

**Effects of environmental factors on growth,
bioactive compounds and cholinergic properties of
hydroponically raised *Salvia* and *Narcissus* species.**

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I hereby declare that this thesis is based on work conducted by myself, and that the work has not been accepted in substance for any other degree. All references to ideas and work of other researchers have been specifically acknowledged.

Abstract

The increasing interest of the pharmaceutical and food industry in exploiting natural products has initiated scientific studies on the so-called aromatic & medicinal plants. However, information regarding their cultivation is scarce, and as bioactive phytochemicals are highly susceptible to environmental regulation, optimization and full control of growth conditions are vital for a cost-effective, standardised and high-quality medicinal product. Thus, *Salvia officinalis* var *purpurea* and *Narcissus* "Ice Follies" were cultivated by means of the hydroponic Nutrient Film Technique (NFT), under various nutrient conditions i.e. nitrate (N-NO₃), phosphorus (P) and various electrical conductivity (EC) regimes. Essential oils from *S. officinalis* and the alkaloid galanthamine from *Narcissus* sp. possess cholinergic activities relevant to the symptomatic treatment of Alzheimer's disease (AD). This study aimed to determine optimum growth conditions for enhanced plant biomass accumulation and improved yield and quality of the bioactive compounds in order to increase the efficacy of the medicinal species. In addition, anti-cholinesterase properties of *S. officinalis* var *purpurea* were investigated.

At the end of each experiment plant biomass accumulation, root to shoot investment, plant height, leaf area and photosynthetic pigments were assessed together with uptake of nitrates, phosphorus, potassium and calcium. Essential oils of sage and alkaloids from *Narcissus* were obtained *via* steam distillation and organic solvent extraction method respectively. Oils were subsequently analysed by means of gas chromatography mass spectroscopy, whereas high performance liquid chromatography was employed for alkaloid analysis. Cholinesterase inhibition was tested *via* the colorimetric Ellman's enzyme assay.

S. officinalis and *Narcissus* species were successfully cultivated in the NFT, for the very first time. In *Salvia* optimum conditions for high shoot biomass production appeared to be the 150 mg/L N-NO₃, 12 mg/L P supply and EC 1.8 mS/cm. Nitrate level marginally affected growth or ontogeny of *Narcissus*, however alkaloid yield was significantly augmented by N-NO₃ supply (180 mg/L) and exhibited much higher values in comparison with the existing literature. In contrast, *Salvia* essential oils revealed weak responses to N-NO₃ and P nutrition in terms of yield and quality, whereas high EC levels considerably altered oil relative composition. Essential oil content was approximately 1% whilst a report for *S. officinalis* raised in Britain indicated only 0.53%. Camphor and α -caryophyllene were abundant in the oil comprising ca the 10-17% and 16-23% respectively. α -Thujone contributed for 9-10%. All other constituents like α and β pinene, 1,8 cineole, ledol, borneol and bornyl acetate appeared with

concentrations less than 4% in the oil mixture. Anti-cholinesterase activity of the essential oils was dual and remained unchanged under all growth conditions exhibiting relatively low IC50 values; approximately 0.055 to 0.07 mg/ml for human acetylcholinesterase (AChE) and 0.07 mg/ml for butyrylcholinesterase (BuChE) enzyme from horse serum that was time-dependant. Competitive type of inhibition was revealed for AChE and non-competitive for BuChE. Finally, the ethanolic extracts of *S. officinalis* var *purpurea* preferentially inhibited BuChE with IC50 significantly lower than the oil i.e. 0.054 mg/ml.

The findings of this project demonstrated that NFT is an important means for successful cultivation of *Salvia* and *Narcissus* species. N-NO₃, P and EC levels significantly affected growth of *Salvia* and EC conditions altered essential oil composition. The yield of galanthamine in bulbs and leaves of *Narcissus* was significantly augmented by N-NO₃ supply. Therefore, standardisation of cultivation methods for medicinal species is essential to assure biochemical consistency and quality. *S. officinalis* var *purpurea* should be considered as an alternative strategy for the treatment of mild to moderate AD.

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List of abbreviations

ABA	abscisic acid
Abs	absorbance
acetyl-CoA	acetyl coenzyme A
ACh	acetylcholine
AChE	acetylcholinesterase
AD	Alzheimer's disease
ADP	adenosine diphosphate
AGR	absolute growth rate of plant
AGRr	absolute growth rate of root
AGRs	absolute growth rate of shoot
Al ⁺³	aluminium cations
ANOVA	analysis of variance
ATCh	acetylthiocholine
ATChI	acetylthiocholine iodide
ATP	adenosine triphosphate
B	boron
BuChE	butyrylcholinesterase
BuTChI	butyrylthiocholine iodide
Ca	calcium
Ca(NO ₃) ₂ *4H ₂ O	Calcium Nitrate Hydrated
Ca ⁺²	calcium cations
ChE	cholinesterase
ChEs	cholinesterase enzymes
Chla	Chlorophyll a
Chlb	Chlorophyll b
CRD	completely randomized design
Cu	copper
CYT	cytokinins
DMAPP,	dimethylallyl diphosphate;
DOXP	1-deoxy-D-xylulose-5-phosphate
DTNB	5,5-dithiobis-2-nitrobenzoic acid
DWB	dry weight of the bulb
DWL	dry weight of the leaves
DWP	dry weight of plant
DWR	dry weight of root
DWS	dry weight of shoots

DXR	DOXP reductoisomerase
DXS	DOXP synthase
EC	electrical conductivity
EC50	median effective concentration
EDTA	ethylenediaminetetracetic acid
ETOH	ethanol
Fe	iron
Fe ⁺³	iron cations
FMN	flavin mononucleotide
FPP	farnasyl diphosphate
<i>FWB</i>	fresh weight of the bulb
<i>FWF</i>	fresh weight of the flowers
<i>FWS</i>	shoot fresh weight
GA-3P	glyceraldehyde 3-phosphate
GAL	galanthamine
GC/MS	Gas Chromatography-Mass Spectrometry
GGPP	geranylgeranyl diphosphate
Glu	glutamine
GPP	geranyl diphosphate
H ₃ BO ₃	boric acid
H ₃ PO ₄	phosphoric acid
His	histidine
HMG-CoA	hydroxymethylglutaryl-coenzyme A
HMGR	hydroxymethylglutaryl-coenzyme A reductase
HNO ₃	nitric acid
HPLC	High Performance Liquid Chromatography
IAA	indole-3-acetic acid
IC50	Concentration value for 50% inhibition
ICP-OES	Inductively Coupled Plasma Optical Emission Spectroscopy
IPP	Isopentenyl diphosphate
K	potassium
K ₂ SO ₄	Potassium Sulphate
KH ₂ PO ₄	Mono Potassium Phosphate
K _m	Michaelis constant
KNO ₃	potassium nitrate
<i>LA</i>	leaf area
LB plot	Lineweaver – Burk plot
LYC	lycorine

MEP	2-C-methyl-D-erythritol 4-phosphate
Mg	magnesium
Mg(NO ₃) ₂ *6H ₂ O	magnesium nitrate hydrated
MgSO ₄ *7H ₂ O	magnesium sulphate hydrated
Mn	manganese
MnSO ₄ *4H ₂ O	manganese sulphate hydrated
Mo	molybdenum
N	nitrogen
N	number of replicates
Na	sodium
NAD	β-nicotinamide adenine dinucleotide
NADP	β-nicotinamide adenine dinucleotide phosphate
NAGREF	National Agricultural Research Foundation
NFT	Nutrient Film Technique
NH ₃	ammonia
(NH ₄) ₆ Mo ₇ O ₂₄	ammonium molybdate
NH ₄ ⁺	cations
N-NO ₃	N from nitrates
NO ₃ ⁻	nitrate anion
OH ⁻	hydroxyl anion
P	phosphorus
P value	indicates significance of slope's deviation from zero
PAL	phenylalanine ammonia lyase
PAR	photosynthetically active radiation
PEP	phosphoenol pyruvate
phe	phenylalanine
Pi	inorganic phosphorus
PLP	pyridoxal-5'-phosphate
PO ₄ ⁻³	inorganic phosphate
PPi	pyrophosphate bonds
r	Pearson product –moment correlation
r ²	indicates goodness of fit of a slope
R _{Sco}	allometric root to shoot co-efficient
RuBPCase 1.5	ribulose biphosphate carboxylase
S	enzyme substrate
S	sulphur
SEM	standard error of the mean
Ser	serine

SLA specific leaf area
SM secondary metabolites
 SO_4^{-2} sulphate cations
SWC % shoot water content
TNB anion of 5-thio-2nitrobenzoic acid,
trp tryptophane
tyr tyrosine
US United states
UV ultraviolet
V enzyme velocity
Vmax maximum enzyme velocity
WHO World Health Organization
Zn zinc
 $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ zinc sulphate

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to my parents

Chapter 1

General introduction

1.1 Natural products or plant secondary metabolites

1.1.1 Definition

Plants produce a remarkably vast and diverse array of low molecular mass compounds known as secondary metabolites (SM) or natural products (Verpoorte, 1998; Wink 2003; Hadacek, 2002). More than 100,000 structures of SM have been described today, whereas estimates of molecules yet to be discovered exceed 500,000. Originally, such compounds were thought to occur exclusively in higher plants. Ongoing research however, and the improvement of analytical techniques has allowed the recovery of more secondary molecules and established their presence in bacteria, lower plants and fungi. Today it is evident that all groups of living organisms synthesize the so-called natural products (Hadacek, 2002).

In contrast to primary metabolites like chlorophyll, nucleic acids, proteins, lipids and carbohydrates secondary metabolites are generally considered non-essential for the basic (primary) metabolic processes of the plant that result in growth and development (Taiz & Zeiger 1991; Dixon, 2001). However, Hadacek (2002) indicated that some secondary molecules may function as co-substrates or co-enzymes serving the needs of primary pathways and therefore distinction between primary and secondary metabolism can be blurred or even meaningless. Hartmann (1996) defined primary metabolism as “universal, uniform, conservative, and indispensable” and secondary metabolism as “singular, diverse, adaptive, dispensable for growth and development, but indispensable for survival”.

1.1.2 Role of secondary metabolites in plants

For many years, the significance of secondary metabolites (SM) was unknown. They were thought to be simply functionless end products of metabolism or metabolic wastes (Taiz & Zeiger 1991; Bennett & Wallsgrove, 1994; Verpoorte, 1998; Wink,

2003), detoxification products, results of shunt and overflow metabolism, degradation products or cell storage compounds (Hadacek, 2002). Nowadays, the term “secondary” represents an unfortunate choice as it implies that SM are not of primary value to the organisms (Bennett & Wallsgrave, 1994; Hadacek, 2002; Singer *et al.*, 2003). Extensive investigations have revealed complex biological activities and functions for SM that render them essential for plant fitness, survival and reproduction (Bennett & Wallsgrave, 1994; Pichersky & Gershenzon, 2002). Secondary metabolites play a major role in the adaptation of plants to their environment by interacting with the ecosystem (Bourgaud *et al.*, 2001). Being sessile organisms, plants cannot run away when attacked by insects, snails or vertebrate herbivores and cannot rely on an immune system when infected by microbes thus evolution of defence chemicals was vital to inhibit their natural enemies (Wink, 2003). Natural products have been attributed with antibiotic, antifungal, antiviral and insecticidal activities that protect plants against pathogens and insects due to the presence of terpene, phenolic or alkaloid secondary molecules. Many secondary compounds described as poisonous, serve for defence against various herbivores i.e. alkaloids (Bennett & Wallsgrave, 1994). Generally, three types of defence compounds exist: those constitutively expressed in cells; those constitutively expressed but requiring biochemical activation and those which are inducible (Verpoorte, 1998; Dixon, 2001; Hadacek, 2002). However, plants need animals and insects for pollination or seed dispersal, so in some cases SM like monoterpenes, anthocyanins carotenoids, phenolics, tannins and saponins function as attractants to these organisms (Wink, 2003; Bennett & Wallsgrave, 1994). In several instances attractant and defensive activities are exhibited by the same molecule (Hadacek, 2002; Wink, 2003) as in the case of anthocyanins and terpenes. Furthermore, the phenomenon of allelopathy is due to the present of specific secondary molecules; alkaloids, cyanogenic glycosides, glucosinolates, terpenes tannins and others (Wink, 2003) that are released in the surrounding environment in order to inhibit germination and / or growth of neighbouring plants (Bourgaud, 2001).

Finally, there are a number of SM that carry out other physiological functions like nitrogen transport and storage in a toxic form (alkaloids), or UV absorption (flavonoids) that protects plants from UV radiation hazards (Taiz & Zeiger 1991; Haborn, 1993; Wink, 2003; Bourgaud, 2001). Gershenzon, (1985) suggested that specific SM can function to reduce transpiration under hot environments by inducing stomatal closure (abscisic acid a triterpenoid) or by providing a vapour shield on the leaf surface (volatile terpenes).

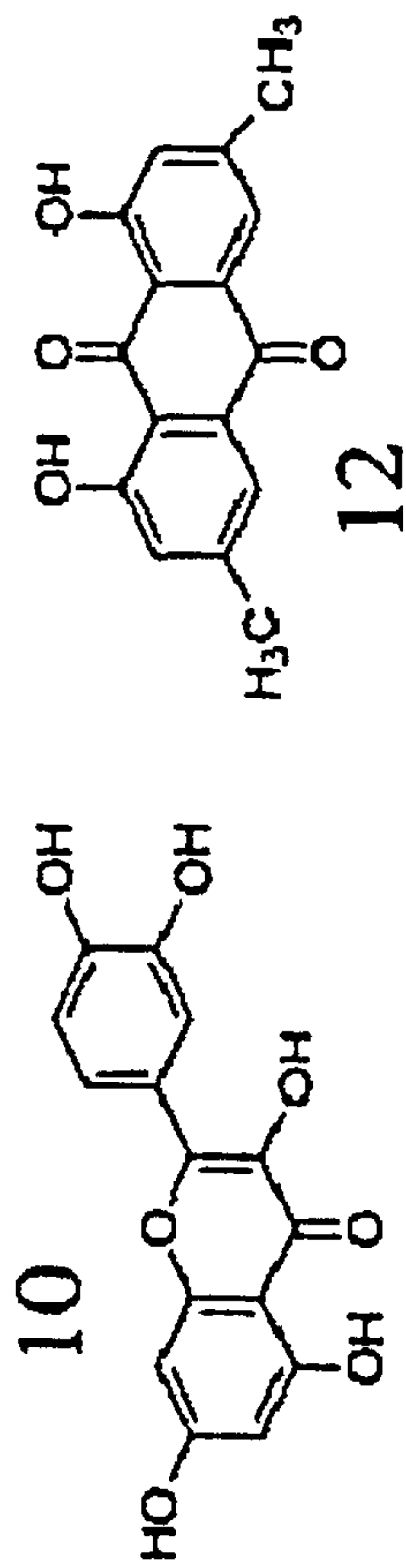
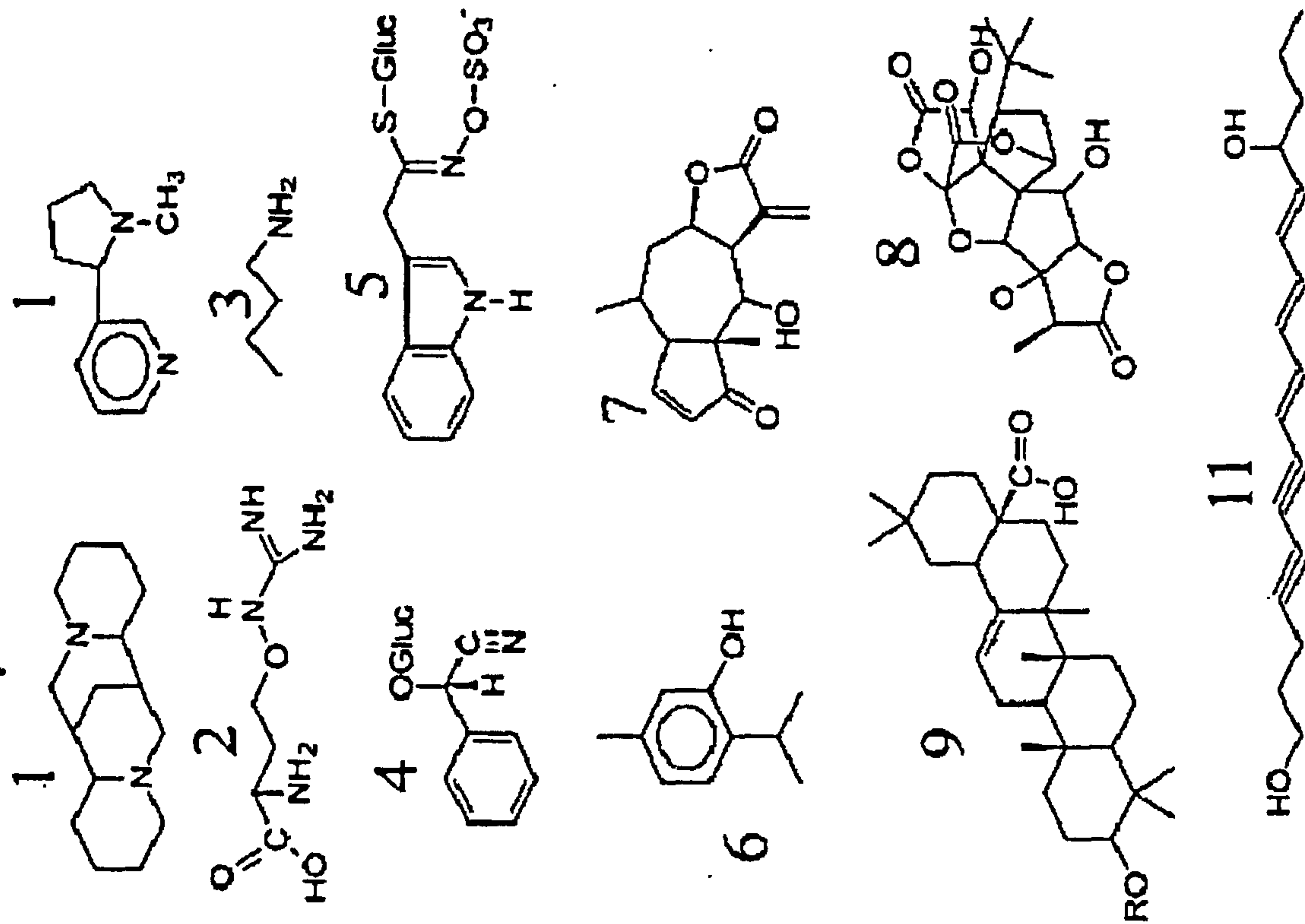
1.1.3 Structural diversity of secondary metabolites

Secondary metabolites (SM) are present in plants in high structural diversity and intraspecific variability (Hadacek, 2002; Wink, 2003), although as a rule a single SM group dominates within a certain taxon (Wink, 2003). The enormous observed variation stems from the evolutionary processes for the acquisition of new, improved defence chemicals for plants survival and fitness (Harborne, 1993; Dixon, 2001; Rausher, 2001; Wink, 2003). Despite the high metabolic investment, it is advantageous for plants to maintain high diversity among SM, in order to increase protection from their enemies. The so called screening hypothesis postulates that plants sustain SM structural diversity to enhance defence by constantly screening against microbe and predators (Hadacek, 2002). Plant SM are usually classified according to their biosynthetic pathways (Bourgaud *et al.*, 2001). In general, four major groups of SM have been identified i.e. terpenoids, phenolics, nitrogen / sulfur containing compounds and fatty acid / polyketide derivatives (Dixon, 2001) (Table 1.1 and Figure 1.1).

Table 1.1: Structural diversity of plant secondary metabolites (Wink, 2003). Main secondary metabolites classes

Number of natural products

<u>With nitrogen</u>	
•Alkaloids (1)	12,000
•Non-protein amino acids (2)	700
•Amines (3)	100
•Cyanogenic glycosides (4)	60
•Glucosinolates (5)	100
•Alkamides	150
<u>Without nitrogen</u>	
•Monoterpenes (incl. Iridoids) (6)	2,500
•Sesquiterpenes (7)	5,000
•Diterpenes (8)	2,500
•Triterpenes, Saponins, Steroids (9)	5,000
•Tetraterpenes	500
•Phenylpropanoids, coumarins, lignans	2,000
•Flavonoids (10)	4,000
•Polyacetylenes, fatty acids, waxes (11)	1,000
•Polyketides (12)	750
•Carbohydrates	>200



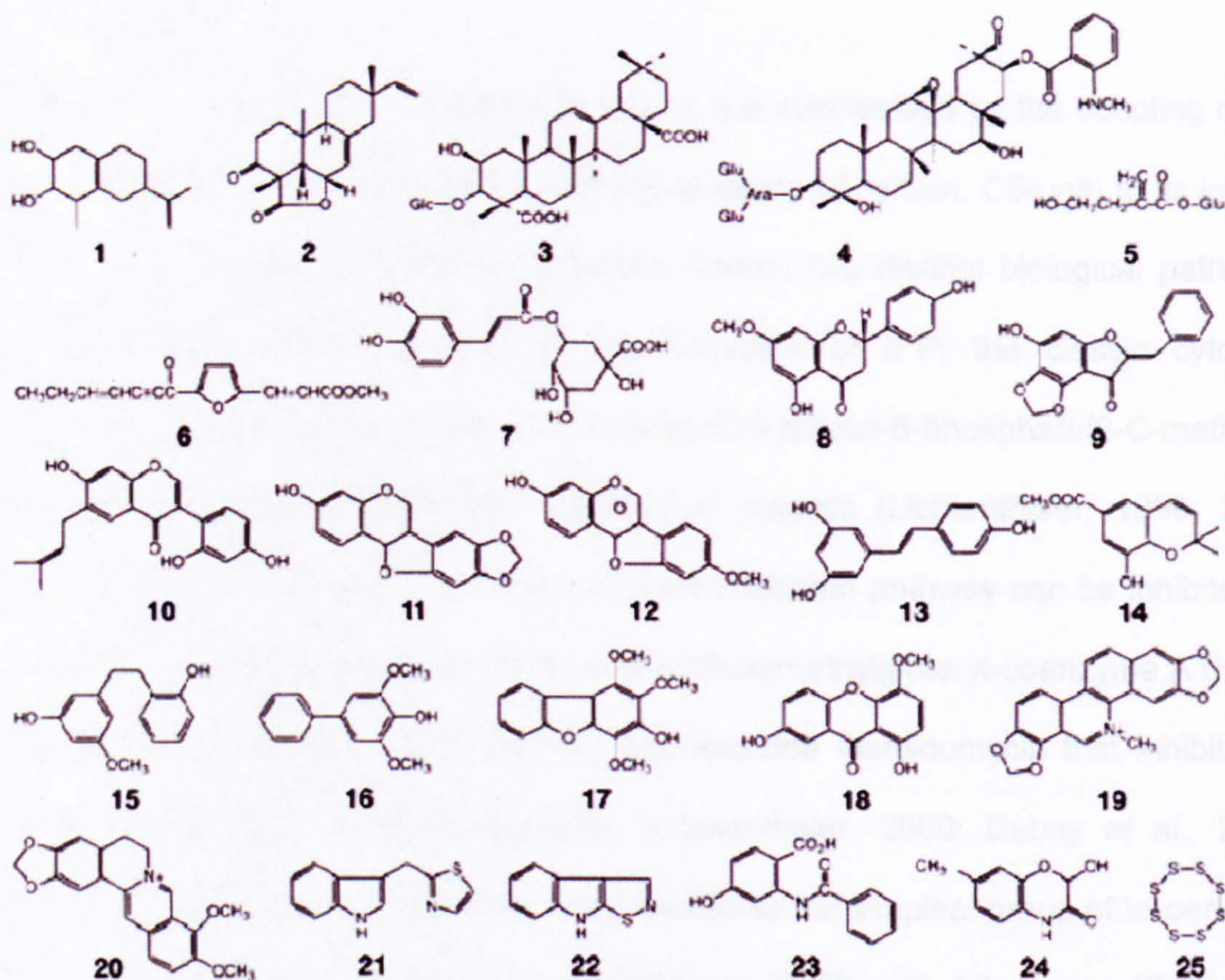


Figure 1.1 Examples of chemical diversity of defence secondary molecules from plants (Dixon, 2001). Secondary metabolite classes are followed in parentheses by the chemical name of the natural product and the plant species that synthesise it.

Terpenoids: 1, sesquiterpene (rishitin, *Nicotiana tabacum*); 2, diterpene (momilactone A, *Oryza sativa*); 3, saponin (medicagenic acid 3-O-glucoside, *Delichos kilimandscharicus*); 4, saponin (avenacin A, *Avena sativa*).

Aliphatic acid derivatives: 5, butyrolactone precursor (tuliposide A, *Tulipa* spp.); 6, furanoacetylene (wyerone, *Vicia faba*).

Phenolics and phenylpropanoids: 7, hydroxycinnamic acid ester (chlorogenic acid, *Nicotiana tabacum*); 8, flavanone (sakuranetin, *Bibes nigrum*; *Oryza sativa*); 9, aurone (*Cephalocereus senilis*); 10, isoflavone (luteone, *Lupinus albus*); 11, pterocarpan (maackiain, *Cicer arietinum*); 12, pterocarpan (medicarpin, *Medicago sativa*); 13, stilbene (resveratrol, *Vitis viniferis*); 14, chromene (*Piper aduncum*); 15, bibenzyl (batatasin IV, *Dioscorea batatas*), 16, biphenyl (aucuparin, *Malus pumila*); 17, benzofuran (*Cotoneaster* spp.); 18, xanthone (*Polygala nyikensis*).

Nitrogen- and/or sulphur-containing compounds: 19, benzophenanthridine alkaloid (sanguinarine, *Papaver bracteatum*); 20, benzylisoquinoline alkaloid (berberine, *Berberis* spp.); 21, indole (camalexin, *Arabidopsis thaliana*); 22, indole (brassilexin, *Brassica* spp.); 23, anthranilamide (*Dianthus caryophyllus*); 24, benzoxazinone (DIMBOA, *Zea mays*); 25, elemental sulphur (*Theobroma cacao*).

1.1.3.1 Terpenes

Terpenes, the largest class of natural products, are synthesised by the coupling of the isoprenoids isopentenyl diphosphate (IPP) (five atoms of carbon, C5-unit) to its isomer dimethylallyl diphosphate (DMAPP, C5-unit). Today, two distinct biological pathways have been recognised that lead to the formation of IPP: the classic cytosolic acetate/mevalonate pathway and the 1-deoxy-D-xylulose-5-phosphate/2-C-methyl-d-erythritol 4-phosphate (DOXP/MEP) present in plastids (Lichtenthaler, 1999; 2000; Dubey *et al.*, 2003) (Figure 1.2). The acetate/mevalonate pathway can be inhibited by mevinolin, a fungal metabolite at the level of hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase and the DOXP/MEP by the herbicide fosmidomycin that inhibits the enzyme DOXP reductoisomerase (DXR) (Lichtenthaler, 2000; Dubey *et al.*, 2003) (Figure 1.2) The monoterpenes (C-10 unit) constitute the simplest group of terpenes. In addition, the terpene family consists of diterpenes (C-20 unit), triterpenes (C-30 unit), tetraterpenes (C-40 unit) and the largest group of terpenes, the sesquiterpenes (C-15 unit) several thousands of which have been identified (Singer, 2003). Oxidation, reduction, isomerisation, conjugation and derivatisation processes have led to the myriad of terpene structural diversity found in nature (Croteau, 1986; Sangwan *et al.*, 2001) (Figure 1.3). Compartmentation of IPP biosynthesis in higher plants between the cytosol (acetate/mevalonate pathway) and plastids (DOXP pathway) has separated terpenes production; sterols, triterpenes and sesquiterpenes are produced in the cytosol whereas monoterpenes, phytol and other diterpenes, carotenoids and plastoquinones are formed in the plastids (Lichtenthaler, 1999; 2000) (Figure 1.2). Compartmentation however, is not absolute and cases exist where one metabolite can be exchanged between pathways (Dubey *et al.*, 2003).

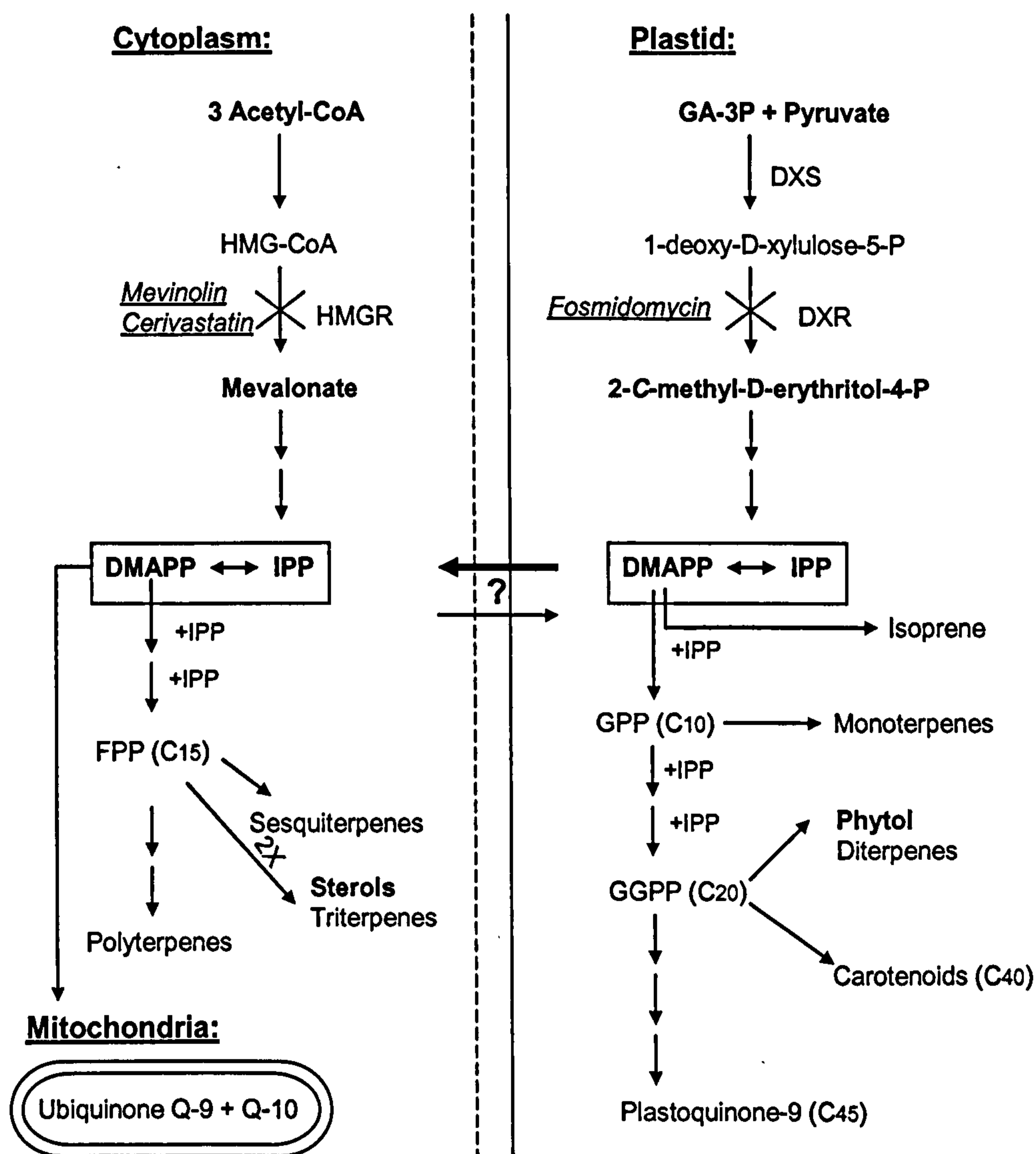


Figure 1.2 Suggested compartmentation of the two independent pathways for IPP (Isopentenyl diphosphate) and terpenoids biosynthesis in higher plants, red algae and chrysophytes (modified from Lichtenthaler, 2000). The arrow in the middle indicates that the partial export of prenyldiphosphates from plastids to the cytosol and / or the import of IPP or farnesyl diphosphate (FPP) into chloroplasts is still uncertain. The antibiotics mevinolin and cerivastatin inhibit the cytosolic pathway for IPP formation and the herbicide fosmidomycin the DOXP/MEP pathway in the plastids. Abbreviations denote:

DOXP, 1-deoxy-D-xylulose-5-phosphate;

DMAPP, dimethylallyl diphosphate;

GA-3P, glyceraldehyde 3-phosphate;

DXS, DOXP synthase ;

DXR, DOXP reductoisomerase;

GPP, geranyl diphosphate;

GGPP, geranylgeranyl diphosphate;

HMG-CoA, hydroxymethylglutaryl-coenzyme A

HMGR, hydroxymethylglutaryl-coenzyme A reductase.

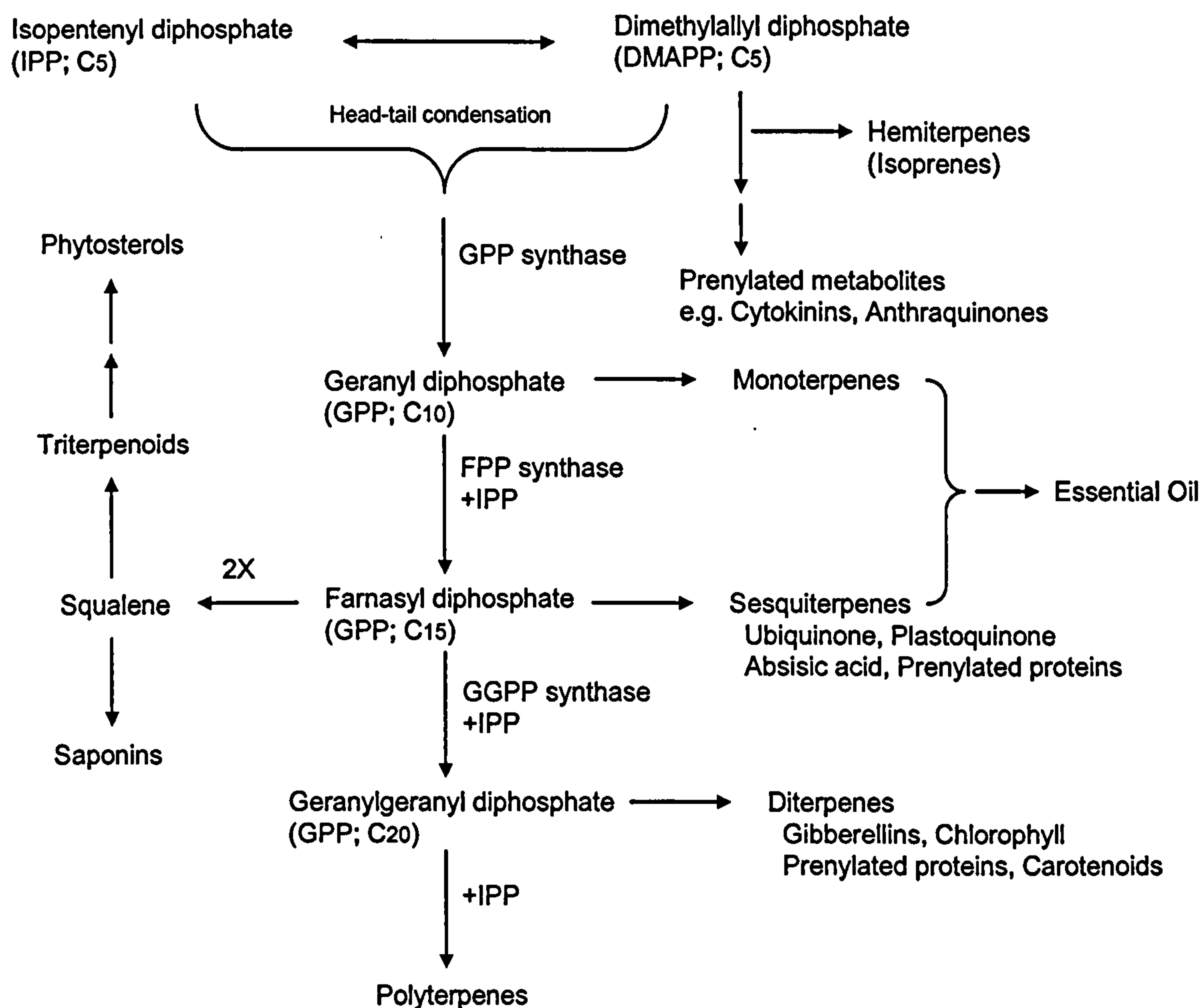


Figure 1.3 Synthesis of various groups of terpenes in higher plants and their inter-relationships. Modified from Dubey *et al.* (2003). Abbreviations stand for:

IPP Isopentenyl diphosphate;

DMAPP dimethylallyl diphosphate;

GPP geranyl diphosphate;

FPP: farnasyl diphosphate and

GGPP geranylgeranyl diphosphate

1.1.3.2 Phenolic compounds

Phenolic molecules characterised by the presence of the phenol group (a hydroxyl function on a benzene ring) in their structure, constitute another major class of secondary metabolites (SM). All phenolic compounds i.e. the phenylpropanoids, formed by a 3-carbon side chain attached to an aromatic ring (Hanson, 2003) and their polymers lignins; the flavonoids, two aromatic rings connected by a 3-carbon bridge,

for example anthocyanins, flavones, flavonols, and isoflavonoids and their polymers tannins (Taiz & Zeiger, 1991) are biochemically synthesised *via* the shikimate pathway (Figure 1.4). The enzyme-catalysed condensation of a phosphoenol pyruvate (PEP) derivative with the aldehyde of erythrose 4-phosphate yields shikimic acid, a precursor in the biosynthetic routes leading to formation of the aromatic amino acids phenylalanine (phe), tyrosine (tyr) and tryptophane (trp) (Hanson, 2003; Sangwan *et al.*, 2001) (Figure 1.4).

1.1.3.3 Nitrogen containing compounds

In contrast to the above molecules, a large variety of plant secondary products entail nitrogen (N) in their structures. This group of secondary metabolites (SM) consists of alkaloids, non-protein amino acids, amines, cyanogenic glycosides, glucosinolates and alkamides (Wink, 2003). Most nitrogenous secondary products are biosynthesised from common amino acids. Alkaloids which are present in approximately 20% of all plant species are derived through decarboxylation of amino acid precursors i.e. ornithine and lysine, phenylalanine and tyrosine, tryptophan or from nicotinic or anthranilic acid (De Luca & Laflamme, 2001; Hanson 2003). The aromatic amino acids (phenylalanine, tyrosine and tryptophan) are derived from the shikimate pathway (see section 1.1.3.2; Figure 1.4), whereas lysine and ornithine biosynthesis is *via* transamination of oxaloacetate (α -ketoacid produced by Krebs cycle) mediated by the enzyme pyridoxal-5-phosphate (PLP) (Diamandithis, 1994). In alkaloids the nitrogen atom is usually part of a heterocyclic ring on which classification is based (Figure 1.5). Today more than 12,000 alkaloid structures have been elucidated (Wink, 2003). Examples of well-known important alkaloids are nicotine, morphine, caffeine and cocaine (Taiz & Zeiger, 1991).

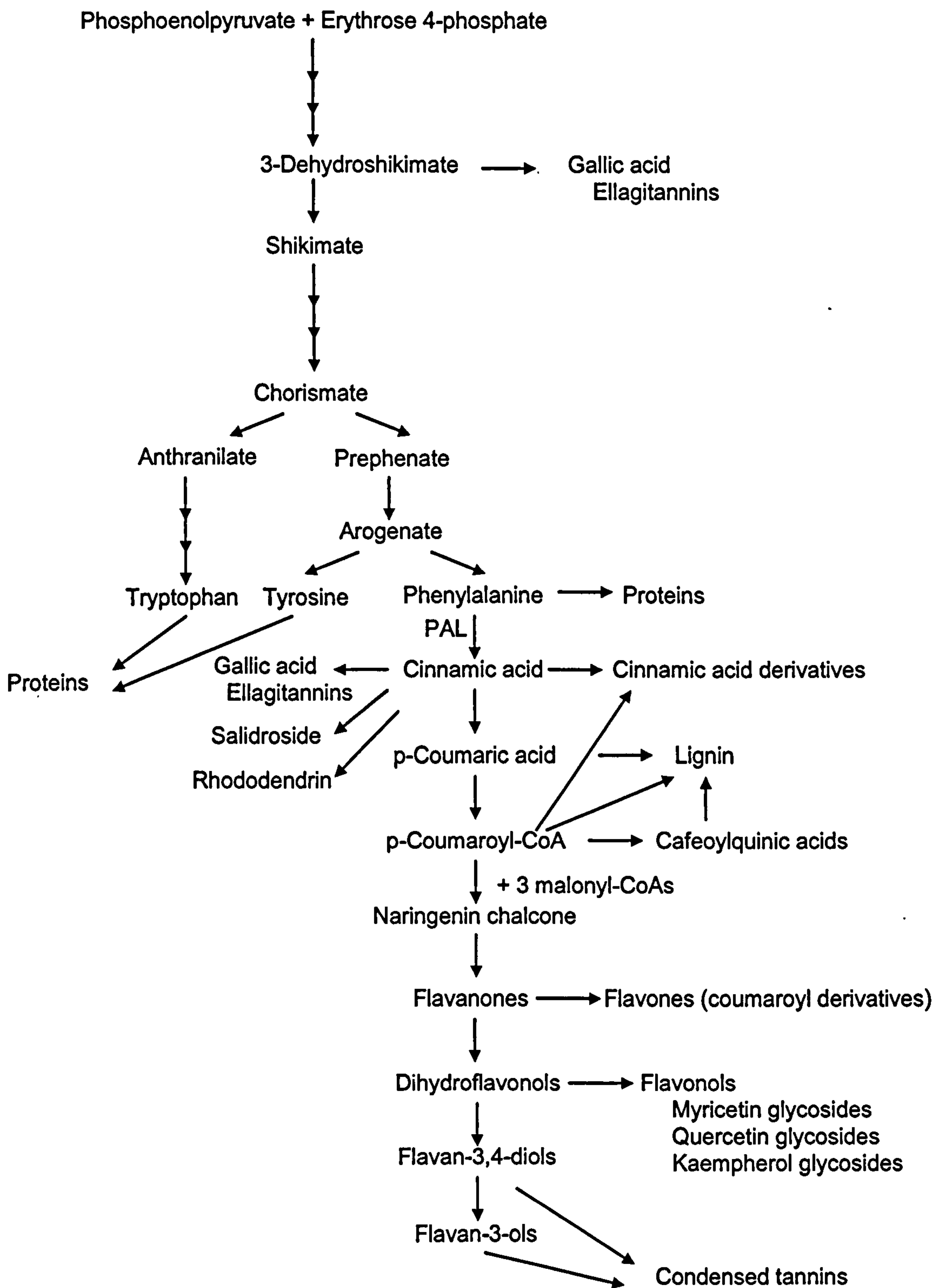


Figure 1.4 Schematic presentation of the shikimic acid pathway leading primarily to the biosynthesis of aromatic amino acids (phenylalanine, tyrosine and tryptophane) and then to different groups of phenolic molecules (modified from Keski-Saari, 2005).

PAL phenylalanine ammonia lyase

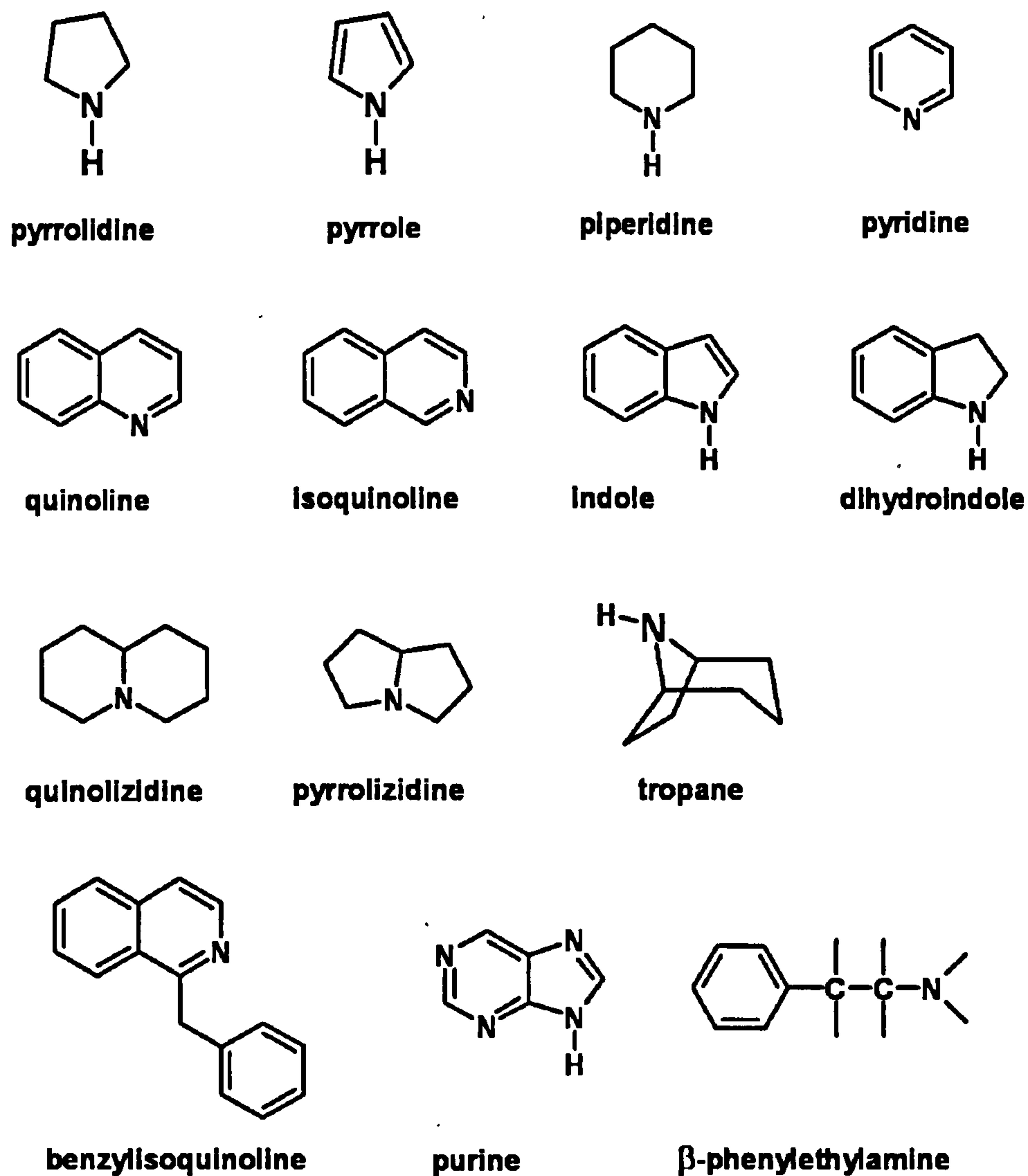


Figure 1.5 Examples of alkaloid structural diversity and the heterocyclic ring system for their classification.

1.2 Secondary metabolites from plant defence to the drug industry

Plant secondary metabolites (SM) exhibit a wide range of amazing biological activities (Bourgaud *et al.*, 2001). Many of these molecules represent valuable chemicals that include drugs, anti-oxidants, flavours, fragrances, cosmetics, dyes and insecticides (Jacobs *et al.*, 2000; Bourgaud *et al.*, 2001). The increasing interest of the food and pharmaceutical industry in using natural plant products (Craker & Simon, 1986; Manukyan, 2005), initiated scientific studies on the so-called aromatic & medicinal plants, with a number of disciplines like pharmacology, botany, chemistry, biology and ecology being involved. Nature has been recognised as an important source of useful active compounds since ancient times and a vast number of aromatic & medicinal plants have been exploited for the treatment of various ailments or other purposes (Verpoorte, 1998; Raskin *et al.*, 2002). Bourgaud *et al.*, (2001) reported that in western countries 25% of the molecules used in the pharmaceutical industry are of natural plant origin. A more recent analysis over the period 1981–2002 concerning natural products as a source for new drugs (Gurib-Fakim, 2006), demonstrated that in fact only 39% of the chemicals used in medicine can be classified as synthetic in origin. In the area of the anti-infectives (anti-bacterial, anti-fungal, parasitic, and viral), approximately 70% were naturally derived or inspired, while for cancer treatment the equivalent number was 67%. World Wide Fund for Nature (WWF), UK research centre (<http://www.wwf.org.uk/researcher/issues/plants>) reported that in industrialised countries, plants have contributed to more than 7,000 compounds produced by the pharmaceutical industry. Natural products have been employed as ingredients in heart drugs, laxatives, anti-cancer agents, hormones, contraceptives, diuretics, antibiotics, decongestants, analgesics and anaesthetics, for ulcer treatments and as anti-parasitic compounds. More than 119 pure chemical substances extracted from higher plants are used today in medicine worldwide (Farnsworth, 1988; Rates, 2001). In developing countries the World Health Organisation (WHO) estimated that about 80% of people depend primarily on traditional medicine, and plant extracts are applied in more than

85% of the treatments. Consequently, between 3.5 and 4 billion people in the world rely on medicinal plants as sources for drugs (Farnsworth *et al.*, 1985). Herbal medicines are an essential and growing part of the international pharmacopeia. Knowledge of medicinal plant properties is accumulating as a result of research and trial and could constitute an increasing alternative in western medicine (<http://www.wwf.org.uk/researcher/issues/plants>). Herbal medicine in richer countries is now very fashionable, with an annual market boost at 10 to 20% in Europe and North America over the last few years. Additionally, there are many other botanical products traded such as health foods, food supplements, herbal teas and others related to health or personal care. The extent to which herbal preparations have been recognised by conventional medicine varies greatly between countries and is for instance higher in Germany than in the UK or USA (Hamilton, 2003). Despite the growing interest in rediscovering natural products (Gurib-Fakim, 2006), no more than 20% of all plant species have been chemically or biologically evaluated to date (Cordell & Colvard, 2005).

