedonia		Krupcolisten	Macedonia
43	PI	358469	78ncai01	SD	37	*Ocimum*	*basilicum*			Donated by L Aladzajkov, Skopje University, Skopje, Macedonia		Siten	Macedonia
44	PI	358470	76ncai01	SD	38	*Ocimum*	*basilicum*			Donated by L Aladzajkov, Skopje University, Skopje, Macedonia		Lokalen	Macedonia
45	PI	358471	78ncai01	SD	39	*Ocimum*	*basilicum*			Donated by L Aladzajkov, Skopje University, Skopje, Macedonia		Sitnolisten	Macedonia
46	PI	358472	89ncai01	SD	40	*Ocimum*	*basilicum*			Donated by L Aladzajkov, Skopje University, Skopje, Macedonia		Divlioki	Macedonia
47	PI	368693	76ncai02	SD	41	*Ocimum*	*basilicum*			Donated by L Aladzajkov, Skopje University, Skopje, Macedonia		Zelen	Macedonia
48	PI	368697	91ncai02	SD	42	*Ocimum*	*basilicum*			Donated by L Aladzajkov, Skopje University, Skopje, Macedonia		Vladimirska	Macedonia
49	PI	368698	76ncai03	SD	43	*Ocimum*	*basilicum*			Donated by L Aladzajkov, Skopje University, Skopje, Macedonia		Srednolisten	Macedonia
50	PI	368699	76ncai03	SD	44	*Ocimum*	*basilicum*			Donated by L Aladzajkov, Skopje University, Skopje, Macedonia		Edec	Macedonia
51	PI	375412	76ncai03	SD	46	*Ocimum*	*basilicum*			Donated by L Aladzajkov, Skopje University, Skopje, Macedonia		Krupen bel	Macedonia
52	PI	375413	91ncai03	SD	47	*Ocimum*	*basilicum*			Donated by L Aladzajkov, Skopje University, Skopje, Macedonia		Krupnolisen	Macedonia
53	PI	375414	76ncai03	SD	48	*Ocimum*	*basilicum*			Donated by L Aladzajkov, Skopje University, Skopje, Macedonia		Tiel krigen	Macedonia
54	PI	414196	76ncai01	SD	49	*Ocimum*	*basilicum*			Unknown origin. Seeds presented by Division of Tobacco, Medical & Special Crops, Plant Industry Station, Beltsville, Maryland, USA		B 49939	US, Maryland
55	PI	414194	91ncai01	SD	50	*Ocimum*	*basilicum*			Unknown origin. Seeds presented by Plant Introduction Station, Glenn Dale, Maryland, USA		Ames 1693	US, Maryland
56	PI	414195	91ncai01	SD	51	*Ocimum*	*basilicum*			Unknown origin. Seeds presented by Plant Introduction Station, Glenn Dale, Maryland, USA		Ames 1694	US, Maryland
57	PI	414196	97ncai01	SD	52	*Ocimum*	*basilicum*			Unknown origin. Seeds presented by Plant Introduction Station, Glenn Dale, Maryland, USA		Ames 1695	US, Maryland
58	PI	414197	89ncai02	SD	53	*Ocimum*	*basilicum*			Unknown origin. Seeds presented by Plant Introduction Station, Glenn Dale, Maryland, USA		Ames 1696	US, Maryland
59	PI	414198	76ncai02	SD	54	*Ocimum*	*basilicum*			Unknown origin. Seeds presented by Plant Introduction Station, Glenn Dale, Maryland, USA		Ames 1697	Maryland
60	PI	414199	89ncai01	SD	55	*Ocimum*	*basilicum*			Unknown origin. Seeds presented by Plant Introduction Station, Glenn Dale, Maryland, USA		Ames 1698	US, Maryland
61	PI	414200	76ncai01	SD	56	*Ocimum*	*basilicum*			Unknown origin. Seeds presented by Plant Introduction Station, Glenn Dale, Maryland, USA		Ames 1699	US, Maryland
62	PI	531396	90ncai02	SD	76	*Ocimum*	*basilicum*			Institute for Plant Production and Qualification, Tapioszelle, Hungary	Cultivated	1429	Hungary
63	PI	176578	82ncai01	SD	7	*Ocimum*	*basilicum*			florist, Aydic, Aydin, Turkey		Fesligen	Turkey, Aydin
64	Ames	7833	97ncai01	SD	5	*Ocimum*	*gratissimum*	*macrophylla*		Collected in Sri Lanka in 1985		Mil 13	Sri Lanka
65	Ames	24448	98ncao01	SD	85	*Ocimum*	*gratissimum*	*gratissimum*		via the National Centre for Agricultural Utilisation Research, Peoria, IL	Wild	NU 61183	Tanzania
66	PI	500952	97ncai01	SD	72	*Ocimum*	*gratissimum*	*gratissimum*		Collected during 1981 Plant Expedition to Zambia.		ZM 2477	Zambia
67	Ames	23154	92ncai01	SD	77	*Ocimum*	*tenuiflorum*					OCI 21593	Denmark
68	Ames	23157	97ncai01	SD	80	*Ocimum*	*tenuiflorum*					OCI 155795	Maldives
69	PI	283779	97ncai01	SD	28	*Ocimum*	*tenuiflorum*		*O. sanctum*	Collected in Bhuj, Kutch District, Gujarat, India in 1562		512	India, Gujarat
70	PI	414201	76ncai01	SD	57	*Ocimum*	*tenuiflorum*		*O. gratissimum*	Received from Beltsville Plant Station, Maryland, USA		B 49933	US, Maryland
71	PI	414204	76ncai01	SD	60	*Ocimum*	*tenuiflorum*		*O. gratissimum*	Received from Beltsville Plant Station, Maryland, USA		B 49936	US, Maryland
72	PI	414205	76ncai01	SD	61	*Ocimum*	*tenuiflorum*		*O. kilimandscharicum*	Received from Beltsville Plant Station, Maryland, USA		B 49937	US, Maryland
73	Ames	23153	97ncai01	SD	78	*Ocimum*	*tenuiflorum*			Seed dealer in Pilonte, Las Marias, Cuba, via Goodwork at Gainesbilton		OCI 82987	Cuba
74	PI	511865				*Ocimum*	*sellof*			Donated by P Armand-Ugon, Catedra de Botanica, Montevideo, Montevideo, Uruguay	Cultivated		Uruguay

Appendix 10 Plant passport information for *Ocimum* spp. in NCRPIS germplasm collection