1.3 The need for medicinal plant cultivation

Medicinal plants have been traditionally collected from their natural habitats. Out of the 52,000 of estimated medicinal plants used currently worldwide, two thirds are harvested from the wild (<http://www.wwf.org.uk/researcher/issues/plants>; Canter, 2005), whereas in Europe only 10% of the commercially used medicinal species are cultivated (Vines, 2004). For generations, many traditional rural societies have harvested medicinal plants from the field at a sustainable level. Changes resulting from population growth, immigration, conversion of land to agriculture, improved transport and accessibility to markets, as well as the global popularity of herbal medicine today, have led to a higher degree of harvesting that threatens species' survival (<http://www.wwf.org.uk/researcher/issues/plants>). Today, aromatic & medicinal plants

are threatened in Europe by over-exploitation, destructive harvesting techniques and habitat degradation (<http://www.wwf.org.uk/researcher/issues/plants>). There is significant concern about diminishing populations, loss of genetic diversity and local extinction (Canter *et al.*, 2005) especially of endemic species (<http://www.wwf.org.uk/researcher/issues/plants>). Estimates indicate that between 4,000 and 10,000 medicinal plant species might now be endangered (Canter *et al.*, 2005). In general, there is little knowledge of the ecology and regeneration abilities of many medicinal & aromatic plants. To ensure conservation and sustainable use of aromatic & medicinal plants Lange (1998), suggested enhancement of cultivation efforts; improved management of wild populations; public awareness; trade monitoring; national and international legislation; better information about wild-harvested plants; and certification of plant material from sustainable sources. Cultivation of aromatic & medicinal plants is a potential way to satisfy the expanding market with concomitant conservation of nature's genetic diversity (Lange, 1998). Stewart & Lovett-Doust, (2003) highlighted as well market's demand for high quality, uniformity and safety guarantee of the medicinal plants, which can be met only under conditions of controlled cultivation. The chance of contaminated field soils with toxic heavy metal or radioactivity and the possible risk of misidentification when harvesting from the wild can result in a significant quality decline (Stewart & Lovett-Doust, 2003). Moreover, as the content of bioactive secondary metabolites is highly susceptible to various environmental factors (see section 1.10) standardisation of the medicinal product is viable only by optimisation and full control of greenhouse-based plant growth (Raskin *et al.*, 2002). General considerations regarding cultivation of aromatic & medicinal plants include the difficulties in growing certain species in the country of origin or elsewhere and the slow-growing, space-demanding or low-yielding species that are less economically appealing to commercial producers. Domestication of a plant can be difficult and expensive. As a result, wild harvesting is likely to remain the only option for certain species (<http://www.wwf.org.uk/researcher/issues/plants>).

1.4 World trade and market of medicinal plants

Europe is a major world trader in medicinal & aromatic plants. At least 2,000 medicinal & aromatic species are traded, of which two thirds (1,200-1,300 species) are native to the continent (Lange, 1998). An estimated 70,000 hectares of land are exploited for the cultivation of medicinal & aromatic plants in the European Union. France, Hungary and Spain have devoted large areas to cultivation (Lange 1998). Major species grown include lavender (*Lavandula* spp.), opium poppy (*Papaver somniferum*), caraway (*Carum carvi*), fennel (*Foeniculum vulgare*) and peppermint (*Mentha x piperita*). Other countries with large scale commercial cultivation are Argentina, Chile, China, India and Poland (<http://www.wwf.org.uk/researcher/issues/plants>). Between 20,000 and 30,000 tonnes of wild plant material are collected annually in Europe (Lange, 1998). As trade in aromatic & medicinal plants is largely unmonitored, effective control over the amounts harvested is not feasible (<http://www.wwf.org.uk/researcher/issues/plants>). Aromatic & medicinal plants are normally traded in a dried form. Alternatively, they could be traded fresh or preserved in alcohol. Plant parts may be traded as a whole, or dissected (cut, rasped or powdered, Lange, 1998). The most important source country for European aromatic & medicinal plants is Bulgaria, with average net exports of 7,000 tonnes *per annum*. Sixty to seventy per cent of what is produced or harvested in Bulgaria is exported, mainly to wholesalers in Germany. The largest global markets for medicinal & aromatic plants are China, France, Germany, Italy, Japan, Spain, the UK and the US. Japan has the highest *per capita* consumption of botanical medicines in the world. In the US and Europe, the trade has been expanding at an average of 10% *per annum*, due to the increasing popularity and recognition of alternative medicine (<http://www.wwf.org.uk/researcher/issues/plants>).

1.5 Secondary metabolites production *in vitro*

Production of secondary metabolites (SM) has traditionally been achieved through conventional field cultivation of aromatic & medicinal plants (Bourgaud *et al.*, 2001). At the end of the 1960s newly introduced plant cell culture technologies constituted a possible tool for the study and synthesis of SM (Bourgaud *et al.*, 2001). However, despite the industrial interest and research efforts on plant *in vitro* cultures a few commercial applications have been achieved (Jacobs *et al.*, 2000; Bourgaud *et al.*, 2001). Problems encountered comprise low yield of the desirable metabolite along with the high cost of the technique (Jacobs *et al.*, 2000; Bourgaud *et al.*, 2001; Gontier *et al.*, 2002). Hughes & Shanks (2002) reported that cell cultures were incapable of synthesising commercially important alkaloids like morphine, codeine, scopolamine, vincristine and vinblastine. Knowledge and interest in secondary metabolite biosynthetic pathways has increased tremendously during the last decade (Hadacek, 2002). In the last 15 years metabolic engineering appeared as the new strategy for augmenting SM yields by “the improvement of cellular activities by manipulation of enzymatic, transport and regulatory functions of the cell with the use of recombinant DNA technology” (Bourgaud *et al.*, 2001). More recently, the science of proteomics has been employed for the identification of the enzymes and other transport or regulatory proteins involved in secondary metabolism biosynthetic pathways (Jacobs *et al.*, 2000). Verpoorte & Memelink, (2002) indicated that despite the ongoing research in SM biosynthesis, characterisation of intermediate molecules and enzymes is still poor and, identification of regulatory and transport proteins has not been achieved (Jacobs *et al.*, 2000). According to an estimation, hundreds of thousands of genes are expected to be involved in plant secondary metabolism pathways (Hadacek, 2002), and only very few have been identified and cloned (Verpoorte & Memelink, 2002). Hughes and Shanks (2002) highlighted the problems in alkaloid pathway metabolic engineering that have not yet been addressed. Among them is the lack of knowledge regarding regulation of alkaloid biosynthesis. Only a few genes have been cloned and the species-specific SM

pathways make the model *Arabidopsis* genome of limited use. Additionally, cofactor limitations, feed-back controls and the influence of subcellular and tissue transport are still far from fully understood (Hughes & Shanks, 2002). The best studied secondary pathways at the genetic level today are those leading to the formation of flavonoids, anthocyanins and some indole and isoquinoline alkaloids (Verpoorte & Memelink, 2002). To date the most successful genetic engineering approach for secondary metabolism improvement was the one employed by Mahmoud & Croteau (2001), who significantly augmented (50%) the essential oil content in peppermint plants by exploiting a gene that encodes the DXR enzyme of the DOXP/MEP pathway for terpenoid biosynthesis (Figure 1.2). Although metabolic engineering has given some promising results in SM enhancement (Bourgaud *et al.*, 2001; Broun & Somerville, 2001; Canter *et al.*, 2005) important constraints still challenge the genetic control of natural product biosynthesis.

1.6 Hydroponics for medicinal plant production

A totally different approach for the production of secondary metabolites (SM) has been followed over the last 30 years by various researchers like Davtyan, (1976); Marapetyan, (1984); Wees & Stewart, (1986); Economakis, (1992); Economakis and Fournaraki, (1993); Economakis, (1993a; 1993b; 1995); Economakis *et al.*, (1999; 2002; 2005); Gontier *et al.* (2002), Karioti *et al.* (2003); Stewart & Lovett-Doust, (2003); Economakis *et al.*, (2005) and Manukyan, (2005), who have employed hydroponic cultivation for aromatic & medicinal plant growth. These studies have demonstrated the efficiency of this technique for improved SM yields, mainly terpenes or in two cases, alkaloids (Gontier *et al.*, 2002; Manukyan; 2005). Canter and colleagues (2005) suggested that cultivation under controlled environments involving hydroponic systems could be a promising way for efficient aromatic & medicinal plant production on a commercial scale. The information available about the hydroponic cultivation of such

plants is scarce (Economakis, 1992; Karioti *et al.*, 2003). Manukyan (2005) pointed out the considerable international interest today in aromatic & medicinal plant culture by means of hydroponics, due to the increasing demand of the pharmaceutical industry for natural medicinal material. Davtyan, already in 1976 had reported that cultivation of various aromatic & medicinal plants in open air hydroponics out-yielded the soil-grown plants in terms of biomass production. Sweet basil (*Ocimum basilicum* L.) and peppermint (*Mentha piperita* L.) plants grown in various soilless media exhibited higher yields than the soil-grown ones (Marapetyan, 1984; in Wees and Stewart, 1986). Wees & Stewart (1986), demonstrated that basil, oregano, parsley and thyme were successfully grown *via* the hydroponic Nutrient Film Technique (NFT, see section 1.7.3). Economakis (1992, 1993a) and Economakis & Fournaraki (1993) indicated a great response of *Origanum dictamnus* L. and *Origanum vulgare* ssp *hirtum* in the NFT respectively. Likewise, cultivation of *Salvia fruticosa* Mill. in the NFT was met by great success (Economakis, 1993b) and Stewart & Lovett–Doust (2003) reported high yields of *Calendula officinalis* L. in solution culture. Furthermore, there is strong experimental evidence to suggest that SM production has been promoted under hydroponic cultivations. Plants of *Pelargonium roseum* Wild and *Ocimum basilicum* raised in open-air hydroponics possessed greater essential oil content than the soil-grown ones (Davtyan, 1976). Hydroponically raised *Origanum dictamnus* L. plants by means of NFT, exhibited higher essential content in bracts and leaves than plants grown in the wild (Economakis, 1992). Experimental outcomes have indicated that yield and composition of *Salvia fruticosa* Mill. and *Origanum dictamnus* SM can be manipulated in a hydroponic NFT system (Economakis *et al.*, 1999; Economakis *et al.*, 2002; Karioti *et al.*, 2003; Economakis *et al.*, 2005). Finally, Manukyan, (2005) reported enhanced production of alkaloids in an open air substrate culture of *Chelidonium majus* L. Accumulated data have strongly suggested the use of hydroponics as a potential tool for the commercial production of aromatic & medicinal plants with high yields of secondary metabolites.

1.7 Hydroponics as a technique for crop production

1.7.1 History of hydroponics

Hydroponics is the science of growing plants without the use of soil, but with the use of water or an inert medium, to which a nutrient solution containing all the essential elements needed for plants' normal growth and development is added (Sr. Deutschmann, 1998; Savvas & Passam, 2002). However, many hydroponic methods might employ types of medium that contain some organic material. In this case the term "soil less culture", is preferred (Sr. Deutschmann, 1998; Savvas & Passam, 2002). Hydroponic growing of plants has in fact preceded soil growing. It is believed to originate in the ancient city of Babylon with its famous hanging gardens, one of the Seven Wonders of the Ancient World. That was probably one of the first successful endeavours to grow plants by means of hydroponics. Hieroglyphic records from ancient Egypt dating several hundred years B.C. provided evidence of plants growing in water along the Nile. In ancient Greece, even before the time of Aristotle, Theophrastus (327-287 B.C.) conducted various experiments in crop nutrition employing hydroponic techniques (Sr. Deutschmann, 1998).

Until the 1930s, raising plants in water and nutrient solutions was a practice restricted to laboratories, where it exclusively served as a tool for the study of plant growth, physiology and root development (Sr. Deutschmann, 1998; Savvas & Passam, 2002). In the late 1920s and early 1930s in the University of California, Gericke for the first time extended his laboratory plant nutrition experiments to the growing of crops outside on a large scale for commercial purposes. Gericke grew vegetables hydroponically, like beets, radishes, carrots, potatoes, and cereal crops, fruits, ornamentals and flowers, using water culture in large tanks and he raised tomatoes that were up to 25 feet height (Sr. Deutschmann, 1998).

The term hydroponics was firstly introduced by Gericke to describe all methods of growing plants in liquid media for commercial purposes (Savvas & Passam, 2002). The word is derived from two Greek words, hydro, meaning water and ponos meaning labour. Gericke's work is considered the basis for all forms of hydroponic growing, even though primarily it was limited to water culture (Sr. Deutschmann, 1998). Many other researchers subsequently developed basic nutrient solutions for the study of plant growth and nutrition; among them were Hoagland (1919), Deutschmann (1932), Arnon (1938) and Robbins (1946) (cited by Cooper, 1979; Sr. Deutschmann, 1998; Savvas & Passam, 2002). Interest in the commercial application of hydroponics emerged for the first time in 1925 when the greenhouse industry turned to hydroponics to overcome conventional culture method problems like degradation of soil structure and fertility as well as pest problems (Sr. Deutschmann, 1998). During the 1950s and 60s, although the scientific and technological standards of that time were adequate for the successful soil less cultivation of crop plants in greenhouses, the areas covered worldwide by horticultural crops grown hydroponically were insignificant and the research activity in this field was relatively low (Savvas & Passam, 2002). In the 1960's the introduction of plastics and their rapid use in horticulture, together with research and development into greenhouses, stimulated the interest in commercial hydroponic production (Hassall & Associates Pty Ltd, 2001). This tendency was more pronounced in the United Kingdom, the Netherlands and some of the Scandinavian countries. In the United Kingdom, the Nutrient Film Technique (NFT), introduced by Cooper (1979), was initially the main hydroponic system adopted by growers on a large scale. Concurrently, Scandinavian and Dutch greenhouse producers, due to serious problems encountered by the continual use of the same soil for many years, tested the possibility of using rockwool, an inert medium, as a soil substitute (Savvas & Passam, 2002).

1.7.2 Hydroponic systems

Seymour (1993) defined two basic types of commercial hydroponic crop production systems:

▶ **Open or 'run to waste' systems;**

In an open hydroponic system nutrient solution is applied to the medium in which the plants are raised and subsequently drained off as waste. This solution is not circulated to the system or reused by plants.

▶ **Closed or 'recirculating' systems;**

Closed systems share the same features with the open ones but differ in one very important point. Running nutrient solution is collected and recirculated in order to be repeatedly used in the system

Moreover hydroponic systems can be divided into two other broad categories:

▶ **Water-based systems;**

All water-based systems are commonly closed systems. They are mainly exploited for short-term crops such as lettuce, tomatoes and herbs. In general water-based systems include predominantly the nutrient film technique (NFT) (see below), water culture, where plants are raised in static, well aerated nutrient solution to ensure oxygen supply and aeroponics; plant roots are in the air and subjected to regular spraying with the nutrient solution (Hassall & Associates Pty Ltd, 2001)

▶ **Media / aggregate systems;**

Longer term vegetable and flower crops are usually raised in media based systems. Media systems are divided into two major categories i.e. inorganic and organic. The inorganic category comprises rockwool, sand, gravel, scoria, perlite, pumice, expanded clay and vermiculite, whereas organic hydroponic media can be sawdust, peat, coconut fibre, bark, foam products, processed wood products, gel products and various mixes of organic and inorganic media (Seymour, 1993).

Until the 1990's virtually all media systems were operated as 'run-to-waste'. Main reasons for this constituted the easier nutrient management and the lower risk of spreading disease. Over the past decade however, environmental concerns have led to a significant increase in the use of the closed / recirculating systems (Hassall & Associates Pty Ltd, 2001). Overall, water-based systems account for only a small proportion of the worldwide hydroponic production, while media systems contribute up to 90% (Donnan, 1998). One explanation for the predominance of media-based systems is that the volume of the necessary recycled nutrient solution is only a tiny fraction of the volume from a continuous flow system such as NFT (Hassall & Associates Pty Ltd, 2001). Table 1.2 illustrates the degree of exploitation of the various systems by the world hydroponic industry (Donnan, 1998).

Table 1.2 Industrial worldwide use of various hydroponic systems (Donnan, 1998).

System Type	System	Proportion of Industry Using System
Water-based Systems	Nutrient Film Technique (NFT)	5%
	Water Culture	3%
	Gravel Culture	1%
	(GFT - gravel flow technique) ^a	
	Aeroponics	0.2%
	<i>Sub-total</i>	<i>9%</i>
Media Systems	Rockwool	57%
	Other inorganic media	22%
	Organic media	12%
	<i>Sub-total</i>	<i>91%</i>

^a GFT stands for gravel flow technique; a modified form of NFT known as gravel culture that involves NFT channels covered with a layer of gravel.

However, in the Australian hydroponic industry NFT is the most prominent in terms of the main systems in use (38%) (Hassall & Associates Pty Ltd, 2001).

1.7.3 The unique Nutrient Film Technique

The Nutrient Film Technique (NFT) system consists of a flat-bottomed channel or gully in which a thin film of water containing all the elements required for plant growth and development, is re-circulated through the bare roots of the plant. The nutrient solution is applied continuously as a shallow stream and recycled from the outflow back to the point of application (Cooper, 1979; Seymour, 1993). The shallow stream of nutrient solution ensures permanent supply of water and nutrients from underneath the rooting system and permanent oxygen supply to the roots from the part of them which is in the air above the nutrient solution (Cooper, 1979). Difficulties that may arise from the use of NFT are firstly, interruption of the flow of nutrient solution even for short periods of time will result in the roots getting very quickly stressed and a second problem is that NFT channels can potentially become blocked by roots of vigorous growing plants (Hassall & Associates Pty Ltd, 2001).

The concept of NFT is attributed to A.J Cooper who highlighted the value of the Nutrient Film Technique and drew international attention to its potential commercial application in 1973 (Papadopoulos, 1991). Cooper (1979) reported a 26% increased yield of NFT grown tomatoes and furthermore demonstrated that plants in NFT can tolerate an extremely wide range of nutrient concentrations without any loss in crop yield or quality. For example, while soil-grown tomatoes require nitrogen ca 200 mg/L to sustain sufficient production, NFT tomato plants gave maximal growth and fruit production with less than 10 mg/L of nitrogen in the nutrient solution. Increasing N concentration to 320 mg/L did not exhibit any significant variation in plants yields. Similarly, the range of tolerance for P supply was greater than 5 to 200 mg/L and for potassium 20 to 375 mg/L (Copper, 1979). Adam (2002) reported that 5 to 243 mg/L of P had little effect on yield of cucumber plants. *Calendula officinalis* grown hydroponically under four levels of P 5 mg/L (shallow hydroponic system) and 10, 100, 200 mg/L (deep flow nutrient solution) demonstrated significantly ($P < 0.001$) greater

vegetative and reproductive biomass at the lowest P concentration (5 mg/L) (Stewart & Lovett-Doust, 2003). These outcomes strongly support the efficiency of shallow hydroponic systems like NFT, where plant needs can apparently be satisfied with minimum nutrient supply and a wide range of nutrient levels can be tolerated before the phenomena of deficiency or toxicity occur (Copper, 1979; Papadopoulos, 1991). NFT has many advantages over other hydroponic systems i.e. absolute control of root environment, uniform nutrient supply across the crop, high control over nutrition and simplified plant watering.

1.7.4 Advantages and disadvantages of hydroponics

The significant expansion of hydroponics in many countries of the world in the last three decades can be ascribed to the independence of hydroponic systems from the soil and all its related problems (Savvas & Passam, 2002). Main drawbacks regarding soil are the presence of soil-borne pathogens and the degradation of soil structure and fertility due to the continual cultivation of the same or related crop species. Moreover, the length of cultivation time in soil is decreased in comparison with hydroponics due to the required soil preparation at the onset of plant culture (Savvas & Passam, 2002). One other major advantage in hydroponics is the reduction in fertilizer application and potential chemical leaching from greenhouses to the environment (Hassall & Associates Pty Ltd; 2001). Furthermore hydroponics has proved to be an excellent alternative to soil sterilization, especially considering the fact that soil sterilants, such as methyl bromide, will soon be forbidden in many more countries, due to high toxicity and adverse effects on the environment (Savvas & Passam, 2002). An additional advantage of hydroponics is the ability offered for precise management of plant nutrition. In hydroponic systems, the root environment is supplied with the optimum oxygen, water and dissolved nutrients at the concentrations and temperature that the plant requires at each stage of growth. The greater control that hydroponics exerts over plants ensures high growth uniformity

(Sr. Deutschmann, 1998; Hassall & Associates Pty Ltd; 2001). Raising plants in a non-soil medium permits the growing of more plants in a limited amount of space. Overall, hydroponics is capable of delivering consistently high quality products that out yield conventional production systems (Sr. Deutschmann, 1998; Hassall & Associates Pty Ltd; 2001). Sr. Deutschmann (1998) stated that hydroponics is a very young science being used on a commercial basis for less than 40 years. However, even in this relatively short period, it has been adapted to many situations. The only restraints for application are sources of fresh water and nutrients. In areas where fresh water is not available, seawater through desalination could be utilised. Therefore, its potential use in providing food in regions of the world with non-arable land needs to be highlighted.

Despite the considerable advantages of hydroponics in commercial horticulture, there are still some disadvantages which restrict the further expansion of such cultivation methods. The main disadvantages of hydroponics today comprise the higher costs normally required for the installation of soil less culture systems, as well as the increased technical skills that are needed to cope with their operation (Savvas & Passam, 2002). Increased costs can also result from the higher energy requirements for running the system (Hassall & Associates Pty Ltd, 2001). In countries where the cultivation of plants in greenhouses has reached industrial dimensions, the above disadvantages could be of minor importance (Savvas & Passam, 2002). Hassall & Associates Pty Ltd (2001) additionally indicated as a main drawback the poor consumer awareness of hydroponic products that could be perceived as unnatural or chemical reliant.

1.7.5 Hydroponic industry worldwide

A report by Hassall & Associates Pty Ltd (2001) on behalf of the Rural Industries Research and Development Corporation (Australia) provided essential information on the role of hydroponics worldwide as an alternative method to conventional soil based agricultural production systems. The key outcomes of this study are summarised below:

- ▶ The commercial hydroponics industry has expanded four to five fold in the last 10 years and is currently estimated at between 20,000 and 25,000 ha;
- ▶ Production is focused in wealthy countries with discerning consumers (The Netherlands, Spain, Canada, Japan, UK, USA, Italy, New Zealand and Australia) or countries which have access to these markets (Mexico and China);
- ▶ The most important commercial producers of hydroponic product in the world are Holland (10,000 ha), Spain (4,000 ha), Canada (2,000 ha), Japan (1,000 ha), New Zealand (550 ha), Australia (over 500 ha), the United Kingdom (460 ha), the USA (400 ha) and Italy (400 ha);
- ▶ Crops grown commercially by means of hydroponics are restricted to tomatoes, cucumbers, lettuce, capsicums and cut flowers;
- ▶ In an effort to minimise the environmental impact of the industry hydroponics is moving towards the use of closed circulating systems for chemical minimisation and the use of Integrated Pest Management;
- ▶ Success in the near future is expected to come from a market focus and not a break through in hydroponics technology;
- ▶ North American expansion is currently driven by 'production boost' but low profits are anticipated unless the Dutch style market is adopted by their industry;
- ▶ International integration of production and marketing will exclude producers who are not sufficiently large scale or part of a bigger collaboration.

1.8 *Salvia officinalis* var *purpurea*

1.8.1 Origin

The genus *Salvia* is the largest one in the Lamiaceae family, which comprises over 5000 species (Gurib-Fakim, 2006). Lamiaceae has contributed some of the most prominent medicinal and culinary species. They are aromatic and have also yielded commercially important (Gurib-Fakim, 2006) mixtures of volatile secondary metabolites namely essential oils (Giannouli & Kintzios, 2000). The genus *Salvia* includes approximately 900 species and has an almost cosmopolitan distribution. Native distribution of *Salvia officinalis* L. is restricted to the west part of the Balkan Peninsula (Albania, Former Yugoslavia and Greece) as well as in northern Italy. The Dalmatian, garden or common sage (*S. officinalis* L.) is among the species of main economic importance (Karamanos, 2000). Many important biological activities have been attributed to sage and today it is widely used in the food, fragrance and drug industry (Karousou *et al.*, 2000). *S. officinalis* var *purpurea* the purple sage has been mainly used as an ornamental.

1.8.2 Plant morphology

S. officinalis var *purpurea* is a perennial shrub with long, angular erect stems, reaching heights of up to 1.60 m. Leaves are opposite, simple, ovate and petiolate (Karamanos, 2000). The inflorescence consists of 4 to 10 flowers. Glandular trichomes, where essential oils are synthesised, accumulated and secreted cover all aerial parts of the plant (Karousou *et al.*, 2000). Two different types of these specialised glandular hairs are found; the capitate and the peltate but only the second type can secrete essential oils (Avato *et al.*, 2005). What distinguishes the variety *purpurea* (purple sage) from *S. officinalis* species is the presence of purple pigments on the surface of only top, young leaves that probably serve defence purposes (Figure 1.6).



Figure 1.6 *Salvia officinalis* var *purpurea* plants raised by the hydroponic Nutrient Film Technique (NFT).

1.8.3 Essential oils of *Salvia officinalis* var *purpurea*

Essential oils are chemically complex mixtures of highly functionalised volatile compounds deriving from plant secondary metabolism. Terpenes, mainly monoterpenes and sesquiterpenes are the predominant constituents of the oils although the presence of other molecules like phenylpropanoids is possible (Sangwan, *et al.*, 2001). Phenylpropanoids offers indispensable and significant flavour and odour to the oil (Sangwan, *et al.*, 2001), however they are not present in *Salvia*. *S. officinalis* essential oils consist of more than 100 individual components, primarily of terpene nature (Giannouli & Kintzios, 2000). Their concentration is maximum in leaves, intermediate in flowers and minimal in stems (Bellomaria *et al.*, 1992). Among *Salvia* species *S. officinalis* is considered to have the highest essential oil yield (Newall *et al.*, 1996). However, Karousou and colleagues, (2000) demonstrated that the essential oil content of three sage species in Greece ranged from: 1.0 to 5.5 % in *S. fruticosa* Mill. 1.3 to 4.2 % in *S. pomifera*; and only 0.9 to 2.3 % (ml / g dry weight) in *S. officinalis* L. The last species (*S. officinalis* L) has exhibited a similar variation of oil content under

different seasons and geographical origins (Putievsky *et al.*, 1986; Chalchat *et al.*, 1998; Perry *et al.*, 1999; Santos-Gomes & Fernandes-Ferreira, 2001). Karioti *et al.* (2003) reported significant differences in oil content and composition of *S. fruticosa* due to plant growth stage.

Major components of *S. officinalis* essential oil (Figure 1.7) are α - and β - thujones (mainly α), camphor, 1,8 cineole, borneol and α -caryophyllene (Kintzios & Gianouli, 2000). *S. officinalis* oil has been described in several studies (Langer *et al.*, 1996; Perry *et al.*, 1999; Piccaglia *et al.*, 1997; Santos-Gomes & Fernandes-Ferreira, 2001; Savelev *et al.*, 2004) and a great variation in its composition has been revealed (Langer *et al.*, 1996). Oil of *S. officinalis* var *purpurea* has been chemically analysed only once by Savelev and colleagues (2004). Data from this study (Savelev *et al.*, 2004) indicated an oil synthesis similar to *S. officinalis* with α -caryophyllene being the predominant constituent (24-32% depending upon plant growth stage).

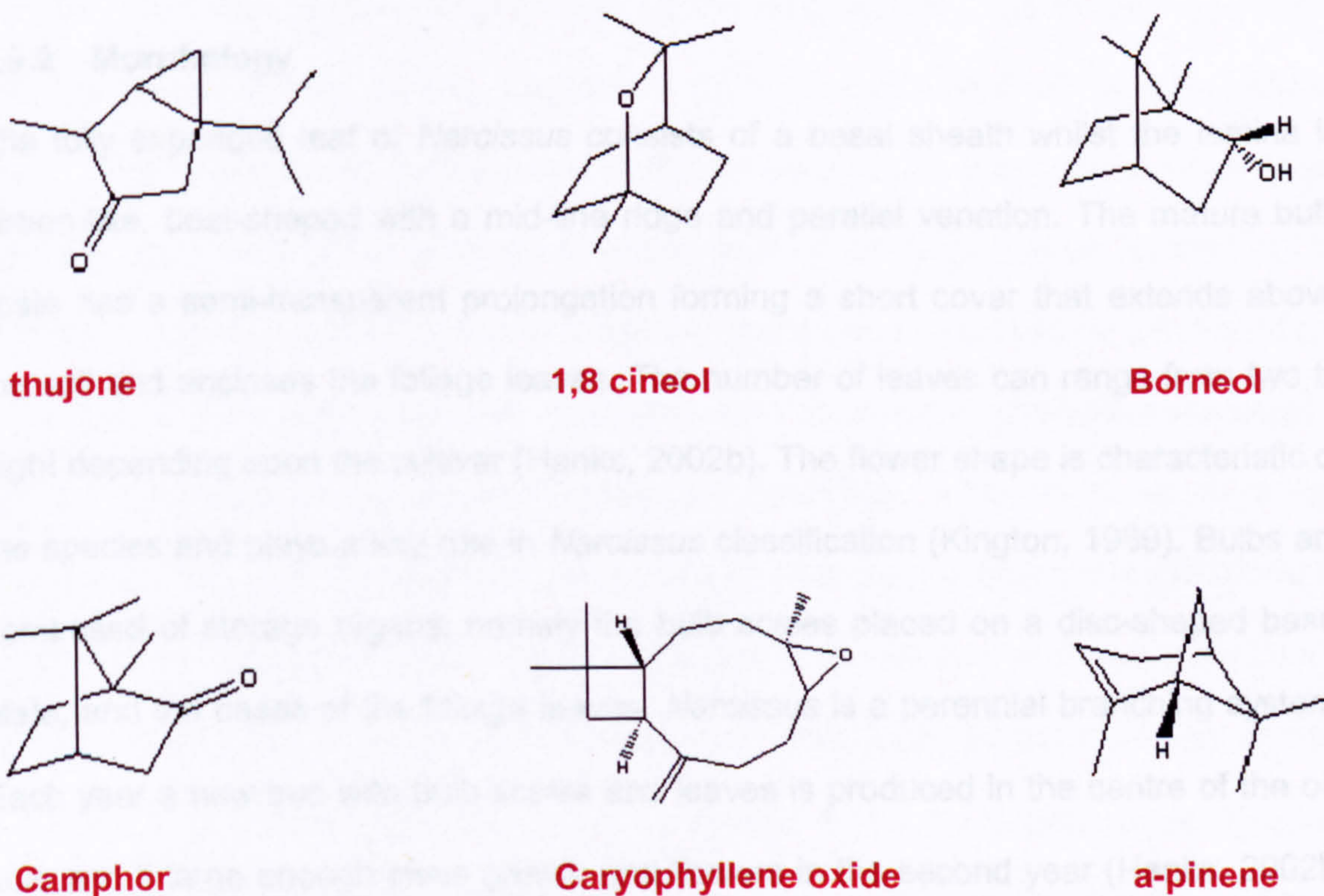


Figure 1.7 Main terpene components of *Salvia officinalis* var *purpurea* essential oil.

1.9 The genus *Narcissus*

1.9.1 Origin

Narcissus was a handsome young Greek man who under the spell of the goddess Venus felt in love with his own reflection in the river that he could never catch. To spare Narcissus from his unfulfilled love, the gods turned him into a beautiful flower (Dweck, 2002). The genus *Narcissus* belongs to the monocotyledon family Amaryllidaceae, to which it contributes about 80 species (Hanks, 2002b). It has a mainly Mediterranean distribution but also occurs in northern Africa and eastwards of Greece, while *N. tazetta* can be found as well in a narrow band to China and Japan (Hanks, 2002b). Plants of the Amaryllidaceae are very important ornamentally. They have been used since ancient times in floriculture and in medicine because of their intrinsic ability to produce the poisonous secondary metabolite class of alkaloids (Cherkasov & Tolkachev, 2002).

1.9.2 Morphology

The fully expanded leaf of *Narcissus* consists of a basal sheath whilst the lamina is ribbon-like, boat-shaped with a mid-line ridge and parallel venation. The mature bulb scale has a semi-transparent prolongation forming a short cover that extends above the soil and encloses the foliage leaves. The number of leaves can range from two to eight depending upon the cultivar (Hanks, 2002b). The flower shape is characteristic of the species and plays a key role in *Narcissus* classification (Kington, 1989). Bulbs are composed of storage organs; namely the bulb scales placed on a disc-shaped basal plate, and the bases of the foliage leaves. *Narcissus* is a perennial branching system. Each year a new bud with bulb scales and leaves is produced in the centre of the old bulb and if large enough gives growth and flowers in the second year (Hanks, 2002b) (Figure 1.8).



Figure 1.8 *Narcissus* “Ice Follies” plants raised hydroponically in an NFT system.

1.9.3 Alkaloids from *Narcissus* sp.

Galanthamine (GAL) and lycorine (LYC) constitute two of the most important alkaloids found in *Narcissus* species, largely used by the drug industry due to their significant biological activities (Figure 1.9). Lycorine is an anti-tumor agent with anti-arrhythmic action. The hydrochloride form is used in Russia as a strong broncholytic while other alkaloids from LYC group possess inhibitory activity on herpes virus (Cherkasov & Tolkachev, 2002). Galanthamine as the hydrobromide has been used for the treatment of various ailments like myasthenia, myopathy, neuritis, infantile paralysis, psychogenic spinal impotence, spastic pareses, muscular dystrophy, antagonistic of muscular relaxants in the case of surgical interventions and for the treatment of schizophrenia (Cherkasov & Tolkachev, 2002). Finally, GAL due to its role as a cholinesterase (ChE) inhibitor constitutes one of the few licensed drugs used today for the treatment of mild to moderate Alzheimer’s disease (AD) (see section 1.11.2.4). Galanthamine, lycorine and the other amaryllidaceae alkaloids are formed *via* the intra-molecular oxidative phenolic coupling of norbelladine derivatives (4-O-methylnorbelladine) (Eichhorn *et al.*,

1998) (Figure 1.9), with the immediate precursor in GAL pathway being narwedine (<http://www.ch.imperial.ac.uk/spivey/teaching/org4biosynthesis/org403alkaloids.pdf>).

Belladine originates from the phenylalanine and tyrosine aromatic amino acids formed via PEP and 4-erythrose phosphate through the shikimic acid pathway (see section 1.1.3.2).

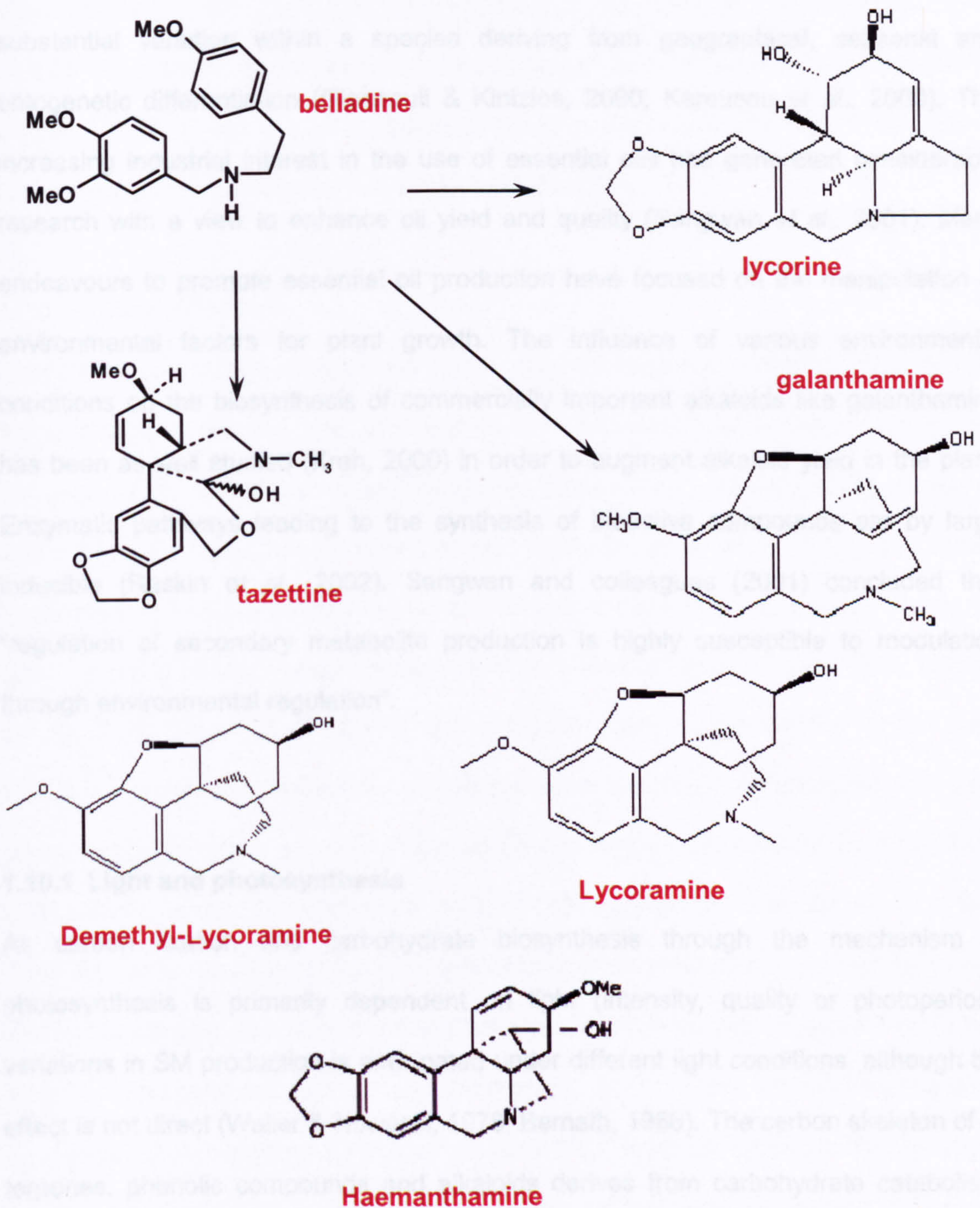


Figure 1.9 Alkaloids isolated from *Narcissus* "Ice Follies" apart from tazettine that occurs in *N. tazetta* species.

1.10 Effect of environmental and ontogenetic factors on plant secondary metabolites production

Bernath (1986) underlined the impact of various environmental factors such as light, photoperiod, temperature, water, nutrition and biotic factors, as well as the influence of plant ontogenetic changes on the biosynthesis of secondary metabolites. Investigations on essential oil content and composition of *Salvia* have revealed substantial variation within a species deriving from geographical, seasonal and ontogenetic differentiation (Giannouli & Kintzios, 2000; Karousou *et al.*, 2000). The increasing industrial interest in the use of essential oils has generated considerable research with a view to enhance oil yield and quality (Sangwan *et al.*, 2001). Many endeavours to promote essential oil production have focused on the manipulation of environmental factors for plant growth. The influence of various environmental conditions on the biosynthesis of commercially important alkaloids like galanthamine has been as well studied (Kreh, 2000) in order to augment alkaloid yield in the plant. Enzymatic pathways leading to the synthesis of bioactive compounds are by large inducible (Raskin *et al.*, 2002). Sangwan and colleagues (2001) concluded that “regulation of secondary metabolite production is highly susceptible to modulation through environmental regulation”.

1.10.1 Light and photosynthesis

As carbon fixation and carbohydrate biosynthesis through the mechanism of photosynthesis is primarily dependent on light (intensity, quality or photoperiod), variations in SM production is anticipated under different light conditions, although the effect is not direct (Waller & Nowacki, 1978; Bernath, 1986). The carbon skeleton of all terpenes, phenolic compounds and alkaloids derives from carbohydrate catabolism. The breakdown of glucose-phosphate produces phosphoenol pyruvate (PEP) (glycolysis) and erythrose-4-phosphate (pentose phosphate pathway) that both lead to

the shikimic acid pathway (phenolic compounds, alkaloids). Glycolysis forms pyruvate (DOXP pathway / terpenes) and acetyl coenzyme A (mevalonic acid pathway / terpenes). All alkaloids, with the exception of those deriving from the aromatic amino acids (shikimate pathway), are produced through the Krebs cycle where acetyl-CoA a product of glycolysis joins oxaloacetate to generate the cycle of tricarboxylic acids (Diamandithis, 1994). Clark & Menary (1980; in Sangwan *et al.*, 2001), hypothesised that oil biosynthesis is controlled by the production and utilisation of photosynthates. Croteau, 1972 (in Bernath, 1986) suggested that an increase in photosynthesis favours terpene formation and inhibits metabolic breakdown.

1.10.2 Water efficiency

Limited water supply suppresses plant growth, induces stomatal closure, the accumulation of solutes and antioxidants and expression of stress-specific genes (Hughes *et al.*, 1989). Secondary metabolite production is believed to be stimulated under stressful environments (Gershenzon, 1983; Sangwan *et al.*, 2001). A possible explanation is that the enhanced glandular trichome density due to stress-induced leaf area reduction results in augmentation of accumulated oil (Gershenzon, 1983; Charles *et al.*, 1990; Simon *et al.*, 1992). Gershenzon, (1983) suggested that under water deficiency conditions monoterpenes can reduce transpiration by stimulating stomatal closure or by providing a vapour shield on the leaf surface by volatilisation. It should be noted that the hormone abscisic acid (ABA) involved in stomatal closure is a sesquiterpene. Alteration in the oil composition can be induced by the need for a rapid decline in transpiration as certain terpenes have lower vapour pressure than others and thus their synthesis is favoured (Gershenzon, 1983). Secondary metabolites serve for defence (see section 1.1.2). Their accumulation under adverse conditions may just be an adaptive strategy for increased resistance against biotic and abiotic factors. It is noteworthy that enhanced production of secondary compounds has been associated with periods of reduced growth (Gershenzon, 1983). On the other hand sufficient water

supply can increase SM yield by a positive effect on the overall growth of the crop (Sangwan *et al.*, 2001; Karamanos, 2000).

1.10.3 Nutrition

By definition, mineral nutrients have specific and vital roles in plant metabolism. Marshner, (1995) classified them according to their functions into three general categories: constituents of organic molecules, activators of enzyme reactions or charge carriers and osmoregulators. The effect of nutrients on SM pathways is still far from full understood and experimental data have revealed contrasting outcomes (Gershenzon, 1983): Despite the importance of natural products, little is known about the effect of plant nutrition on plant secondary metabolite production. Nevertheless, numerous studies have strongly indicated the control exerted by nutrition on SM production (Waller and Nowacki, 1978; Gershenzon, 1983; Bernath, 1986; Hornok, 1986; Sangwan *et al.*, 2001).

1.10.4 Ontogeny

Qualitative and quantitative changes of alkaloids and essential oils in various species have been attributed to developmental stage/phase of the plant (Bernath, 1986; Sangwan *et al.*, 2001). Bernath (1986) strongly indicated that secondary metabolite accumulation is closely connected to plant growth and development. Outcomes from various field experiments have provided evidence that the oil yield of sage is considerably less at the vegetative stage whereas it reaches a maximum during seed formation (Putievsky *et al.*, 1986; Karousou *et al.*, 2000; Santos-Gomes & Fernandes-Ferreira, 2001; Karioti *et al.*, 2003;). Differentiation in oil composition of *Salvia fruticosa* Mill. due to ontogenetic phase i.e. full bloom and seed formation stage has been as well demonstrated (Karioti *et al.*, 2003). Kreh (2002) reported that alkaloid content (Galanthamine) in *Narcissus* bulbs fluctuated throughout the life cycle of the plants.

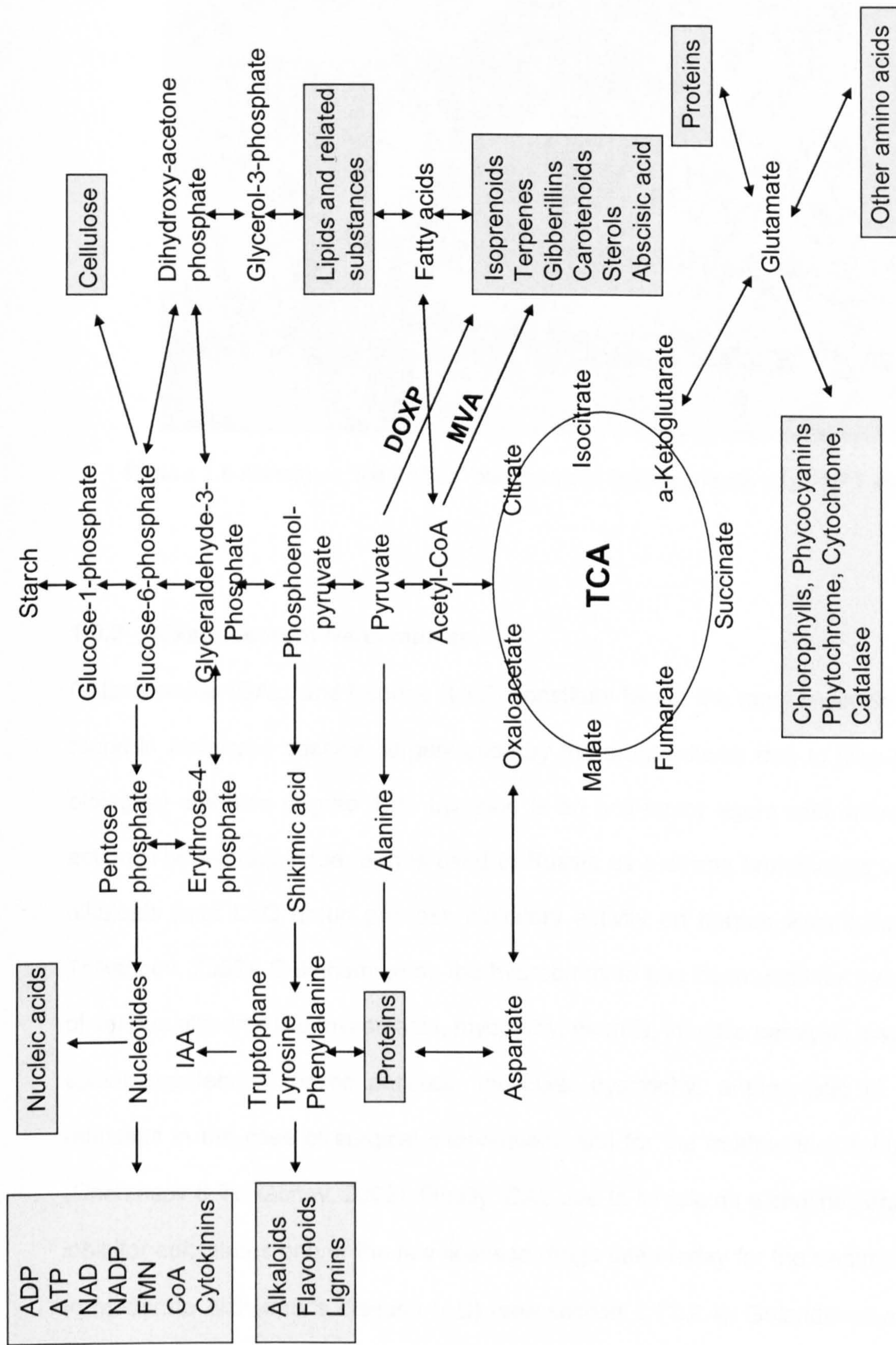


Figure 1.10 The metabolic big picture (modified from Taiz & Zeiger, 1991).

1.11 Terpenes and alkaloids for the treatment of Alzheimer's Disease

1.11.1 Alzheimer's disease

1.11.1.2 Definition and epidemiology

Alzheimer's disease (AD) is a form of dementia; an acquired impairment of intellectual and memory functioning caused by a disease of the brain, not associated with disturbances in the level of consciousness. Although the disease can potentially affect adults of any age, it usually appears in the elderly population (Friedland & Wilcok, 2000). The neurodegenerative nature of AD primarily induces a decline in the short-term memory (Perry *et al.*, 1996). Later symptoms in AD are progressive memory loss and general deterioration of cognitive functions leading to agnosia, aphasia, apraxia, depression, psychosis and sometimes development of neurological disorders (Wake, 2001; Howes, *et al.*, 2003). Alzheimer's disease constitutes the most common type of dementia in the elderly (Klafki *et al.*, 2000; Oddo and LaFerla, 2006). In the United States and Europe, AD is responsible for 50 to 60% of dementia cases (Francis *et al.*, 1999). Based on research of the older population in East Boston, US, Evans and colleagues (1989), reported that more than 45% of people aged 85 year or over suffered from AD. The outcomes of the previous study are corroborated by Skoog and co-workers (1993), who demonstrated very similar figures for a representative sample of 85 years old adults in Sweden; 44% of the individuals were diagnosed with AD. According to the WHO, around 35 million people in industrialised countries will suffer from AD by 2010 (Heinrich & Teoh, 2004). The second most common form of dementia is vascular dementia. Estimations of its prevalence in Europe vary widely from 10 to 50% of all dementia cases (Rocca, *et al.*, 1991), mainly because of difficulties in defining the criteria for the recognition of the disease (Ritchie & Lovestone, 2002). More types of dementia are dementia with Lewy bodies (DLB), vascular dementia (VaD), frontotemporal dementia (FTD) and others (Friedland & Wilcok, 2000). Determining the type of dementia is not always a

straight forward task as an accurate diagnosis may not be feasible during the patient's life. Final diagnosis can only be given when changes in the structure of the brain can be directly observed in a post-mortem examination (Heinrich & Teoh, 2004).

1.11.1.3 The pathology of AD

Alois Alzheimer in 1907 demonstrated two protein formations in the diseased brain associated with the pathophysiology and pathomorphology of AD; the neuritic plaques and the neurofibrillary tangles (NFTs), the most certain diagnostic features of Alzheimer's (Strange, 1992). Neuritic plaques are microscopic extra-cellular depositions of β amyloid protein, connected with injury in the axonal and dendritic parts of neurons and are found in large numbers in the limbic and associated cortices (Dickson, 1997). Neuritic plaques occur as well in the normal ageing brain tissue. Their number being augmented in AD, is positively correlated with the degree of cognitive impairment (Perry *et al.*, 1978b). Neurofibrillary tangles (NFTs) constitute intracellular bundles of abnormal fibres consisting of paired helical filaments that are composed by hyperphosphorylated *tau* proteins. NFTs are commonly found in the hippocampal and pyramidal cortical cell cytoplasm and are generally absent from the cortex of the healthy aged brain (Strange, 1992).

1.11.1.4 Cholinergic brain system and AD

Neurochemical changes have been consistently evident in the Alzheimer's diseased brain. Perry (1986), reported that memory impairments in patients suffering from AD result from a defect in the cholinergic system (cholinergic hypothesis). The human brain has several groups of cholinergic neurons located within the basal forebrain, striatum and brainstem (Figure 1.11) (Geula & Mesulam, 1999).

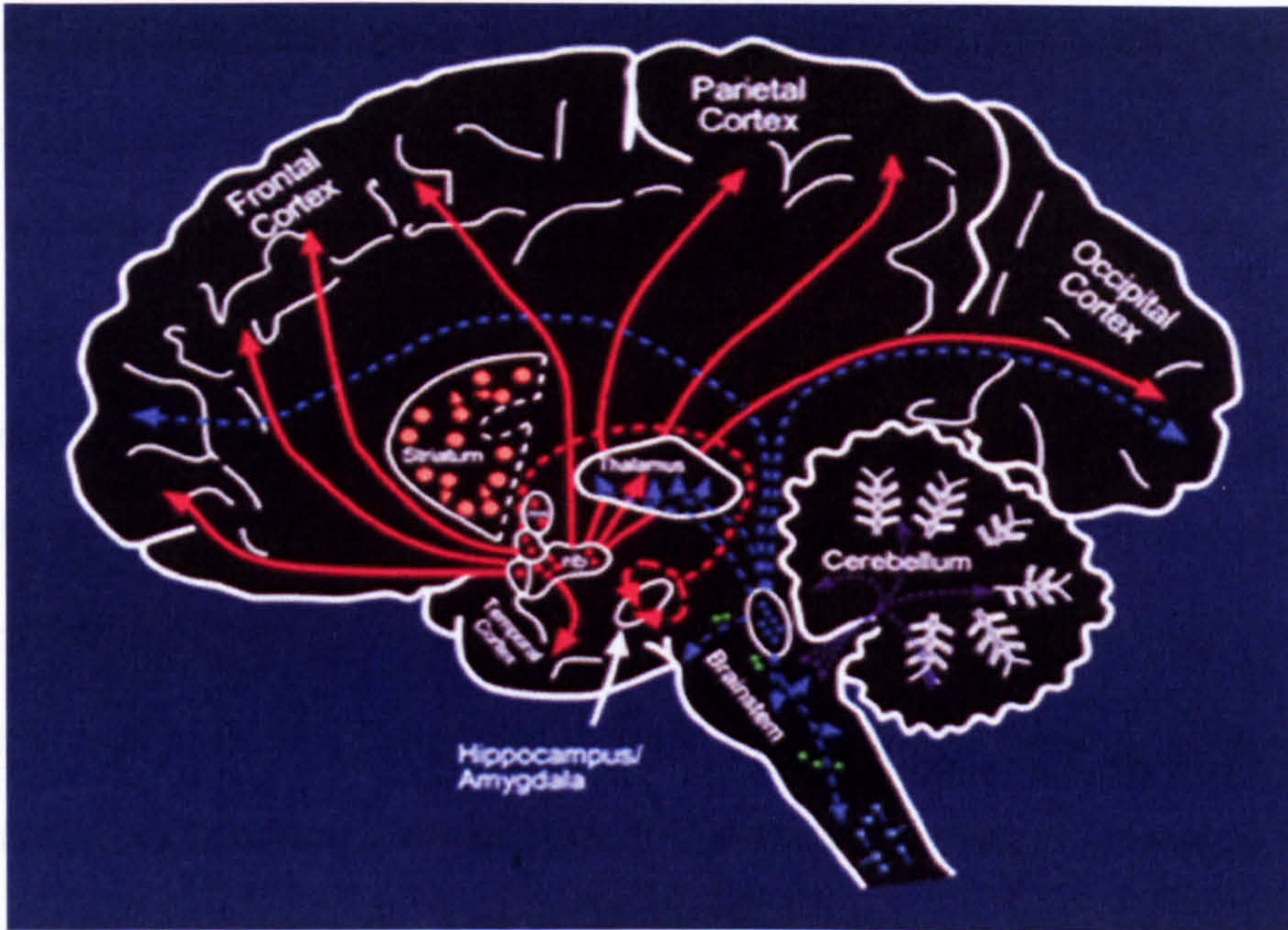


Figure 1.11 Cholinergic system in the human brain

A neuron is considered cholinergic when it produces acetylcholine (ACh), the neurotransmitter molecule (Figure 1.12). ACh exists in three isoforms: G1, present in the brain; G4, in the brain and neuromuscular endplate; and G2, in the skeletal muscle and blood-forming cells (Ellis, 2005). Non-cholinergic or cholinceptive neurons in the brain include glutamatergic, gabaergic, dopaminergic, histaminergic, serotonergic and noradrenergic (Mesulam, 2000).

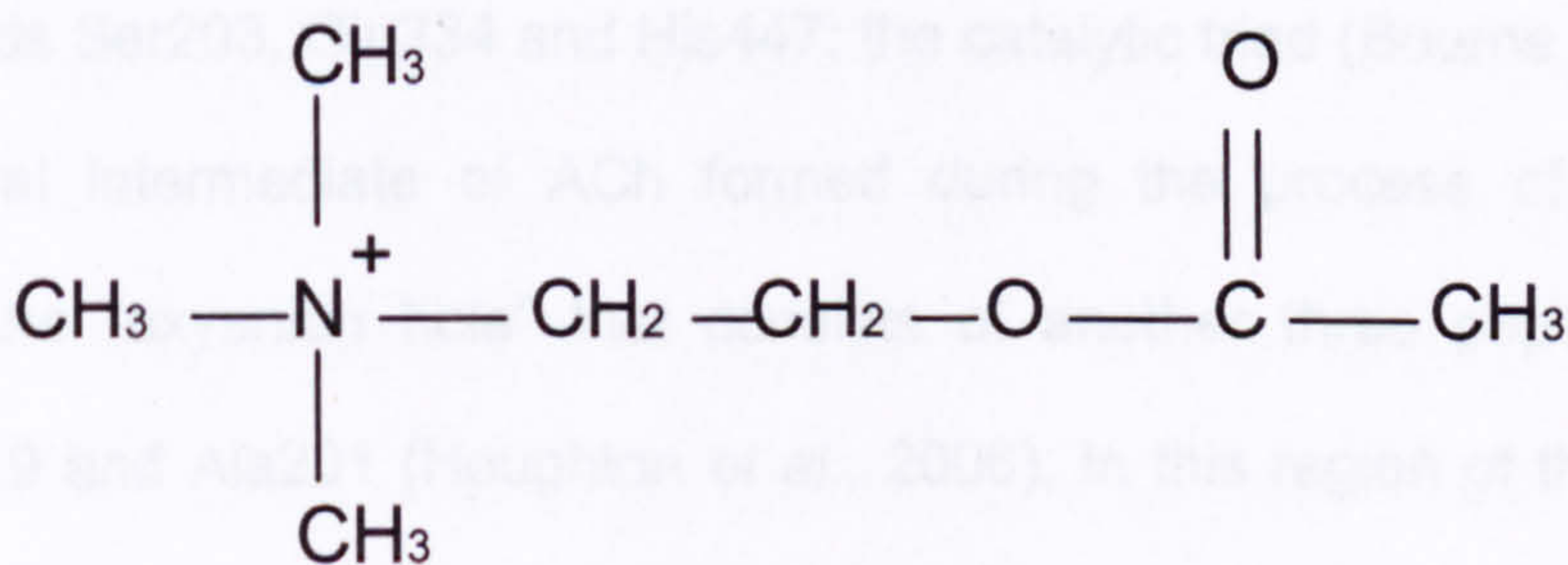


Figure 1.12 The neurotransmitter molecule acetylcholine (ACh)

Acetylcholinesterase

Additionally, all cholinergic neurons of the human basal forebrain and brainstem contain choline acetyltransferase (ChAT) the enzyme that synthesizes ACh, and the enzyme acetylcholinesterase (AChE). AChE is known to exert non-cholinergic functions as well for example in morphogenesis during embryonic development, in modulation of neuronal activity and in the defence against toxic compounds (Massoulié *et al.*, 1993). The major role of AChE (EC 3.1.1.7) is to terminate the action of ACh through rapid catalytic hydrolysis essential for cholinergic neurotransmission (Massoulié *et al.*, 1993; Taylor and Radic, 1994; Mesulam, 2000). Analysis of the three-dimensional structure of the enzyme revealed that AChE belongs to the α/β hydrolase fold family that comprises α/β proteins sharing this common fold, in which a central β -sheet is surrounded by loops and helices (Sussman *et al.*, 1991) (Figure 1.13). The active site of the enzyme consists of a 20 Å deep and narrow cavity named the active-site gorge or the aromatic gorge, since 60% of its surface is lined up by the rings of conserved aromatic residues. The gorge contains two sites for ligand binding; an anionic site at the bottom of the gorge and a peripheral anionic site (PAS) at the entrance, approximately 10-20 Å from the previous one. At the anionic subsite or choline-binding subsite the positively-charged quaternary nitrogen of ACh binds *via* π -cation interactions (Silman & Sussman, 2000). Catalysis of ACh at the catalytic or esteratic site involves interaction with three key amino acids Ser203, Glu334 and His447; the catalytic triad (Bourne *et al.*, 2003). The tetrahedral intermediate of ACh formed during the process of catalysis is stabilised at the “oxyanion hole” that consists of another three peptide residues Gly118, Gly119 and Ala201 (Houghton *et al.*, 2006). In this region of the gorge two other large amino acids Phe295, Phe297 form the “acyl pocket” that restricts entering of large substrates to the active site (Greig *et al.*, 2001). Three more aromatic residues are present at AChE peripheral active site Tyr70, Trp279 and Tyr121, (Silman and Sussman, 2000). AChE constitutes 90% of the total cholinesterases

(ChEs) in the temporal cortex of the normal brain (Perry *et al.*, 1978a). In the human brain the enzyme exists mainly in two forms; as a membrane bound globular tetramer G4 form, and a more soluble monomer G1 form. The dimeric G2 and other asymmetric forms have minimal contribution (Attack *et al.*, 1986).

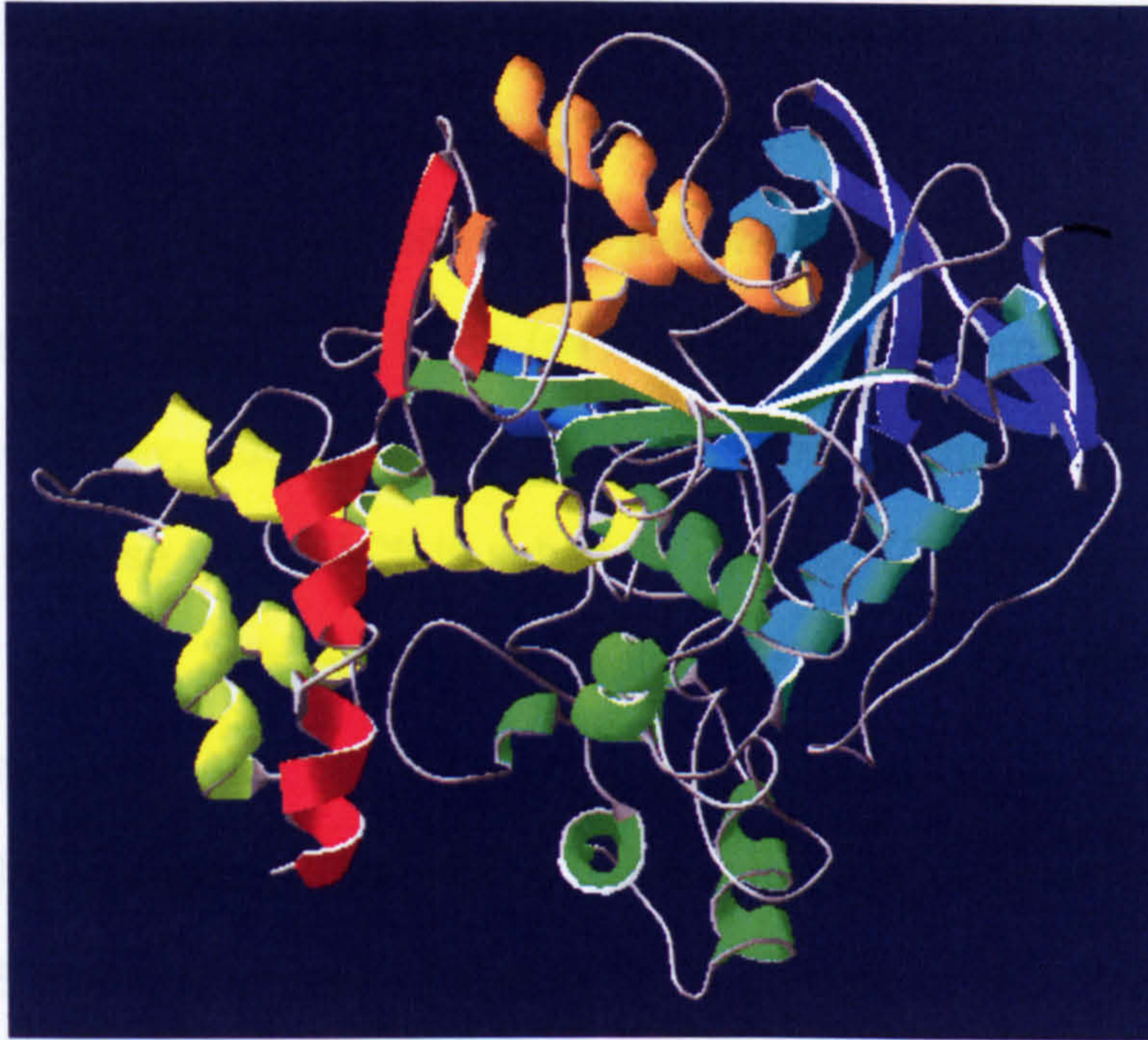


Figure 1.13 Acetylcholinesterase enzyme (AChE)

(<http://en.wikipedia.org/wiki/Acetylcholinesterase>)

Butyrylcholinesterase

The human cerebral neurocortex contains another enzyme that can hydrolyse ACh i.e. butyrylcholinesterase (BuChE) (EC 3.1.1.8). In the normal brain BuChE activity is detected in all regions that receive cholinergic innervation; located mainly in glial cells in contrast to AChE that is primarily associated with neurons (Giacobini, 2001; Mesulam *et al.*, 2002a). While AChE accounts for 80% of the total ChE activity, BuChE is responsible for the remaining 20% (Greig *et al.*, 2001). AChE and BuChE share 65% amino acid homology (Soreq & Zaku, 1993). AChE exhibits high selectivity for ACh hydrolysis, however BuChE being less substrate specific facilitates the metabolism of several different molecules including various neuroactive peptides

(Taylor & Radic, 1994). The aromatic gorge of BuChE is relatively larger with less aromatic residues. At the bottom of the gorge in the “acyl pocket” phenylalanines are replaced with two smaller amino acids valine and leucine allowing thus the binding of bigger substrates (Ekholm & Konschin, 1999). Moreover, at the peripheral anionic site (PAS) aromatic amino acids are substituted by aliphatic ones thus affecting the overall size and hydrophobicity of BuChE. The kinetics of BuChE further distinguish it from AChE. AChE is more efficient at low substrate concentrations (0.5 mM), and becomes inhibited at higher ACh levels (ca 2 mM, personal communication with Prof E. Perry). Conversely, BuChE is less efficient at low substrate concentrations but very efficient at those where ACh is substrate inhibited (Greig *et al.*, 2001). Due to the proximity of glial cells with the synaptic gap where the neurotransmitter is released, extracellular ACh can be effectively hydrolysed by BChE enzyme. Thus, Giacobini (2000) suggested that the possible physiological role of BuChE is to support AChE in the hydrolysis of excess ACh, (Giacobini, 2001; Giacobini *et al.*, 2002).

Cholinergic receptors

Cholinergic neurotransmission is facilitated by two types of neuronal transmembrane protein arrays that serve as the receptors of the ACh molecule. Muscarinic acetylcholine receptors (MAChRs) consist of seven peptide helices and responses to ACh binding are mediated by G-proteins (Mesulam *et al.*, 1995). The name muscarinic derives from the affinity of its active site for muscarine, a hallucinogenic compound occurring in the Basidiomycete fungus *Amanita muscaria*. Disruption or blocking of the receptor causes mnemonic effects and confusional states, possibly by interference in the activation and coupling of the receptor with its G-protein (Wake, 2001). The other family of ACh receptors, the nicotinic acetylcholine receptors (NAChRs) operate as ligand-gated ion channels and constitute a pentameric structure formed by a combination of the five same or different subunits (α_2 to α_{10}

and $\beta 2$ to $\beta 4$) organised around a central channel (Gotti & Clementi, 2004; Oddo & La Ferla, 2006). Each of the subunits of the receptor comprises four α – helical peptides (Court *et al.*, 1994). The sites for ACh-binding are located on the α subunits. Successful binding of ACh molecules at separate sites on the α subunits of the receptor induces conformational changes that open the central channel and allow the entrance and exit of Na^+ and K^+ or Ca^{2+} cations, causing thus depolarization of the cell membrane that generates action potential. The high binding affinity of NACHRs for nicotine alkaloid is associated with the $\alpha 4\beta 2$ subunits (Wake *et al.*, 2000) the most abundant nicotinic receptor in the brain (Gotti & Clementi, 2004).

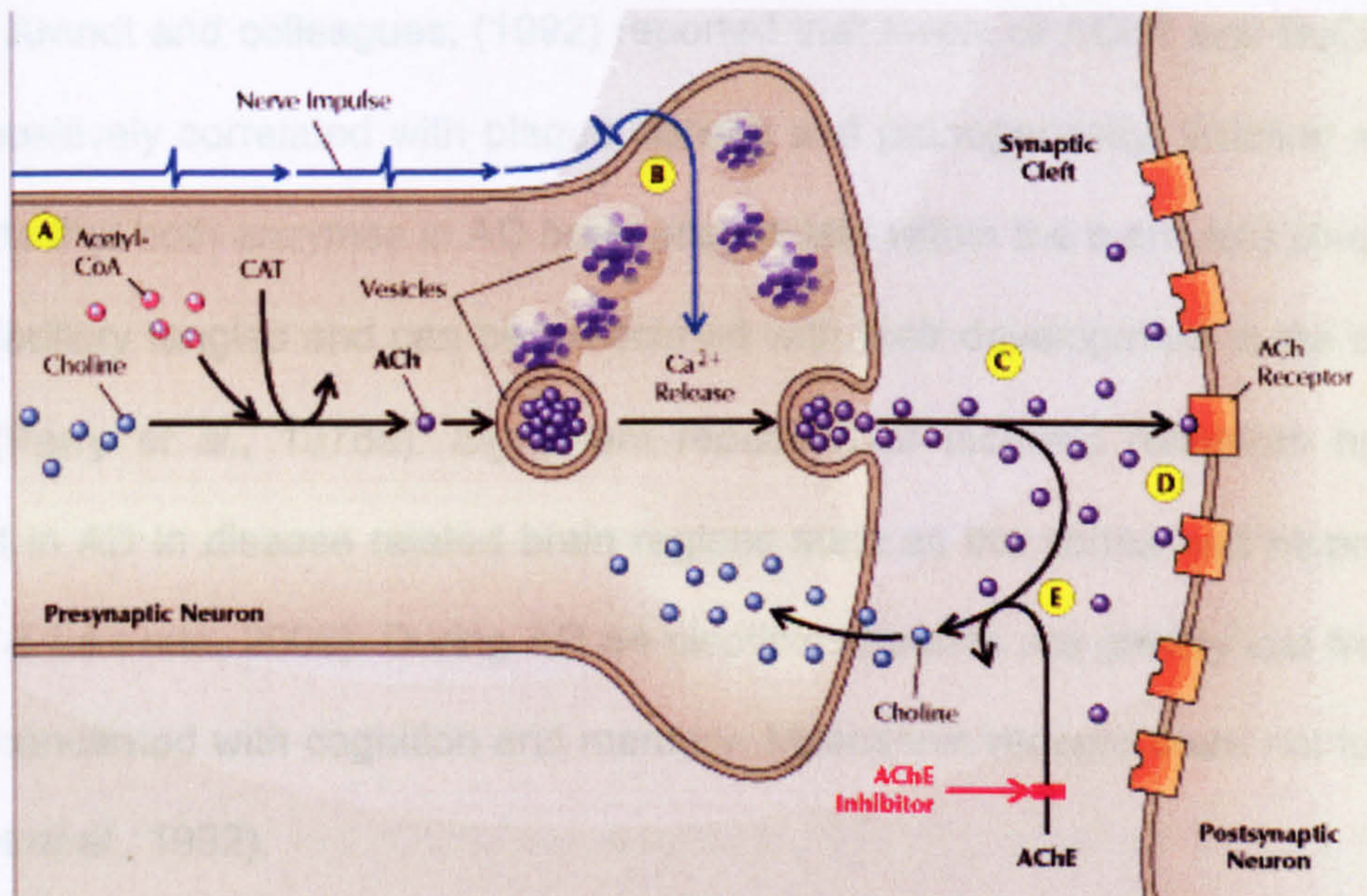


Figure 1.14 Cholinergic neurotransmission. The neurotransmitter molecule ACh is synthesised in pre-synaptic cholinergic neurons by choline acetyltransferase enzyme (ChAT) by the transfer of an acetyl group from acetyl-coenzyme A to choline (A). ACh molecules are stored in discrete vesicles at the end of the pre-synaptic neurons. Arrival of a nerve impulse triggers the release of Ca^{2+} ions, which activate actin microfilaments that in turn pull the storage vesicles into position for ACh release. In a single event the vesicles empty their contents into the synaptic cleft (C). Most of these molecules bind to cholinergic receptors on adjacent postsynaptic neurons (D). Any that remain unbound are rapidly hydrolysed by AChE (E). The choline released in the process is reused in synthesising new ACh. Inhibition of AChE and BuChE increase the amount of ACh available for neurotransmission.

In Alzheimer's disease (AD), a consistent neuropathological finding associated with memory loss is the decline of ChAT activity (Perry *et al.*, 1977). The greatest deficits of ACh occur in the brain areas concerned with memory and cognition, the hippocampus and temporal, frontal and parietal cortices (Geula & Mesulam, 1996). Cholinergic deficits, especially at the temporal and parietal lobe are strongly correlated with dementia scores (Perry *et al.*, 1978b). Other neurochemical changes include alterations in the levels of cholinesterase enzymes. In advanced AD cases, AChE activity may be reduced to 55-67% in specific brain regions, whereas BuChE activity remains constant, increases (Giacobini, 2001), or decreases (Kuhl *et al.*, 2006). Arendt and colleagues, (1992) reported that levels of AChE and BuChE have been positively correlated with plaque density and pathogenesis. Existing evidence supports that both enzymes in AD brain accumulate within the β amyloid plaques and neurofibrillary tangles and can be associated with their development in the diseased brain (Perry *et al.*, 1978a). Significant reduction of nicotinic receptors has been evident in AD in disease related brain regions such as the cortex and hippocampus (Oddo & La Ferla, 2006). During AD $\alpha 4$ nicotinic subunits are greatly lost from brain areas concerned with cognition and memory. Muscarinic receptors are not lost in AD (Aubert *et al.*, 1992).

1.11.2 Current methods for AD treatment

The cholinergic hypothesis (Perry, 1986) led to the development of licensed drugs aiming to enhance cholinergic activity. These drugs targeted the inhibition of ChEs (Enz *et al.*, 1993) in order to augment the declining levels of ACh available for neurotransmission and compensate for the loss of cholinergic function. Drugs approved by the U.S. Food and Drug Administration (FDA) for the symptomatic treatment of patients with AD are: tacrine, donepezil, rivastigmine, galanthamine (Giacobini, 2000) and another, not a ChE inhibitor, agent memantine (see below). In

the case of other types of dementia like VaD and DLB no FDA approved treatment exists (Ellis, 2005). Treatment with AChE inhibitors results in a significant improvement in cognitive function (Levy *et al.*, 1999; Jann, 2000; Coyle & Kershaw, 2001; Gruntzender & Morris, 2001) and may also retard progression of the disease (Farlow *et al.*, 2000). At present, no cure or prevention for dementia of the AD type has been found, leaving the symptomatic relief offered by ChE inhibition as the major therapeutic option. However, other therapeutic approaches have focused on the combined therapy of ChE inhibitors and memantine (Klafki *et al.*, 2006), nicotinic agonists (Gotti & Clementi, 2004) and strategies targeting β -amyloid and hyperphosphorylated tau protein pathologies (Klafki *et al.*, 2006).

Licensed ChE inhibitors are either non-selective (rivastigmine) or preferentially inhibit AChE enzyme. A growing body of evidence has indicated the therapeutic role of BuChE inhibition in Alzheimer's disease. AChE knockout mice not only survived to adulthood but exhibited structural and physiological integrity of the central and peripheral cholinergic pathways indicating that BuChE is capable of substituting at least to some extent for AChE, and plays an important role in cholinergic transmission (Xie *et al.*, 2000; Mesulam *et al.*, 2002b). Selective BuChE inhibition has been shown to elevate extracellular ACh levels and improve learning in rats (Greig *et al.*, 2001; Giacobini, 2004). The degree of BuChE inhibition has been significantly correlated with cognitive improvement in patients with AD (Giacobini *et al.*, 2002). Dual inhibition of ChEs resulted in improved glucose metabolism in cortical brain regions associated with memory and attention (Greig *et al.*, 2001). BuChE as well as AChE activity is involved in the development of amyloid plaques and neurofibrillary tangles in the diseased brain and thus BuChE inhibition along with AChE can be beneficial for the symptomatic treatment of AD.

1.11.2.1 Tacrine

Tacrine was the first marketed acetyl cholinesterase (AChE) inhibitor approved by FDA in 1993 (Figure 1.15). Tacrine preferentially inhibits AChE in the hippocampus and cortex (Enz *et al.*, 1993) and is a non-competitive type of inhibitor. Pacheco and co-workers (1995) demonstrated that tacrine is non-selective and readily inhibits both AChE and BuChE in a mixed, non-competitive way. Moreover, tacrine is believed to stimulate nicotinic receptors *via* binding on an allosteric site other than the ACh binding site (Nordberg, 2000). Nevertheless, tacrine never achieved widespread use in patients with AD due to its hepatotoxicity and severe side-effects (Thal, 1999). At the present it has been practically withdrawn from clinical trials (Lopez-Pousa *et al.*, 2006).

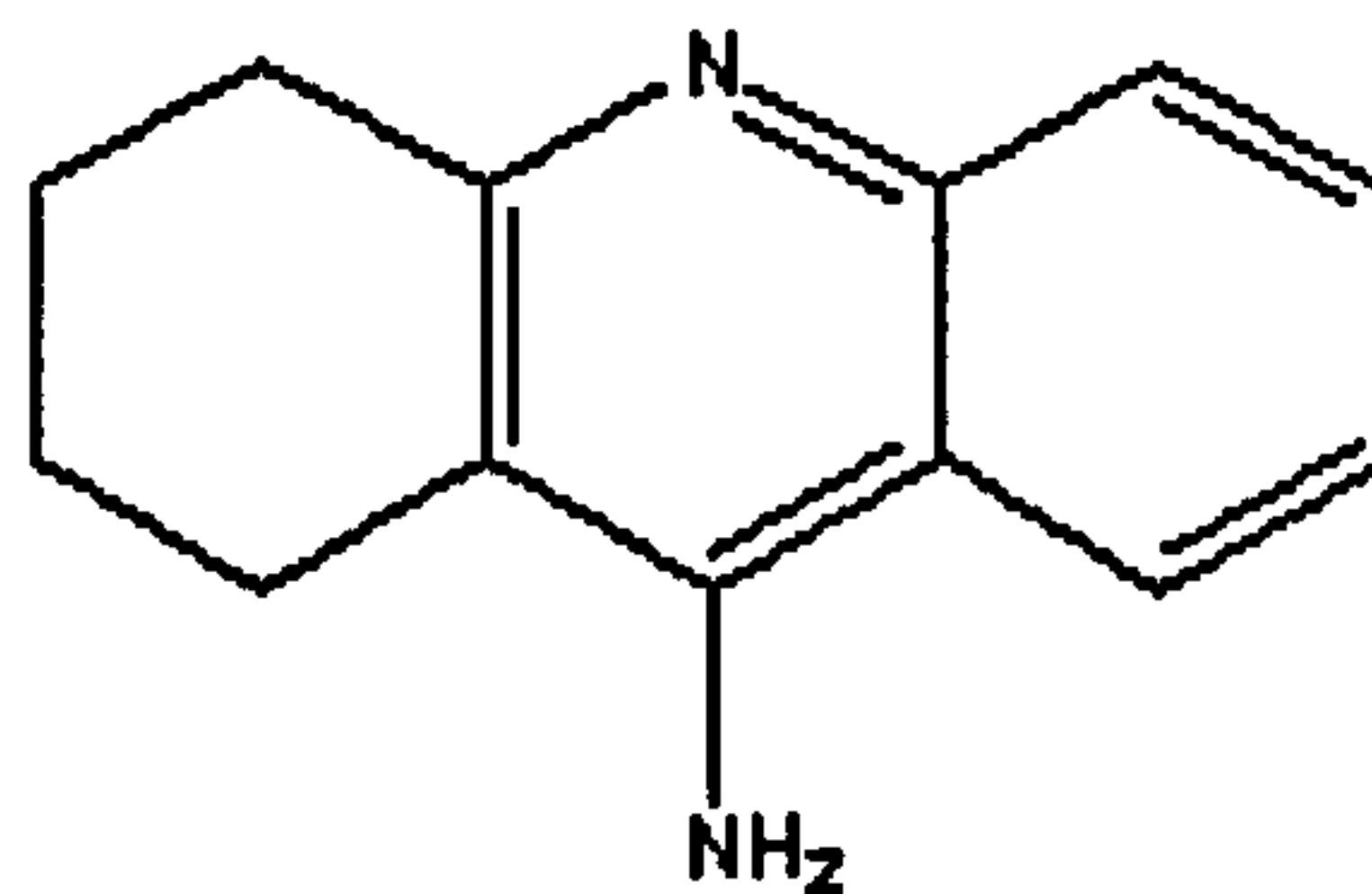


Figure 1.15 Tacrine (C₁₃H₁₄N₂)

1.11.2.2 Donepezil

In 1996, FDA approved donepezil (E2020), an inhibitor with a high degree of affinity and selectivity for AChE, contrary to BuChE (Snape, *et al.*, 1999) (Figure 1.16). Giacobini, (2000) reported that the ratio of BuChE inhibition to AChE is around 1000. Donepezil, readily penetrates the blood brain barrier (BBB) in rats (Kosasa *et al.*, 2000) and exhibits non-competitive, reversible type of inhibition (Snape *et al.*, 1999). Zhao and Tang (2002) reported that donepezil (E2020) is more selective for the G1 human form of AChE in striatum and hippocampus than the G4 form. Conversely, in the cortex both forms are inhibited to a similar degree. Giacobini, (2000) summarised the side-effects caused by donepezil treatment. Hepatotoxicity is virtually non-existent with donepezil and side effects occur in much lower percentages (6-13%)

than with tacrine (40-58%). In addition, longer half life (70 hours) and only once a day dosing schedule have enabled donepezil to go beyond tacrine in clinical use. This drug has received widespread acceptance in the U.S. for AD patients (Thal, 1999).

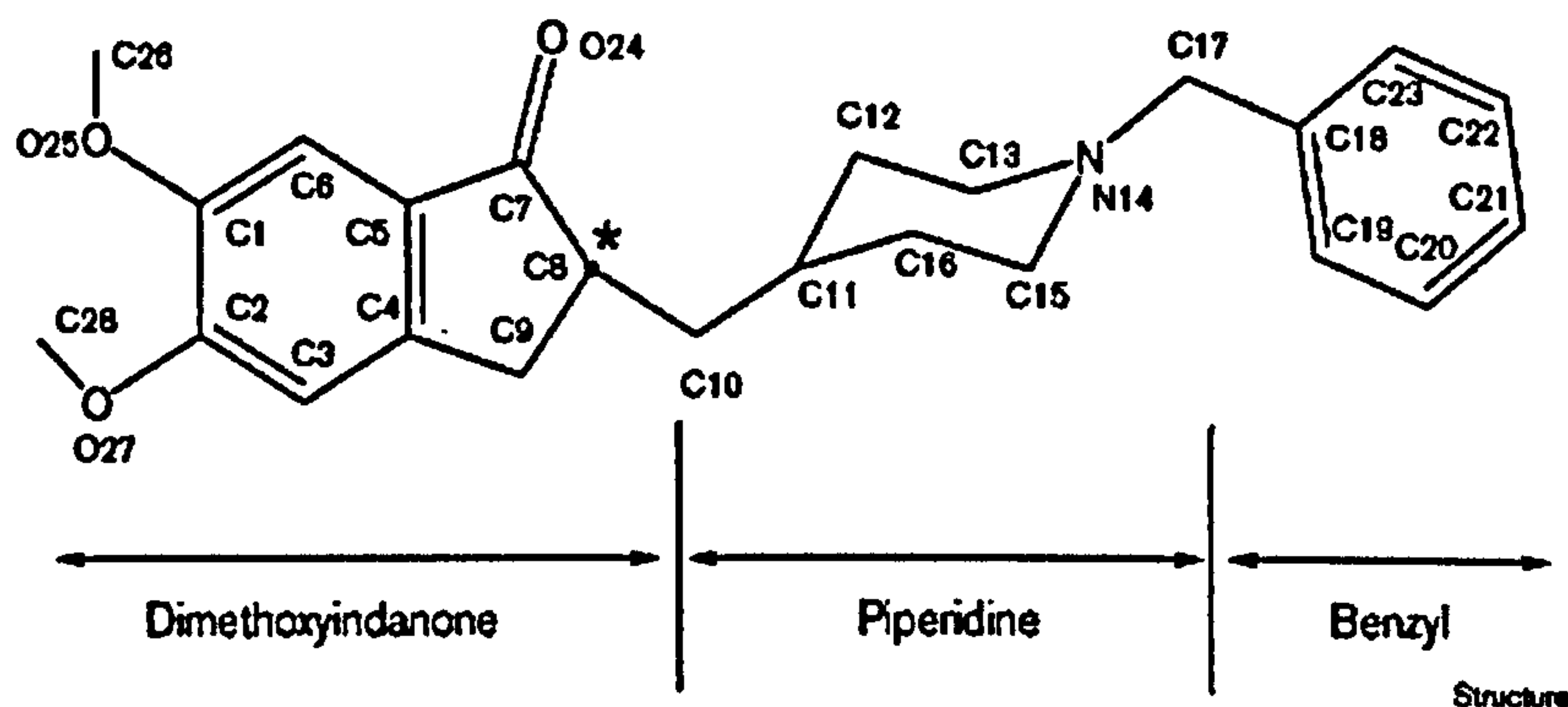


Figure 1.16 Donepezil ($C_{24}H_{29}NO_3$), *C8 is a chiral carbon

1.11.2.3 Rivastigmine

Rivastigmine is a member of the carbamate class of AChE inhibitors and was approved by the FDA in 2000 (Bar-On *et al.*, 2002) (Figure 1.17). Rivastigmine is a non-selective inhibitor of ChEs, with a slight preference for AChE (Enz *et al.*, 1991; Giacobini, 2000). Another report (Bar-On *et al.*, 2002) indicates higher inhibition of human BuChE than AChE. Lopez –Pousa & Lombardia (1999) reported that rivastigmine is a competitive, slowly reversible inhibitor for AChE. Rivastigmine preferentially inhibits the G1 form of the enzyme in cortex, hippocampus and striatum contrary to the G4 form that is also inhibited but to a lesser degree (Zhao & Tang, 2002). The level of G1 form does not decline in AD and is found to be present in plaques and tangles (Greig, *et al.*, 2001). A clinical trial in 725 patients with mild to moderate probable AD, conducted in 45 centres in Europe and in North America for 26 weeks, provided evidence that rivastigmine is a well tolerated and effective drug in the treatment of the disease (Rosler *et al.*, 1999).

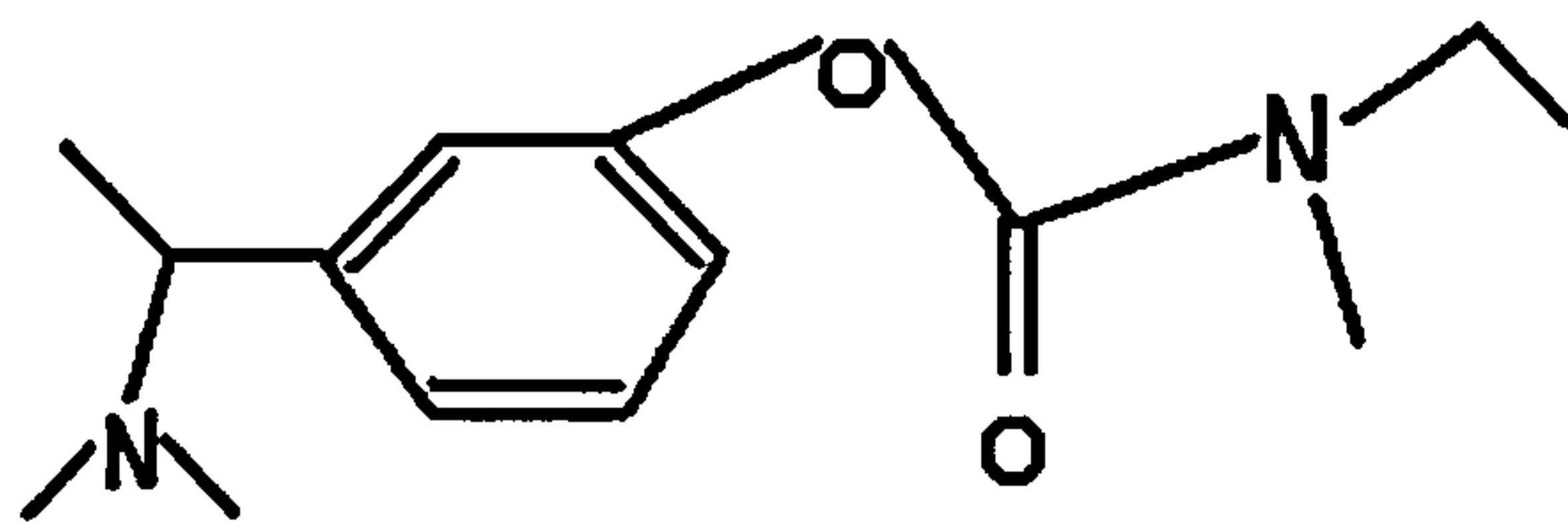


Figure 1.17 Rivastigmine (C₁₄H₂₂N₂O₂)

1.11.2.4 Galanthamine

Galanthamine is a naturally occurring alkaloid of the Amaryllidaceae family, commercially obtained by *Narcissus* and *Leucojum* species, as well as synthetically (Moraes, 2002; Heinrich & Teoh, 2004) (see Figure 1.9). It is the most recent FDA approved ChE inhibitor for the treatment of AD (February, 2001), under the name Reminyl®. Moraes, (2002) indicated that constant supply at an affordable price can only be achieved by systematic cultivation of the medicinal plants. As the numbers of patients with AD in industrialised countries are vast, stable production and augmentation of GAL yields have become essential to cover the needs of the pharmaceutical industries. Galanthamine is a potent, competitive ChE inhibitor, demonstrating 53-fold selectivity for AChE over BuChE. IC₅₀ values reported for human erythrocyte AChE is 0.35µM and 18,6mM for plasma BuChE. It has also a 10-fold lower potency for human brain AChE than its red blood cell equivalent (Tomesn & Kewitz, 1990). Galanthamine, does not exhibit selectivity for any ChE isoforms (Inglis, 2002). Moreover, galanthamine is a non-competitive allosteric ligand for nicotinic ACh receptors (NACHRs), promoting neurotransmission by allosteric modulation of the receptor. It binds at the pre- and post- synaptic NACHRs, at a binding site different to ACh, thus amplifying the response of the receptor on the post-synaptic neuron and increasing the release of ACh. Activation of the pre-synaptic NACHRs also increases the release of other neurotransmitters believed to

have an important role in memory, such as glutamate (Heinrich & Teoh, 2004). Galanthamine has proved to be an effective well-tolerated drug for the symptomatic treatment of AD that improves cognition, function and activities of patients with mild to moderate AD. The cognition benefits are sustained for at least 12 months. Moreover, development of behavioural disturbances and psychiatric symptoms are delayed (Scott & Goa, 2000; Corey-Bloom, 2003). Side effects associated with galanthamine are predominantly gastrointestinal, like nausea, vomiting, dizziness headache and anorexia, usually mild to moderate in severity (Heinrich & Teoh, 2004). Nevertheless, galanthamine is considered as a first-line treatment for patients with mild to moderate AD (Corey-Bloom, 2003).

1.11.2.5 Memantine

Memantine was approved by US FDA in 2003 for the treatment of patients with moderate to severe AD. Memantine is not a ChE inhibitor, it is an antagonist at the N-methyl-D-aspartate receptor, which is involved in the glutamatergic neurotransmitter system. Memantine is well tolerated, can be used in monotherapy or along with ChE inhibitors and has proven clinical effectiveness in patients with cognitive impairments (Ellis, 2005). Patients treated with memantine and donepezil concurrently showed statistically significant improvement in cognitive function after 4 months especially in the area of memory, language and praxis in comparison with those who received placebo / donepezil (Schmitt & Wichems, 2006).

In February 2005, the Food and Drug Administration (FDA) published an “Alert for Healthcare Professionals” concerning galanthamine. This alert stated that the preliminary results of two clinical trials with galanthamine that lasted for 24 months indicated three times higher risk of death to patients with mild cognitive impairment treated with the drug than those who were given placebo (FDA ALERT 02/23/05). A

comparative study on the effects of donepezil, galanthamine and rivastigmine in every day clinical practice for a period of 6 months demonstrated mortality of 6.2% (95%CI= 2.9-9.4) among all ChE inhibitors (Lopez-Pousa *et al.*, 2006). Nevertheless, Lopez-Pousa and colleagues (2006) in a comparative analysis of mortality in patients treated with galanthamine and donepezil concluded that mortality rates were not associated with the ChE inhibitor, the dose or the duration of the treatment. However, severe adverse side effects by the FDA approved drugs, mainly peripheral cholinergic (gastrointestinal) are evident. Today, new guidelines in the UK strongly recommend ChE inhibitors therapy only for moderate to severe AD cases (www.nice.org.uk; 2006-052 Lanch of dementia guideline 1).

1.11.3 Herbs with central nervous system (CNS) activities

Throughout history, the plant kingdom has provided the fundamental *materia medica* to all human cultures (Farnsworth, 1990). A wide range of plant species traditionally documented “good for the memory”, have been investigated as sources of chemicals with cholinergic activities. Such investigations yielded plenty of molecules with CNS effects (Perry *et al.*, 1999; Houghton & Howes, 2005; Houghton *et al.*, 2006). A lot of these cholinergic phytochemicals, deriving from plant secondary metabolism are alkaloids, like galanthamine, and toxic therefore at even low concentrations. Consequently, their potential use in dementia therapeutic approaches is restricted in terms of dosage and chronic application (Perry *et al.*, 1999). Ethnopharmacology however, has revealed that terpene-containing herbs are consistently mentioned for memory enhancement. Herbs due to their traditional use are considered to be “safe” at least in non excessive dosages (Perry *et al.*, 1996; 1999). While no evidence for their effectiveness existed other than their reputation, it was speculated that cholinergic activity could be involved (Perry *et al.*, 1996). Further bio-activity screening was thus required to corroborate or eliminate this putative basis for the

plants reputation (Attur-ur-Rahman & Choudhary, 1999). Herbs with reported cholinergic effects *in vitro* or *in vivo* comprise: *Melissa officinalis* L, from Labiatae family (Perry *et al.*, 1999; Wake *et al.*, 2000; Kennedy *et al.*, 2002; 2003); *Mentha* sp (Labiatae) (Miyazawa *et al.*, 1998); *Artemisia absinthium* L. from Compositae (Wake *et al.*, 2000); *Origanum majorana* L, (Labiatae) (Chung *et al.*, 2001); *Centella asiatica* L, (Umbelliferae), *Gingo biloba* L (Coniferae), *Salvia miltiorrhiza* (Labiatae) (Howes *et al.*, 2003) and *Huperzia serrata* (Lycopodiceae) from which huperzine A a potent AChE inhibitor is isolated (Wang & Tang, 2005).

1.11.4 *Salvia* species with cholinergic activities

The demonstrated anti-cholinesterase activity of *Salvia* oils and extracts reflects at least to some extent, the reputation of sage to affect the central nervous system (CNS). Cholinergic properties of *Salvia* species have been under investigation *in vitro* (Perry *et al.*, 1996; Perry *et al.*, 2000; Savelev *et al.*, 2003; Savelev *et al.*, 2004) and *in vivo* (Perry *et al.*, 2002; 2003; Akhondzadeh *et al.*, 2003; Tildesley *et al.*, 2003, Kenedy *et al.*, 2005; Tildesley *et al.*, 2005) for the last 10 years. *In vitro* outcomes, which revealed inhibitory activity for AChE concerned predominantly oils and / or extracts from *S. lavandulaefolia* (Perry *et al.*, 1996; Perry *et al.*, 2000; Savelev *et al.*, 2003; 2004) and *S. officinalis* (Perry *et al.*, 1996; Savelev *et al.*, 2004), whereas only one report involves the anti-cholinesterase activity of *S. officinalis* var *purpurea* or *S. fruticosa* oil (Savelev *et al.*, 2004). Specifically, Perry and co-workers (1996) reported inhibition (0.07µg/ml) of human brain AChE (autopsy tissue) from *S. officinalis* and *S. lavandulaefolia* (Spanish sage) essential oils. Anti-cholinesterase activity of the Spanish sage for human erythrocytes AChE was confirmed later *in vitro*, by Perry *et al.* (2000), and by Savelev *et al.* (2003; 2004). Savelev and colleagues (2004) investigated cholinergic activities of essential oils and oil constituents / terpenes from various *Salvia* species. Commercially obtained terpenes were tested individually or in combinations of two and eight and possible synergistic and antagonistic interactions

among oil compounds were reported (Savelev *et al.*, 2003). The apparent cholinesterase inhibitory activity of *Salvia in vitro* has been further corroborated by *in vivo* outcomes that demonstrated beneficial effects on the participants from sage intake. Kennedy *et al.*, (2005), indicated that acute administration of *S. officinalis* can positively affect mood in young healthy volunteers. Supporting evidence is provided by Tildesley *et al.* (2003; 2005) who reported enhanced mood and mnemonic performance in young participants from *S. lavandulaefolia* oil consumption. A pilot tolerability clinical trial involving 11 patients, which did not include a placebo control suggested significant improvement in patients' condition after 6 weeks, while tolerability of the oil was proved excellent (Perry *et al.*, 2003). Perry and colleagues (2002) indicated that *S. lavandulaefolia* oil or its metabolites can cross the blood brain barriers and induce decline in AChE activity of select brain areas in rats. Recent studies have indicated the necessity for exploration of BuChE inhibition along with AChE as accumulating findings suggested its relevance in the treatment of AD (see paragraph 1.11.2). The effect of *Salvia* species on BuChE enzyme of the cholinergic system has been investigated only once (Savelev *et al.*, 2004), however inhibitory activity was evident.

Herbs produce a diverse array of terpenes, phenolics and / or alkaloids (see section 1.1), the pharmaceutically active compounds in the brain (Houghton & Howes, 2005). Cholinergic properties of *Salvia* species have been mainly attributed to terpenes. Terpene molecules due to their lipophilic nature can potentially cross the brain blood barrier or the highly volatiles ones i.e. mono- and sesquiterpenes can be taken in by inhalation. Over the last decade research outcomes have provided evidence for AChE and / or BuChE cholinesterase inhibitory activity by individual terpene molecules, inhibitory activity weaker than the alkaloids (Houghton & Howes, 2005; Houghton *et al.*, 2006). Terpenes act synergistically in the extract and no single

compound has ever exceeded the activity of the whole mixture (Miyazawa *et al.*, 1998; Savelev *et al.*, 2003).

The search for novel ChE inhibitors is still of great interest. Natural products are an important potential source of such compounds (Ingkaninan *et al.*, 2002), particularly if they derive from plants used traditionally for anti-aging or memory enhancing purposes (Perry *et al.*, 1999). Today, herbal drugs have been systematically tested in animals and cell models of AD but not so extensively in clinical trials. Nevertheless, *Salvia officinalis* and *S. lavandulaefolia* clinical trials have shown very interesting results for the use of herbs in mood and memory improvement (Perry *et al.*, 2003; Tildesley *et al.*, 2003; 2005; Kenedy *et al.*, 2005). Herbals are relatively less toxic, can readily cross the blood brain barrier, have good bioavailability and can improve cognitive and cholinergic function, appearing thus to be a good alternative to current AD treatment (Anekonda & Reddy, 2005).

1.12 Aims and objectives

The current project involves two medicinal plant species with reported cholinergic activities i.e. *Salvia officinalis* var *purpurea* and *Narcissus* “Ice follies” that have potential (*Salvia*) or established (*Narcissus*) use for the symptomatic treatment of Alzheimer’s disease (AD) due to bioactive compounds intrinsically produced by plant secondary metabolism. Furthermore, *Salvia officinalis* var *purpurea* and *Narcissus* “Ice follies” constitute important ornamentals with wide commercial recognition in UK and elsewhere.

This project aimed to:

- ▶ achieve efficient cultivation of *S. officinalis* var *purpurea* and *Narcissus* “Ice Follies” via hydroponics, especially when knowledge for medicinal plant production is significantly limited;
- ▶ investigate possible effects of nitrogen (N) and phosphorus (P), and the effect of electrical conductivity (EC) on plant growth in order to define optimum nutrient and EC conditions for their cultivation;
- ▶ investigate possible effects of N, P and EC on secondary metabolite production, with the view to enhance i) galanthamine content in *Narcissus* and ii) essential oil yield and quality in *Salvia*. Oil quality is defined in terms of toxic thujone content and ChE inhibition properties;
- ▶ Corroborate the single report that demonstrated low IC50 values of *S. officinalis* var *purpurea* for both ChEs, and AChE competitive type of inhibition for the first time by an essential oil.

Endeavours to define optimum nutrient and EC conditions for *S. officinalis* var *purpurea* and *Narcissus* “Ice Follies” growth, and concomitantly induce alterations in plant secondary metabolites profile employed:

- ▶ application of various nutrient conditions (N, P) and EC by means of the Nutrient Film Technique (NFT). Hydroponics and specifically NFT, was used because of its advantages over the conventional soil-culture. NFT has been demonstrated as an excellent tool to apply nutrition with high degree of control and specificity;
- ▶ harvest of plants and i) measurement of growth parameters including biomass accumulation, root to shoot investment, plant height, leaf area as well as ii) assessment of photosynthetic pigments
- ▶ determination of nutrient consumption and correlation with growth parameters
- ▶ extraction of essential oils *via* steam distillation, and extraction of alkaloids utilising organic solvents;
- ▶ quantitative and qualitative analysis of essential oils and alkaloids *via* chromatographic techniques (GC/MS and HPLC respectively);
- ▶ performance of enzyme bioassays to test inhibitory activity of essential oils produced under different nutrient and EC conditions.

Finally, it was hypothesised that:

- ▶ hydroponics / NFT is an efficient method for commercial production of herbs and ornamentals and a useful tool for nutrient studies, so experimental outcomes could be extrapolated to conventional soil cultures;
- ▶ application of factors like N, P and EC will alter secondary metabolite content and quality
- ▶ *S. officinalis* var *purpurea* is a relatively potent, competitive ChE inhibitor with dual inhibitory activity on the cholinergic enzymes

This is the first study of *S. officinalis* and *Narcissus* species cultivation via the Nutrient Film Technique and the first investigation of the N, P and EC effects on *S. officinalis* growth and essential oils, in hydroponics.

Chapter 2

General Materials & Methods

2.1 Plant sources and propagation

2.1.1 *Salvia officinalis* var. *purpurea*

Authenticated young plants of *Salvia officinalis* var *purpurea* were purchased from a commercial supplier (Reed and Thoresby t/a Yorkstock, Yorkshire, UK). Plants were propagated for the purpose of this project employing standard practices, except for the use of fertilisers or other chemical substances used for crop protection. Vegetative propagation was preferred to propagation with seeds in order to minimize genetic variation among individual plants. Softwood cuttings from a limited number of supplier's mother plants, were placed in a mixture of fine peat, vermiculite and bark (2:1:1) in a mist propagation unit with basal heat of 18 °C. Rooted cuttings were subsequently transplanted into standard plastic pots of 9 cm diameter with peat and wood fibre (3:1) and kept in the supplier's glasshouse. Once the plants had reached a size of 15 cm approximately they were transferred to Newcastle University's experimental greenhouse at Moorbank Gardens.

2.1.2 *Narcissus* "Ice Follies"

Authenticated bulbs of *Narcissus* "Ice Follies" were purchased from R.A. Scamp Quality Daffodils (14 Roscarrack Close, Falmouth, Cornwall TR11 4PJ). Bulbs were produced under standard commercial practices.

2.2 Plant culture

2.2.1 Nutrient Film Technique

Nutrient Film Technique (NFT) was employed for plant cultivation in the experiments. The NFT system set up at Moorbank greenhouse (section 2.1.2), consisted of 12 independent NFT subunits. Each autonomous subunit comprised three components; a flat-bottomed channel, a reservoir or catchment tank and one water pump attached to plastic black tubing. Styrene light prismatic diffusers of 2.4 mX10 cmX6 cm (Newey & Eyre, electrical distributors) were utilized as hydroponic channels, once covered inside with black and white co-extruded polythene sheeting (LBS horticultural and irrigation supplies, UK) extensively used in hydroponic cultures. The white side of the plastic was folded along the channels after plant installation for three main purposes; to minimize root heating, protect rooting system from light exposure and prevent nutrient solution contamination from immediate contact with the light diffuser. Sage plants were previously subjected to root washing under a mild stream of water to achieve removal of the compost mixture from the roots, while *Narcissus* bulbs were installed untreated inside 3 cm net pots. Channels of the NFT were placed above ground-level on a 10X2X0.8 m metal constructed bench, at 40 cm intervals, with a slope fixed at 2% to facilitate nutrient solution circulation in the system. The solution's circulation in the NFT was primarily accomplished by the exploitation of a water pump MJ750, MAXI-JET ® Universal pumps (Ultimate Discount Aquatics, UK) immersed in the nutrient solution of the reservoir tank (80 L capacity, Wilkinson's stores, UK). The pump *via* appropriate tubing (12 mm, Ultimate Discount Aquatics, UK) fed the top (entrance) of the channel at a rate of 2 L/min. Water level in the channel did not exceed 1 cm height. Consequently the flow and fall of the solution from the end of the channel (exit) into the catchment tank located directly underneath was 50 cm (vertical distance between the channel and solution's surface in the tank) ensuring adequate oxygenation of the nutrient solution.



Figure 2.1 Nutrient Film Technique (NFT) system comprised 12 independent subunits each consisted of a channel covered by black/white plastic sheet, reservoir tank of 80 L and a pump-tubing system for the circulation of nutrient solution in the unit. Water pumps are immersed in the nutrient solution of reservoir tanks. Channels support the growth of six plants at 40 cm intervals.

2.2.2 Greenhouse conditions

The NFT unit was set up in the first compartment of the Moorbank heated glasshouse; temperature range 15 to 25 °C. Controlled air temperature, was recorded daily *via* a min-max thermometer. Standard floodlights fixed 1.5 meters above the bench level were employed for light emission *via* 400 Watt (SON-T NAV) high pressure sodium discharge lamps, one per square meter. Photoperiod was regulated by a central electric system with a timer incorporated. Light was emitted for a period of 10 h per day. Photosynthetic active radiation (PAR, $\mu\text{mol m}^{-2} \text{s}^{-1}$) was measured at plant-level (Skye, Q. Ir light meter) during all the experiments conducted and ranged from 200 to 350 $\mu\text{mol m}^{-2} \text{s}^{-1}$ depending upon the environmental conditions in the region at the time.

2.2.3. Nutrient solutions

Water soluble fertilizers along with chemical reagents were incorporated in 50 L of tap water to produce a balanced concentrated nutrient solution (stock solution) consisting of all the essential macro and micronutrients for plant growth and development. Hydroponic recipes / elemental composition of nutrient solutions expressed in mg/L, were designed from Dr Costas Economakis, (Laboratory of Hydroponics and Medicinal and aromatic plants, National Agricultural Research Foundation, NAGREF) and are fully described in the following experimental chapters under the materials and methods sections. Taking into account the percentage of the element of interest, amounts of corresponding fertiliser or chemical reagent (g) were converted to mg/L of element in the stock solution and *via versa* (Appendix 1, section 1.1). Fertilisers were purchased from a commercial supplier Hortiffeds (Park Farm, Kettlethorpe, Lincoln LN1 2LD, UK) and chemical reagents were obtained from Sigma Co, UK. In order to avoid chemical interactions resulting in pellet formation in the nutrient mixture, stock solution was prepared in two individual containers denoted as A (calcium nitrate, potassium nitrate) and B (remaining chemicals) (Table 2.1). Nutrient solutions were derived from stocks after 100-fold dilution with tap water. Elemental contribution of water was taken into account before designing hydroponic recipes (Appendix 1, section 1.2).

Table 2.1 Chemicals components of nutrient solutions adopted for the experiments and their elemental contribution

Fertilizers	Type	Macronutrient
Calcium Nitrate Hydrated	$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	Ca, N- NO_3
Potassium Nitrate	KNO_3	K, N- NO_3
Magnesium Nitrate Hydrated	$\text{Mg}(\text{NO}_3)_2 \cdot \text{H}_2\text{O}$	Mg, N- NO_3
Magnesium Sulphate Hydrated	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	Mg, S
Mono Potassium Phosphate	KH_2PO_4	K, P
Potassium Sulphate	K_2SO_4	K, S
Chemical Reagents	Type	Micronutrient

EDTA*Fe*Na		Fe
Manganese sulphate Hydrated	MnSO ₄ *4H ₂ O	Mn
Boric acid	H ₃ BO ₃	B
Zinc sulphate Hydrated	ZnSO ₄ *7H ₂ O	Zn
Copper sulphate Hydrated	CuSO ₄ *5H ₂ O	Cu
Ammonium molybdate Hydrated	(NH ₄) ₆ Mo ₇ O ₂₄	Mo

2.2.4 Typical measurements in hydroponics

Electrical conductivity (EC, mSiemens cm⁻¹) and pH values of the nutrient solution contained in the reservoirs changed over time due to plant activity, with the rate of change depending on plant biomass and total volume of nutrient solution in the NFT unit. EC and pH levels were therefore monitored every other day by means of a portable EC and pH meter (HANNA, VWR International Ltd, UK) and subsequently readjusted to the targeted values. Decreased EC was corrected by addition of stock solution, while 5% nitric acid was utilised for the pH adjustment. Nutrient solution temperature was recorded concurrently with EC utilising the thermometer incorporated in the same instrument. Water removal from the nutrient solution due to plant consumption, was also measured employing a common ruler of 1 m. The ruler had been previously scaled in litres (L) by repeatedly adding the same volume of water (2 L) in the reservoir and marking its level on the ruler. For correcting water removal the ruler was placed vertically inside the tank and the level of the remaining nutrient solution was directly read off the scale. The assumption that water and nutrient solution have the same density was made. Reservoirs were afterwards manually refilled up to 50 L (standard solution volume in the tanks chosen for all the experiments conducted) with tap water. Nutrient solution of 50 L volume always in relation to plant biomass, is sufficient to provide plants with the necessary amount of elements and water and maintain the levels of EC and pH constant for longer periods resulting in improved hydroponic practice.

2.3 Elemental analysis of nutrient solution

2.3.1 Inductively Coupled Plasma – Optical Emission Spectroscopy

Plant nutrient uptake was monitored throughout cultivation by analysis of the nutrient solution at equal time intervals. Samples of nutrient solution (100 ml) were collected every fortnight from all NFT reservoirs and kept in darkness at 7 °C prior to analysis. Elemental analysis for P, K and Ca was conducted in the laboratory by means of Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) (UNICAM 701 Series Emission Spectrometer) with conditions applied: plasma power 1 KW, argon gas flow 13 L/ min, nebuliser 38 psi and auxiliary 0.5 L/min. Once argon gas is passed through the ICP torch, free electrons break argon down into atoms, ions and electrons forming what is known as plasma. Plasma is sustained in an electromagnetic field created on top of the torch *via* a copper coil connected to a radio frequency generator. The temperature of plasma can reach more than 6000 °C and when a sample is introduced as a fine mist of droplets, evaporation of the solvent, decomposition into individual molecules and dissociation into atoms occurs. The atoms that originate from the sample by collision with plasma particles absorb energy resulting in excitation or even ionization. Once atoms or ions are in excited states, they decay to lower energy states with concurrent emission of electromagnetic radiation (light). ICP–OES measures the intensity of light emitted at specific wavelengths for qualitative and quantitative determination of elements with detection limits from parts per billion to parts per million for each element (Boss & Fredeen, 1989)

ICP analysis was conducted additionally for tap water utilized during hydroponic cultivation (Appendix 1, section 1.2). The outcome of this analysis was taken into account before designing the composition of stock solutions in the relevant experiments.

2.3.2 Nitrate measurement

N from nitrate (N-NO₃) determination in the laboratory employed a bench spectrophotometer Jenway 6405UV/Vis (light source: tungsten halogen lamp for visible and deuterium for UV radiation; wavelength range: 190 – 1100 nm). The analytical method adopted from a Unicam protocol for the determination of nitrates in aqueous solutions by Unicam SP500, SP1800, SP3000, SP8000 spectrophotometers, was obtained from researcher Costas Economakis, (Laboratory of Hydroponics and Medicinal and aromatic plants, National Greek Research Foundation, NAGREF) (for the protocol see Appendix 1, section 1.3). Exactly 2 ml of 50-fold diluted nutrient solution in deionised water was placed in a quartz cuvette and absorbance was read at 210 nm against deionised water. Because organic constituents of natural water also absorb at the same wavelength correction of the above value became necessary. The protocol's approach to this problem was determination of the absorbance of the organic matter at 275 nm that is related to the 210 nm absorbance with a factor of 4 (four times less absorbance in this wavelength than the 210 nm). Subtraction of the value at 275 nm multiplied by a factor of 4, from the original value at 210 nm revealed the absorbance of N-NO₃ (equation 2.1). The method made the assumption that the concentration of the organic matter corresponds to only 5 or 10% of total N. Concentration of N-NO₃ (mg/L) in nutrient solution finally derived from a calibration curve that associated absorbance of standard solutions of potassium nitrate (KNO₃) with their known concentration (range 1 to 6 mg/L).

$$\text{Abs N-NO}_3 = \text{Abs 210 nm} - 4 \times \text{275 nm} \text{ (equation 2.1)}$$

2.3.3 Nutrient uptake curves

Nutrient uptake curves for nitrogen (N), phosphorus (P), potassium (K) and calcium (Ca) were constructed employing the elemental analysis data. Plotted curves described the mean consumption by plants of the corresponding element at regular intervals during hydroponic cultivation. Elemental consumption for one period was estimated by calculating the difference in concentration values of the element in the hydroponic solution at two subsequent measurements, summed up by the concentration of the corresponding element added through nutrient solution during that period and divided by the number of plants in the channel (equation 2.2)

$$\text{Plant consumption} = \frac{(\text{Value period } n + \text{Conc. added via nutrient solution} + C - \text{Value period } n+1) \cdot 50}{\text{Number of plants in the channel}} \quad (\text{equation 2.2})$$

where C represents the amount of N added from 5% nitric acid for pH correction or the concentration of Ca gained from water when reservoirs were refilled.

Plant consumption is expressed in mg / plant

During hydroponic cultivation there is the potential for unaccountable losses from the solution due to precipitation and acquisition by algae present in the system (Cooper, 1979). Algal growth was minimised by the frequent addition of 0.0056% active chloron (2 ml in 50 L when chloron is 14%) in the nutrient solutions.

2.4 Plant growth parameters

2.4.1 Fresh and dried weights

At the end of each experiment 6 *Salvia* or 16 *Narcissus* plants were harvested. Plant material, after dissociation into component parts was weighed with the aid of a Salter electronic balance FX-3200 (0.00g – 3100g) and fresh weights were recorded. Plant parts were placed in paper bags and subsequently transferred into an oven at 30 °C and 40 °C for *Salvia* and *Narcissus* species respectively, where they remained until constant weight was reached. Dry weights were then determined utilising a Mettler PC 440 (Delta Range) (0.00g – 420g) balance. Fresh and dry weights of 10 plants and partitioning plant parts were also recorded prior to the beginning of the cultivation (initial plant weights, Mettler AC 100 balance).

2.4.2 Absolute growth rate (AGR)

The difference between initial and final dry weights was used for the estimation of the AGR of plant and component plant parts (equation 2.3). AGR is a valuable tool when comparing data that belong to the same system i.e. species (Hunt, 1990).

$$\text{AGR (g/week)} = (w_2 - w_1) / t \text{ (equation 2.3)}$$

w_2 = plant dry weight at the end of the experiment (g)

w_1 = initial mean dry weight of 10 plants (g)

t = duration of the experiment in weeks

2.4.3 Root to shoot allometric co-efficient (RSco)

The absolute growth rate participates in the measurement of the allometric root to shoot co-efficient (RSco, equation 2.4), which is a convenient indicator of the balance of partitioning between the root and shoot of the plant (Hunt, 1990).

$$\text{RSco} = (\text{AGR}_r / \text{AGR}_s) \times 100 \text{ (equation 2.4)}$$

AGR_r = Absolute growth rate of root

AGR_s = Absolute growth rate of shoot

2.4.4 Specific leaf area (SLA)

The youngest fully expanded pairs of *Salvia* leaves were employed for leaf area measurements *via* a Delta T area meter (Delta T Devices, Cambridge, UK), which was calibrated against a 1 cm² surface piece of paper. Dry weights of leaves were recorded utilising a Mettler AC 100 balance and specific leaf area was calculated as the ratio of leaf area to dry leaf weight (equation 2.5)

$$\text{SLA (cm}^2/\text{g)} = \text{Area of the leaves (cm}^2\text{)} / \text{leaves dry weight (g)} \quad (\text{equation 2.5})$$

The youngest fully expanded pair of leaves was usually represented by the third pair of leaves from the top of the plant stem. One pair of leaves was randomly selected from all plants within each channel.

2.5 Leaf pigments

Pairs of the youngest fully expanded leaves of *Salvia* were randomly selected from three plants within each channel at the end of hydroponic experiments, in order to determine photosynthetic pigment content in leaves. Leaf samples were wrapped in aluminium foil and immediately transferred to the laboratory for analysis. Chlorophylls a and b (chl_a, chl_b) and total carotenoids measurements were made employing a bench spectrophotometer Jenway 6405UV/Vis (light source: tungsten halogen lamp for visible and deuterium for UV radiation; wavelength range: 190 – 1100 nm). Analysis was performed under diffused lighting to avoid pigment degradation due to direct light exposure. Fresh leaf discs (four) of 1 cm diameter and total surface area 3.14 cm² were weighed (*via* a Mettler AC 100 balance, 60-70 mg) prior to grinding in 5 ml 80% acetone (Sigma Aldrich Co, UK) with the aid of a mortar and pestle. Removal of the remaining plant tissue employed 3 min centrifuging at 13000 rpm utilising 10 ml eppendorf tubes. Once the supernatant was transferred into quartz cuvetes, absorbance was read at 663, 646 and 470 nm against a blank of 80% acetone. Chl_a, Chl_b and total carotenoid concentrations were derived by applying the equations

described in Wellburn (1994) for the corresponding extraction solvent (equations 2.6, 2.7, 2.8). Aqueous acetone has been traditionally used for the extraction of chlorophylls from plant material (Porra, 2002). In addition the ratio of Chla to Chlb (Chla/Chlb) was calculated.

$$\text{Chla } (\mu\text{g}) = 12.21 \cdot A_{663} - 2.81 A_{646} \quad (\text{equation 2.6})$$

$$\text{Chlb } (\mu\text{g}) = 12.13 \cdot A_{646} - 5.03 \cdot A_{663} \quad (\text{equation 2.7})$$

$$\text{Carotenoids } (\mu\text{g}) = (1000A_{470} - 3.27\text{Chla} - 104\text{Chlb})/198 \quad (\text{equation 2.8})$$

2.6 Essential oil extraction from *Salvia officinalis* var *purpurea*

Steam distillation was adopted for the extraction of essential oils in the apparatus constructed (Figure 2.2). Sample for essential oil isolation (36 to 38 g) consisted of *Salvia* dried leaves only, obtained from a mixture of all plants in the channel pooled together (six). Distilled water was boiled by means of an electric heating mantle, in a 2 L volume, three-necked, round bottomed flask and generated steam that passed through a second flask containing the sample. High temperature steam extracted volatile constituents from the leaves and a mixture of condensed vapour and oil, via a 40 cm condenser with a drop tip, was collected in a 2 L glass bottle. For the separation of the two phases, oil was extracted from the water with an organic solvent (diethyl ether, purum, Sigma Co, UK) employing a 1 L separating funnel. One part of ether was added for every four parts of the mixture and the funnel was shaken vigorously. The two phases were then allowed to stand and ether was collected in a different flask from the water. The procedure was repeated three times until all the oil extracted from leaves was contained in the organic solvent.

Prior to ether evaporation, anhydrous magnesium sulphate (Sigma Co, UK) was incorporated with ether to absorb any water molecules remaining in the organic phase. The mixture was stirred for 10 min (model HB502, Bibby Sterilin Ltd) and filtered with the aid of a glass filtration apparatus under vacuum. Ether was subsequently subjected to evaporation *via* a rotary evaporator (type 349/2, J. Bibby Science Products Limited) accompanied by a heatable water bath to maintain the solvent temperature constant (30 °C) during the evaporation process. Ether is a solvent with a very low boiling point, and so was evaporated under vacuum, trapped by a condenser and collected in a flask for disposal. The oil-containing flask was weighted utilizing a Mettler AC 100 balance. The flask had been pre weighed prior to the evaporation process and the difference in the two values determined the oil yield in mg. Essential oils were stored in 2 ml sealed amber glass vials (Hichrom Ltd, UK), under oxygen-free nitrogen, at -20 °C, prior to analyses.

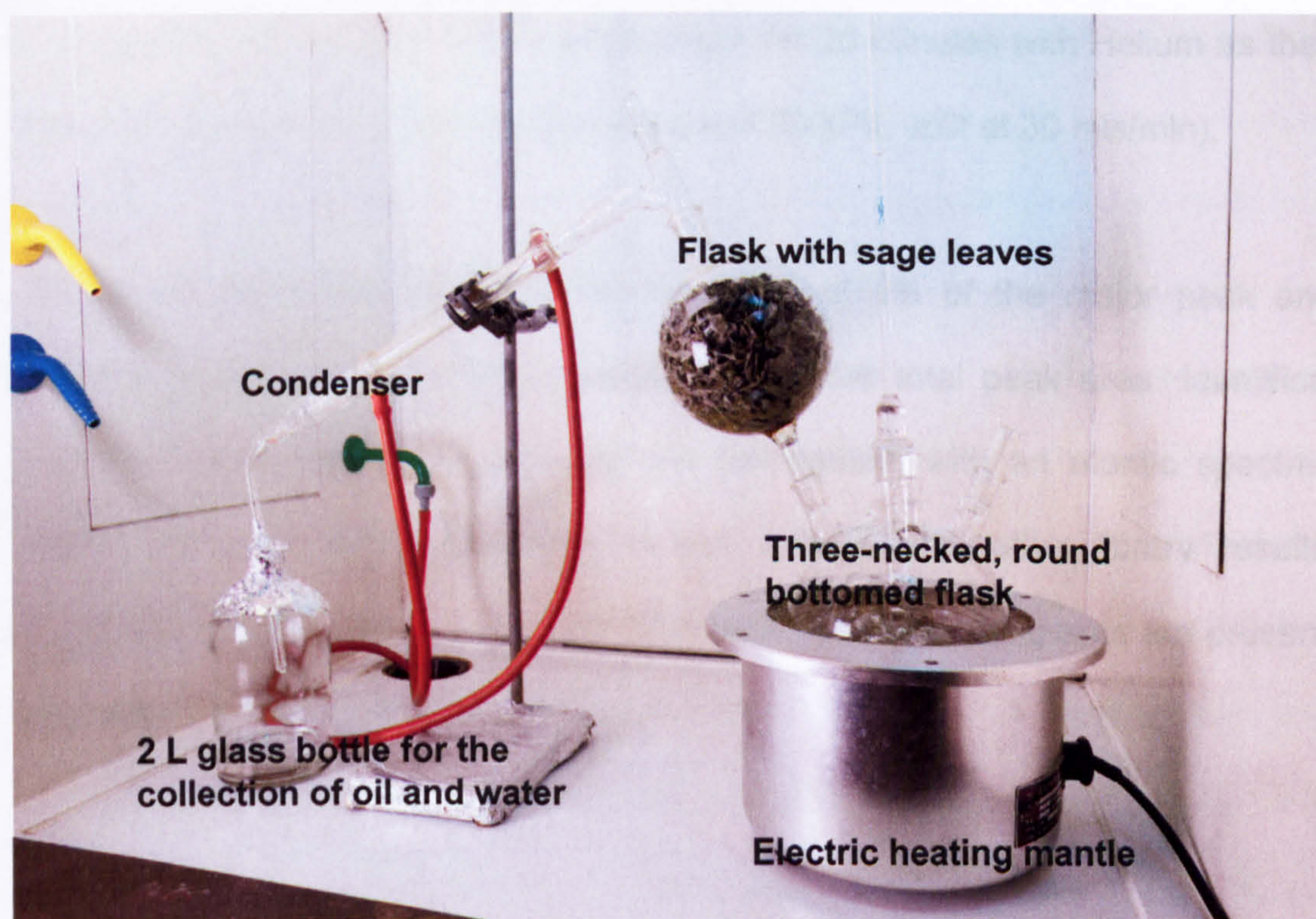


Figure 2.2 Steam distillation apparatus for the extraction of oils from *Salvia* plants

2.7 Essential oil analysis by GC/MS

Gas Chromatography-Mass Spectrometry (GC/MS) analysis of essential oil was performed on Hewlett-Packard 6890 GC with a split/splitless injector (280 °C) linked to a Hewlett-Packard 5973 mass selective detector (electron voltage 70eV, filament current 220 μ A, source temperature 230 °C, quad temperature 150 °C, multiplier voltage 2000 V, interface temperature 310 °C). Data acquisition was controlled by a HP Kayak xa pc chemstation computer, initially in full scan mode 50-550 amu/sec for greater sensitivity.

A sample (1 μ l) diluted in dichloromethane was injected by an HP7683 auto sampler and the split opened after one minute. After the solvent peak had passed the GC temperature programme and data acquisition commenced. Compound separation was performed on Zebron (ZB-5) (30 m x 0.25 mm ID x 0.25 μ m df) capillary column coated with 5% phenyl polysiloxane. The GC was temperature programmed from 50-300 °C at 4 °C per min and held at a final temperature for 20 minutes with Helium as the carrier gas at flow rate of 1 ml/min (initial pressure of 50 kPa, split at 30 mls/min).

Total peak area was calculated on the basis of 1% of the major peak and each compound was expressed as a percentage of the total peak area. Identification of individual compounds was achieved *via* comparison with an atomic spectra library (NIST, atomic spectra database Version 2.0). Comparative library results were accepted when prediction was more than 90%. GC chromatograms are present at the Appendix 4.

2.8 Alkaloid extraction from *Narcissus* “Ice Follies”

After plants were harvested, *Narcissus* bulbs were separated from roots, cut into pieces and dried inside paper bags in an oven at 40 °C until constant weight was reached. Foliage in paper bags was dried under the same conditions. Dried plant material was powdered separately employing a Mulinex TM grinder prior to extraction. Randomly, two ground plants were pooled together; total number of final samples per channel was eight.

Alkaloids were obtained from powdered plant material adopting a one-step acid extraction method for the isolation of alkaloids from plant tissue (Mustafa *et al.*, 2003; Moraes-Cerdeira *et al.*, 1997a). From each channel two random bulb and leaf samples were extracted. The method was optimised as: 1 g of bulb or leaf tissue was macerated in 30 ml aqueous solution of phosphoric acid (0.1 M H₃PO₄ 85%, Analar, BDH chemicals Ltd, Poole, England) of 0.1 M. The 1 to 30 ratio (1 g dry matter in 30 ml solvent) was considered to be adequate for efficient extraction without diluting the desirable compounds to the degree where extract concentration would be necessary. Extracts were shaken for 8 hours, at room temperature and sonicated for 30 min twice with a 4 hour interval, utilising an ultrasonic bath (Decon Ultrasonics Ltd, UK). Sediment was separated by pouring off the aqueous phase that was subsequently centrifuged for 15 min, (supernatant re-centrifuged; 15 min) at 14,000 rpm and 4 °C prior to HPLC analysis (see section 2.9). Aliquots (1 ml) of the supernatant were freeze – dried in order to determine their dry weight equivalents. Mustafa and co-workers (2003) strongly indicated the acid extraction as more suitable for quantitative analysis in comparison with the common acid/base procedure for the isolation of amines from plant material (Kreh & Matusch, 1994; Selles *et al.*, 1997; Machocho *et al.*, 1999; Mustafa *et al.*, 2003). The simplicity of the method minimized analytical errors while resulting in a relatively pure extract.

2.9 Quantitative & qualitative analysis of alkaloids by High Performance Liquid Chromatography (HPLC).

Reversed-phase High Performance Liquid Chromatography analysis was adopted for the identification and quantification of galanthamine (GAL) and lycorine (LYC) alkaloids from *Narcissus* “Ice Follies” crude extracts. The HPLC equipment comprised two Shimadzu model LC-10AS pumps, a Shimadzu model SIL-10A auto-injector/sampler, a Pye Unicam PU 4021 multichannel UV detector and a Shimadzu C-R5A integrator for recording and integrating the chromatographic peaks. Chromatographic separation was performed on a Hyperclone BDS, C18 column (250 x 4.6 mm i.d; 5 μ m) protected by a 7.5x4.6 mm i.d; 5 μ m, C18 guard column (Phenomenex, UK).

High purity solvents were utilised for HPLC analysis; HPLC-grade acetonitrile (Fisher Scientific, Ltd, UK), deionised water (Milli-Di, Millipore Corporation) and analar H₃PO₄ (85%, BDH chemicals Ltd, Poole, England). Elution of the alkaloids (volume injected, 5 μ l) was achieved *via* an isocratic system of 5:95 acetonitrile:water v/v mobile phase of pH 2.5 in presence of H₃PO₄, pumped at 0.8 ml/min flow rate, for a 30 min run time. Detection was performed at 210 nm and identification was accomplished by comparing analytes peak shape and retention time with peaks from a standard solution of GAL and LYC (150 μ M each in aqueous H₃PO₄) previously run under the same conditions (Appendix 5). Two bulb and leaf extracts were run from each channel. Mean values per channel were employed for further statistical analysis.

2.10 Measurement of human erythrocyte acetylcholinesterase activity

2.10.1 Assay

Assessment of inhibition of human erythrocytes acetylcholinesterase (AChE; EC3.1.1.7) from plant extracts (Sigma Co, UK) was carried out adopting the spectrophotometric assay described by Ellman *et al.*, (1961) modified for micro plate (Savelev *et al.*, 2004, Okello *et al.*, 2004). For the enzyme assay, sodium (Na) phosphate buffer pH8, enzyme AChE, substrate acetylthiocholine iodide solution in distilled water (ATChI), plant extract and 5,5-dithiobis-2-nitrobenzoic acid (DTNB) prepared in sodium (Na) phosphate buffer pH7 in presence of sodium bicarbonate, were incorporated at the volumes and concentrations shown in Table 2.2. The reactants were mixed in a 96-well, flat bottom, polystyrene microtitre plate (Greiner Bio-One Ltd, UK), by automatic shaking for 5 sec every 1 min, throughout the assay. The mixture, with the exception of ATChI was pre-incubated at 30 °C for a period of 15 min and the reaction was initiated by adding the substrate. Change in absorbance (Abs/min) at 405 nm was measured on a microplate reader (Multiskan Ascent; ThermoLabsystems) and Ascent Software version 2.6 for a period of six minutes. All reagents utilised were purchased from Sigma Co., UK.

Table 2.2 Volumes and concentrations of the reagents comprising the assay for inhibitory activity plant extracts on human erythrocyte acetylcholinesterase.

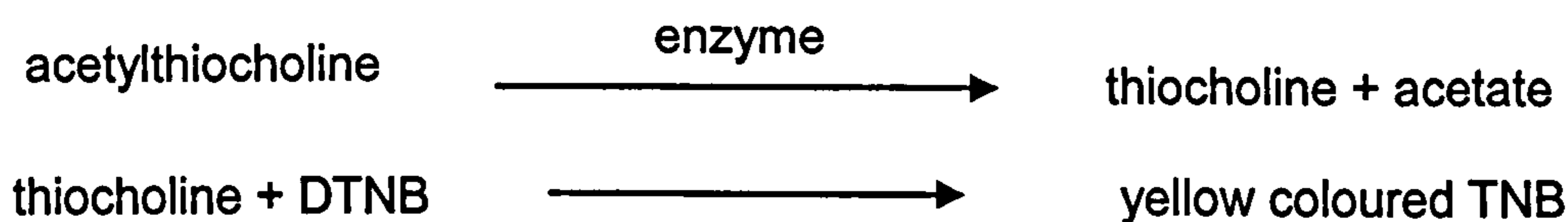
Reagent	Assay concentration ^a	Assay volume
Na-Phosphate Buffer pH 8	0.091 M	200 µl
DTNB in Na-Phosphate Buffer pH 7 with Sodium bicarbonate	0.27 mM 0.12 M	5 µl
AChE	0.05 Units/ml ^b	5 µl
ATChI in distilled water	0.5 mM	5 µl
Plant extract in 53% ethanol	0.5 mg/ml	5 µl
Total		220 µl

- a. Concentration of reagents in the final reaction mixture according to which original solutions concentrations (stocks) were calculated.
- b. Enzyme activity is expressed in units per ml of enzyme solution. By definition, one unit of enzyme will hydrolyse one μ mole of substrate to acetate and thiocholine per min at pH 8, 37 °C.

In order to determine the inhibitory activity of the plant extracts, controls and blanks were included in each run of the assay. A control (5 μ l of 53% ethanol substituted for the plant extract) was employed in every assay of cholinesterase inhibition to measure the activity of the uninhibited enzyme. Galanthamine (GAL) being a standard inhibitor was used as a positive control in place of the assayed extract (calibration curve with GAL concentrations: 15, 30, 60, 120, 240 and 480 μ M). A substrate blank (5 μ l buffer pH 8 substituted for the enzyme) was run in order to reveal any non-enzymatic substrate hydrolysis and the absorbance value produced was subtracted from the rest for inhibition calculations. Enzyme blank (5 μ l buffer pH 8 substituted for the substrate) determined the degree of binding of DTNB to DTNB reactive substances in the enzyme like sulphurhydryls. Since this was found to be minimal the enzyme blank was not needed on a regular basis. At each run, samples, control and blanks were replicated four times.

2.10.2 The principle of Ellman's spectrophotometric method

The colorimetric method developed by Ellman *et al.*, (1961) is the most widely adopted assay (Ingkaninan *et al.*, 2002) for determining ChE activity. The method is based on the production of a colour compound (anion of 5-thio-2nitrobenzoic acid, TNB) that can be quantified by means of spectroscopy. ATCh is hydrolysed enzymatically to yield acetate and thiocholine. Thiocholine rapidly reacts with DTNB, producing the yellow coloured anion (TNB) that has a maximum absorbance at 412 nm. Absorbance value of TNB per min, directly related to the rate of substrate hydrolysis in the reaction mixture can be employed for calculating enzyme activity (Appendix 3).



When the enzyme hydrolyses the substrate uninhibited, the maximum yield of TNB is achieved. In the presence of an inhibitor, substrate hydrolysis is partially suppressed, reducing the release of the yellow anion with a consequent decrease in absorbance values. Percentage of the inhibition is derived by applying equation 2.10.

$$\% \text{Inhibition} = \frac{\text{Abs/min without inhibitor} - \text{Abs/min with inhibitor}}{\text{Abs/min without inhibitor}} - (\text{Abs/min blank}) \times 100\% \quad (\text{equation 2.10})$$

However, any potential interference between the reagents and the inhibitor would reduce the production of yellow colour introducing an experimental error that needs to be addressed.

2.10.3 Ethanol interference

Assessment of *Salvia officinalis* var *purpurea* essential oils for AChE inhibitory activity required dilution of the extracted oil in a non-polar solvent. Ethanol (EtOH) was considered the most relevant, due to relatively low toxicity for human recipients in comparison with other solvents such as methanol. Savelev *et al.* (2004) confirmed significant inhibition of human AChE enzyme by the presence of 1.9% ETOH in the reaction mixture and established a final concentration of 1.2% and a six minute waiting period before running the assay, for minimizing the inhibitory effect. Employing the approach adopted from the previous author, *Salvia* oils were dissolved in 53% of ETOH (analar, BDH chemicals Ltd, Poole, England) and subsequently sonicated in a sonic bath for 15 min to enhance solubility (Decon Ultrasonics Ltd, Uk). The waiting period though was not found to be necessary.

2.10.4 Bovine cholinesterase inhibitory activity

AChE from bovine erythrocytes (Sigma Co., UK) was employed as well for the measurement of AChE inhibition adopting the assay described before (2.10.1). Due to solubility problems of bovine membrane-bound protein a final concentration of 0.01 U/ml was employed for the assay and stock solutions were sonicated in sonic bath (Decon Ultrasonics Ltd, UK) for 10 min. Bovine enzyme showed no ETOH sensitivity, and utilizing 80% of the solvent for oils preparation was thus permitted.

2.11 Measurement of butyrylcholinesterase activity

Assessment of butyrylcholinesterase (BuChE; EC 3.1.1.8) from horse serum inhibition was performed as described in 2.10.1 with the exception that butyrylthiocholine iodide (BuTChI) was adopted as substrate and the enzyme final concentration was 0.02 U/ml (Table 2.3). BuChE was not inhibited by the presence of 80% ETOH that was employed for dilution of the plant extract. Reactant mixture was incubated for both 15 and 30 min.

Table 2.3 Volumes and concentrations of the reagents comprising the assay for inhibitory activity on BuChE

<i>Reagent</i>	<i>Assay concentration</i> ^a	<i>Assay volume</i>
Na-Phosphate Buffer PH 8	0.091 M	200 µl
DTNB in Na-Phosphate Buffer pH 7 with Sodium bicarbonate	0.27 mM 0.12 M	5 µl
BuChE	0.02 Units/ml ^b	5 µl
BuTChI in distilled water	0.5 mM	5 µl
Plant extract in 80% ethanol	0.5 mg/ml	5 µl
Total volume		220 µl

a. Concentration of reagents in the final reaction mixture according to which original solutions concentrations (stocks) were calculated.

b. Enzyme activity is expressed in units per ml of enzyme solution. By definition, one unit of enzyme will hydrolyse one µmole of substrate to acetate and choline per min at pH 8, 37°C.

2.12 Dose - response curves and equations of enzyme inhibition

Plant extracts were tested over a range of concentrations obtained *via* serial dilutions. Each concentration was replicated four times, on two individual plates. Mean values \pm SEM (n=4) from different plates were used for the construction of two separate dose – response curves. Adopting Prism graph pad (version 4) software, percentage of inhibition from the series of dilutions of the same plant extract was plotted against the logarithm of plant extract concentration (sigmoidal curve, or four parameters logistic equation) (see Appendix 2). The concentration value (mg/ml) where 50% inhibition was achieved represented the IC50 value of the corresponding inhibitor and is an indicative measure of the strength of inhibitory activity. The average of the two IC50 values determined for one plant extract was employed for further statistical analysis, as from each of the three experimental treatments four plant extracts were produced.

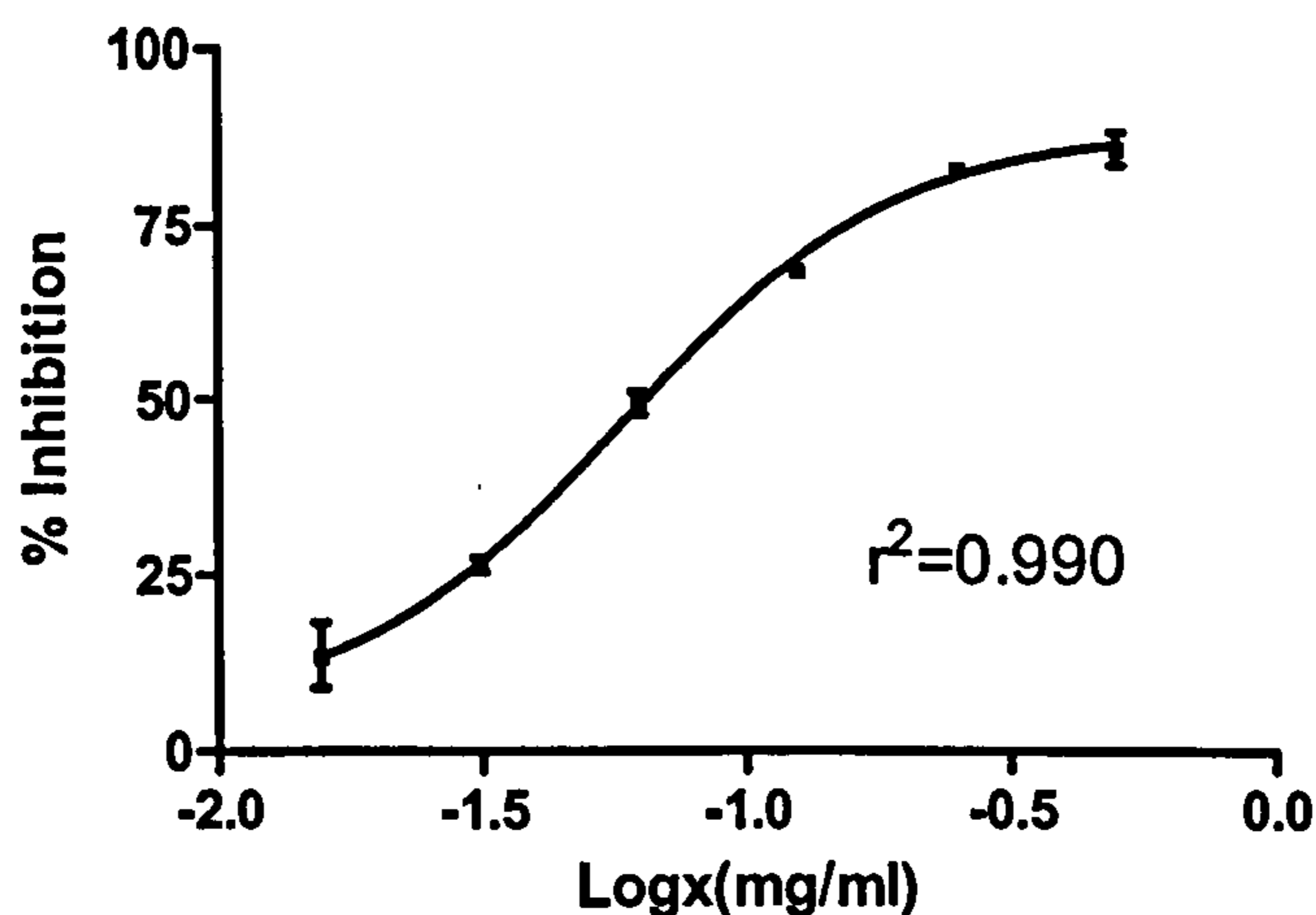


Figure 2.3 The typical dose-response curve presented is described by the general equation: $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{(\text{LogEC50} - X) * \text{HillSlope}})$

Four parameters are involved:

Min, or bottom of the curve (the baseline response)

Max, or top of the curve (the maximum response)

EC50: median effective concentration. That is, the concentration that provokes a response half way between the bottom and the maximum response of the curve

Hillslope: characterizes the slope of the curve at its midpoint.

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2.13 Kinetics for enzyme inhibition

Kinetic parameters of cholinesterase inhibition were determined for both acetyl- and butyryl- cholinesterase enzymes. Employing the Lineweaver – Burk plot (LB plot), Michaelis constant (K_m) and initial enzyme velocity (V_{max}) were directly determined from the intercepts of the regression line with the axes. K_m value is equal to $-1/S$ and V_{max} to $1/V$ where S and V are the intercepts on the abscissa and ordinate respectively (S stands for enzyme substrate and V denotes enzyme velocity) (Cornish-Bowden, 1995; see Appendix 3). Characterization of the type of inhibition was made according to Engel (1981) (Table 2.4).

Table 2.4 Changes in K_m and V_{max} with different type of inhibitions

K_m	V_{max}	Type of inhibition
↑	–	competitive
↑	↓	Non competitive (mixed)
–	↓	Non competitive (single)
↓	↓	uncompetitive

↑ denotes increase in the presence of the inhibitor

↓ denotes decrease in the presence of the inhibitor

– indicates no change in the presence of the inhibitor

Reagents from Ellman's method (paragraph 2.10.1 and 2.10.4) were incubated with two distinct concentrations of a cholinesterase inhibitor / plant extract i.e. 0.016 mg/ml and 0.031 mg/ml. The assay comprised a control, where the inhibitor was replaced by equal volumes of 53% or 80% ethanol and a substrate blank (buffer pH 8 substituted for the enzyme). The reaction was initiated by adding six different substrate concentrations (from 0.5 to 0.0156 mM) obtained *via* serial dilutions. Its concentration on the plate was replicated four times ($n=4$). The assay was repeated twice for every plant extract tested.

2.14 Experimental design and statistical analysis

Experiments from the present study are single-factor experiments as only one factor varies, while all others are kept constant. Experiment layout was based upon the completely randomized design (CRD) (Gomez & Gomez, 1984). According to CRD treatments in each experiment are randomly assigned so that each experimental unit has the same chance of receiving any one of the treatments. The present experiments comprised three treatments with four replicates / individual NFT sub-units in each treatment. For statistical analysis mean values deriving from all plants in the channel were employed as measurements on individual plants within the same sub-unit are not independent from each other. For the CRD, any difference among experimental units receiving the same treatment is considered as experimental error. CRD is only appropriate for experiments with homogeneous experimental conditions, where environmental factors are relatively easy to control (Gomez & Gomez, 1984) and has been used before for hydroponic experiments (Andrews *et al.*, 1999). Schematic layout of the hydroponic experiments is presented in Figure 2.4.

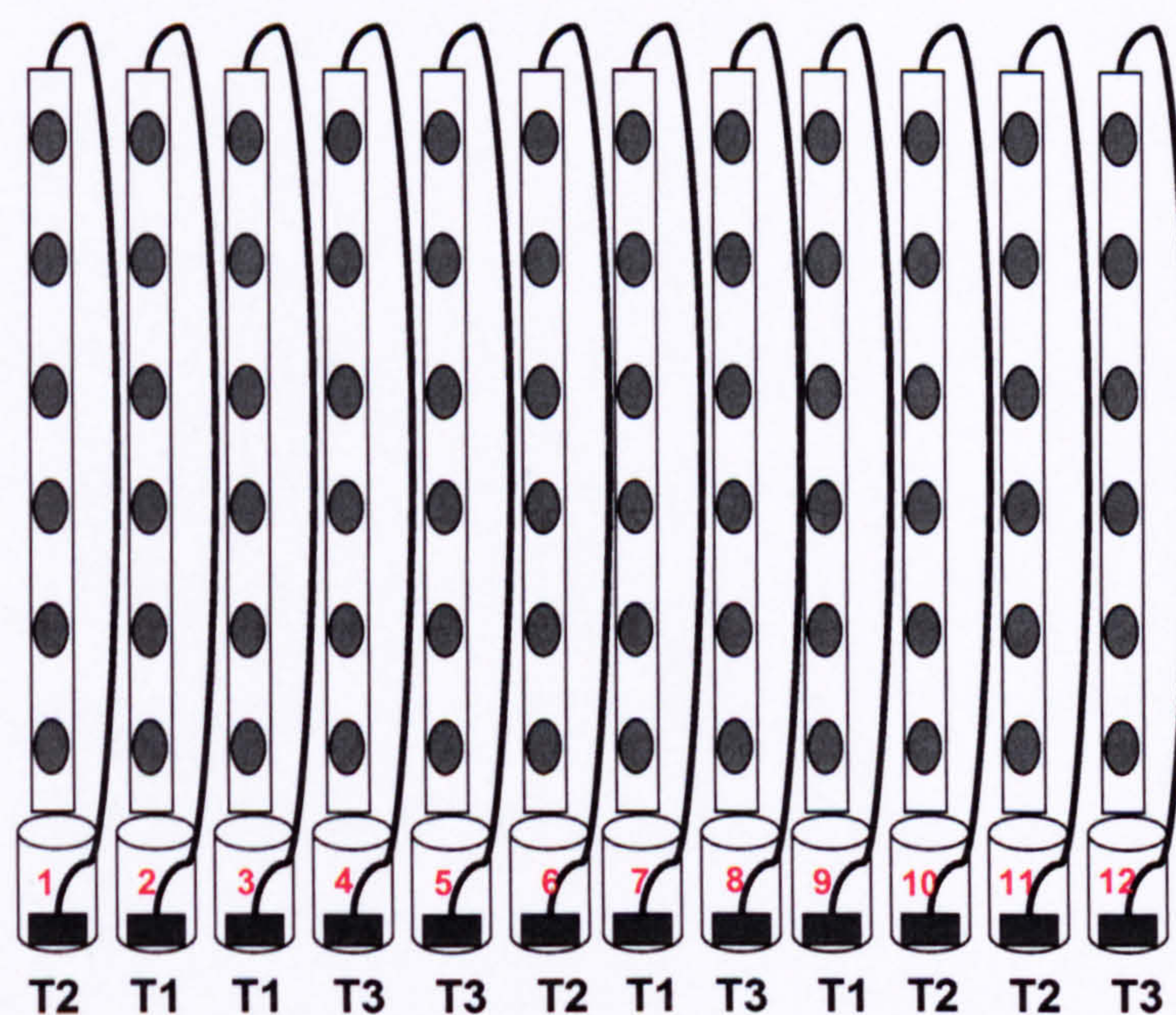


Figure 2.4 Layout of the hydroponic experiment, based on a complete randomised design (CRD; Gomez and Gomez, 1984). T1, T2 and T3 denoted the three different types of treatment in each experiment.

Data were tested for normality of distribution and homogeneous variance using Levene tests, prior to ANOVA. Significant differences between means were determined with the Tukey's Multiple Comparison Test at the 5% level. Regression analyses and Pearson product-moment correlation were used to evaluate the strength of the relationships. All statistical analyses were performed using SPSS version 12.0 (SPSS Inc., Chicago, USA) while graphs were constructed with the aid of SIGMA PLOT version 9 and Graph Pad Prism[®] version 4.

Chapter 3

Effects of phosphorus on medicinal properties and growth of *Salvia officinalis* var *purpurea* cultivated under hydroponic conditions.

3.1 Introduction

Salvia officinalis var *purpurea* (common purple sage) has been demonstrated *in vitro* (Savelev *et al.*, 2004) to possess inhibitory activity on the cholinesterase enzymes (ChEs) present in the human brain. To date cholinesterase inhibitors constitute the most effective approach to the treatment of Alzheimer's disease (AD) (Levy *et al.*, 1999; Jann, 2000; Coyle & Kershaw, 2001; Gruntzender & Morris, 2001), a progressive neurodegenerative disorder characterised by a deficit in the cholinergic brain system (see section 1.11.1). Whereas *Salvia lavandulaefolia* and *S. officinalis* cholinergic activities have been under investigation *in vitro* and *in vivo* for the last 10 years, the purple sage has been considered only once (see section 1.11.4). Savelev and co-workers (2004) indicated relatively potent inhibitory activity for acetyl-cholinesterase (AChE) and butyryl-cholinesterase (BuChE) enzyme, which recent studies strongly suggested that could be relevant for the symptomatic treatment of AD (see section 1.11.2).

Cholinergic properties of *Salvia* species have been attributed to essential oils (Perry *et al.*, 1996; Perry *et al.*, 2000, 2002, 2003; Savelev, *et al.*, 2003; Tildesley *et al.*, 2003; Savelev *et al.*, 2004 and Tildesley, *et al.*, 2005) constitutively produced by the secondary metabolism of sage plants. Manipulating therefore essential oil production, would be of major importance in terms of ChE inhibition. Numerous endeavours to enhance yield and quality of essential oils have employed the application of various nutrients, including phosphorus (P). Contrary to the well investigated impacts of P nutrition on plants primary metabolism the influence of P on secondary pathways has not been so extensively studied. Nevertheless, strong evidence exists for effects of P on secondary metabolites biosynthesis. Experimental outcomes have revealed P impacts on essential oil content and composition for various species. Foliar treatment with 10% diammonium phosphate increased essential oil content in *Mentha arvensis* by 19% (Ram *et al.*, 2003). P fertilizers combined with mycorrhizal inoculation for

improved P-acquisition enhanced oil yield of *Foeniculum vulgare* seeds by 78% (Kapoor *et al.*, 2004). Economakis and co-workers (2002) reported altered oil content and volatile constituents from *Origanum dictamnus* bracts and leaves under differing P levels in the nutrient solution (5, 30 and 60 mg/L) of a hydroponic cultivation. Moreover, soil and foliar application of P and nitrogen (N) affected essential oil composition of fennel in a preferential manner. Increased concentration of two phenylpropanoids was demonstrated at the expense of terpenes, by diverting a common precursor molecule (PEP) into the phenylpropanoids biosynthetic pathway (Khan *et al.*, 1999) (see general introduction). However, a number of other existing studies suggested no evidence for an effect of P on secondary metabolism deriving compounds. Economakis (1995) reported no variation in the oil content of *Salvia fruticosa* Mill. plants cultivated by means of hydroponics under three distinct P concentrations (17, 34 and 68 mg/L). In agreement with that, application of different P doses (0.5, 1, 10 and 30 mg/L) in the nutrient solution did not affect oil content of *Mentha x villosa* Huds (Ramos *et al.*, 2005). However, essential oils constitute terpene mixtures that derive from PEP and acetyl-CoA through the DOXP and mevalonic acid pathway (see general introduction) and ATP / NADPH are required for their biosynthesis. Thus inorganic P status of the plant can potentially affect SM production (Loomis & Corneau, 1972 in Kapoor 2004).

Phosphorus nutrition has a major impact on plant growth. The assimilated and highest oxidized form of phosphorus Pi (inorganic phosphate, PO_4^{-3}) is indispensable for important cell functions (Ticconi & Abel, 2004). Phosphate esters constitute intermediates in metabolic pathways of biosynthesis and catabolism, where energy transduction depends upon phosphorylated molecules and the energy-rich pyrophosphate bonds (PPi) (Marchener, 1995). Phosphorus regulates key enzyme activity (e.g. phosphorylation of PEP carboxylase in C3 and C4 plants) (Theodorou &

Plaxton, 1995). Moreover, Pi ensures the stability of nucleotides *via* its presence in the structure of these macromolecules and the stability of biomembranes that contain phospholipids (Abel *et al.*, 2002). Thus, availability of Pi has profound consequences for plant metabolism and growth.

Despite its abundance in the nature, the physiochemical properties of Pi make it hardly accessible for plants. Phosphorus is extremely insoluble in most soils as it forms salts with calcium and magnesium or complexes with iron and aluminium. Moreover a high proportion of Pi in the soil (80-90% in the rhizosphere) is bound to the organic matter and requires mineralization before being assimilated (Marchner 1995). In conventional agriculture, the problem of P deficiency is alleviated by costly fertilizer application that provides soluble phosphorus, readily utilized by plants. To cope with the common situation of inadequate Pi supply, plants have evolved sophisticated responses to maintain internal cytoplasmic Pi irrespective of external concentrations, while maximizing extracellular P acquisition (Raghothama & Karthikeyan, 2005). When Pi is insufficient, plant catabolism adjusts to minimize adenylate- and Pi-dependant reactions i.e. alternative pathways for glycolysis and mitochondrial electron transport (Theodorou & Plaxton, 1995). Activity of nucleases and phosphatases is induced in order to liberate and recycle Pi from nucleic acids and other intracellular phosphorous containing molecules (Ticconi & Abel, 2004, Abel *et al.*, 2002; Vance *et al.*, 2002). To conserve further Pi, phospholipid content of biomembranes is reduced *via* galacto- and sulfo-lipids replacement (Frentzen, 2004; Ticconi & Abel, 2004; Abel *et al.*, 2002; Vance *et al.*, 2002; Dörman & Benning, 2002). The predominant galacto-lipids of plant cell membranes *in lieu* of phospholipids probably represent plants evolutionary adaptation to tackle P environmental deficiencies (Frentzen, 2004; Dörman & Benning, 2002). Under P limitation, biochemical modification of the rhizosphere *via* induced synthesis and extrusion of intracellular products is associated with enhanced Pi acquisition from the soil. A set of phosphohydrolase enzymes like acid phosphatases,

secreted by the rooting system release Pi from organic substrates in the soil, whereas exudation of organic acids like citrate, malate, oxalate and protons facilitates chelation of Fe⁺³, Al⁺³ and Ca⁺² cations and liberates Pi from insoluble salts and other inorganic complexes (Raghothama & Karthikeyan, 2005; Ticconi & Abel, 2004; Abel *et al.*, 2002; Tarafdar & Claassen, 2003; Vance *et al.*, 2002; Marchner, 1995; Lynch, 1995).

The absorption of Pi from the growth medium and translocation to the shoot is mediated by a system of low and high affinity Pi transporters on the plasma membrane (Raghothama & Karthikeyan, 2005; Ticconi & Abel, 2004; Abel *et al.*, 2002; Bucher *et al.*, 2001; Smith *et al.*, 2003). The low affinity transport system is constitutively expressed in plants, while the high affinity is strongly enhanced under P starvation (Raghothama & Karthikeyan, 2005). Secretion of root exudes, which are responsible for the mineralization of P in the soil can be coupled with Pi starvation-inducible high-affinity Pi transporters, revealing a well coordinated mechanism of P regulation in plants (Abel *et al.*, 2002).

P deprivation is well known to exert profound changes in root morphology and architecture (Ticconi & Abel, 2004). Under P deficiency enhanced root hair proliferation and density have been reported (Raghothama & Karthikeyan, 2005; Ticconi & Abel, 2004; Abel *et al.*, 2002; Bate & Lynch, 2000; Lopez-Bucio, 2003; Ma *et al.*, 2001; Bucher, 2001). Inhibition of primary root growth and promotion of lateral root proliferation leads to the development of a shallower more branched rooting system that facilitates exploration of the top soil where Pi availability is higher (Raghothama & Karthikeyan, 2005; Lopez-Bucio *et al.*, 2003; Williamson *et al.*, 2001; Lynch 1995). Reduced shoot growth as a consequence of increased biomass allocation to the root under inadequate P supply is a typical plant response to P limitation (Raghothama & Karthikeyan, 2005; Ticconi & Abel, 2004; Abel *et al.*, 2002; Marchner 1995; Lynch, 1995). Finally, the species-specific formation of cluster-proteoid roots, main sites of

organic acid exudation constitutes another plant strategy to cope with low P availability (Shane *et al.*, 2004a and 2004b; Neumann *et al.*, 2000; Gilbert *et al.*, 2000).

Nutrient deficiency as well as other biotic and abiotic adverse environmental factors can alter pigment content (chlorophyll a and b and carotenoids) in plant tissue. Reduced pigment content can be an indication of stress condition in plants (Richardson *et al.*, 2002; Shevchenko *et al.*, 2004).

The need to define optimum P conditions for plant growth and development can be satisfied by nutrient uptake analysis. Measurement of P nutrient uptake *via* its depletion from the growth medium is feasible in solution cultures. Bar-Tal *et al.* (1995) reported that nutrient uptake is highly correlated to the nutrient content in plant tissues. Hydroponics constitutes a powerful tool for nutrient studies (Wilcox, 1982). Moreover, existing evidence confirms that herbs have been successfully cultivated by means of Nutrient Film Technique (NFT) (Wees & Stewart, 1986; Economakis, 1992, 1993a, 1993b, Economakis & Fournaraki, 1993; Economakis, 1995). Cultivation of herbs is a relatively new approach developed under the pressure of high quality controlled products but contrary to vegetable crops our knowledge of hydroponics application for herbs production is restricted (see general introduction).

Salvia officinalis var *purpurea* (purple sage) is an important medicinal and ornamental plant. Hydroponic or soil cultivation of *Salvia officinalis* var *purpurea* has never been studied before, while only one report exists regarding P effect on sage growth (*Salvia fruticosa*, Miller; Economakis, 1995). Hence, purple sage plants were hydroponically raised under various P levels with the view to increase essential oil content and quality. In parallel, defining optimum P supply for high herbage yields was attempted.

3.2 Materials and methods

3.2.1 Plant cultivation, nutrient experiment and environmental conditions

Young plants of *Salvia officinalis* var *purpurea* (purple sage) were obtained in March 2005 (section 2.1.1) for the purpose of hydroponic culture. Six plants were randomly assigned to individual NFT subunits and established in the channels at 40 cm intervals. Prior to their installation they had been subjected to root washing under a mild stream of water to achieve removal of the compost mixture from the root environment (see section 2.2.1 for NFT system). Air temperature, PAR, EC and pH of the nutrient solutions were recorded as described before (section 2.2).

S. officinalis var *purpurea* was cultivated under three different levels of phosphorus (P) i.e. 12, 34 and 60 mg/L. Each treatment was replicated four times (n = 4 individual NFT sub-units) (see section 2.11 for experimental design). The hydroponic recipe for stock solution preparation (g of fertilisers in 50 L of stock solution) and target elemental composition of nutrient solutions in catchment tanks are presented in Table 3.1. Stock solution contributed only for the 12 mg/L of P while its concentration was raised at 34 and 60 mg/L by adding 1:1 phosphoric acid to nitric acid (5%) and only phosphoric acid (5%) during pH adjustments. Nitric acid on its own was utilized in the treatment with the lower level of phosphorus (12 mg/L) for pH correction. Addition of nitric acid in the treatments with 12 and 34 mg/L P increased the levels of nitrates (N-NO₃) by 20 and 13 mg/L respectively in comparison with the 60 mg/L P treatment (Table 3.1). Target pH in all NFT sub units was 6 and EC 1.8 mS/cm. Plants were raised for a period of four months.

Actual 10 days mean pH and EC values of nutrient solutions in catchment tanks, before the correction with acid or stock solution are presented below (Figure 3.1 and 3.2). pH fluctuated between 6.3 and 7.2 while EC ranged from 1.54 to 1.86 mS/cm. Increases in pH of the nutrient solution are principally attributed to NO_3^- fixation into ammonia in plant roots, a process that releases OH^- in the medium (Cooper, 1979).

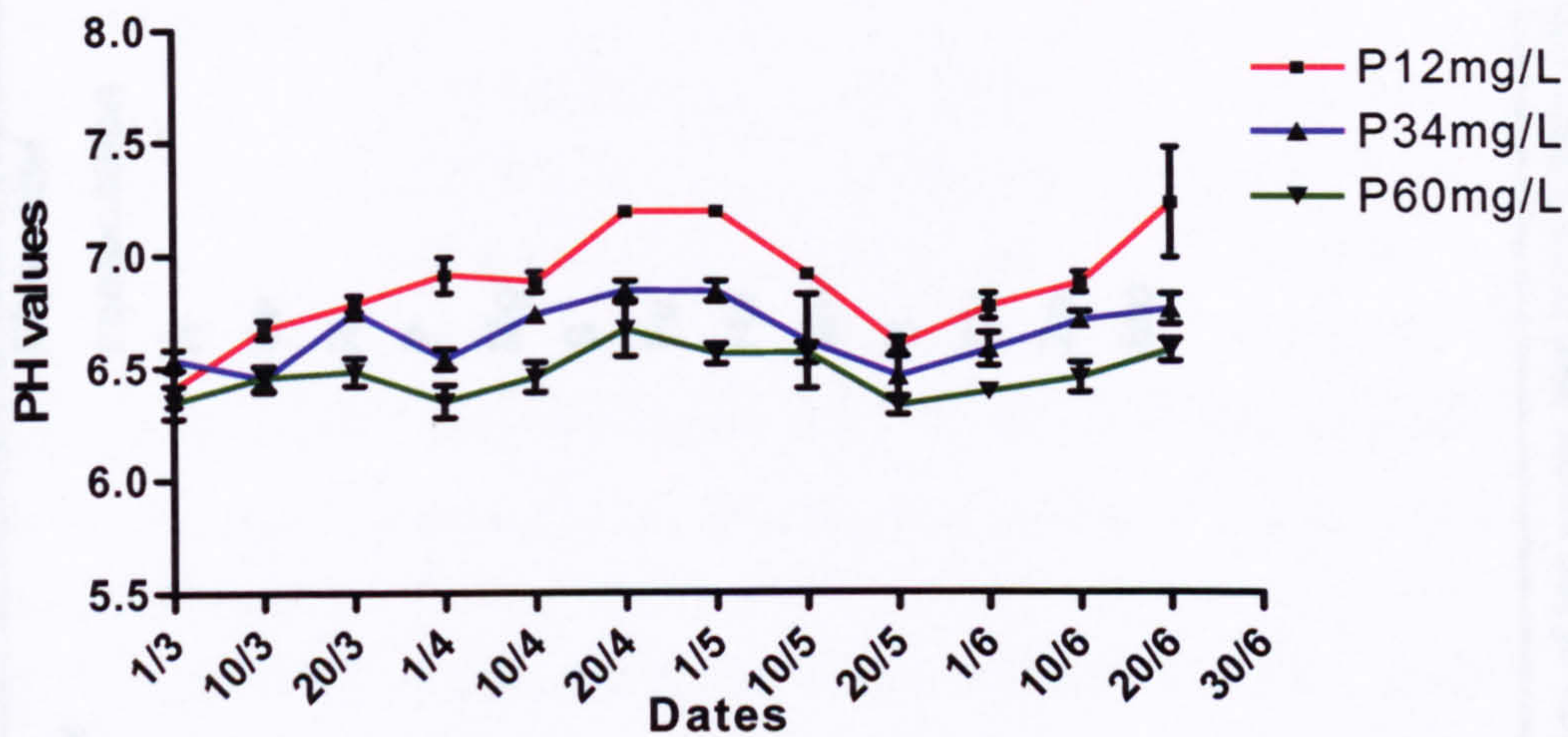


Figure 3.1 Average pH at ten day intervals for the three P treatments (12, 34 and 60 mg/L) during the cultivation period. Target pH was 6. Values represent the means and vertical bars indicate standard errors of the mean (n=4 NFT sub-units)

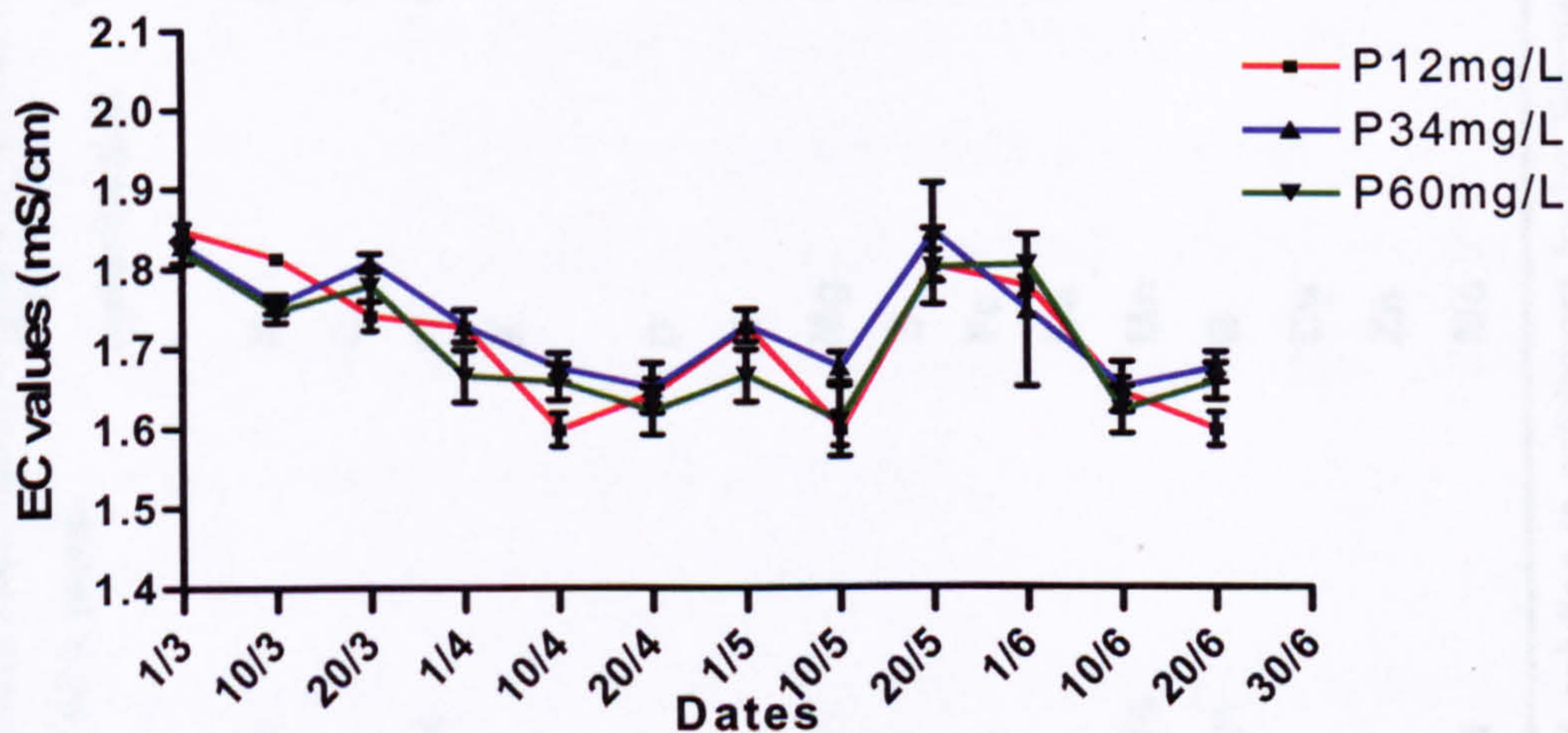


Figure 3.2 Mean EC at ten day intervals for the three P levels in the nutrient solution (12, 34 and 60 mg/L) during the cultivation period. Target EC was 1.8 mS/cm. Fluctuations are related to plant nutritional demands. Values represent the means and vertical bars the standard errors of the mean (n=4 NFT sub-units)

Table 3.1 Target composition of stock and nutrient solutions for phosphorus experiment

Chemical	g in 50 L stock	Elemental contribution	mg/L in stock	Elemental composition	Total mg/L in solution a
Stock A					
Ca(NO₃)₂*4H₂O	2371	N	6876.20	Ca	148.85
KNO₃	2564	Ca	8977.00	K	213.68
		N	6923.84	P	13.67
		K	19668.83	Mg	55.18
Stock B					
KH₂PO₄	301	P	1366.64	Fe	8.40
		K	1699.42	Na	15.63
MgSO₄	2555	Mg	4929.63	Mn	1.48
		S	6663.33	B	0.43
EDTA FeNa	323	Fe	840.00	Cu	0.10
		Na	665.00	Zn	0.09
MnSO₄*H₂O	22.75	Mn	148.00	Mo	0.07
H₃BO₃	12.01	B	42.00		
CuSO₄*5H₂O	2	Cu	10.00		
ZnSO₄*7H₂O	2	Zn	9.00		
(NH₄)₆Mo₇O₂₄	0.64	Mo	7.00		

^a Final composition of nutrient solution in catchment tanks. Elemental contribution of tap water has been taken into account (Appendix 1, section 1.2)

Actual concentrations of N-NO₃, P, potassium (K), and calcium (Ca) of the nutrient solutions and their fluctuations determined at the beginning of the experiment, are presented in Table 3.2.

Table 3.2 Actual concentrations of elements N, P, K, and Ca in nutrient solutions

Treatment	P14 mg/L±SEM	P32 mg/L±SEM	P 60mg/L±SEM	P
Element (mg/L)^d				
N	141 ± 0.82a	128 ± 0.6b	121 ± 0.5c	P<0.000
K	175 ± 2.0a	171 ± 1.8a	173 ± 1.7a	P>0.5
P	12 ± 1.2a	34 ± 1.5a	59 ± 2.0a	P<0.000
Ca	123 ± 5.5a	124 ± 7.8b	123 ± 7.8c	P>0.5

Values within rows followed by the same letter are not significantly different

^d N was analysed by means of UV spectroscopy while K, P and Ca concentrations were determined via ICP.

Values represent the mean and standard error of the mean (SEM), calculated from four replicates (n=4 NFT sub-units)

Total stock solution and 5% acid consumption (HNO₃ for P 12mg/L, 1:1 HNO₃:H₃PO₄ for P 34 mg/L and H₃PO₄ for P 60 mg/L treatment) are illustrated in Table 3.3 One way ANOVA revealed significant differences (P<0.001) among treatments in acid consumption. Stock solution uptake was higher in P 60 mg/L treatment but the increase was not statistically significant (P>0.05).

Table 3.3 Total acid and stock solution consumption by sage plants during the cultivation period

Treatment	P 14 mg/L	P 32 mg/L	P 60 mg/L
Consumption			
Acid total (ml) ± SEM	672.5 ± 2.5 a	525.3 ± 22.1 b	506.3 ± 12.5 b
Stock solution total (ml) ± SEM	1205 ± 17.1 a	1293 ± 61.2 a	1413 ± 77.6 a

Values within rows not followed by the same letter are significantly different (P<0.001)

Standard error of the mean (SEM) was calculated from n=4 NFT sub-units

The average min and max air temperature ranged from 16 to 20 and 26 to 38 °C respectively during the four months of cultivation (Figure 3.3).

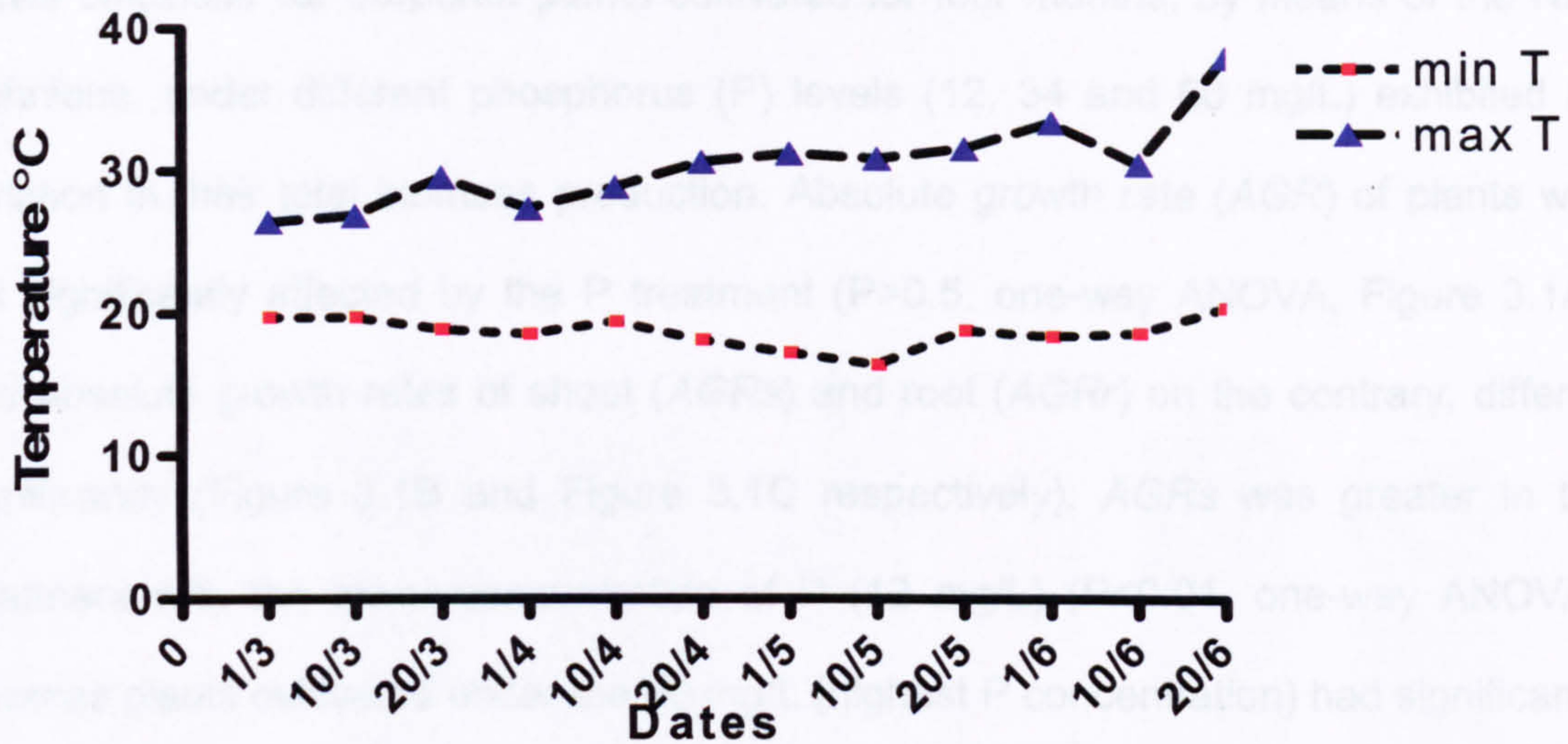


Figure 3.3 Average values at 10 day intervals for min and max air temperature during the cultivation period.

3.3 Results

3.3.1 Growth response to phosphorus levels

Salvia officinalis var *purpurea* plants cultivated for four months, by means of the NFT technique, under different phosphorus (P) levels (12, 34 and 60 mg/L) exhibited no variation in their total biomass production. Absolute growth rate (AGR) of plants was not significantly affected by the P treatment ($P > 0.5$, one-way ANOVA, Figure 3.1A). The absolute growth rates of shoot (AGRs) and root (AGR_r) on the contrary, differed significantly (Figure 3.1B and Figure 3.1C respectively). AGRs was greater in the treatment with the lower concentration of P (12 mg/L) ($P < 0.01$, one-way ANOVA), whereas plants cultivated under the 60 mg/L (highest P concentration) had significantly increased AGR_r ($P < 0.001$, one-way ANOVA). The different trend in biomass allocation of plants as influenced by P supply was reflected by the root to shoot allometric coefficient (R_{Sco}), being significantly greater at the 60 and 34 mg/L of P in comparison with the lowest P concentration i.e. 40% ($P < 0.001$) and 30% increase ($P < 0.01$) respectively (Figure 3.1D). As R_{Sco} did not follow a normal distribution (Anderson-Darlington test, $P < 0.05$) one-way analysis of variance and multiple comparison Tukey's test was performed with the ln-transformed values. Original mean values are presented in Figure 3.1D without the error bars.

The preferential biomass partitioning to the shoots at 12 mg/L P and to the roots at the higher P levels, at a constant total plant biomass are clearly illustrated by the absolute dry weight of plant (DWP) ($P > 0.05$, one-way ANOVA, Figure, 3.2C), dry weight of shoots (DWS) ($P < 0.01$, Figure 3.2A, one-way ANOVA) and roots (DWR) ($P < 0.001$, one-way ANOVA, Figure 3.2B). Irrespective of treatment all *Salvia* plants cultivated for four months in the NFT had well-sized shoots, the commercially relevant part of sage as indicated by their fresh weight, although the 12 mg/L P treatment produced plants with enhanced shoot fresh biomass (FWS, Figure 3.3A). FWS ranged from 436 ± 55 g

to 615 ± 70 g (average lowest and highest replicate value \pm SEM). Plant height was significantly affected by the level of P ($P < 0.01$, one-way ANOVA) with plants being taller at the lowest P concentration (Figure 3.3B) where enhanced shoot growth occurred.

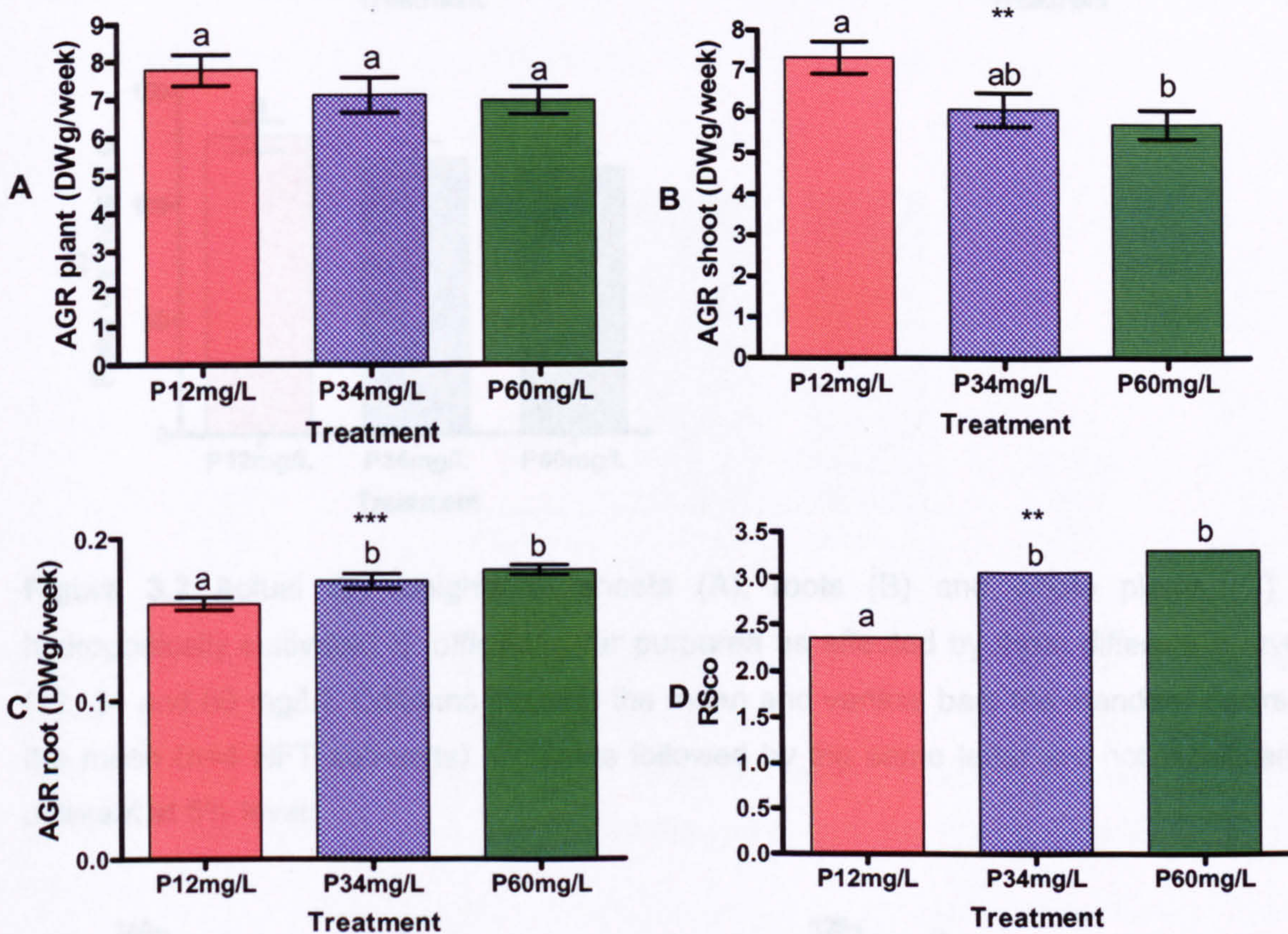


Figure 3.1 Impact of P nutrition on AGR of whole plant (A), shoot (B), root (C) and RScO (D) for *S. officinalis* var *purpurea* plants cultivated hydroponically under three levels of P (12, 34 and 60 mg/L). Columns indicate the mean and vertical bars the standard errors of the mean (n=4 NFT sub units). Columns followed by the same letter are not significantly different at 5% level. Asterisks denote level of significance ** $P < 0.01$, *** $P < 0.001$.

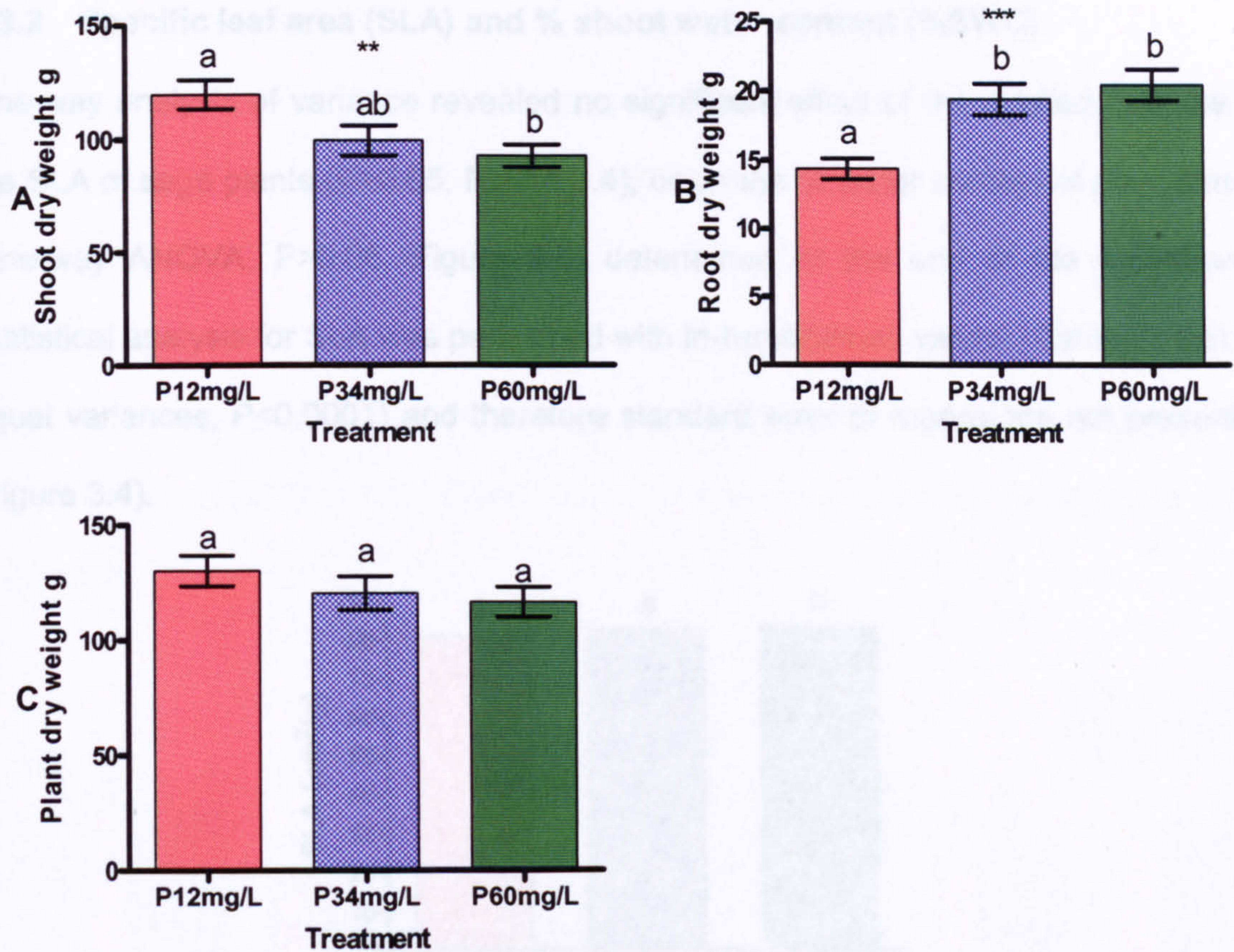


Figure 3.2 Actual dry weights of shoots (A), roots (B) and whole plants (C) of hydroponically cultivated *S. officinalis* var *purpurea* as affected by three different P levels (12, 34 and 60 mg/L). Columns indicate the mean and vertical bars the standard errors of the mean (n=4 NFT sub-units). Columns followed by the same letter are not significantly different at 5% level.

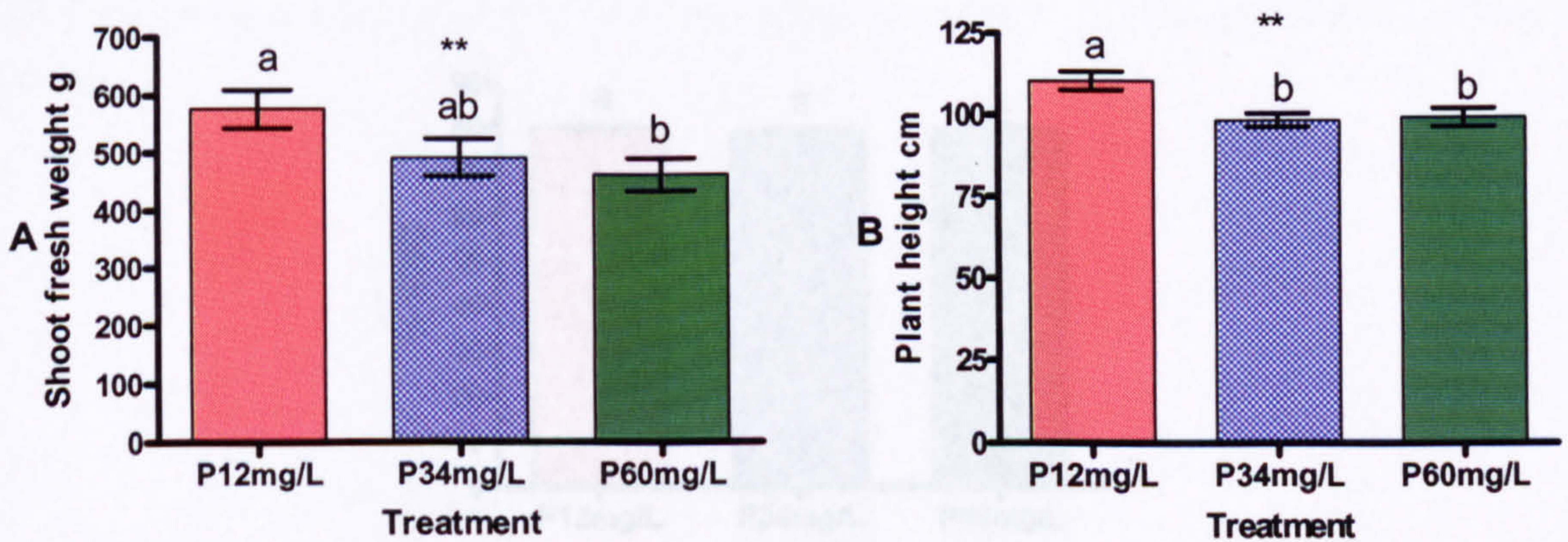


Figure 3.3 Shoot fresh weight (A) and plant height (B) of *S. officinalis* var *purpurea* plants cultivated hydroponically under three levels of P (12, 34 and 60 mg/L). Columns indicate the mean and vertical bars the standard errors of the mean (n=4 NFT sub-units). Columns followed by the same letter are not significantly different at 5% level. Asterisks denote level of significance ** P<0.01, *** P<0.001.

3.3.2 Specific leaf area (SLA) and % shoot water content (%SWC)

One-way analysis of variance revealed no significant effect of the applied P levels on the SLA of sage plants ($P > 0.05$, Figure 3.4), or on the % water content of plant shoots (one-way ANOVA, $P > 0.05$, Figure 3.5) determined at the end of the experiment. Statistical analysis for SLA was performed with In-transformed values (Bartlett's test for equal variances, $P < 0.0001$) and therefore standard error of means are not presented (Figure 3.4).

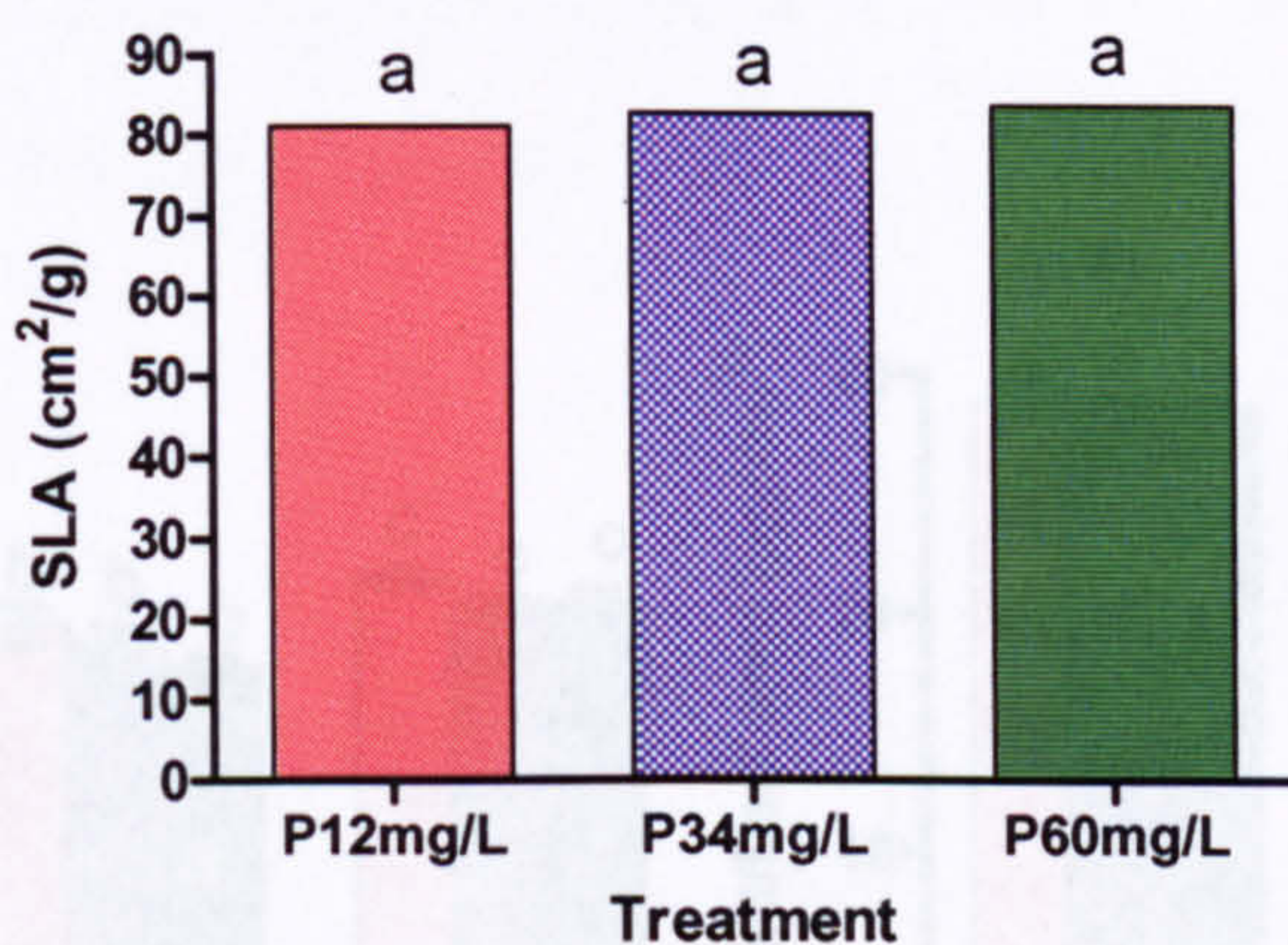


Figure 3.4 Effect of P nutrition on specific leaf area (SLA) of *S. officinalis* var *purpurea* plants grown hydroponically under different P supply (12, 34 and 60 mg/L). SLA was determined via the youngest pair of fully expanded leaves. Columns represent the mean ($n=4$ NFT sub-units). Columns followed by the same letter are not significantly different at 5% level.

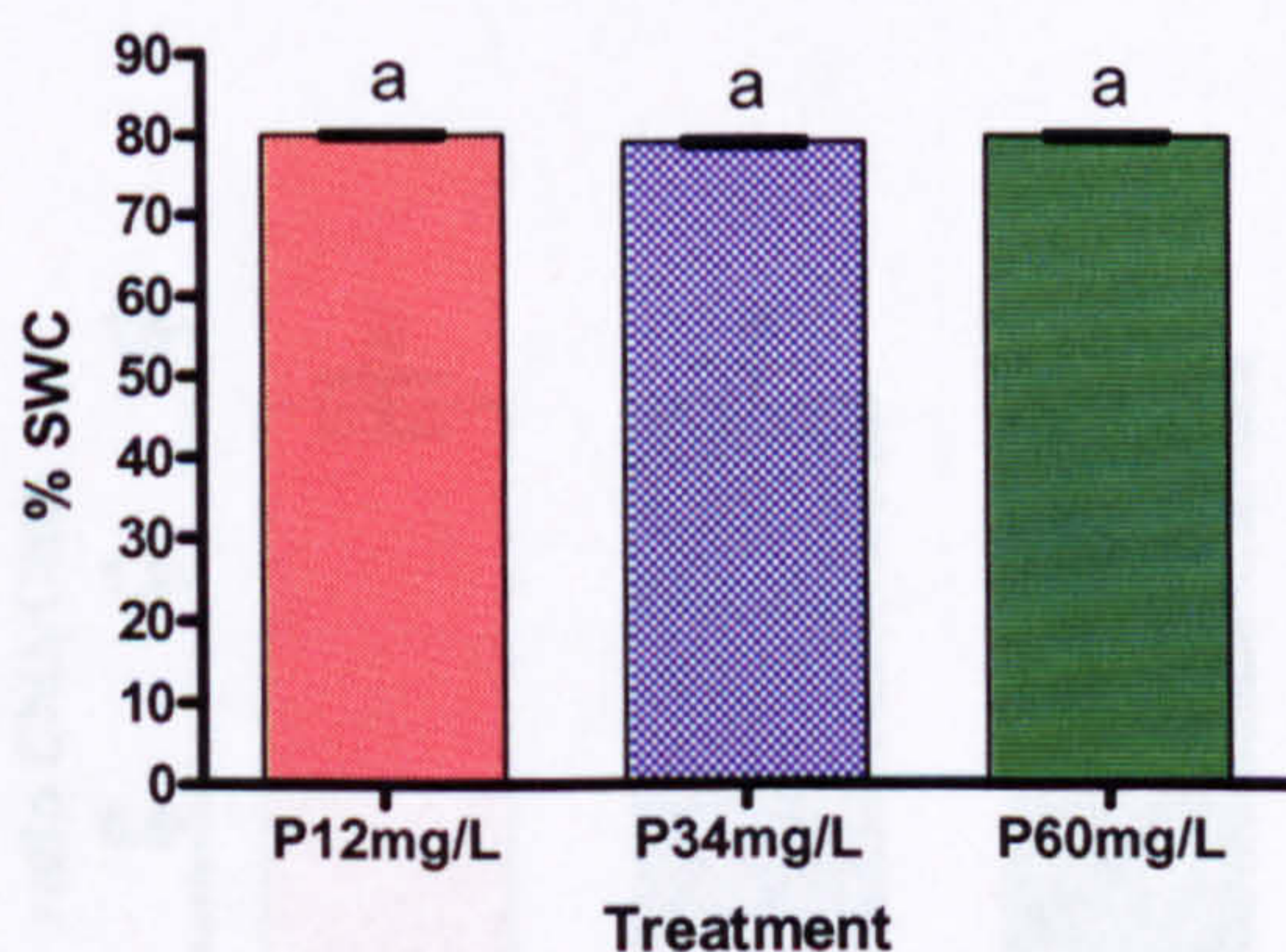


Figure 3.5 % Shoot water content of *S. officinalis* var *purpurea* plants grown under three levels of P (12, 34 and 60 mg/L). Columns represent the mean and vertical bars indicate standard errors of the mean ($n=4$ NFT sub-units). Columns followed by the same letter are not significantly different at 5% level.

3.3.3 Leaf pigments

Chlorophyll a (Chla) and b (Chlb) and total carotenoid content of young fully expanded sage leaves was measured one day before harvest. Amount of pigments (μg) was expressed in relation to fresh weight of leaf (Figure 3.6A) and the leaf area (Figure 3.6B). Growth under different levels of P resulted in no significant differences in Chla, Chlb and total carotenoids as revealed by one-way analysis of variance ($P>0.05$). The ratio of Chla to Chlb (ratio Chla/Chlb) was hence maintained under different P treatments.

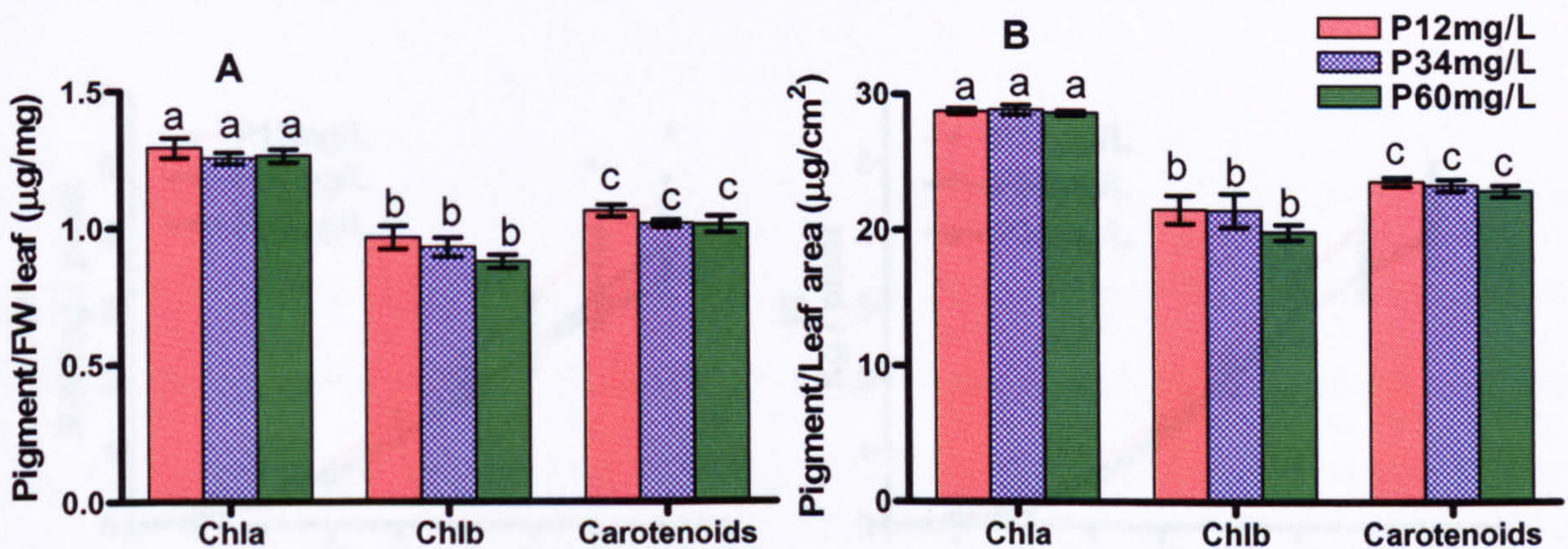


Figure 3.6 Chla, Chlb and total carotenoid content of fresh leaves expressed per leaf fresh weight (A) or leaf area (B) of *S. officinalis* var *purpurea* plants cultivated under three levels of P (12, 34 and 60 mg/L). Vertical bars indicate standard errors of the mean (n=4). Columns within a group of three, followed by the same letter are not significantly different 5% level.

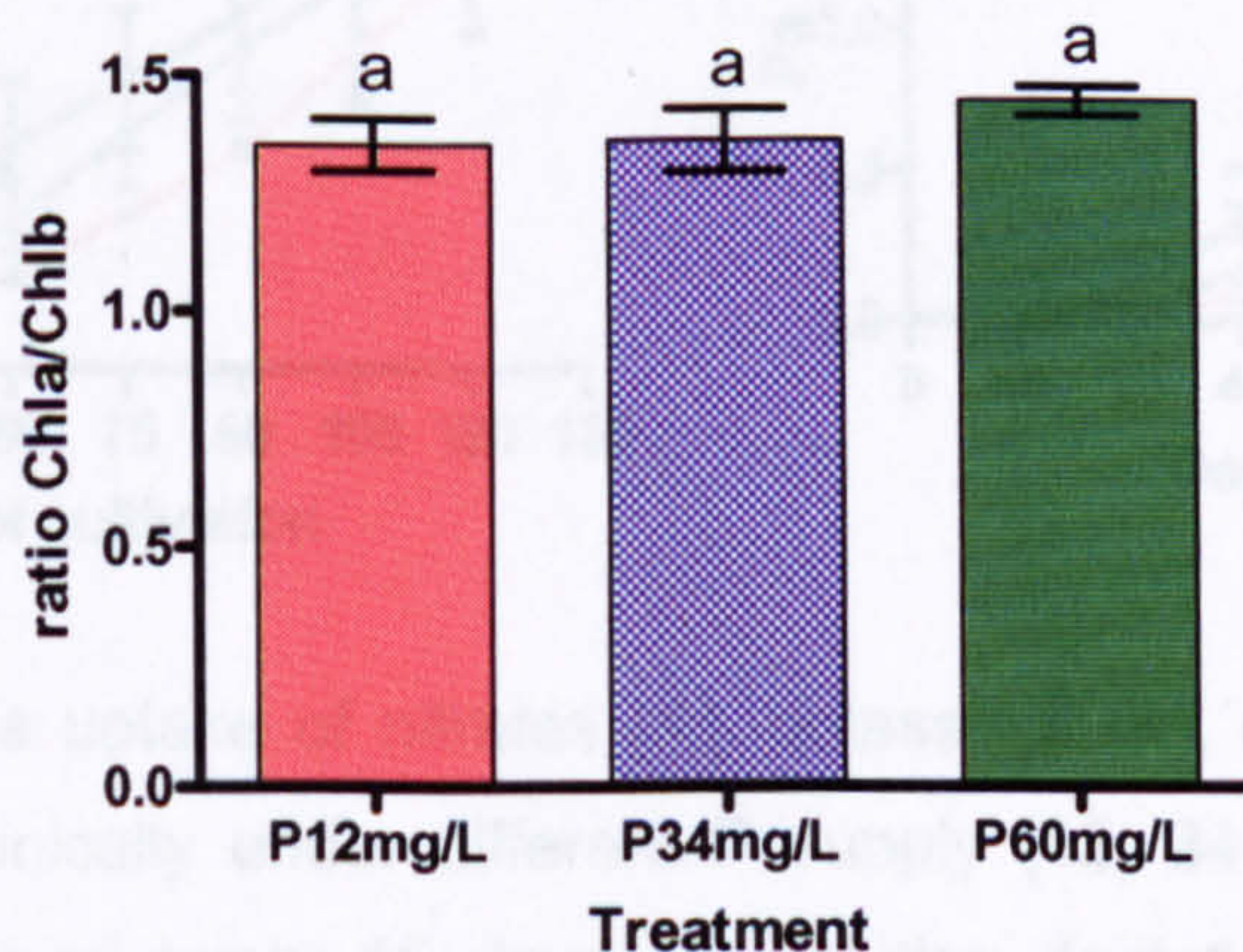


Figure 3.7 Ratio of Chla to Chlb under three levels of P (12, 34 and 60 mg/L). Vertical bars indicate standard error of the mean (n=4). Columns followed by the same letter are not significantly different at 5% level.

3.3.4 Nutrient uptake

The uptake of nitrates (N-NO₃), P, potassium (K) and calcium (Ca) was determined by depletion of the corresponding nutrient from the solution culture throughout the hydroponic cultivation with a 15 day interval. Cumulative uptake of the above elements and water are presented in Figure 3.8 (A, B, C, D) and Figure 3.9 respectively. The last measure on each graph (120 days) represents the mean total uptake per plant (in g) of the corresponding nutrient for the duration of the experiment. As nutrient depletion studies and plant tissue analysis have good correlation, nutrient uptake by solution analysis solely is justified (Bar-Tal *et al*, 1995).

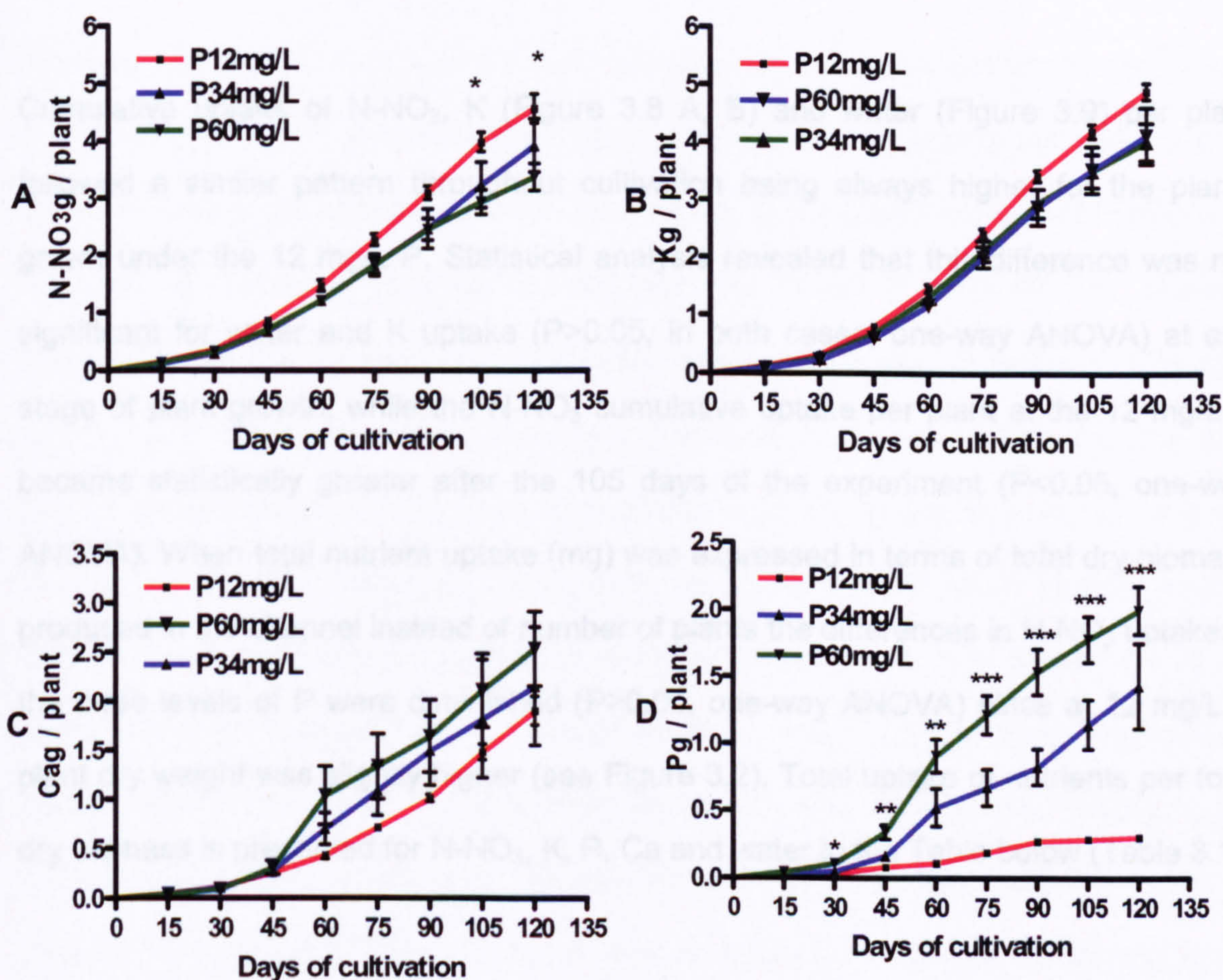


Figure 3.8 Cumulative uptake of nitrates (A), potassium (K), calcium (Ca) and P (D) of plants grown hydroponically under different P supply (12, 34 and 60 mg/L). Uptake of nutrients was determined every 15 days *via* solution depletion studies. The 120 days measure corresponds to the total nutrient uptake of the relevant element. Vertical bars indicate standard errors of the mean (n=4 NFT sub-units). Asterisks denote level of significance * P<0.05, ** P<0.01, *** P<0.001.

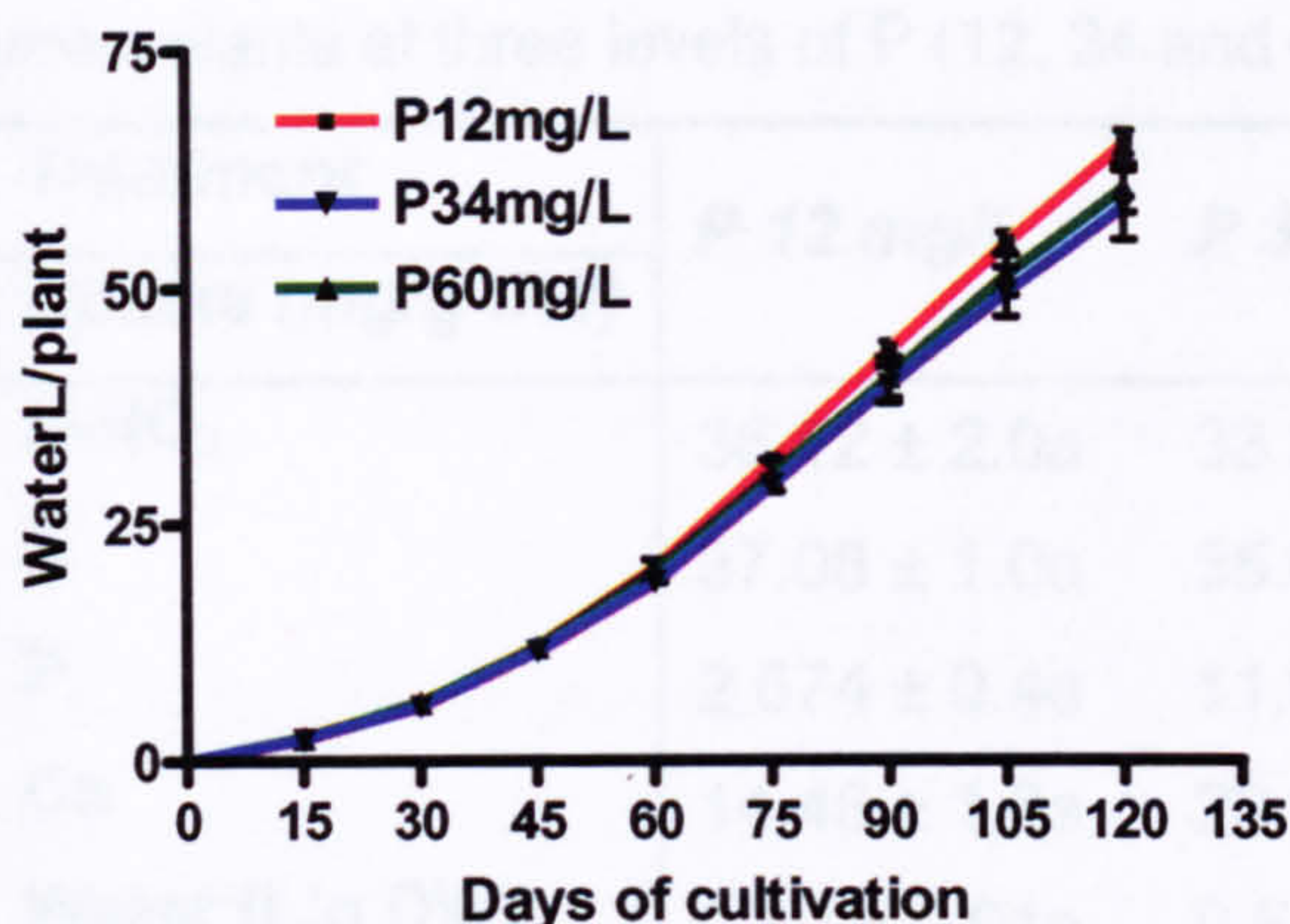


Figure 3.9 Cumulative uptake of water from hydroponically cultivated plants under three levels of P (12, 34 and 60 mg/L). The 120 days measure corresponds to the total water uptake. Vertical bars indicate standard errors of the mean (n=4 NFT sub-units).

Cumulative uptake of N-NO₃, K (Figure 3.8 A, B) and water (Figure 3.9) per plant followed a similar pattern throughout cultivation being always higher for the plants grown under the 12 mg/L P. Statistical analysis revealed that this difference was not significant for water and K uptake ($P > 0.05$, in both cases, one-way ANOVA) at any stage of plant growth, while the N-NO₃ cumulative uptake per plant at the 12 mg/L P became statistically greater after the 105 days of the experiment ($P < 0.05$, one-way ANOVA). When total nutrient uptake (mg) was expressed in terms of total dry biomass produced in the channel instead of number of plants the differences in N-NO₃ uptake at the three levels of P were diminished ($P > 0.05$, one-way ANOVA) since at 12 mg/L P plant dry weight was slightly higher (see Figure 3.2). Total uptake of nutrients per total dry biomass is presented for N-NO₃, K, P, Ca and water in the Table below (Table 3.1).

Table 3.1 Total uptake of channel (mg) per total plant dry biomass (g) of hydroponically grown plants at three levels of P (12, 34 and 60 mg/L)

<i>Treatment</i>	<i>P 12 mg/L</i>	<i>P 34 mg/L</i>	<i>P 60 mg/L</i>	<i>P</i>
<i>Uptake (mg/g DW)</i>				
N-NO₃	36.12 ± 2.0a	33.31 ± 2.2a	29.21 ± 1.4a	P>0.5
K	37.08 ± 1.0a	35.08 ± 1.9a	35.00 ± 2.2a	P>0.5
P	2.674 ± 0.4a	11.95 ± 1.5b	17.36 ± 1.1c	P<0.0001
Ca	14.48 ± 1.3a	23.92 ± 4.7a	21.92 ± 2.6a	P>0.5
Water (L/g DW)	0.50 ± 0.01a	0.51 ± 0.02a	0.52 ± 0.03a	P>0.5

Values represent the mean ± standard error (n=4 NFT sub-units). Values within a row followed by the same letter are not significantly different. P indicates the level of significance.

The opposite trend to N-NO₃ and K cumulative uptake per plant was shown for Ca and P (Figure 3.8C and D respectively). P uptake by plants significantly increased with P concentration in the nutrient solution. The impact of P supply was already obvious at 15 days of cultivation between the highest and lowest P level (P<0.5, one-way ANOVA), while after 75 days of the experiment there was a highly significant difference among all P treatments (P<0.001, one-way analysis of variance). The disparity in P uptake became even higher when total uptake was expressed in terms of total dry biomass (P<0.0001, Table 3.1), as plants under 60 mg/L P were slightly smaller (Figure 3.2). Cumulative Ca uptake, in a similar manner to P, was greater for plants grown under the highest P level, intermediate at 32 mg/L P and lower for the lowest P concentration. This order of cumulative Ca uptake was maintained throughout the cultivation. Analysis of variance though showed no significant difference among treatments at all growth stages, which was probably due to a large variation in Ca uptake within the treatment.

Employing the total uptake per channel at the end of the experiment the ratio of N-NO₃ to the rest of the nutrients was calculated (Table 3.2). As values did not comply with the assumptions of analysis of variance log-transformation was necessary. One-way

ANOVA of the transformed values revealed that the ratio of N to K (N/K) was considerably higher at 12 and 34 mg/L of P, the N to Ca ratio (N/Ca) was increased only at the 12 mg/L P, while under the 34 and 60 mg/L of P plants had 4-fold and 7-fold lower N to P ratio (N/P) than those cultivated with the 12 mg/L P concentration.

Table 3.2 Total uptake ratio of N-NO₃ to K, P and Ca of hydroponically grown plants under three levels of P (12, 34 and 60 mg/L).

Treatment	P 12 mg/L	P 34 mg/L	P 60 mg/L	P
Uptake ratio				
N/K	0.9721a	0.9484a	0.8369c	P<0.01
N/P	12.25a	2.857b	1.686c	P<0.001
N/Ca	2.524a	1.499b	1.365b	P<0.01

Mean uptake values are presented (n=4). Values within a row not followed by the same letter are significantly different. According to Tukey's Multiple Comparison Test all differences between 12 and 60 mg/L P were significant at 0.01 level and any other combination at 0.5.

When Pearson product –moment correlation was performed significant relationships among nutrients uptake became evident. Correlation analysis between N-NO₃ total uptake per plant and K, revealed a strong positive relationship among the nutrients ($r=0.962$, Table 3.3). Significant negative relationships were exhibited between P and N-NO₃ ($r=-0.829$) or K uptake ($r=-0.868$) (Table 3.3,). On the other hand, no relationship was established for Ca and N-NO₃, K or P as illustrated by the very low r values (Table 3.3). Finally, correlation analysis demonstrated robust relationships between the applied P treatment (12, 34 and 60 mg/L) and total N-NO₃, K and P uptake. Especially in the case of phosphorus a highly significant positive correlation with the treatment was indicated by an $r=0.956$

Table 3.3 Correlation matrix illustrating relationships between total uptake of different nutrients (N-NO₃, P, K and Ca) or between applied P treatment and nutrients uptake of *S. officinalis* var *purpurea* plants grown hydroponically under three P levels (12, 34 and 60 mg/L).

x	N-NO ₃	P	K	Ca	P supply
y					
N-NO ₃		r=-0.829 P<0.01	r= 0.962 P<0.000	r= 0.217 P> 0.05	r= -0.820 P<0.01
P	r=-0.829 P<0.01		r= -0.868 P<0.01	r= 0.663 P>0.05	r= 0.956 P<0.000
K	r= 0.962 P<0.000	r=-0.868 P<0.01		r= 0.221 P> 0.05	r= -0.853 P< 0.01
Ca	r= 0.217 P> 0.05	r= 0.663 P>0.05	r= 0.221 P> 0.05		r= 0.475 P>0.05

r represents the Pearson product –moment correlation

P value indicates significance of the correlation

x, y represents the nutrient uptake variables of the correlation Number of x, y pairs tested was n=12 NFT sub-units / replicates

P supply is the concentration of phosphorus in the nutrient solutions (12, 34 and 60 mg/L)

3.3.5 Nutrient uptake and growth parameters

Dry biomass production of shoots (*DWS*) and roots (*DWR*) was significantly associated with total nutrient uptake of P and N-NO₃ as demonstrated by regression analysis. N-NO₃ uptake explained 77% in the variation of shoot growth ($r^2=0.77$, $P<0.001$, Figure 3.11A). The curve-linear relationship of N-NO₃ with the root appeared less strong ($r^2=0.58$, $P<0.5$, Figure 3.11B). A positive significant association was revealed between total P uptake and root dry weight; P was responsible for the 76% of the increase in *DWR* ($r^2=0.76$, $P<0.001$, Figure 3.11C). A non-linear regression best described the relationship of *DWS* versus P total uptake. *DWS* decreased with increasing P uptake up to 2 g per plant and then slightly increased at higher values of P uptake ($r^2=0.75$, $P<0.01$, Figure 3.11D). Finally, significant relationships were established between K uptake and *DWS* and *DWR* (Figure 3.11E and 3.11F respectively).

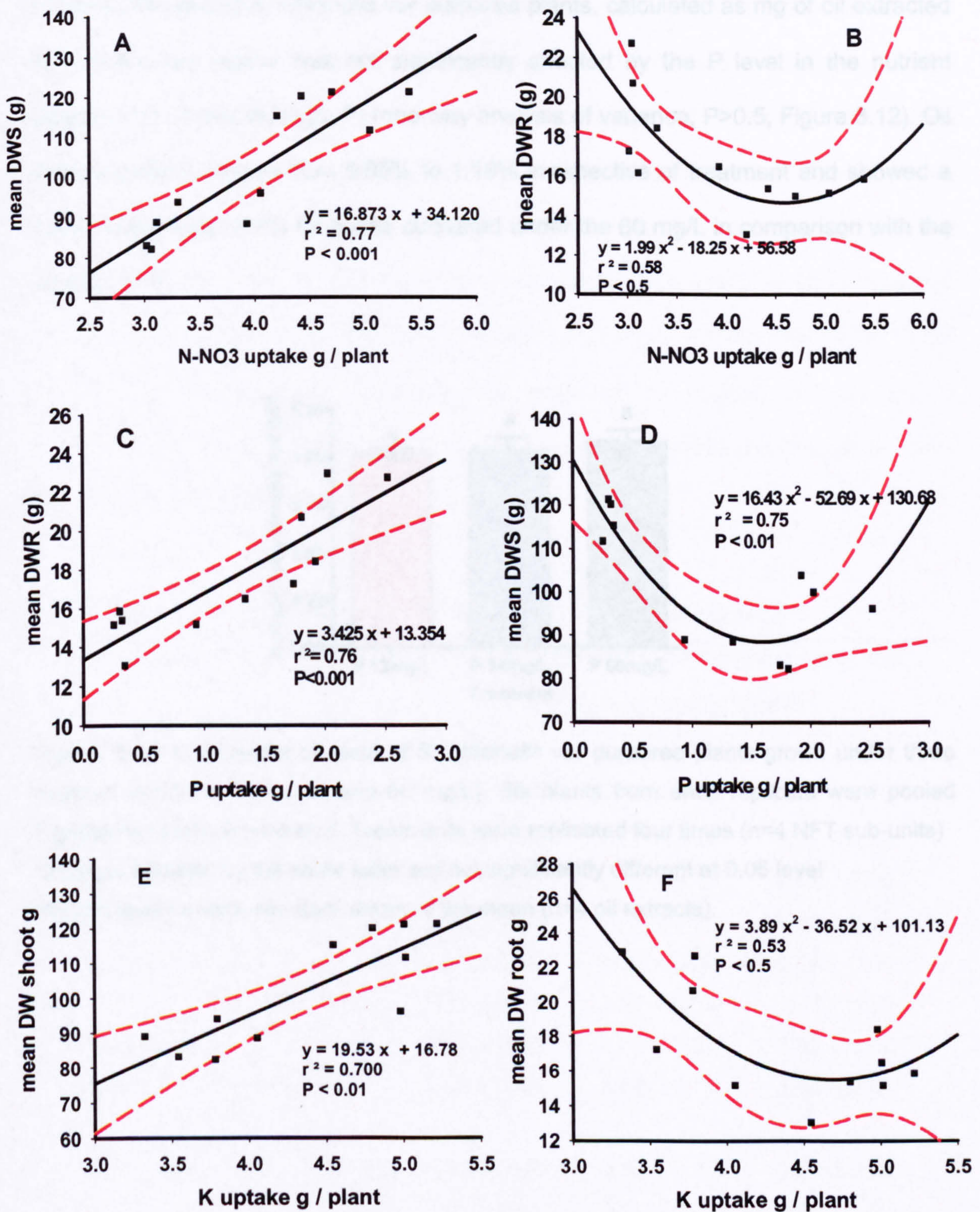


Figure 3.11 Regression analysis of N-NO₃ uptake with shoot growth (*DWS*) (A), root growth (*DWR*) (B); P uptake with *DWR* (C) and *DWS* (D) and finally K uptake and *DWS* (E) and *DWR* (F). Red dotted lines represent the 95% confidence band of the regression. r^2 indicates goodness of fit of the slope for linear and quadratic regressions. P value indicates significance of slope's deviation from zero

3.3.6 Essential oil yield and composition of *Salvia officinalis* var *purpurea*

Essential oil yield of *S. officinalis* var *purpurea* plants, calculated as mg of oil extracted from 100 g dry leaves was not significantly affected by the P level in the nutrient solution (12, 34 and 60 mg/L P) (one-way analysis of variance, $P > 0.5$, Figure 3.12). Oil yield in general ranged from 0.95% to 1.18% irrespective of treatment and showed a marginal increase of 7% for plants cultivated under the 60 mg/L in comparison with the 12 mg/L of P.

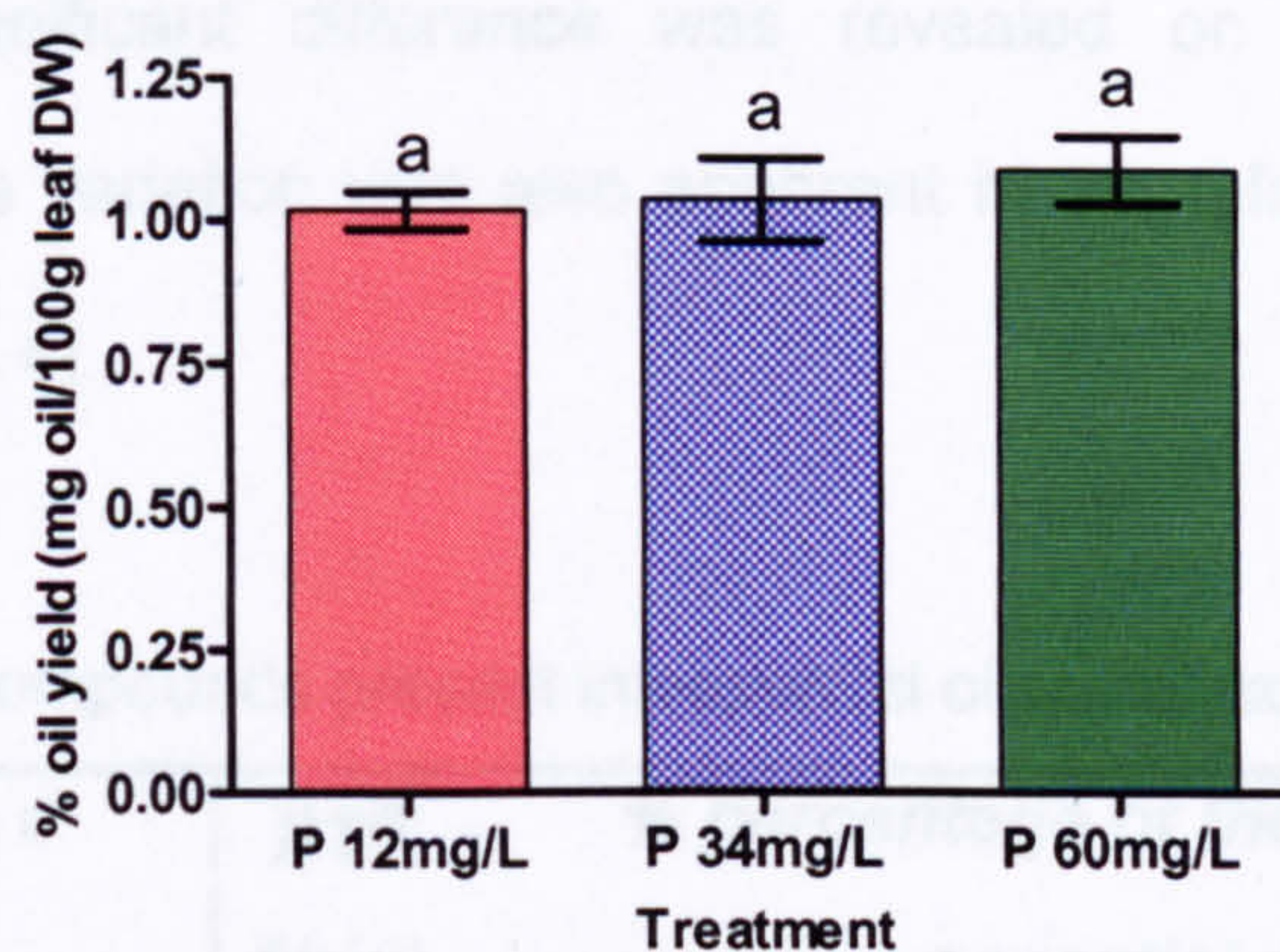


Figure 3.12 % Essential oil yield of *S. officinalis* var *purpurea* plants grown under three different levels of P (12, 34 and 60 mg/L). Six plants from each replicate were pooled together for each oil produced. Treatments were replicated four times (n=4 NFT sub-units) Columns followed by the same letter are not significantly different at 0.05 level Vertical bars indicate standard errors of the mean (n=4 oil extracts).

GC/MS analysis revealed 37 compounds present in the oils of all treatments out of which 25 were identified (Table 3.4). Camphor and α -caryophyllene were abundant in the oil comprising 17% and 19% respectively of the oil mixture. α -Thujone the third major compound contributed for 9% of the oil while the rest of the constituents like α and β pinene, 1,8 cineole, ledol, borneol and bornyl acetate appeared with concentrations less than 4% in the mixture. Phosphorus treatment did not have any major effect on the quality of the oil as demonstrated by one-way analysis of variance. Camphor content was slightly enhanced under the 12 mg/L of P from 16.1% to 18.5% ($P < 0.5$) but no significant difference was revealed on thujone isomers and α caryophyllene. Some variation was also apparent in the relative contribution of minor compounds (Table 3.4).

Table 3.4 Chemical compounds present in essential oils analysed by GC/MS

Compound, % C	RT ^d (min)	% percentage of the compounds in the essential oil \pm SEM ^e		
		P 12 mg/L	P 34 mg/L	P 60 mg/L
NI 9	5,5	0.38 \pm 0.05	0.40 \pm 0.05	0.31 \pm 0.06
α Pinene 96%	9,9	2.93 \pm 0.57	2.62 \pm 0.20	2.59 \pm 0.28
camphene 97%	10,5	2.61 \pm 0.35	2.49 \pm 0.13	2.62 \pm 0.09
β Pinene 97%	11,7	3.64 \pm 0.43	4.17 \pm 0.7	4.48 \pm 0.35
β mycrene 83%	12,6	0.28 \pm 0.04	0.30 \pm 0.04	0.28 \pm 0.01
NI	13,6	0.22 \pm 0.01	0.22 \pm 0.01	0.23 \pm 0.03
D-limonene 90%	14,0	0.92 \pm 0.08	0.88 \pm 0.03	0.89 \pm 0.02
eucalyptol (1,8 cineole) 98%	14,1	3.46 \pm 0.13	3.43 \pm 0.15	3.45 \pm 0.17
3 carene 91%	15,3	0.36 \pm 0.04	0.40 \pm 0.02	0.39 \pm 0.02
NI	16,5	0.22 \pm 0.01	0.22 \pm 0.02	0.20 \pm 0.01
thujone α 95%	17,1	9.13 \pm 0.48	9.47 \pm 0.63	9.12 \pm 0.61
thujone β 98%	17,6	1.63 \pm 0.05	1.58 \pm 0.08	1.62 \pm 0.06
camphor 98%	18,5	18.39 \pm 0.43b	16.13 \pm 0.52a	16.59 \pm 0.60a
Bicyclo(3,1,1)heptan-3- one,2,6,6-trimethyl 95%	19,2	2.01 \pm 0.03d	1.89 \pm 0.01de	1.77 \pm 0.01e

Borneol 95%	19,4	1.32 ± 0.04	1.38 ± 0.08	1.42 ± 0.06
Isopinocampone 91%	19,7	0.70 ± 0.25	0.44 ± 0.02	0.45 ± 0.02
D-Verbenone 90%	21,2	0.31 ± 0.02	0.29 ± 0.01	0.26 ± 0.02
Bornylacetate 98%	23,7	1.43 0.06b	1.08 ± 0.12a	1.35 ± 0.06ab
Ylangene 98%	26,6	0.57 ± 0.03	0.56 ± 0.07	0.53 ± 0.05
Copaene 99%	26,7	1.46 ± 0.08	1.44 ± 0.20	1.35 ± 0.12
NI	27,8	0.76 ± 0.02	0.75 ± 0.03	0.79 ± 0.01
caryophyllene 99%	28,1	1.90 ± 0.09	1.94 ± 0.05	1.96 ± 0.09
Germacrene D 97%	28,4	0.41 ± 0.11	0.45 ± 0.12	0.53 ± 0.12
1H-Cyclopropeazulene	28,7	1.61 ± 0.04	1.46 ± 0.11	1.58 ± 0.07
decahydro-1,1,7 99%				
a- caryophyllene 97%	29,1	19.06 ± 0.63	19.25 ± 0.36	19.00 ± 0.35
NI	29,4	0.51 ± 0.05	0.56 ± 0.05	0.54 ± 0.04
Naphthalene 97%	29,9	3.05 ± 0.09	3.01 ± 0.17	3.03 ± 0.08
NI	30,4	1.62 ± 0.04	1.63 ± 0.09	1.69 ± 0.05
NI	30,6	0.97 ± 0.03	0.92 ± 0.11	0.91 ± 0.07
Butylated	31,0	2.83 ± 0.07	2.88 ± 0.08	2.64 ± 0.011
Hydroxytoluene 97%				
NI	31,3	5.27 ± 0.13	5.23 ± 0.41	5.34 ± 0.13
NI	32,6	1.02 ± 0.04	1.09 ± 0.52	1.11 ± 0.06
Ledol	33,3	3.49 ± 0.11 b	3.92 ± 0.03 a	3.87 ± 0.13 a
NI	33,6	0.69 ± 0.02 b	0.78 ± 0.01a	0.78 ± 0.03a
NI	39,1	0.72 ± 0.12 b	1.22 ± 0.07 a	1.29 ± 0.05 ab
NI	45,1	2.02 ± 0.29	2.88 ± 0.07	2.41 ± 0.21

c, Identification was accomplished via NIST database. Percentage represents matching of the compound with library's spectrum

d, Retention time of the peak

e, Relative percentages of the compounds in the oil mixture, calculated from a total area of peaks (100%).

g, Not identified compound

j Plants were cultivated hydroponically under three levels of P (12, 34 and 60 mg/L). Six plants from each replicate were pooled together for each oil extracted. Treatments were replicated four times (n=4 NFT sub-units)

a, b Where there is a statistically significant difference is indicated by the letters a, b. Values followed by the same letter within a row are not significantly different at P<0.5. Values in each row were tested for equality of variances and normality of distribution prior to ANOVA. Means and SEM were determined from four replicates (n=4 oils analysed)

3.3.7 Acetyl and butyryl - cholinesterase inhibition by *Salvia officinalis* var *purpurea*

3.3.7.1 Cholinesterase inhibition by the sage essential oils

Anti – cholinesterase activity of *Salvia officinalis* var *purpurea* essential oils produced hydroponically under three levels of P is presented for both cholinesterase enzymes (acetyl- and butyryl- cholinesterase) (Table 3.5). *S. officinalis* var *purpurea* oils possessed obvious dual anti-cholinesterase properties. The mean IC50s for human AChE ranged from 0.082 mg/ml (12 mg/L P) to 0.059 mg/ml (60 mg/L P). Although the inhibitory activity of the oil appeared to be enhanced under the 60 mg/L P, differences were not statistically significant ($P>0.05$, one-way ANOVA). Similarly, BuChE inhibition potency was not affected by the P treatment. An increase in the incubation time of BuChE with the oils from 15 to 30 min, induced a significant decline in the IC50 values from 0.11 mg/ml to 0.078 mg/ml respectively ($P<0.01$, unpaired t test, n=12 oil extracts). IC50 values were not normally distributed; hence analysis of variance was performed employing logarithms. Sigmoidal dose-response curves for 50% inhibition determination are presented in the Appendix 2, section 2.1.

Table 3.5 AChE and BuChE inhibition of oils expressed by their IC50 values

Treatments^e	IC50 (mg/ml)^f	
	Oil AChE inhibitory activity ^g	
P 12 mg/L	0.082a	
P 34 mg/L	0.075a	
P 60 mg/L	0.059a	
	Oil BuChE inhibitory activity ^d	
	15 min incubation	30 min incubation
P 12 mg/L	0.099b	0.071c
P 34 mg/L	0.112b	0.068c
P 60 mg/L	0.115gb	0.079c

^e Oils were extracted from *S. officinalis* var *purpurea* plants cultivated hydroponically under three levels of P (12, 34 and 60 mg/L). Treatments were replicated four times, resulting in the production of four oils per treatment. Six plants from each replicate /NFT sub-unit were pooled together for every oil/inhibitor.

^f Concentration of oil required for 50% enzyme inhibition, as calculated from a sigmoidal dose-response curve equation. IC50 values represent the mean of four oils. Values within columns followed by the same letter are not significantly different at 0.05 level. IC50 values were not normally distributed, analysis of variance was hence performed employing logarithms and standard errors of the means are not presented

^g AChE enzyme from human erythrocytes 0.05 U/ml

^d BuChE enzyme from horse serum 0.02 U/ml

a, b, c Values within columns followed by the same letter are not significantly different (n=4)

3.3.7.2 Cholinesterase inhibition by the sage extracts

Inhibitory activity of 80% ethanol extracts of *S. officinalis* var *purpurea* appeared to be relatively strong for BuChE enzyme (Table 3.6) with IC50 values significantly lower than the oils ($P < 0.01$, unpaired t-test, $n = 12$). Preliminary results gave no evidence of human AChE inhibition, therefore data for the activity of extracts activity on this enzyme are not presented.

Table 3.6 BuChE inhibition from 80% ethanol extracts expressed by their IC50 values

Treatments^e	80% extract BuChE inhibitory activity^c IC50 (mg/ml)^b
P 12 mg/L	0.041a
P 34 mg/L	0.063a
P 60 mg/L	0.061a

^a Values within columns followed by the same letter are not significantly different ($n = 4$ sage extracts)

^b Concentration of oil required for 50% enzyme inhibition, as calculated from a sigmoidal dose-response curve equation. IC50 values represent the mean of four oils. Values within columns followed by the same letter are not significantly different at 0.05 level. IC50 values were not normally distributed, analysis of variance was hence performed employing logarithms and standard errors of the means are not presented

^c BuChE enzyme from horse serum 0.02 U/ml

^e Extracts were obtained from *S. officinalis* var *purpurea* plants cultivated hydroponically under three levels of P (12, 34 and 60 mg/L). Treatments were replicated four times, resulting in the production of four extracts per treatment. Six plants from each replicate were pooled together for every extract/inhibitor.

3.3.8 Kinetic studies

Kinetic parameters of cholinesterase inhibition were determined for butyryl- and acetylcholinesterase enzyme. Employing the Lineweaver – Burk plot, Michaelis constant (K_m) and initial enzyme velocity (V_{max}) were directly determined from the intercepts of the regression line with the axes (Appendix 3). As replicate oils irrespective of P treatment, possessed very similar quality (see GC/MS analysis) a number of them were randomly chosen for the purpose of kinetic studies. *S. officinalis* var *purpurea* oils demonstrated non-competitive (mixed) type of inhibition for horse serum BuChE while the same oils were competitive inhibitors of human AChE. Characterisation of the type of inhibition was made according to Engel (1981). Actual and V_{max} and K_m values for all oils tested and controls and the equivalent Lineweaver – Burk plots are presented in the Appendix 3.

3.4 Discussion

3.4.1 Plant biomass production and biomass allocation

Outcomes of the present study strongly suggested that under NFT cultivation plants needs in phosphorus (P) can be satisfied with a minimum nutrient supply. The 12 mg/L P level was sufficient for high biomass production of *Salvia officinalis* var *purpurea* plants. High growth rates achieved at this concentration, remained constant as P was elevated from 12 to 60 mg/L. This seems to be in consist with de Groot *et al.* (2003) who demonstrated that relative growth rate of tomato plants increased sharply with P fertilisation under inadequate P conditions, which for *Salvia* presumably is less than 12 mg/L, and quickly levelled off at adequate P levels. NFT grown plants exhibit a wide range of tolerance to P; 5 to ca 200 mg/L of P had little effect on yield of tomato (Copper, 1979) or cucumber plants (Adams, 2002). Stewart & Lovett-Doust, (2003) reported that *Calendula officinalis* grown in a shallow flow hydroponic system exhibited significantly greater vegetative and reproductive biomass under 5 mg/L P than 10, 100, 200 mg/L (deep flow nutrient solution system).

Whilst total plant biomass was not affected by P concentration changes in biomass allocation to shoots and roots were evident under the different treatments. Preferentially biomass partitioning to roots and reduced shoot growth was demonstrated from *S. officinalis* var *purpurea* plants grown under the higher P level (60 mg/L). Root dry biomass was positively correlated with applied P in the nutrient solution, a fact that initially contradicts the general rule of reduced root to shoot ratio under high P supply and enhanced root growth in limiting P environments (Raghothama & Karthikeyan, 2005; Ticconi & Abel, 2004; Abel *et al.*, 2002; Marchner 1995; Lynch, 1995). However, when chickpea plants grown in an NFT system (P, 1mM KH_2PO_4) were subjected to P stress (P, 0.01mM KH_2PO_4) for two weeks (Alloush, 2003), contrary to what is usually evident, P depletion did not promote root growth. As

the response of *Salvia* to P nutrition has been considered only once before (Economakis, 1995) no optimum concentration of the nutrient has yet been established. Thus, P levels higher than 60 mg/L might be required to induce a decline in root development and apparently P levels lower than 12 mg/L are required to provoke P deficiency. However, in agreement with the present study Economakis (1995) reported that *S. fruticosa* Miller shoot growth was also suppressed at P concentrations exceeding 34 mg/L, while the greatest root to shoot ratio was obtained under 68 mg/L P. The stimulated rooting system of *S. officinalis* var *purpurea* at 60 mg/L of P could reflect enhanced effort to capture and store the excess P from the growth medium, as in their natural habitats many plants are P deprived. A similar strategy has been adopted by *Hakea prostrata* species (Shane *et al.*, 2004a; b), when sensing available P in the environment (see section 3.4.3).

Promoted root growth due to high P concentration was accompanied by a reduction in shoot biomass, an effect associated with increased partitioning of carbohydrates towards the roots to sustain their enhanced activity (Lopez-Cantarero *et al.*, 1998). In contrast, improved shoot biomass production was evident under the 12 mg/L P and was positively correlated with the enhanced nitrate (N-NO₃) uptake observed at this treatment (de Groot *et al.*, 2003). Increased P uptake seemed to occur at the expense of N-NO₃ and K (negative correlation, see results for nutrient uptake). Altered root growth and root to shoot ratios are mediated by the action of plant hormones that include auxin (IAA), ethylene and cytokinins (CYT). P supply has been shown to induce changes in the level of CYT and auxin (Raghothama & Karthikeyan, 2005; Vance *et al.*, 2003; de Groot *et al.*, 2003; Lopez-Buccio *et al.*, 2002; Martin *et al.*, 2000; Neumann *et al.*, 2000; Gilbert *et al.*, 2000). CYT are negative regulators of root development while they promote growth of young shoots. An opposite role to that of CYT is demonstrated by IAA (Aloni *et al.*, 2005). Whereas there is strong evidence for induced CYT decline under P starvation (de Groot *et al.*, 2003; Martin *et al.*, 2000), an

earlier study on P supply and CYT levels in hydroponically cultivated *Platanus* seedlings (Dhillon, 1978) revealed decreased CYT at P concentration exceeding the 60 mg/L P, which could explain the improved root growth of *S. officinalis* var *purpurea* at this P treatment.

3.4.2 Leaf attributes under phosphorus variation

At the end of the cultivation period *S. officinalis* var *purpurea* plants shared similar leaf features under the three different levels of P. Specific leaf area (*SLA*), a measure of leaf density and thickness was not altered due to P treatment. This is consistent with the fact that shoot water content was unaffected, considering that *SLA* is primarily determined by water status in plant (Meziane & Shipley, 2001). P treatment caused no apparent variation in pigment production. Given that uniform *SLA* can indicate similar photosynthetic activities; for example thicker leaves have lower net photosynthetic rate per dry mass of leaf due to light attenuation mainly from the leaves upper surface (Reich *et al.*, 1999), and given the fact that chlorophyll content remained the same, it could be concluded that plants at different P levels had potentially equal photosynthetic rates, under uniform environmental conditions. This can be supported by the consistent total plant biomass production.

3.4.3 Nutrient uptake analysis

Nutrient uptake of N-NO₃, K and Ca throughout the cultivation period appeared to be the same, providing a good explanation for the consistency in total plant biomass production under all P treatments. However P uptake, increased significantly with the applied P concentration in the nutrient solution, an outcome consistent with experimental data for the effect of P on *S. fruticosa* Mill. during vegetative growth (Economakis, 1995). P-sufficient plants persist in taking up P, even when growth and

metabolic demands for the nutrient have been satisfied and store P in the cell vacuoles as inorganic phosphorous (Pi) (Theodorou & Plaxton, 1995). This trend agrees with the augmented Pi content in shoot and root tissue, observed under elevated P in the growth medium (Shane *et al.*, 2004b; Yemane & Skjelvag, 2003, Lopez-Cantarero *et al.*, 1998). The fact that high P supply did not promote total biomass production of *Salvia* plants indicates that the excess P absorbed under the 60 mg/L treatment was stored and not directed to primary metabolic processes. Hydroponically grown *Hakea prostrata* like *S. officinalis* var *purpurea*, exhibited enhanced P uptake under increased P level in the nutrient solution to the extent that P toxicity developed in the leaves. In contrast, other species have demonstrated a decline in net P uptake rates at elevated P supply (Shane *et al.*, 2004a). Regulation of P influx is possible *via* a system of high- or low- affinity P transporters present on the cell plasma membrane (Raghothama & Karthikeyan, 2005; Ticconi & Abel, 2004; Abel *et al.*, 2002; Bucher *et al.*, 2001; Smith *et al.*, 2003). *Hakea prostrata* plants grown naturally in xerophytic environments tend to accumulate Pi during the winter when the nutrient in the soil is more accessible and utilize it during vegetative growth stage in summer (Shane *et al.*, 2004a and 2004b). The response of sage to P nutrition is largely unknown but as *Salvia* species naturally inhabit the hot and dry Mediterranean environment a similar trend to that might be possible.

S. officinalis var *purpurea* plants cultivated under the range of P concentrations used in the present work exhibited no apparent symptoms of P deficiency/toxicity. Data from *S. officinalis* var *purpurea* obtained in the present work for total plant biomass, nutrient uptake, *SLA* and photosynthetic pigments suggests no evidence of plant stress at any level of P (12, 34 and 60 mg/L).

3.4.4 Effect of phosphorus levels on yield and composition of essential oil

S. officinalis var *purpurea* plants grown hydroponically under different P supply for a period of four months, showed oil contents from 0.95% to 1.18%. This is the first report of oil yield for *S. officinalis* var *purpurea* and as expected is similar to *S. officinalis*, which displayed a variation of 0.9 to 2.3% under different seasons and geographical origins (Karousou *et al.*, 2000). These findings are consistent with several other authors who have reported oil yield of the same species (Chalchat *et al.*, 1998; Perry *et al.*, 1999; Putievsky *et al.*, 1986; Santos-Gomes & Fernandes-Ferreira, 2001). The lower oil content obtained from *S. officinalis* var *purpurea* in this study reflects at least to some extent the strong influence of plant growth stage on essential oil production that can be considerably less at the vegetative growth stage and reach its maximum during seed formation (Karousou *et al.*, 2000; Putievsky *et al.*, 1986; Karioti *et al.*, 2003; Santos-Gomes & Fernandes-Ferreira, 2001). The influence of the environmental origin of plants on oil content should also be considered. A low 0.53% oil content was revealed for *S. officinalis* obtained *via* supercritical fluid extraction from plants grown in Britain (personal communication with Dr S. Savelev).

P concentration in the nutrient solution did not cause any significant variation in the yield or relative composition of *S. officinalis* var *purpurea* essential oil. Contrasting results from numerous other experiments have highlighted P impacts on oil content and quality from various species (Ram *et al.*, 2003; Kapoor *et al.*, 2004; Economakis, 2002 and Khan *et al.*, 1999). However, the outcome of the present study is consistent with Economakis (1995) who reported no effect of P level on the oil content of *S. fruticosa* cultivated by means of hydroponics under similar P levels and agrees with Ramos *et al.*, 2005 and Gershenzon (1983). Terpenes seem to exhibit a variety of responses to nutrient application with no obvious trend (Gershenzon, 1983). Kapoor and co-workers, (2004) reported that essential oil biosynthesis is dependent on

inorganic P level in the plant. However, the excess of P absorbed at the 60 mg/L treatment was not utilised in the synthesis or metabolism of terpenes. The strong influence of genetic attributes on terpene production could be a good explanation for the consistency in oil biosynthesis under different nutrient regimes (Hamilton *et al.*, 2001). Nevertheless, GC/MS analysis demonstrated a distinct composition of *S. officinalis* var *purpurea* essential oil from other sage species like *S. lavandulaefolia* and *S. fruticosa* (Langer *et al.*, 1996; Savelev *et al.*, 2004; Kintzios & Gianouli, 2000) and a similar quality to *S. officinalis* (Langer *et al.*, 1996; Perry *et al.*, 1999a; Piccaglia *et al.*, 1997; Kintzios & Gianouli, 2000; Santos-Gomes & Fernandes-Ferreira, 2001; Savelev *et al.*, 2004). The main compound of *S. officinalis* var *purpurea* oil α -caryophellene comprised 19% as in the case of *S. officinalis*. Camphor, a non-competitive antagonist to nicotinic ACh receptors (Park *et al.*, 2001) was within the same range of other *Salvia* species or even less (16-18%), while α -thujone molecule with reported toxic properties (Holder, 2000) appeared to be relatively low (9%). Other oil constituents that exhibit cholinesterase inhibition (Perry *et al.*, 2000; Savelev *et al.*, 2003; 2004) were at minor concentrations i.e. 1.8 cineole (3.5%), α - and β -pinene (2.8 and 4% respectively), 3-carene (0.4%) and β -caryophyllene (1.9%).

3.4.5 *Salvia officinalis* var *purpurea* anti-cholinesterase activity

The present study revealed that oils of *S. officinalis* var *purpurea* obtained from hydroponically grown plants under controlled nutritional conditions, possessed dual anti-cholinesterase activity, in contrast to *S. lavandulaefolia* and *S. officinalis* that preferentially inhibited AChE (Savelev *et al.*, 2004). BuChE inhibitory activity of *S. officinalis* var *purpurea* oil was time dependant as suggested by Savelev and co-workers (2004) for the same species, and increased incubation improved the potency of the oil. Average IC₅₀ values of the 12 oils extracted were 0.072 mg/ml and 0.073 mg/ml for AChE and BuChE respectively. The observed decline in the inhibitory activity in comparison with other data produced for *S. officinalis* var *purpurea* (Savelev *et al.*,

2004) is possible due to a difference in the final concentration of the enzymes utilized in both studies. The elevated amount of acetyl- and butyryl- cholinesterase of the present study renders the enzyme more competitive to the inhibitor. P concentration in the nutrient solution caused no variation in ChE inhibitory activity as oil quality was unaffected by P treatment.

80% ethanol extracts from *S. officinalis* var *purpurea* revealed selected inhibition for BuChE with significantly lower IC₅₀ values than the oils (0.054 mg/ml) most probably due to the different chemical compositions of distilled and organic solvent extracts from the same species (Langer *et al.*, 1996). 80% ethanol extracts are expected to contain not only terpenes, but also phenolic compounds which are abundant in sage species, as well as other molecules more polar than the oil constituents. Preliminary results suggested no evidence of human AChE inhibition by the ethanol extracts; therefore extracts activity on AChE enzyme was not further investigated. Data for the inhibitory activity of *S. officinalis* var *purpurea* ethanolic extracts on BuChE is demonstrated here for the first time and is consistent with Kennedy *et al.*, 2005 who reported the same IC₅₀ values for *S. officinalis* BuChE inhibition (IC₅₀ 0.056mg/ml) and very weak inhibitory activity for AChE (IC₅₀ 0.398mg/ml). Contrasting inhibition by the sage extracts for AChE and BuChE possibly reflects differences in the structure of the two enzymes (Silman & Sussman, 2000). In AChE, access to the active site is limited to relatively small and elongated molecules, whereas larger molecules could remain bound to the peripheral anionic site (PAS) due to interactions with aromatic amino acids at the gorge rim or electrostatic interactions with the active site, thus exerting indirect inhibitory effects on the enzyme. In BuChE however, larger molecules are allowed to enter the gorge while different amino acids present at the PAS generate differential reactions with potential inhibitors. Considering the inhibitory activity of the oils (non polar metabolites), it could be suggested that AChE enzyme structure is more resistant to relatively polar mixtures that probably because of lower hydrophobicity

cannot cross the enzyme gorge. Fractionated analysis of the extract could shed light on this direction.

3.4.6 Kinetics

Kinetic studies of inhibition employing Lineweaver-Burk plot, revealed that *S. officinalis* var *purpurea* essential oil is a competitive inhibitor for human AChE. To date, competitive inhibition from an oil has never been reported. This finding is supported by previous work of Savelev on ChE inhibition from various *Salvia* species (personal communication with Dr S. Savelev), who confirmed the same type of inhibition, only by the oil of *S. officinalis* var *purpurea*. Oils of *Salvia lavandulaefolia*, *S. officinalis* and *S. fruticosa*, species with potent cholinergic activities exhibited non-competitive type of inhibition for both AChE and BuChE (Perry *et al.* 2000, Savelev *et al.*, 2004). Kinetic outcomes for BuChE inhibition by *S. officinalis* var *purpurea* oil of the present study demonstrated non-competitive type of inhibition. Differential type of inhibition for the two cholinergic enzymes by the same oil most probably reflects variations in the structure of acetyl- and butyryl-cholinesterase as explained above. This is supported by experimental data demonstrating that single oil constituents (Savelev *et al.*, 2004) or terpene combinations (Savelev *et al.*, 2003) exhibit differing abilities for inhibiting the two ChEs.

3.4.7 Conclusions

Cultivation of *S. officinalis* var *purpurea* in an NFT system under different levels of P (12, 34 and 60mg/L) resulted in the production of healthy, vigorous plants of high biomass irrespective of P concentration. However, herbage yield was improved at less than 34 mg/L whereas no effect of P treatment was evident on essential oil content and quality. Oils of *S. officinalis* var *purpurea* plants exhibited dual inhibitory activity for AChE and BuChE and competitive type of inhibition for human AChE was revealed. Leaf ethanolic extracts preferentially inhibited BuChE with relatively low IC₅₀ values.

Chapter 4

Effects of nitrogen on medicinal properties and growth of *Salvia officinalis* var *purpurea* cultivated in solution culture.

4.1 Introduction

Salvia officinalis var *purpurea* (commonly purple sage) constitutes one of the *Salvia* species that have been demonstrated (*in vitro* experiments, Savelev *et al.*, 2004) to exert inhibitory effects on enzymes present in the human central nervous system namely the cholinesterases (ChEs). Since cholinergic activities of sage oils are due to the relative composition of their terpene compounds, manipulating oil production with a view to enhance yield and quality, is a major goal in terms of ChE inhibition. There have been numerous attempts to improve the quality and content of essential oils through the application of various nutrients, including nitrogen (Gershenzon, 1983; Hornok, 1986;; Economakis *et al.*, 1999). Nitrogen is an indispensable element for plant growth and development. Its importance for plant function is demonstrated in the vast number of metabolic molecules i.e. amines, amides, amino acids, peptides, proteins, coenzymes, nucleic acids, chlorophyll pigments and secondary compounds like alkaloids, which entail N in their structure (Marchner, 1995). Up to 75% of leaf organic N is located in the chloroplasts primarily as enzyme protein or incorporated in chlorophyll molecule suggesting a positive relationship between assimilated N and photosynthesis (Van der Werf & Nagel, 1996; Andrews *et al.*, 1999; Meziane & Shipley, 2001) and consequently plants growth. Chlorophyll content in the leaf has been associated with plant yield (Richardson *et al.*, 2002) and is closely linked to inorganic N supply from the roots. The amount of chlorophyll and pigment content in general (chlorophyll a and b and carotenoids) is altered under adverse environmental factors, including nutrient deficiency (Richardson *et al.*, 2002; Shevchenko *et al.*, 2004).

Inorganic N is taken up from the growth medium in the form of nitrate (NO_3^-) or ammonium (NH_4^+). NO_3^- and NH_4^+ ions comprise the 80% of the total anion or cation uptake by roots (Marchner, 1995). While ammonium is readily incorporated into organic structures, for nitrate reduction into nitrite and ammonia (NH_3) a set of

enzymes (nitrate reductase and nitrite reductase respectively) is required. When NO_3^- is assimilated into organic matter, excretion of hydroxyl groups (OH^-) increases the pH of the growth medium (Cooper 1979; Papadopoulos, 1991). Conversely, NH_4^+ incorporation is accompanied by proton H^+ release and can reduce pH to very low undesirable values inducing toxicity in plant tissues at even very low concentrations. Due to the pH effects that can accompany ammonium uptake, nitrate is the preferable form of N for plants cultivation (Marchner, 1995).

NO_3^- absorption by roots and N content in plants is closely related to nitrate supply. Commonly, the amount of N in plant tissues increases with nitrate availability in the growth medium; however, a number of studies suggest that elevated nitrate levels do not induce analogous increases in plant growth (Economakis, 1993b; Karamanos, 1995; Schenk, 1996; Andrews *et al.*, 1999; Akanbi & Togun, 2001; Omidbaigi & Arjmandi, 2002; Baranauskiene *et al.*, 2003; de Groot *et al.*, 2003; Martin *et al.*, 2006). Marschner (1995) indicated that elevated amounts of nitrates are of limited use for plant metabolism. The need to define optimum nutrient conditions for plant growth and development can be satisfied by nutrient uptake analysis. Measurement of nutrient uptake *via* depletion studies of the corresponding nutrient from the solution growth medium is highly correlated to the nutrient content in plant tissues (Bar-Tal *et al.*, 1995).

Hydroponics constitutes a powerful tool for nutrient studies (Wilcox, 1982). Moreover, existing evidence confirms that herbs have been successfully cultivated in NFT (Wees & Stewart, 1986; Economakis, 1992, 1993a, 1993b, Economakis & Fournaraki, 1993; Economakis, 1995). Cultivation of herbs is a relatively new approach developed under the pressure to obtain high quality controlled product (Stewart & Lovett-Doust, 2003). Contrary to vegetable crops our knowledge of the application of hydroponics for herbs production is restricted (Karioti *et al.*, 2003). Hydroponic or soil cultivation of *Salvia*

officinalis var *purpurea* has never been studied before while a limited number of reports exist regarding N effect on sage culture in soil (*S. farinacea*, Knowles *et al.*, 1993; *S. officinalis*, Caramanos, 2000) or under hydroponic conditions (*S. fruticosa*, Economakis, 1993b, 1995).

It is of particular interest to establish if the yield and quality of essential oils in *Salvia officinalis* var *purpurea* can be manipulated by varying N concentration in hydroponics. Karioti and co-workers (2003) reported qualitative and quantitative differences in oil compounds from *S. fruticosa* Mill. cultivated by means of NFT, due to nitrate level in the nutrient solution (100, 150 and 200 mg/L) and growth stage. This study in particular (Karioti *et al.*, 2003) demonstrated decreased oil yield under the 200 mg/L N-NO₃ and total absence of 1.8-cineole at this nitrate level, along with variations in other constituents. In coherence with the previous author, Khan *et al.*, (1992; 1999) reported changes in essential oil content and quality of fennel seeds (*Foeniculum vulgare*) under the application of N and P fertilisers. Essential oil content of *Thymus vulgaris* was also affected by N and P supply (Omidbaigi & Arjmandi, 2002), while oil yield of *Tagetes minuta* L. was significantly increased at the highest dose of N fertiliser in comparison to the controls in a more recent field trial (Singh & Ganesha, 2005). However, contradictory outcomes were revealed for *Thymus vulgaris* cultivated in the field under different regimes of N fertilisers that did not exert significant effects on oil content or composition (Baranauskiene *et al.*, 2003). Economakis *et al.* (1999) reported that N had no effect on oil content and quality of the medicinal *Origanum dictamnus*. Moreover, N and P fertilisers did not significantly alter oil quality of *S. officinalis* and *Mentha piperita* in a field trial in northern Italy (Piccaglia & Marioti, 1993). Karamanos (1995) demonstrated no influence of N fertilisers on oil content of *S. officinalis* over a range of 80 to 240 kg/ha. A recent study (Singh, *et al.*, 2005) revealed no influence by N, P and K fertilisers application on lemongrass oil yield and quality.

The aim of the present chapter was to raise *S. officinalis* var *purpurea* plants under various nitrate levels with a view towards increasing the content and quality of essential oils in terms of cholinergic activity. In parallel, the experiment was designed to define optimum nitrate levels for obtaining high herbage yields.

4.2 Materials and methods

4.2.1 Plant cultivation, nutrient and environmental conditions

Young plants of *Salvia officinalis* var *purpurea* were obtained in August 2004 for a hydroponic experiment. Randomly, six were assigned to individual NFT subunits and established in channels at 40 cm intervals. Prior to their installation plants had been subjected to root washing under a mild stream of water to achieve removal of the compost mixture from the roots (see section 2.2.1 for NFT system). Air temperature, PAR, EC and pH of the nutrient solutions were recorded as described before (section 2.2.2 and 2.2.4).

Plants were cultivated under three nitrate regimes i.e. 100, 150 and 200 mg/L nitrogen from nitrates (N-NO₃). Each treatment comprised four replicates (individual NFT subunits) (see paragraph 2.11 for experimental design). Hydroponic recipes for stock solution preparation (g of fertilisers/chemical reagents in 50 L of water) and target elemental composition of nutrient solutions in reservoir tanks are presented in Table 4.1. Concentration of sulphur (SO₄⁻²) differed among treatments to achieve desired nitrate concentration (Table 4.1). Variation of SO₄⁻² constitutes a common practice in hydroponics as sulphate anion is well known not to interfere in the uptake of other ions (Alloush 2002, Savvas & Passam, 2002). Target pH in all NFT sub units was 6 with nitric acid utilised for pH corrections. EC was arranged at 1.8, 1.85 and 1.9 mS/cm for the 100, 150 and 200 mg/L treatment respectively. *S. officinalis* var *purpurea* plants were grown for a period of three months commencing on the 20th of August 2004. At the end of the experiment three plants were randomly harvested from each NFT sub-unit.

Actual 10 day mean pH and EC values of nutrient solutions before the correction with acid or stock solution are depicted in Figure 4.1 and 4.2. EC ranged from 1.54 to 1.86 mS/cm. pH values fluctuated between 6.3 and 7.2. Increases in pH of the nutrient solution are principally attributed to NO_3^- fixation into ammonia in plant roots, a process that releases OH^- in the medium (Cooper, 1979)

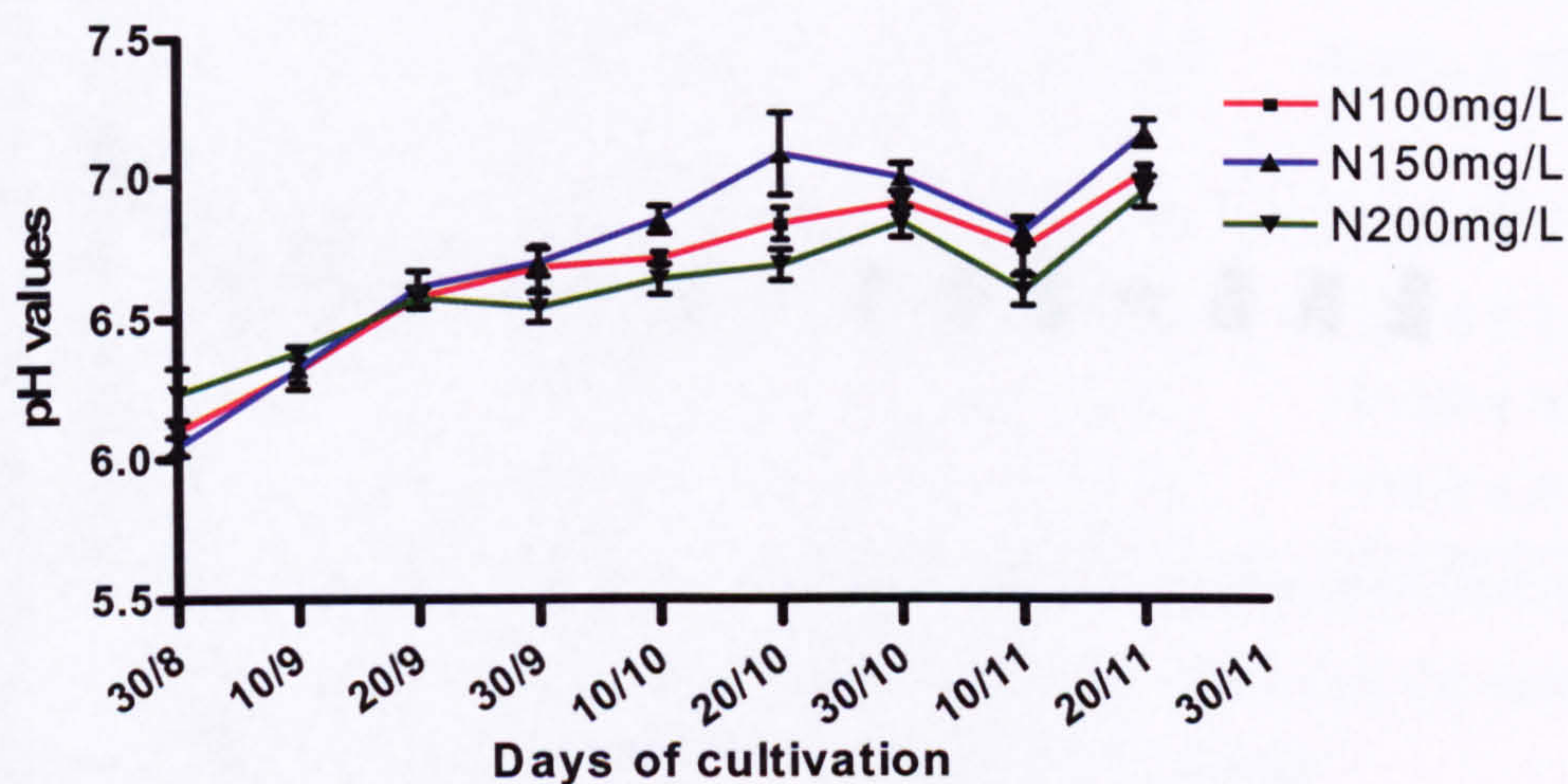


Figure 4.1 Average pH over ten day intervals for the three N- NO_3 treatments (100, 150 and 200 mg/L) during the cultivation period. Target pH was 6. Values represents the means and vertical bars indicate standard errors of the mean (n=4 NFT sub-units)

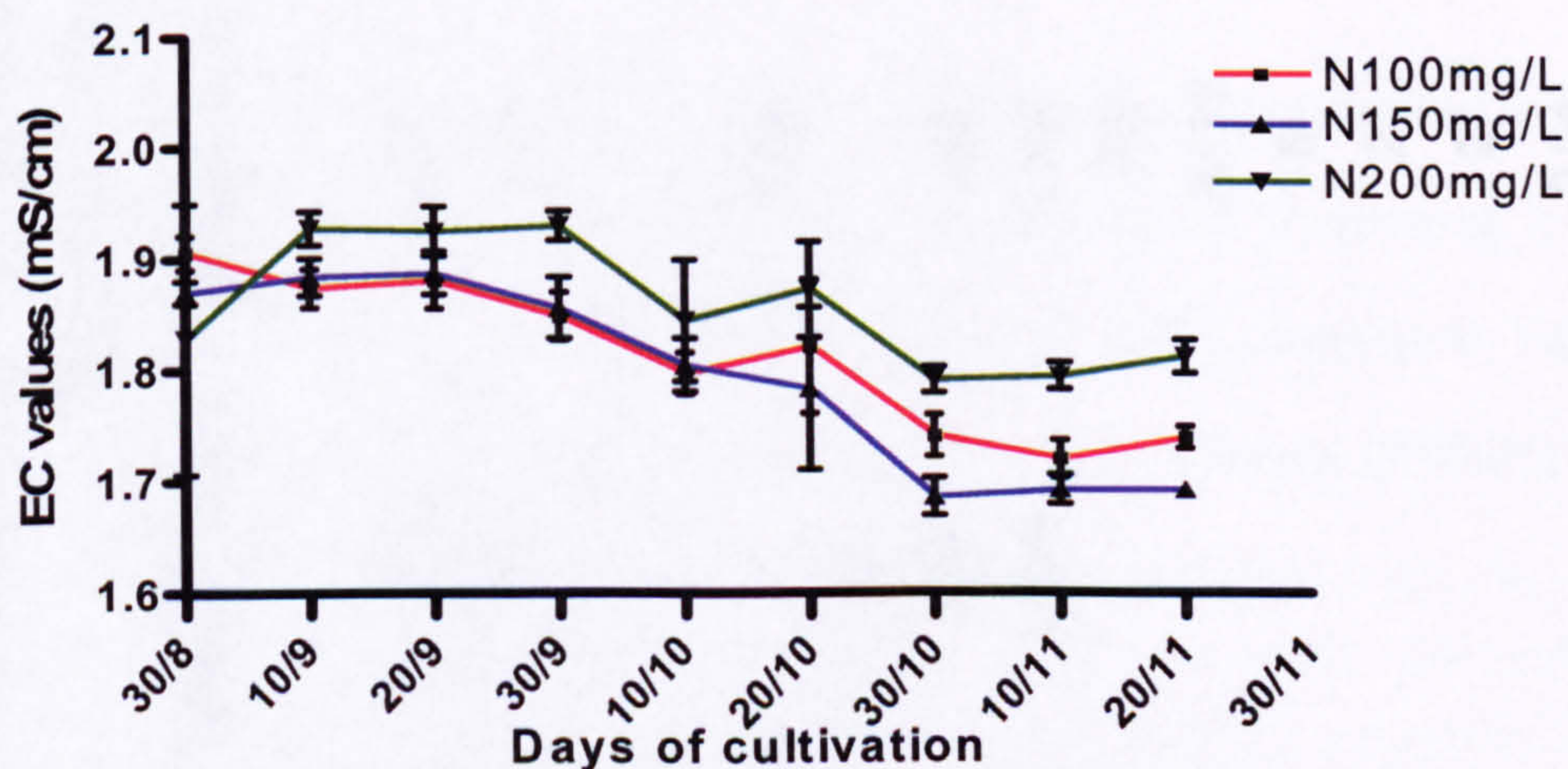


Figure 4.2 Mean EC over ten day intervals for the three N- NO_3 levels in the nutrient solution (100, 150 and 200 mg/L) during the cultivation period. Target EC was 1.8, 1.85 and 1.9 for the 100, 150 and 200 mg/L nitrate treatment respectively. Values represent the means and vertical bars the standard errors of the mean (n=4 NFT sub-units)

Table 4.1 Stock recipe and elemental composition of nutrient solutions for nitrate experiment. Three levels of nitrates (N-NO₃) were applied i.e. 100, 150 and 200 mg/L. Stock solutions consisted of commercially obtained fertilizers (Hortifeeds) and chemical reagents (SIGMA, Co, UK)

Chemical	Amount (g) in 50 L stock			Total mg/L in solution ^a		
	100mg/L N-NO ₃	150mg/L N- NO ₃	200mg/L N- NO ₃	N-NO ₃	N-NH ₄	189
Stock A						
Ca(NO₃)₂*4H₂O	2.21	2.21	2.21			4.41
KNO₃	1.32	2.11	2.11			142.5
Stock B						
Mg(NO₃)₂		0.86	2.64			200.5
K₂SO₄	0.7					32
MgSO₄	2.74	1.96	0.21			60.7
KH₂PO₄	0.705	0.705	0.705			122.6
EDTA FeNa		323				76.4
MnSO₄*H₂O		22.75				30.9
H₃BO₃		8				8.4
CuSO₄*5H₂O		2				0.98
ZnSO₄*7H₂O		2				1.48
(NH₄)₆Mo₇O₂₄		0.64				0.28

^a Final composition of nutrient solution in catchment tanks. Elemental contribution of tap water has been taken into account (Appendix 1, section 1.2)

Actual concentrations of nitrates, potassium (K), phosphorus (P) and calcium (Ca) of the nutrient solutions determined at the beginning of the experiment, and their fluctuations are presented in Table 4.2.

Table 4.2 Actual concentrations of N-NO₃, P, K and Ca in nutrient solutions

<i>Treatment</i>	<i>100 mg/L</i>	<i>150 mg/L</i>	<i>200 mg/L</i>	<i>P</i>
<i>Element (mg/L)^d</i>	<i>N-NO₃ ± SEM^e</i>	<i>N-NO₃ ± SEM^e</i>	<i>N-NO₃ ± SEM^e</i>	
N-NO₃	106.3 ± 1.4c	151.0 ± 2.5b	198.3 ± 1.9a	P<0.001
K	184.4 ± 2.7a	177.5 ± 4.7a	181.5 ± 2.9a	P>0.05
P	34.27 ± 0.3a	28.04 ± 2.3a	31.00 ± 0.7a	P>0.05
Ca	119.3 ± 0.8a	107.5 ± 8.2a	115.4 ± 4.3a	P>0.05

Values within rows followed by the same letter are not significantly different

^d N was analysed by means of a spectroscopy while K, P and Ca concentrations were determined *via* ICP-OES (see general materials and methods).

^e Standard error of the mean (SEM) was calculated from four replicates (n=4 NFT sub-units)

Total stock solution and 5% nitric acid consumption are presented in Table 4.3. One way ANOVA revealed considerable differences among treatments in stock solution consumption (P<0.05) being higher for 150 mg/L N-NO₃ treatment. Total acid utilised for the 200 mg/L N-NO₃ treatment was significantly reduced (P<0.01) compared with the lower N-NO₃ treatments (100 and 150 mg/L).

Table 4.3 Total acid and stock solution consumption by *Salvia officinalis* var *purpurea* plants during the cultivation period of three months.

<i>Treatment</i>	<i>100 mg/L</i>	<i>150 mg/L</i>	<i>200 mg/L</i>
<i>Consumption</i>	<i>N-NO₃ ± SEM^e</i>	<i>N-NO₃ ± SEM^e</i>	<i>N-NO₃ ± SEM^e</i>
Acid total (ml) ± SEM	251.5 ± 8.4 a	275.0 ± 11.9 a	199.5 ± 9.4 b
Stock solution total (ml) ± SEM	447.5 ± 12.5 a	515.0 ± 17.8 b	452.5 ± 16.52 a

Values represent the means and standard error of the mean (SEM) calculated from n=4 NFT sub-units

Stock solution values not followed by the same letter are significantly different at 5% level (P<0.05). Total acid values not followed by the same letter are significantly different at 1% level (P<0.01)

Min and max air temperature ranged from 18 to 19 and 26 to 30 °C respectively during the three months of cultivation (Figure 4.3).

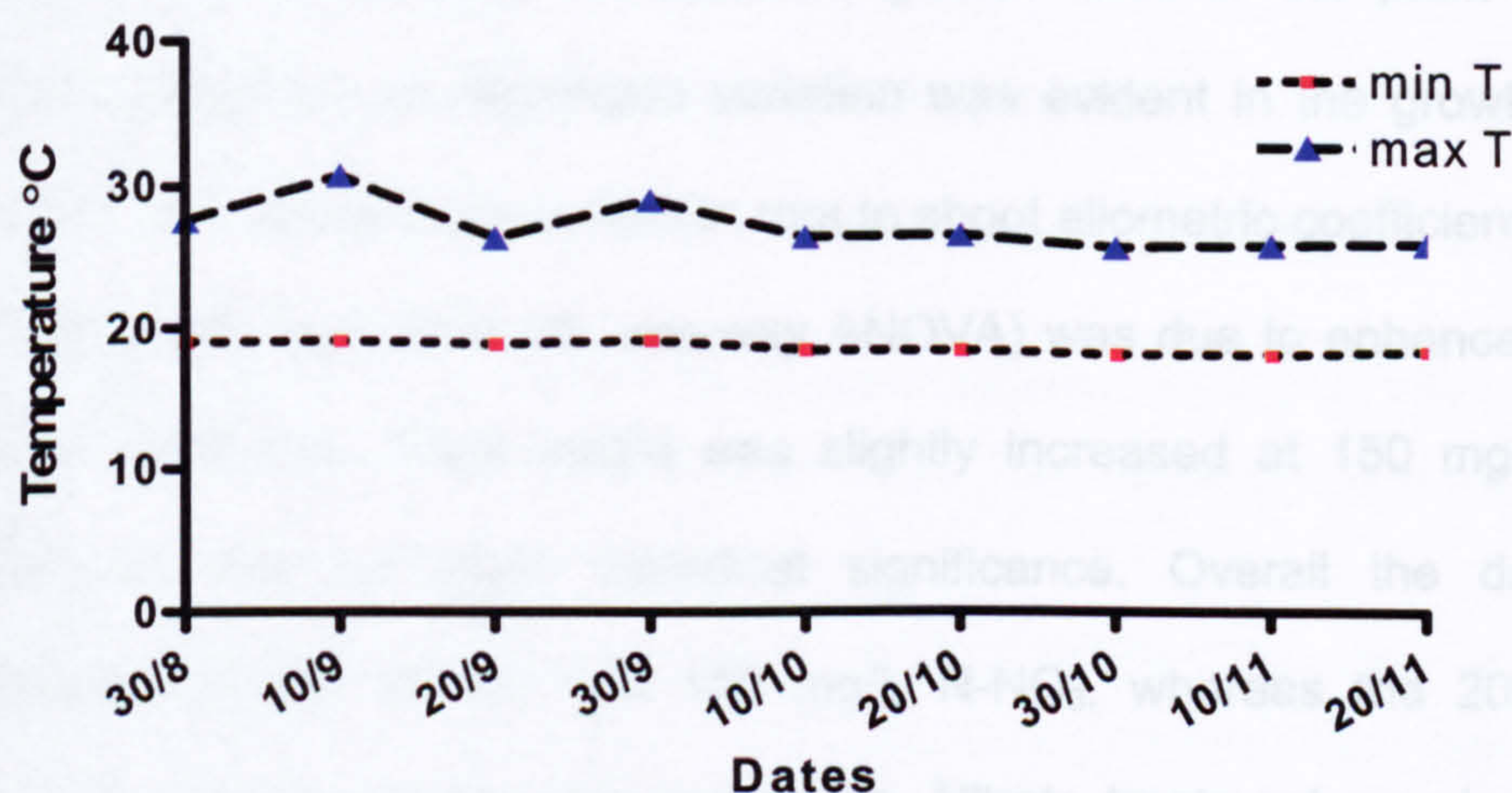


Figure 4.3 Average values over 10 day intervals for min and max air temperature throughout the cultivation period of *Salvia officinalis* var *purpurea* plants raised under 100, 150 and 200 mg/L of N-NO₃ by means of a hydroponic (NFT) system installed in a heated glass-house.

4.3 Results

4.3.1 Growth response to nitrates

The impact of nitrate treatment on growth attributes of *Salvia officinalis* var *purpurea* is illustrated in Table 4.4. Dry biomass (*DWP*) of *S. officinalis* var *purpurea* plants hydroponically cultivated under 100, 150 and 200 mg/L of nitrogen from nitrates (N-NO₃) in the nutrient solution was considerably enhanced under the 100 and 150 mg/L in comparison to 200 mg/L level. Shoot dry matter production (*DWS*) appeared to be equally influenced by the concentration of nitrates (see Table 4.4). The effect of 100 mg/L nitrate treatment on *DWP* and *DWS* although less pronounced, did not significantly differ from the 150 mg/L level ($P>0.05$, one-way ANOVA). Nitrate concentration in the nutrient solution did not affect root growth (*DWR*), as revealed by one-way analysis of variance ($P>0.05$). Additionally, nitrate supply induced considerable changes in the absolute growth rates of the plant (*AGR*) and shoot (*AGRs*), whereas no significant variation was evident in the growth rate of the root (*AGRr*). The observed reduction in root to shoot allometric coefficient (*RSc_o*) under the 100 and 150 mg/L ($P<0.05$, one-way ANOVA) was due to enhanced shoot growth at these treatments. Plant height was slightly increased at 150 mg/L N-NO₃ but the difference did not attain statistical significance. Overall the data demonstrated improved growth at 100 and 150 mg/L N-NO₃, whereas the 200 mg/L treatment induced a decline in biomass production. Nitrate treatment marginally reduced shoot water content (%*SWC*) of sage plants raised at 150 mg/L N-NO₃ from 80 to 78%, a statistically significant decrease at the 5% level. Analysis of variance revealed a considerable reduction in the leaf area (*LA*) of plants raised under the same treatment (150 mg/L N-NO₃). In general, *S. officinalis* var *purpurea* plants grown for three months with various concentrations of nitrates (100, 150 and 200 mg/L N-NO₃) were healthy but rather small. The fresh weight of the shoot (*FWS*), the commercially important part of sage, ranged roughly from 150 to 260 g (Table 4.4).

Table 4.4 Impact of N-NO₃ nutrition on the growth of *S. officinalis* var *purpurea* plants

Growth attributes ^b	Treatment ^a			P ^c
	100 mg/L N-NO ₃	150 mg/L N-NO ₃	200 mg/L N-NO ₃	
<i>FWS</i> (g)	203.7 ± 13.33 a	241.5 ± 19.50 a	170.0 ± 23.47 a	
<i>DWP</i> (g)	51.64 ± 3.69 ab	61.29 ± 4.09 a	44.27 ± 5.09 b	*
<i>DWS</i> (g)	40.76 ± 3.00 ab	53.07 ± 4.287 a	34.00 ± 5.16 b	*
<i>DWR</i> (g)	10.88 ± 1.011 a	12.65 ± 1.424 a	10.28 ± 0.708 a	
<i>AGR</i> (g/week)	3.762 ± 0.308 ab	4.983 ± 0.508 a	3.148 ± 0.425 b	*
<i>AGRs</i> (g/week)	3.156 ± 0.25 ab	3.982 ± 0.208 a	2.592 ± 0.43 b	*
<i>AGRr</i> (g/week)	0.59 ± 0.08 a	0.7373 ± 0.12 a	0.5397 ± 0.06 a	
<i>RScO</i>	18.01 ± 1.56 ab	15.40 ± 2.02 a	25.56 ± 3.03 b	*
<i>Height</i> (cm)	56.56 ± 3.28 a	66.80 ± 6.19 a	59.94 ± 2.67 a	
<i>LA</i> (cm ²)	17.38 ± 0.98 a	14.39 ± 0.41 b	17.34 ± 0.92 a	*
<i>%SWC</i>	79.67 ± 0.46 a	77.73 ± 0.47 b	79.75 ± 0.28 a	**

^a *Salvia officinalis* var *purpurea* plants were cultivated hydroponically in an NFT unit under three levels of N-NO₃ in the nutrient solution (100, 150 and 200 mg/L). Treatments were replicated four times (n=4 NFT sub-units)

^b *FWS*, *DWP*, *DWS* and *DWR* indicate the fresh weight of the shoot and the dry weight of plant, shoot and root respectively. *AGR*, *AGRs*, *AGRr* and *RScO* represent the absolute growth rate of the plant, shoot and root and the root to shoot allometric coefficient respectively, determined according to Hunt (1990). *LA* stands for leaf area measured via the youngest fully expanded pairs of leaves and *%SWC* for the % water content of the shoots. Values represent the mean and standard errors of the mean (n=4 NFT sub-units/replicates). Means followed by the same letter are not significantly different

^c Asterisks denote level of significance * (P<0.05) and ** (P<0.01)

4.3.2 Leaf pigment content

Nitrate treatment exerted no change on the chlorophyll a (Chla) and b (Chlb) concentration when chlorophylls were expressed in relation to leaf fresh weight or leaf area ($P > 0.05$, Figure 4.4 A and B respectively). The same ratio of Chla to Chlb (ratio Chla/Chlb) was therefore maintained under the different nitrate concentrations (100, 150 and 200 mg/L N-NO₃) (Figure 4.4 C). Conversely, a significant increase in total carotenoid content was revealed (one-way ANOVA, $P < 0.05$ Figure 4.4 A and B) for plants raised under the 150 mg/L of N-NO₃.

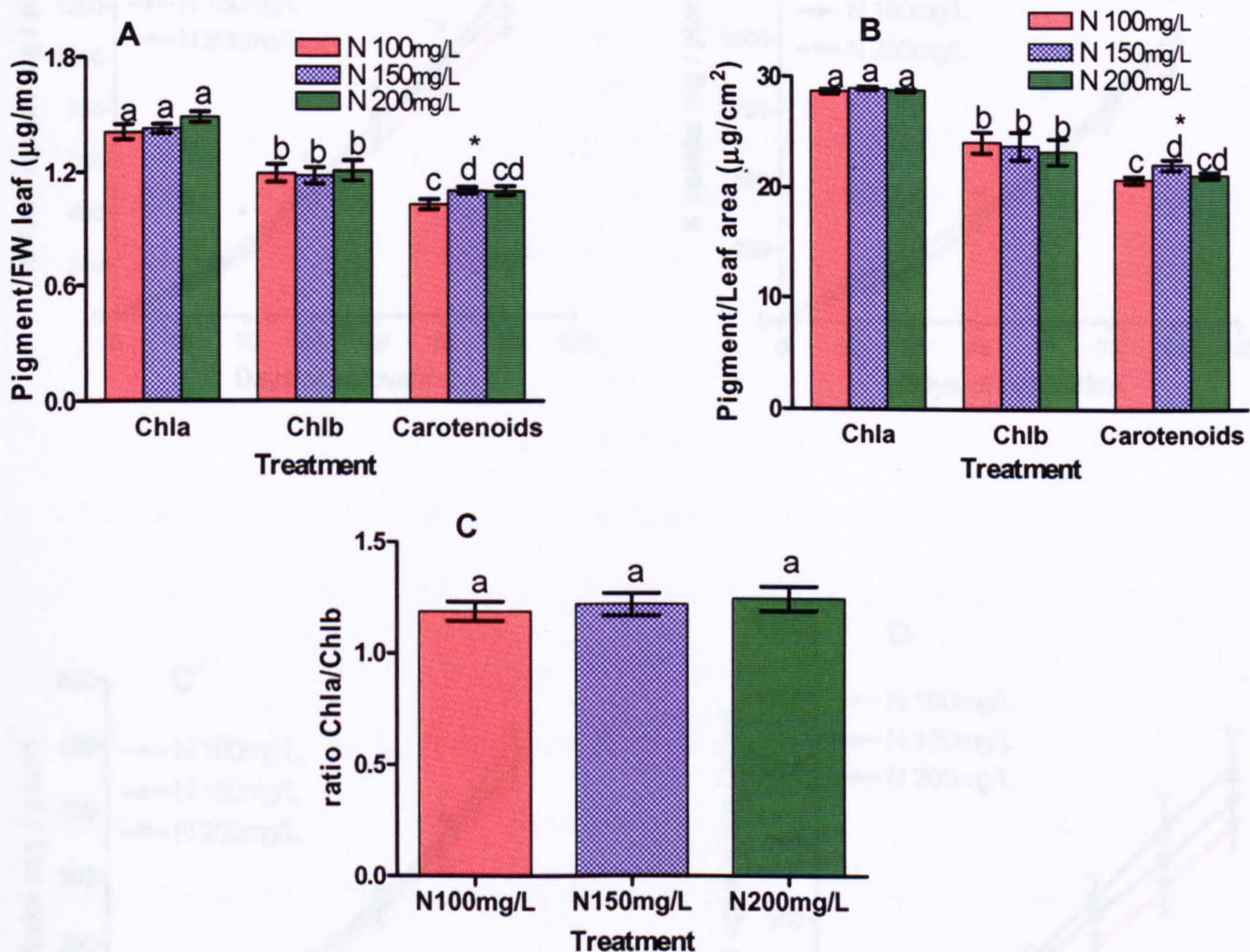
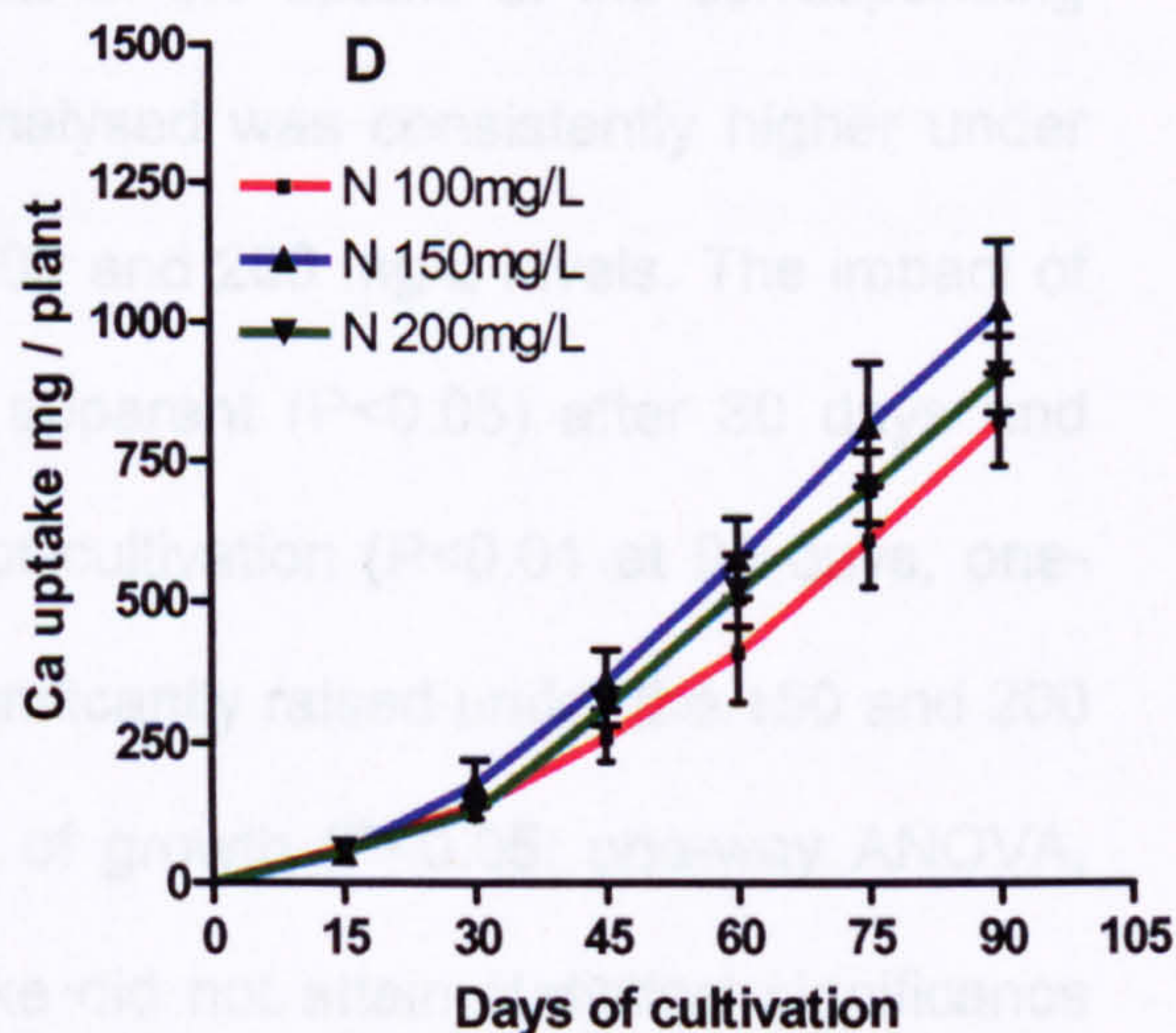
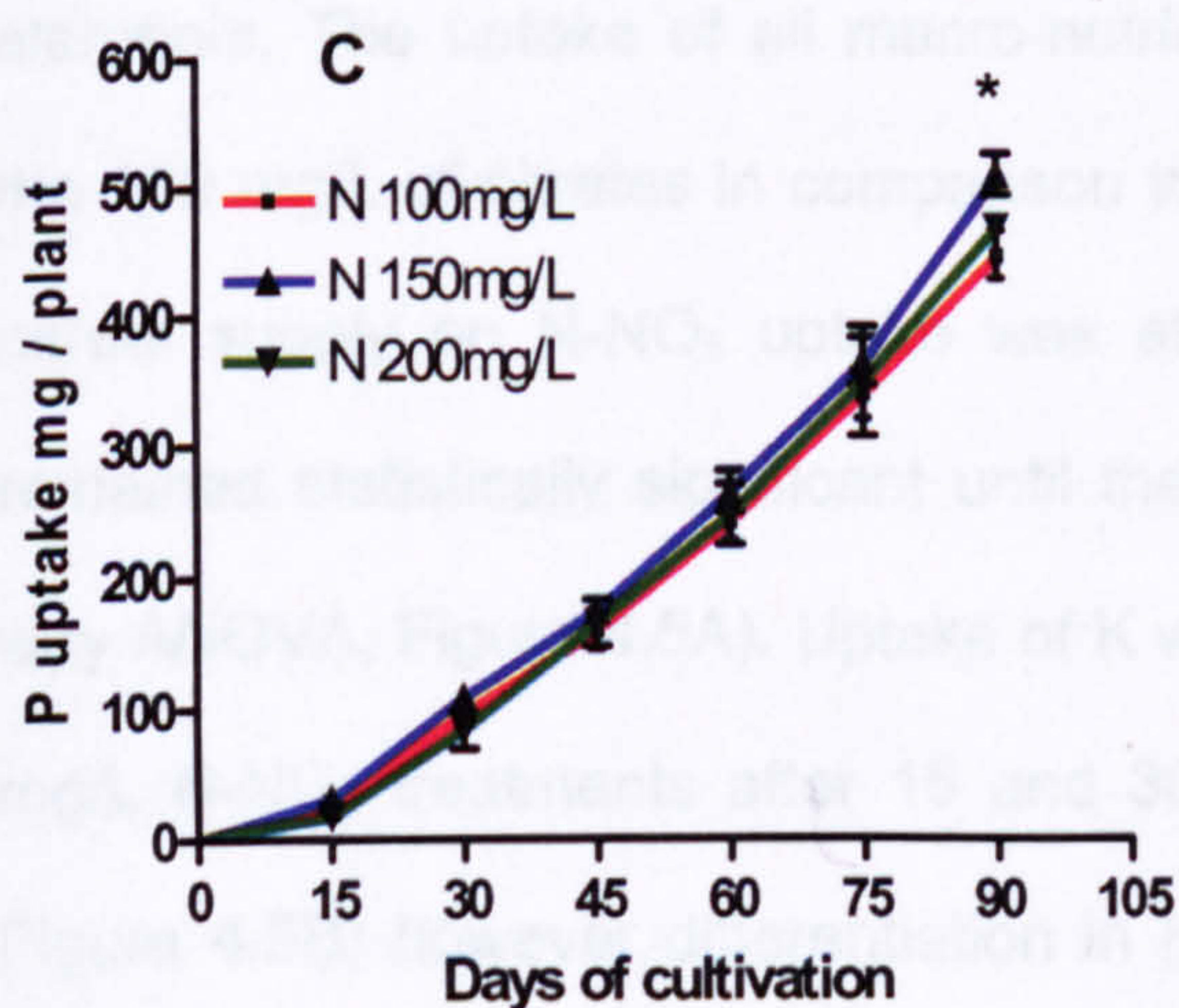
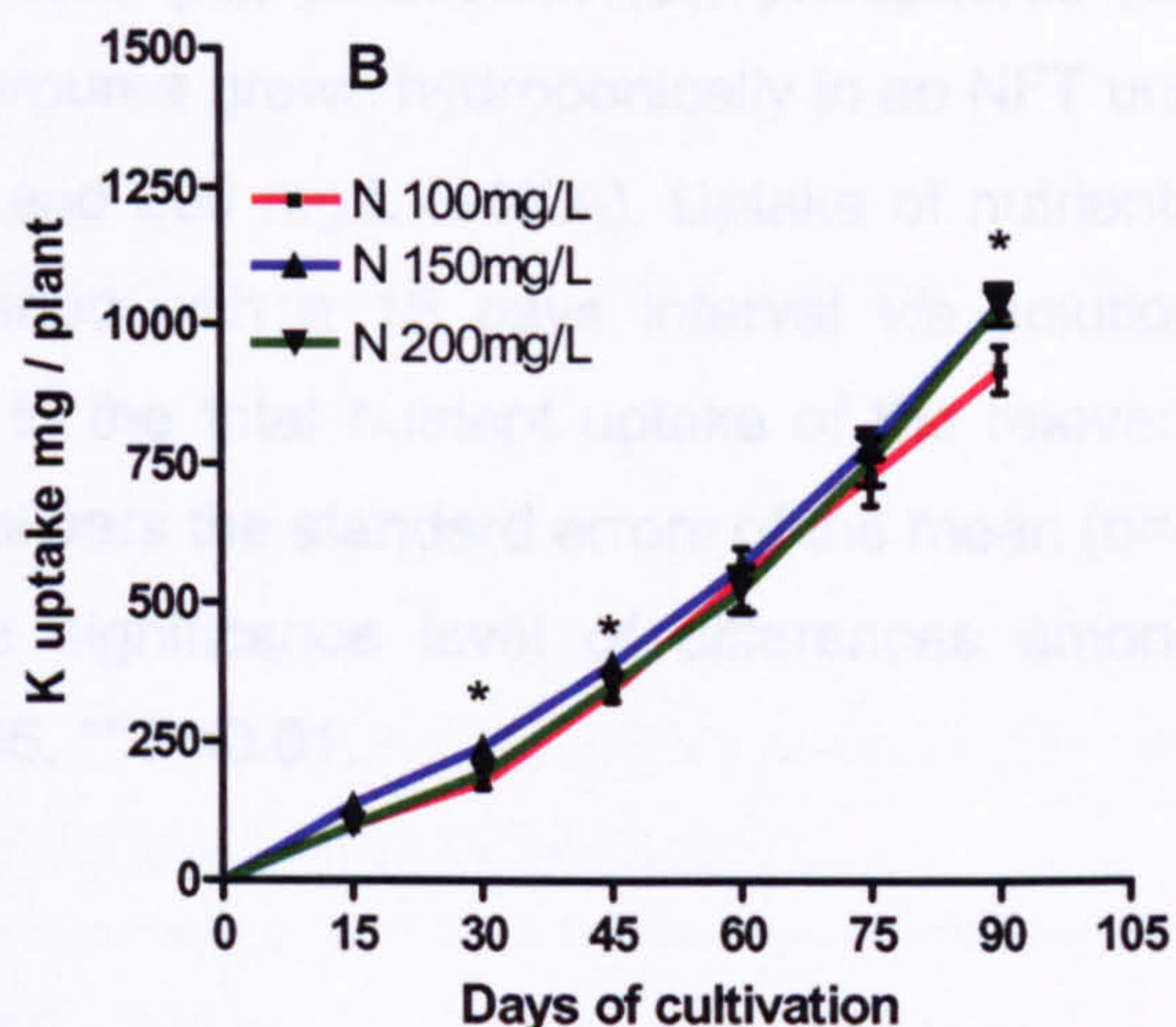
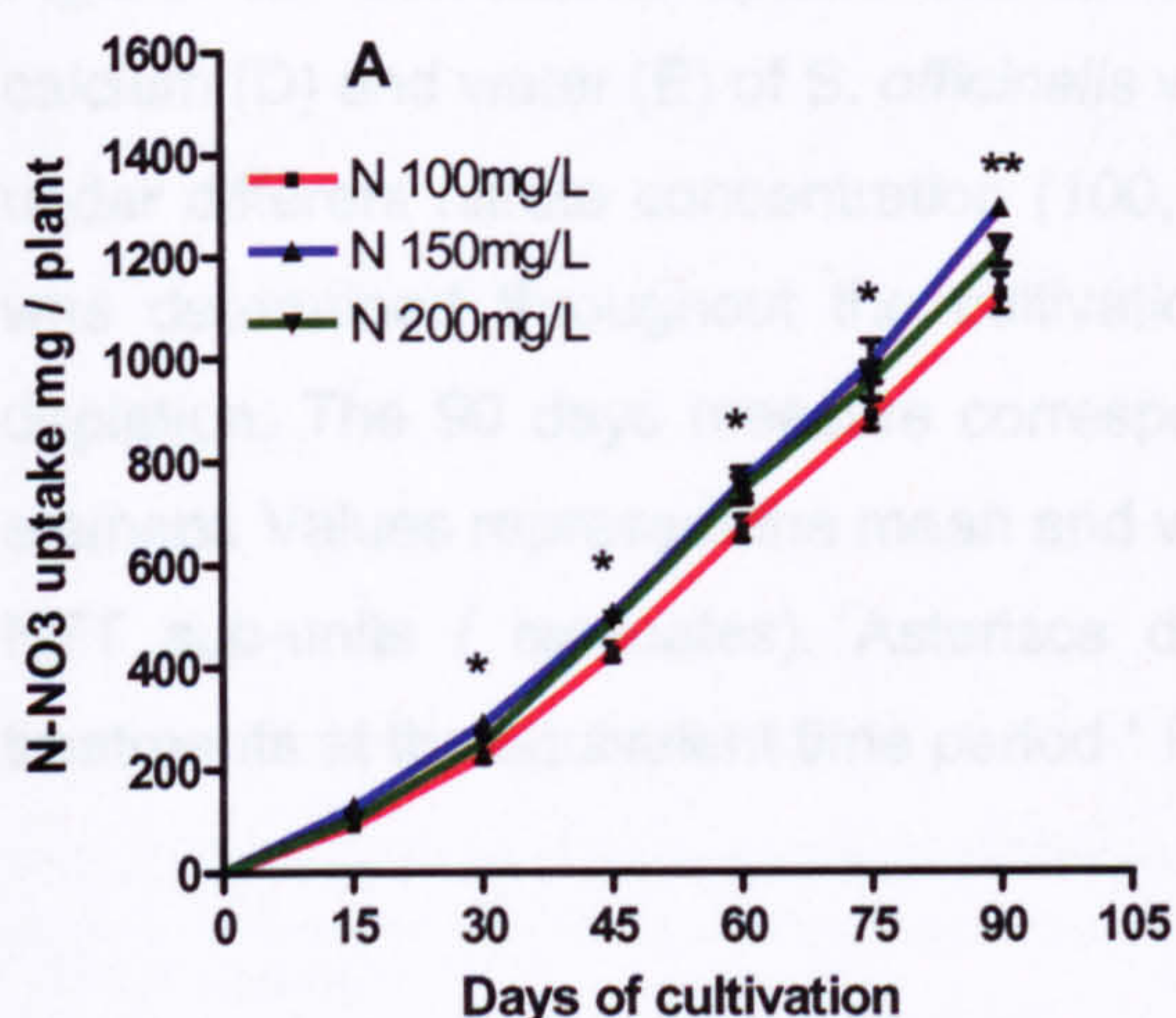


Figure 4.4 Chla, Chlb and total carotenoids content of fresh leaves expressed per leaf weight (A) or leaf area (B) of *S. officinalis* var *purpurea* plants cultivated under three levels of N-NO₃ (100, 150 and 200 mg/L). Pigment content (µg) was determined from young fully expanded sage leaves harvested one day before the end of the experiment.

Vertical bars indicate standard errors of the mean ($n=4$ NFT sub-units / replicates). Columns within a group of three, followed by the same letter are not significantly different at 5% level. Asterisks denote level of significance * $P < 0.05$.

4.3.3 Nutrient uptake as affected by nitrate level

Cumulative nutrient uptake of *S. officinalis* var *purpurea* plants (in mg) for nitrates, phosphorus (P), potassium (K) and calcium (Ca), and water uptake are depicted in Figure 4.5 A, B, C, D and E respectively. The last value on the uptake curve represents the mean total uptake per plant of the corresponding element and water for the entire cultivation period.



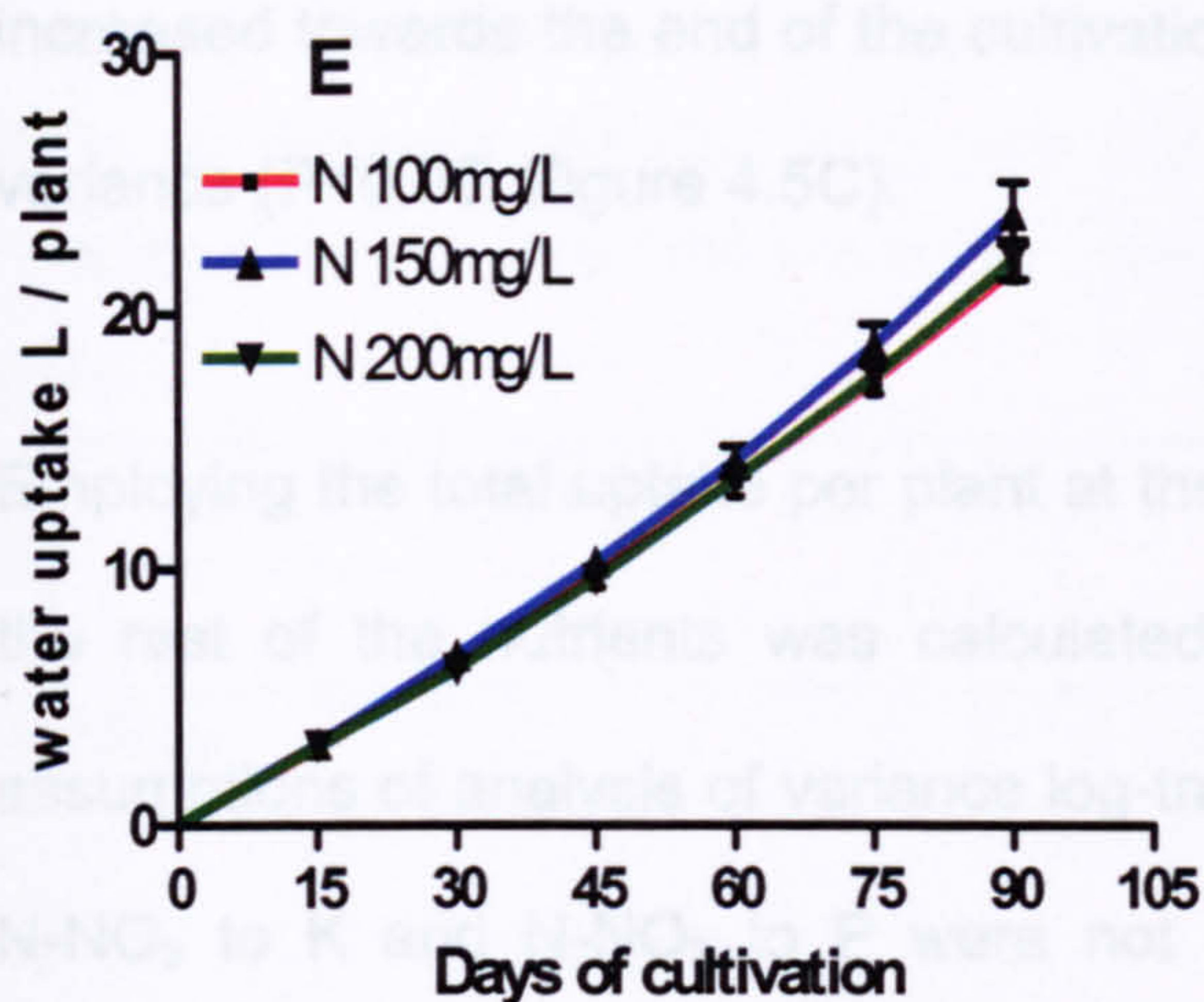


Figure 4.5 Cumulative uptake curves for nitrates (A), potassium (B), phosphorus (C) calcium (D) and water (E) of *S. officinalis* var *purpurea* grown hydroponically in an NFT unit under different nitrate concentration (100, 150 and 200 mg/L N-NO₃). Uptake of nutrients was determined throughout the cultivation period with a 15 days interval *via* solution depletion. The 90 days measure corresponds to the total nutrient uptake of the relevant element. Values represent the mean and vertical bars the standard errors of the mean (n=4 NFT sub-units / replicates). Asterisks denote significance level of differences among treatments at the equivalent time period * P<0.05, ** P<0.01.

Cumulative uptake curves of *S. officinalis* var *purpurea* plants constructed for N-NO₃, K, P and Ca uptake under three nitrate levels (100, 150 and 200 mg/L N-NO₃) demonstrated the same trend among treatments in the uptake of the corresponding elements. The uptake of all macro-nutrients analysed was consistently higher under the 150 mg/L of nitrates in comparison to the 100 and 200 mg/L levels. The impact of nitrate supply on N-NO₃ uptake was already apparent (P<0.05) after 30 days and remained statistically significant until the end of cultivation (P<0.01 at 90 days, one-way ANOVA, Figure 4.5A). Uptake of K was significantly raised under the 150 and 200 mg/L N-NO₃ treatments after 15 and 30 days of growth (P<0.05, one-way ANOVA, Figure 4.5B) however differentiation in K uptake did not attain statistical significance after 45 days of growth. N-NO₃ level caused no significant variation in the uptake of Ca although higher values were observed under the 150 and 200 mg/L N-NO₃ treatment (P>0.05, one-way ANOVA, Figure 4.5D). P uptake was found to be significantly

increased towards the end of the cultivation period as revealed by one-way analysis of variance ($P < 0.05$, Figure 4.5C).

Employing the total uptake per plant at the end of the experiment the ratio of N-NO₃ to the rest of the nutrients was calculated (Table 4.5). As values complied with the assumptions of analysis of variance log-transformation was not necessary. The ratio of N-NO₃ to K and N-NO₃ to P were not affected by the nitrate level in the nutrient solution suggesting analogous responses of N, K and P to nitrate treatments. In the same manner the N-NO₃ to Ca ratio appeared to be the same ($P > 0.05$) under all nitrate levels (Table 4.5), however standard errors indicated great variation within treatments.

Table 4.5 Uptake ratio of N-NO₃ to K, P and Ca of hydroponically grown *Salvia officinalis* var *purpurea* under three levels of nitrates (100 150 and 200 mg/L of N-NO₃).

Treatment	N-NO₃ 100 mg/L	N-NO₃ 150 mg/L	N-NO₃ 200 mg/L
Uptake ratio			
N/K	1.22 ± 0.069a	1.21 ± 0.052a	1.18 ± 0.030a
N/P	2.52 ± 0.122a	2.42 ± 0.124a	2.61 ± 0.099a
N/Ca	1.41 ± 0.161a	1.25 ± 0.210a	1.32 ± 0.06a

Values represent the mean and standard errors of the mean (n=4 NFT sub-units / replicates). Values within a row followed by the same letter are not significantly different at 5% level.

When Pearson product-moment correlation was performed among nutrients total uptake, significant relationships became evident. N-NO₃ and K uptake were strongly and positively associated ($r=0.74$, $P < 0.01$). Significant positive relationships were also revealed between N-NO₃/P and K/P uptake ($r=0.66$, $P < 0.05$ and $r=0.61$, $P < 0.05$ respectively). On the other hand, no firm relationship was established for Ca and any other of the three elements (N-NO₃, P, or K). All pre-mentioned correlations are illustrated in Table 4.6.

Table 4.6 Correlation matrix illustrating potential relationships between total uptake of different nutrients (N-NO₃, P, K and Ca) of *S. officinalis* var *purpurea* plants grown hydroponically for three months under different levels of nitrates (100,150 and 200 mg/L N-NO₃)

x	N-NO₃	P	K	Ca
y				
N-NO₃		r = 0.656 P<0.05	r = 0.743 P<0.01	r = 0.310 P> 0.05
P	r = 0.656 P<0.05		r = 0.614 P<0.05	r = 0.238 P>0.05
K	r = 0.743 P<0.01	r = 0.614 P<0.05		r = 0.025 P>0.05
Ca	r = 0.310 P> 0.05	r = 0.238 P>0.05	r = 0.025 P>0.05	

r represents the Pearson product –moment correlation

P value indicates significance of the correlation

x, y represents the nutrient uptake variables of the correlation

Number of xy pairs tested was n=12 NFT sub-units / replicates

Regression analysis revealed significant relationship between nitrate concentration in the nutrient solution and the uptake of N-NO₃, K and P (Figure 4.6). N-NO₃ uptake was strongly influenced by the nitrate supply ($r^2=0.68$) in a dose-response manner. At the 100 mg/L treatment it appeared to be relatively low but with the rise of N-NO₃ concentration in the solution to 150 and 200 mg/L a considerable increase in nitrate uptake became evident. N-NO₃ uptake under the 200 mg/L nitrate supply never exceeded the values observed at 150 mg/L (Figure 4.6A). The same pattern was followed by the rest of the nutrients with their uptake being greater at the higher nitrate treatments (150 and 200 mg/L). The impact of nitrate treatment on K and P uptake is illustrated in Figures 4.6B and 4.6C respectively. The effect of nitrate level was less obvious for Ca uptake with the N-NO₃ concentration of the nutrient solution explaining only 43% of the uptake of Ca (Figure 4.6D).

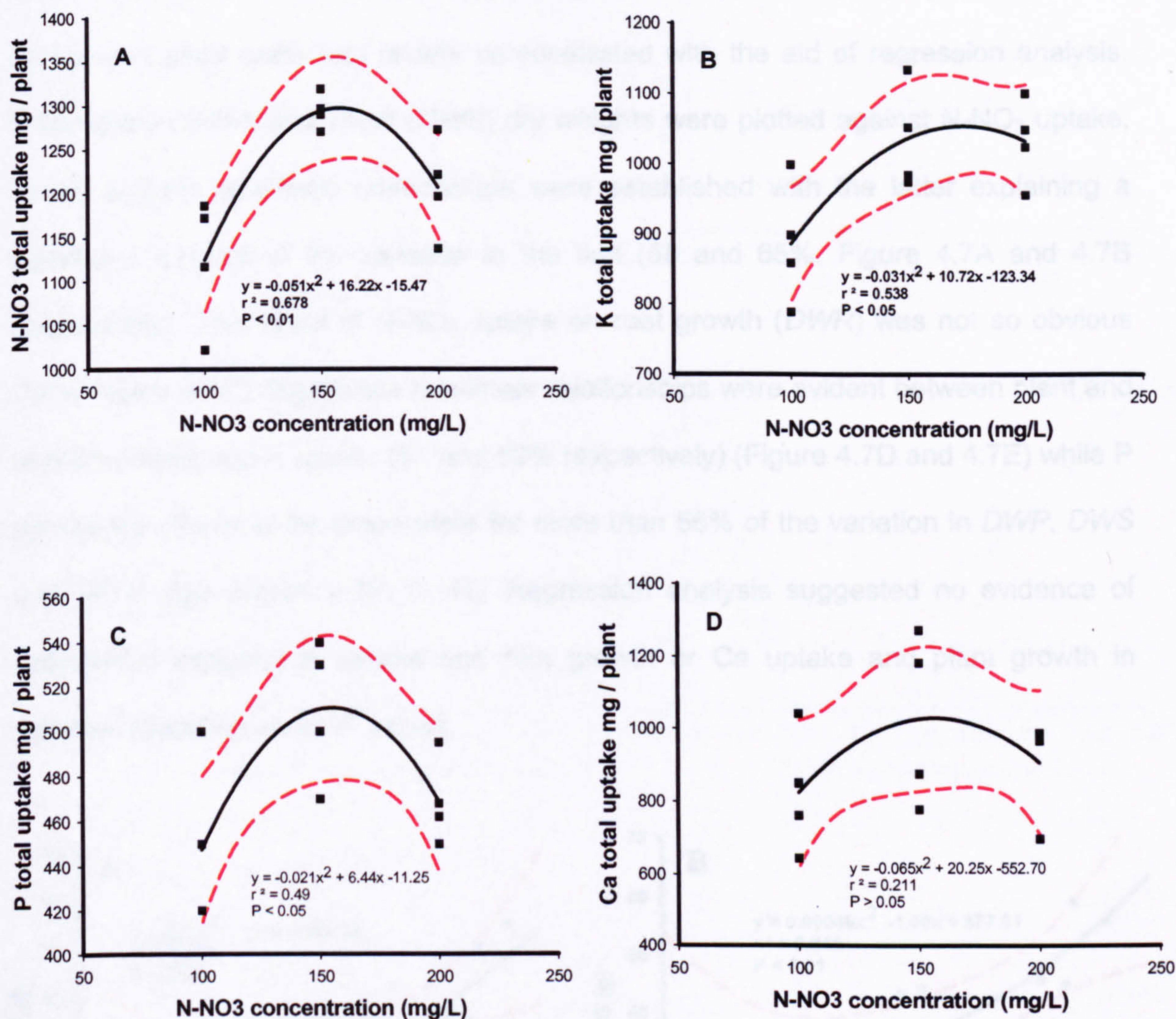


Figure 4.6 Regression analysis performed between N-NO₃ level in the nutrient solution versus total uptake per plant of the nutrients: N-NO₃ (A), K (B), P (C) and Ca (D).

S. officinalis var *purpurea* plants were hydroponically raised under three nitrate treatments (100, 150 and 200 mg/L N-NO₃) for a period of three months.

Red dotted lines represent the 95% confidence band of the regression (quadratic).

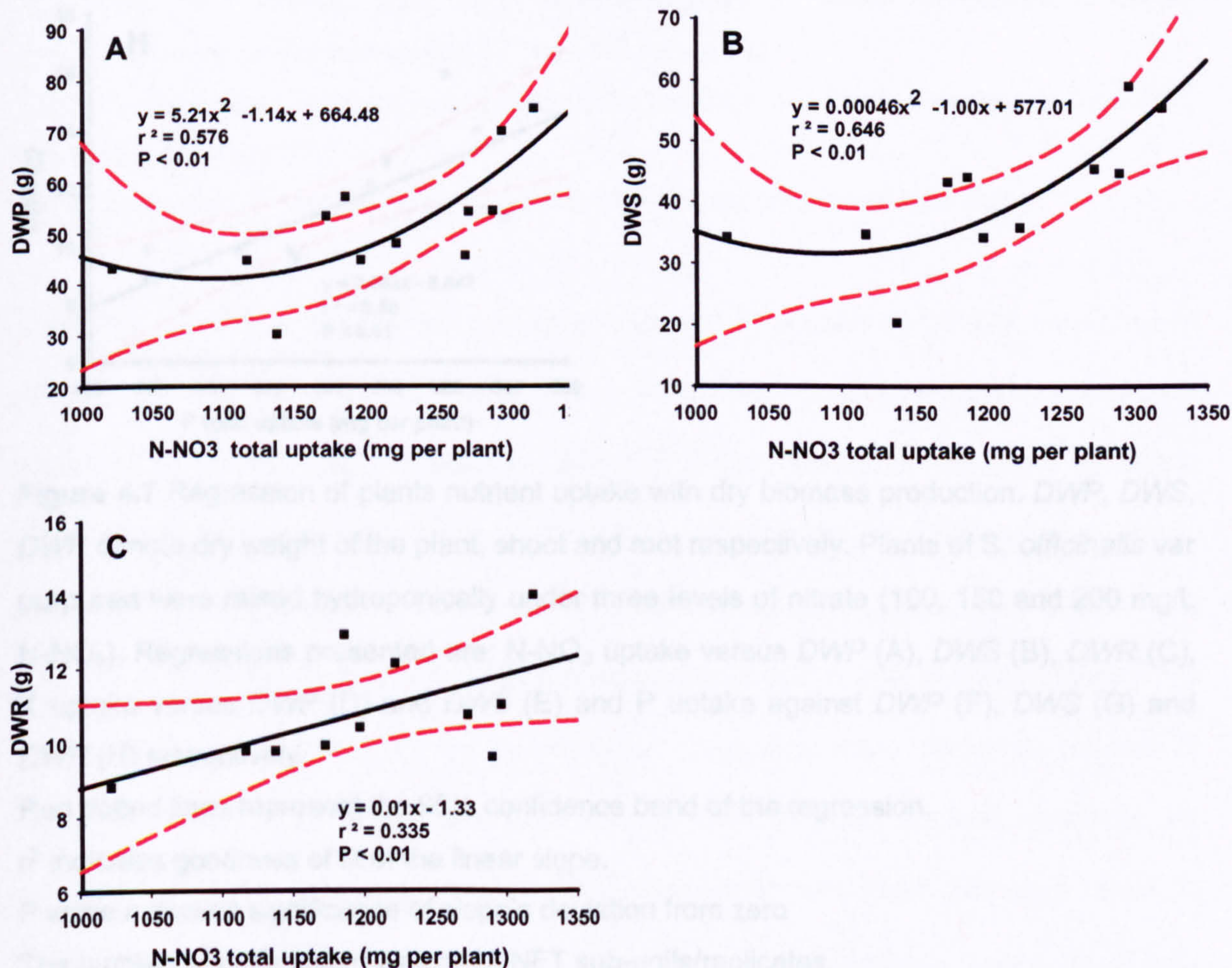
r^2 indicates goodness of fit the quadratic slopes.

P value indicates the significance of slope's deviation from zero

The number of pairs tested was $n=12$ sub-units/replicates

4.3.4 Nutrient uptake relation to plant growth

The profound effect of macro-nutrients uptake on plant biomass production and component plant parts was clearly demonstrated with the aid of regression analysis. When plant (*DWP*) and shoot (*DWS*) dry weights were plotted against N-NO₃ uptake, strong positive quadratic relationships were established with the latter explaining a significant amount of the variation in the first (58 and 65%, Figure 4.7A and 4.7B respectively). The effect of N-NO₃ uptake on root growth (*DWR*) was not so obvious (34% Figure 4.7C). Significant non-linear relationships were evident between plant and shoot biomass and K uptake (51 and 52% respectively) (Figure 4.7D and 4.7E) while P uptake was found to be responsible for more than 56% of the variation in *DWP*, *DWS* and *DWR* (see Figure 4.7F, G, H). Regression analysis suggested no evidence of association between K uptake and root growth or Ca uptake and plant growth in general, revealing minor r^2 values.



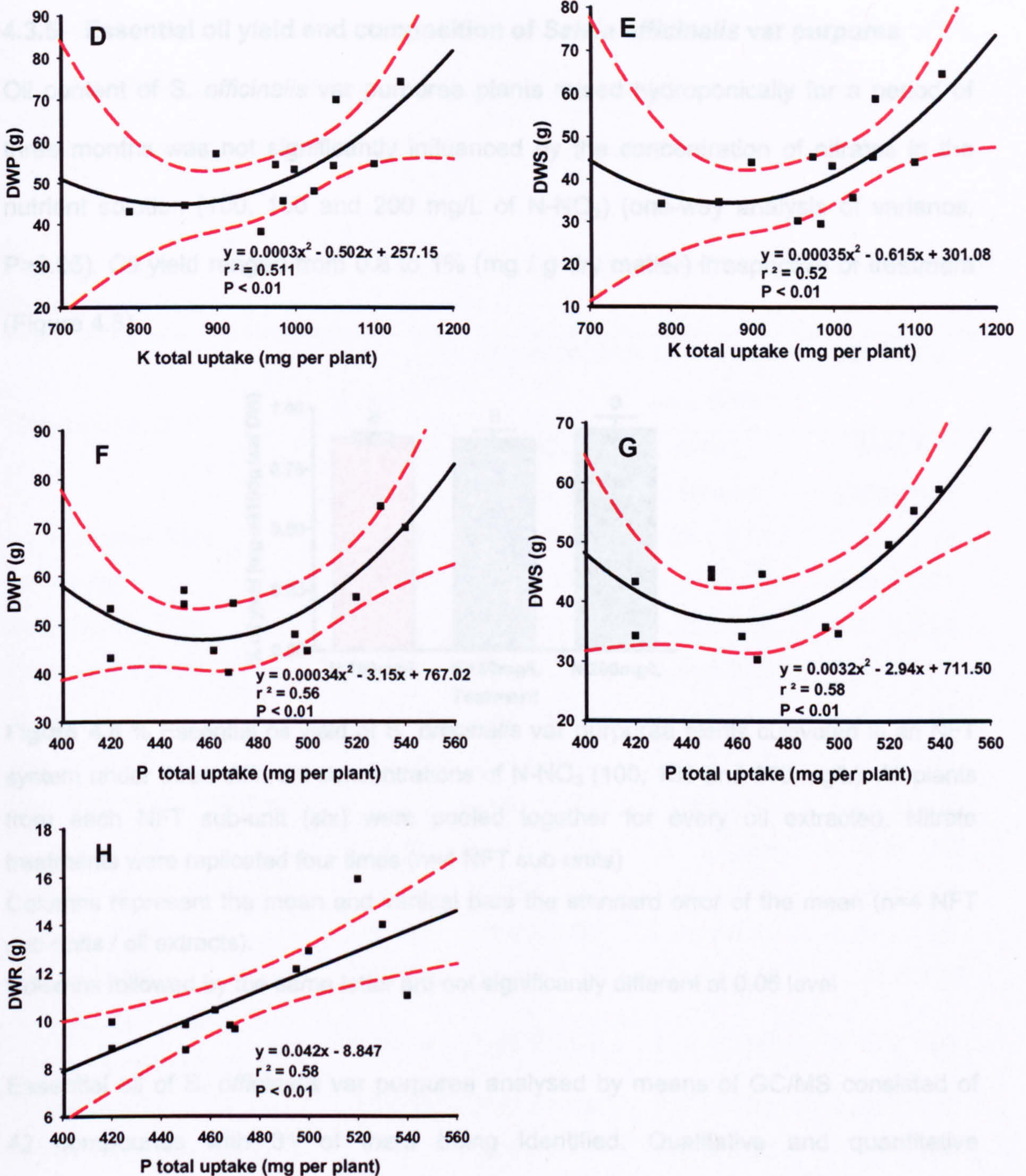


Figure 4.7 Regression of plants nutrient uptake with dry biomass production. *DWP*, *DWS*, *DWR* denote dry weight of the plant, shoot and root respectively. Plants of *S. officinalis* var *purpurea* were raised hydroponically under three levels of nitrate (100, 150 and 200 mg/L $N-NO_3$). Regressions presented are: $N-NO_3$ uptake versus *DWP* (A), *DWS* (B), *DWR* (C), K uptake versus *DWP* (D) and *DWS* (E) and P uptake against *DWP* (F), *DWS* (G) and *DWR* (H) respectively.

Red dotted lines represent the 95% confidence band of the regression.

r^2 indicates goodness of fit of the linear slope.

P value indicates significance of slope's deviation from zero

The number of pairs tested was $n=12$ NFT sub-units/replicates

4.3.5 Essential oil yield and composition of *Salvia officinalis* var *purpurea*

Oil content of *S. officinalis* var *purpurea* plants raised hydroponically for a period of three months was not significantly influenced by the concentration of nitrates in the nutrient solution (100, 150 and 200 mg/L of N-NO₃) (one-way analysis of variance, P>0.05). Oil yield ranged from 0.8 to 1% (mg / g dry matter) irrespective of treatment (Figure 4.8).

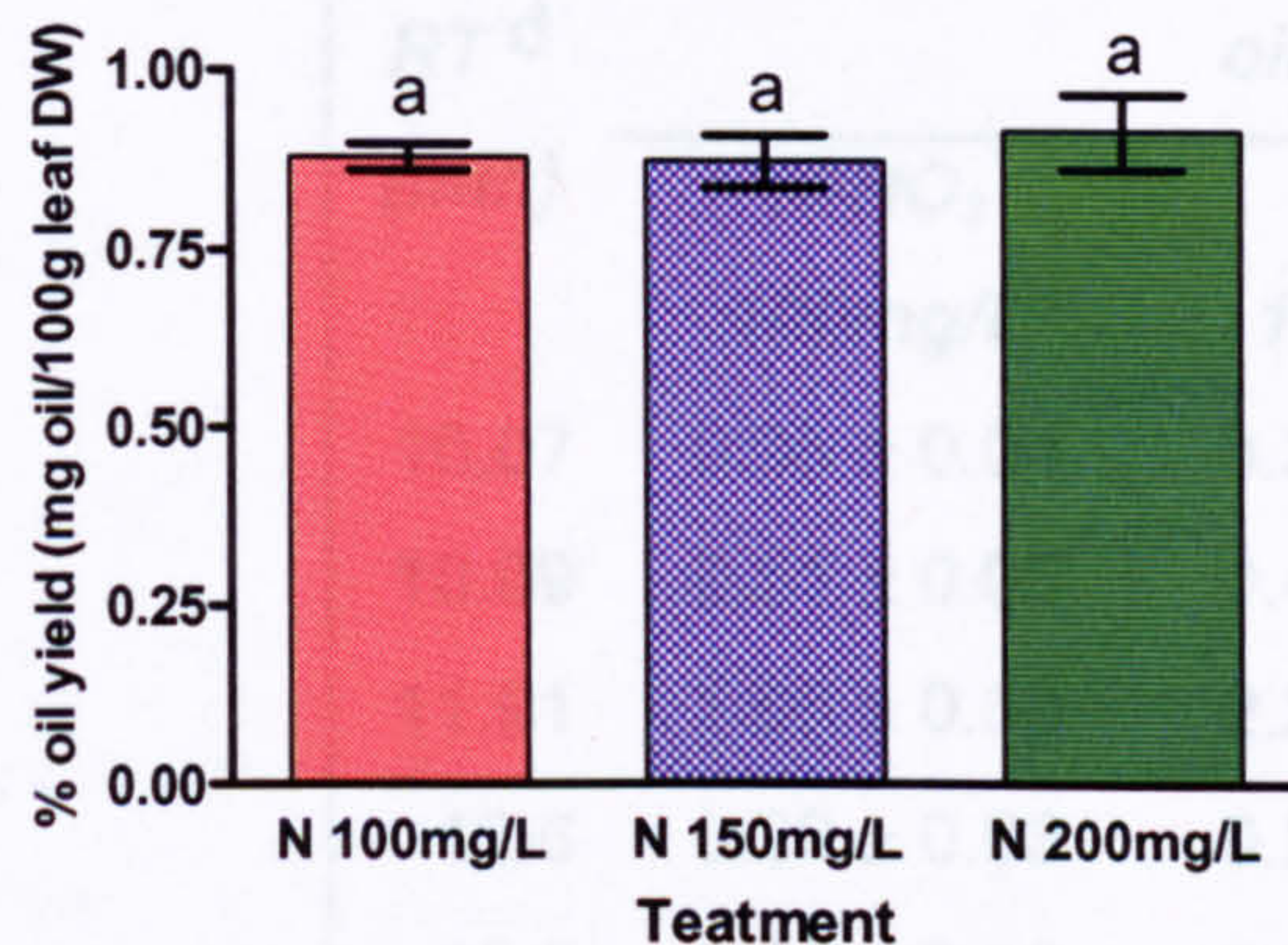


Figure 4.8 % Essential oil yield of *S. officinalis* var *purpurea* plants cultivated in an NFT system under three different concentrations of N-NO₃ (100, 150 and 200 mg/L). All plants from each NFT sub-unit (six) were pooled together for every oil extracted. Nitrate treatments were replicated four times (n=4 NFT sub-units)

Columns represent the mean and vertical bars the standard error of the mean (n=4 NFT sub-units / oil extracts).

Columns followed by the same letter are not significantly different at 0.05 level

Essential oil of *S. officinalis* var *purpurea* analysed by means of GC/MS consisted of 42 compounds with 31 of them being identified. Qualitative and quantitative composition of the oil is illustrated in Table 4.7. α -Caryophyllene was found to be the most abundant constituent in the oil mixture contributing the 23% of the whole. Camphor, β -thujone and ledol also constituted major compounds accounting for 11, 10 and 5% of the total respectively. With the exception of 1.8 cineole that represented 3% of the mixture, *S. officinalis* var *purpurea* oil comprised many terpenes present in quantities from 1 to 2.5% like beta pinene, D-limonene, borneol, germacrene, isocamphone, ylangene and caryophyllene. The rest of the compounds were minor, in no case exceeding 0.5%. Oil quality appeared to be uniform under all N-NO₃

concentrations (100, 150 and 200 mg/L N-NO₃) signifying that nitrate treatment of the hydroponically raised *S. officinalis* var *purpurea* plants did not induce changes in oil production (one-way ANOVA) (Table 4.7).

Table 4.7 Chemical compounds present in essential oils of *Salvia officinalis* var *purpurea* analysed by GC/MS

Compound, % ^c	RT ^d (min)	% percentage of the compounds in the		
		oil ± SEM ^e		
		N-NO ₃ 100mg/L	N-NO ₃ 150mg/L	N-NO ₃ 200mg/L ^j
alpha Pinene 95%	10.07	0.35 ± 0.04	0.32 ± 0.01	0.32 ± 0.03
camphene	10.69	0.51 ± 0.05	0.47 ± 0.02	0.43 ± 0.07
beta Pinene 97%	11.91	1.65 ± 0.53	2.26 ± 0.08	2.05 ± 0.32
beta mycrene 83%	12.6	0.29 ± 0.03	0.29 ± 0.04	0.32 ± 0.03
NI ^g	13.6	0.33 ± 0.12	0.32 ± 0.01	0.35 ± 0.01
D-limonene 90%	14.19	0.71 ± 0.06	0.89 ± 0.04	0.77 ± 0.02
eucalyptol 98% (1,8 cineole)	14.26	2.96 ± 0.24	3.09 ± 0.05	2.93 ± 0.22
3 carene 91%	15.83	0.25 ± 0.001	0.27 ± 0.004	0.26 ± 0.005
thujone a 94%	17.19	10.05 ± 0.38	9.52 ± 0.56	9.86 ± 0.49
thujone b 98%	17.31	0.34 ± 0.01	0.36 ± 0.01	0.37 ± 0.02
camphor 98%	17.74	11.41 ± 0.23	11.48 ± 0.92	11.46 ± 0.32
Bicyclo(3,1,1)heptan-3-one,2,6,6-trimethyl 95%	18.76	1.24 ± 0.04	1.14 ± 0.06	1.19 ± 0.06
Borneol 95%	19.39	2.67 ± 0.15	2.21 ± 0.42	2.85 ± 0.12
Isopinocampone 91%	19.62	1.05 ± 0.03	0.99 ± 0.11	1.14 ± 0.04
NI	19.91	0.76 ± 0.04	0.73 ± 0.04	0.84 ± 0.04
D-Verbenone 90%	20.79	0.33 ± 0.01	0.33 ± 0.02	0.37 ± 0.01
Bornylacetate 94%	21.28	0.37 ± 0.01	0.40 ± 0.03	0.40 ± 0.04
2-Methoxy-4-vinyl phenol	23.98	0.55 ± 0.01	0.56 ± 0.03	0.60 ± 0.02
alpha- cubebene 95%	24.94	0.58 ± 0.03	0.57 ± 0.1	0.56 ± 0.1
Ylangene 98%	26.12	0.65 ± 0.03a	0.74 ± 0.02b	0.74 ± 0.02b
Copaene 96%	26.84	0.25 ± 0.02	0.30 ± 0.01	0.29 ± 0.0
NI	26.98	0.69 ± 0.04	0.77 ± 0.04	0.76 ± 0.03
1H-Cyclopropeazulene 99%	27.46	0.29 ± 0.01a	0.36 ± 0.01b	0.35 ± 0.02ab
caryophyllene 99%	28.08	0.94 ± 0.03	1.02 ± 0.06	1.05 ± 0.03

Germacrene D 97%	28.39	2.44 ± 0.01	2.55 ± 0.07	2.61 ± 0.05
1H-Cyclopropeazulene decahydro-1,1,799%	28.69	0.88 ± 0.04	1.03 ± 0.04	1.00 ± 0.04
alpha- caryophellene 97%	29.50	23.2 ± 0 0.8	23.61 ± 0.66	24.00 ± 0.73
NI	29.70	0.28 ± 0.01	0.27 ± 0.03	0.28 ± 0.01
NI	30.20	2.34 ± 0.13	2.60 ± 0.11	2.54 ± 0.06
NI	30.31	0.81 ± 0.06	1.48 ± 0.37	1.32 ± 0.1
Naphthalene octahydro-7- methyl 99%	30.75	1.68 ± 0.08	1.97 ± 0.09	1.81 ± 0.07
NI	30.92	0.48 ± 0.03	0.53 ± 0.03	0.51 ± 0.03
Butylated Hydroxytoluene 97%	31.13	0.29 ± 0.02	0.30 ± 0.003	0.30 ± 0.02
Naphthalene 1,2,3,5,6,8a hexahydro-4,7-dimethyl 96%	31.27	5.48 ± 0.28	5.18 ± 0.54	5.67 ± 0.37
Isolongifolene 9,10,-dehydro	32.92	1.31 ± 0.05	1.36 ± 0.3	1.29 ± 0.05
Azulene octahydro 98%	33.20	0.34 ± 0.02	0.42 ± 0.03	0.37 ± 0.02
Ledol	33.63	5.59 ± 0.25	5.21 ± 0.24	5.00 ± 0.23
NI	35.40	0.31 ± 0.01	0.32 ± 0.03	0.29 ± 0.01
NI	39.45	2.05 ± 0.21	2.07 ± 0.25	1.76 ± 0.02
NI	45.51	7.86 ± 1.51	5.87 ± 0.99	5.34 ± 0.76
NI	46.23	0.65 ± 0.05	0.46 ± 0.08	0.38 ± 0.06

c, Identification was accomplished via NIST database. Percentage represents matching of the compound with library's spectrum

d, Retention time of the constituents peak in the chromatogram

e, Relative percentages of the compounds in the oil mixture, calculated from a total area of peaks (100%).

g, Not identified compound

j, Plants were cultivated hydroponically under three levels of N-NO₃ (100, 150 and 200 mg/L). Six plants from each replicate were pooled together for each oil produced. Treatments were replicated four times (n=4 NFT sub-units)

a, b, Where there is a statistically significant difference is indicated by the letters a, b. Values followed by the same letter within a row are not significantly different at P<0.05. Values in each row were tested for equality of variances and normality of distribution prior to ANOVA. Means and standard errors were determined from four replicates (n=4 oils analysed deriving from four NFT sub-units)

4.3.6 Acetyl and butyryl - cholinesterase inhibition by the essential oils

Essential oils from hydroponically grown *S. officinalis* var *purpurea* plants with different nitrate treatments possessed obvious dual anti-cholinesterase properties (Table 4.8). Inhibitory activity expressed by mean IC50 values ranged from 0.086 to 0.12 mg/ml for bovine acetylcholinesterase (AChE) and 0.096 to 0.13 mg/ml for butyryl cholinesterase (BuChE) enzyme under 15 min incubation with the inhibitor / oil, irrespective of growth treatment (Table 4.8). Increase in incubation time significantly enhanced the potency of the oil ($P < 0.001$, one way ANOVA) for BuChE reducing the IC50 value at 0.070-0.079 mg/ml (Figure 4.9, Table 4.8). Different N-NO₃ concentration in the nutrient solution (100, 150 and 200 mg/L N-NO₃) did not induce variation in *S. officinalis* var *purpurea* the inhibition of cholinesterases suggesting no stimulation of cholinergic activities in oils extracted from *S. officinalis* var *purpurea* by the nitrate treatment ($P > 0.05$, one-way ANOVA). Comparison of IC50 values for both cholinergic enzymes and incubation periods irrespective of treatment is depicted in Figure 4.9.

Table 4.8 AChE and BuChE inhibition of *S. officinalis* var *purpurea* oils expressed as IC50 value

<i>Treatment^a</i>	<i>IC50 ± SEM (mg/ml)^b</i>	
	<i>AChE inhibitory activity^c</i>	
<i>N-NO₃ 100 mg/L</i>	0.086 ± 0.01a	
<i>N-NO₃ 150 mg/L</i>	0.120 ± 0.01a	
<i>N-NO₃ 200 mg/L</i>	0.101 ± 0.02a	
	<i>BuChE inhibitory activity^d</i>	
	<i>15 min incubation</i>	<i>30 min incubation</i>
<i>N-NO₃ 100 mg/L</i>	0.118 ± 0.02a	0.077 ± 0.006b
<i>N-NO₃ 150 mg/L</i>	0.096 ± 0.008a	0.070 ± 0.005b
<i>N-NO₃ 200 mg/L</i>	0.131 ± 0.011a	0.075 ± 0.006b

a Oils were extracted from *S. officinalis* var *purpurea* plants cultivated hydroponically under three levels of N-NO₃ (100, 150 and 200 mg/L). Treatments were replicated four times, resulting in the production of four oil extracts per treatment. Six plants from each replicate / NFT sub-unit were pooled together for every oil/inhibitor.

b Concentration of oil required for 50% enzyme inhibition, as calculated from a sigmoidal dose-response curve equation (Appendix 2, section 2.2). IC₅₀ values represent the mean of four oils and standard error of the means. Values within columns followed by the same letter are not significantly different at 5% level (n=4 NFT sub-units)

c AChE (acetylcholinesterase) enzyme from bovine erythrocytes

d BuChE (butyrylcholinesterase) enzyme from horse serum.

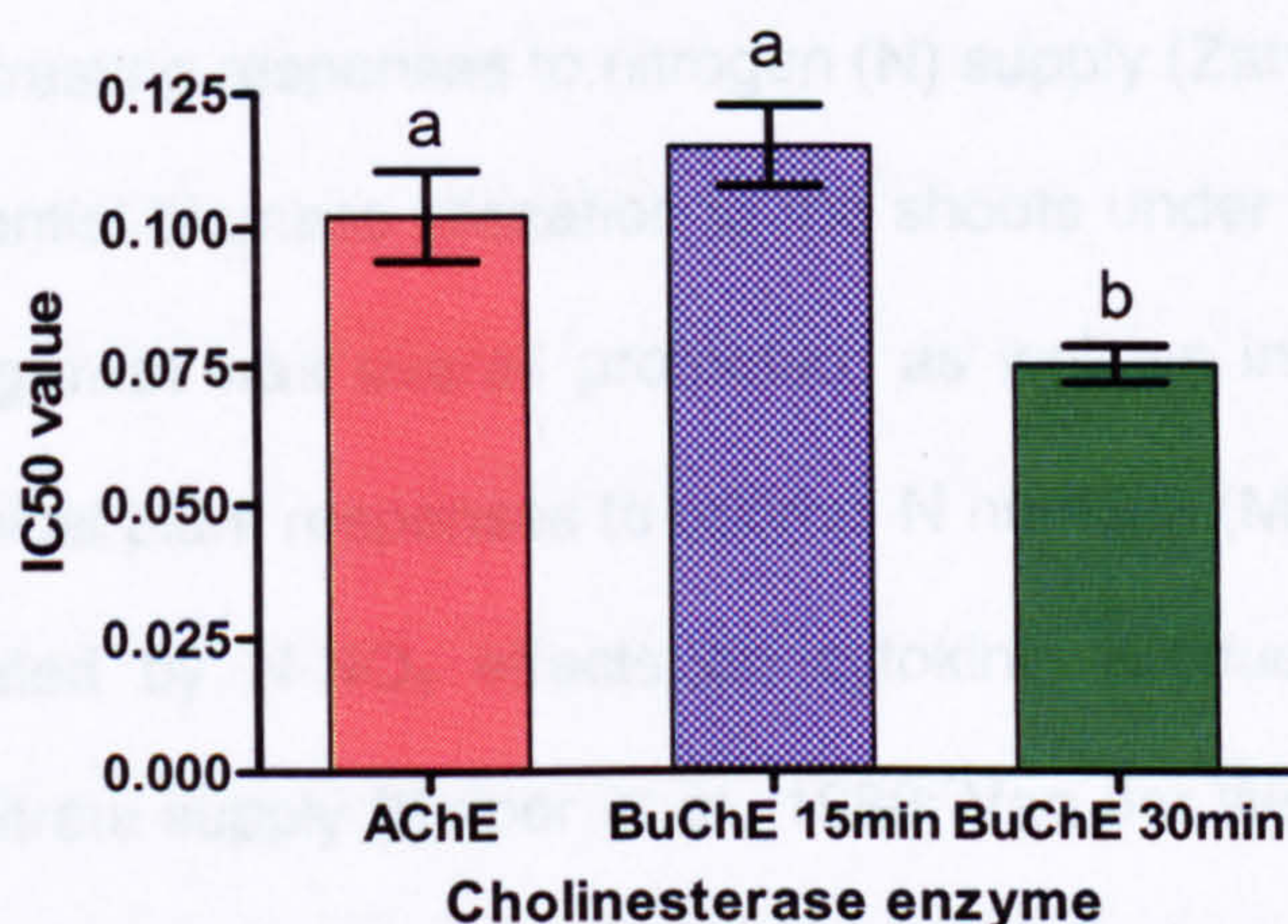


Figure 4.9 Comparison of inhibitory activity (expressed as an IC₅₀ value) for bovine AChE (15 min incubation) and BuChE (15 and 30 min incubation) enzymes from 12 oils of *S. officinalis* var *purpurea* plants raised hydroponically under different nutrient conditions. Columns represent the means and vertical bars the standard error of the mean (n=12 oils). Columns not followed by the same letter differed statistically at 0.001 level.

4.4 Discussion

4.4.1 *Salvia officinalis* var *purpurea* growth responses to nitrogen nutrition

The present study attempted to define optimum nitrate (N-NO₃) nutritional levels for the cultivation of *S. officinalis* var *purpurea*, an important ornamental and medicinal plant. Outcomes confirmed the impact of nitrates on plant growth. Dry weight of the plant (*DWP*) and shoot (*DWS*) as well as absolute growth rates of plant (*AGR*) and shoot (*AGRs*) were significantly affected by the N-NO₃ concentration (100, 150 and 200 mg/L) in the nutrient solution of the hydroponically raised *S. officinalis* var *purpurea*. The 100 and 150 mg/L nitrate supply were sufficient for maximum biomass production, whereas the higher N-NO₃ level (200 mg/L) induced a decline in plant yield. Nitrate treatment (100, 150 and 200 mg/L) did not cause significant variation in plant root size. Roots in general exhibit contrasting responses to nitrogen (N) supply (Zatylny & St-Pierre, 2006). Conversely, preferential biomass allocation to the shoots under the 100 and 150 mg/L treatments where growth was overall promoted, as well as increased shoot to root ratios constitute typical plant responses to optimal N nutrition (Marschner, 1995) that is likely to be mediated by N-NO₃ effects on cytokinin production in plants that is enhanced due to nitrate supply (Kuiper *et al.*, 1988; Van der Werf & Nagel, 1996) The reduced shoot biomass observed at the higher N-NO₃ supply has been shown in other studies where elevated amounts of N did not promote or even suppressed growth; for example the findings of Economakis (1993b) for *Salvia fruticosa* Mill. in solution culture under the same N-NO₃ concentration. Field experiment with *S. officinalis* L. and N fertiliser application over a range of 0 to 240 Kg N/ha (Karamanos, 1995) demonstrated enhanced plant biomass at 80 Kg N/ha in comparison with higher N supply that did not further promote plant yield. Outcomes of the last study (Karamanos, 1995) are in agreement with Singh and co-workers (2005) who reported increased growth of medicinal *Withania somnifera* with N application from 0 to 90 Kg/ha. Other reports providing supporting evidence for the present experiment include Khan *et al.*,

1995a; Lata & Sadowska, 1996; Schenk, 1996; Andrews *et al.*, 1999; Akanbi & Togun, 2001; Omidbaigi & Arjmandi, 2002; Baranauskiene *et al.*, 2003; de Groot *et al.*, 2003; Martin *et al.*, 2006. It is noteworthy that growth of *S. officinalis* var *purpurea* plants in this study was not linearly correlated to uptake of nutrients as the highest N-NO₃ concentration (200 mg/L) revealed increased nutrient absorption in comparison with the 100 mg/L level, despite the induced decline in plant biomass production. Andrews and colleagues (1999) and de Groot *et al.*, (2003) suggested that accumulated nitrates in plant do not improve growth rate, but solely increase N concentration in plant tissues (Marschner, 1995; Andrews *et al.*, 1999). In the present experiment nitrate, phosphorus (P) potassium (K), or calcium (Ca) uptake was not linearly related with the applied nitrate dose in the nutrient solution. This is in agreement with previous studies that demonstrated no correlation between nitrates in plant tissue (or nitrate influx) and N fertilisation level (Akanbi & Togun, 2001; Baranauskiene *et al.*, 2003; Martin *et al.*, 2006). The maximum nitrate uptake per unit of roots is not a constant value but for a certain species, plant age and environmental conditions depends on N status in plant and nitrate concentration at the root surface (Schenk, 1996). It can be concluded that for *Salvia officinalis* var *purpurea* and *S. fruticosa* Mill. (Economakis, 193b) maximum inflow rate of nitrates occurs at approximately 150 mg/L N-NO₃. The similar uptake values of nitrates at 200 mg/L N-NO₃ with the 150 mg/L supply indicate a negative feedback regulation of N-NO₃ uptake (Marschner, 1995; Cardenas-Navarro *et al.*, 1998; Glass *et al.*, 2001) at doses higher than 150 mg/L. Down regulation of nitrate influx constitutes a protective mechanism against osmotic damage or potential ion toxicity as a result of excessive accumulation (Glass *et al.*, 2001). Uptake of nutrients that are actively absorbed like N, P and K is regulated by circulation of amino acids (glutamine; Glass *et al.*, 2001) from shoot to root and *vice versa* (Schenk, 1996). Similar N-NO₃ uptake values to 150 mg/L treatment with a concomitant induced decline in shoot growth at 200 mg/L nitrate level indicates apparently lower incorporation of N into proteins in plant leaves and translocation of amino acids from shoot to root that may be

the cause for feedback inhibition of nitrate uptake (de Groot *et al.*, 2003). Down regulation of nitrate transporters can also occur through effects exerted by nitrate itself i.e. allosteric effects or transporter phosphorylation (Glass *et al.*, 2001).

Water uptake was higher for the 150 mg/L N-NO₃ treatment but the increase did not attain statistical significance. It has been suggested that water uptake is closely related to nitrate supply (Cardenas-Navarro *et al.*, 1998). The observed decline in leaf area (LA) of *S. officinalis* var *purpurea* plants raised under the 150 mg/L N-NO₃ treatment is consist with the reduced shoot water content under the same nitrate supply (Meziane & Shipley, 2001). Taub (2002) indicated reduced specific leaf area with increased N supply, an outcome that is in agreement with the results of the present study.

The amount of chlorophyll was not influenced by the N-NO₃ treatment in this experiment. Chlorophyll content in leaves is considered an estimate of leaf photosynthetic capacity and could be related to primary production (Richardson *et al.*, 2002). Singh and colleagues (2005) reported significantly increased levels of chlorophylls for *Withania somnifera* under the highest N dose application (90kg/ha) in comparison with the control. However, no significant difference was evident among the N treatments (30, 60, 90 kg/ha; Singh *et al.*, 2005).

4.4.2 Nitrogen effect on yield and composition of *Salvia officinalis* var *purpurea* essential oils

In parallel with growth, *S. officinalis* var *purpurea* essential oil yield and composition was studied. Plants raised hydroponically, for a period of three months under different N-NO₃ supply (100, 150 and 200 mg/L) had a mean oil content of 0.9% (g / 100 g *DWL*). This is the first report concerning oil yield of *S. officinalis* var *purpurea*. Sage plants belonging to the same species (*S. officinalis* L.) have exhibited a variation of 0.9 to 2.3% oil content under different seasons and geographical origins (Putievsky *et al.*,

1986; Chalchat *et al.*, 1998; Perry *et al.*, 1999a; Karousou *et al.*, 2000; Santos-Gomes & Fernandes-Ferreira, 2001). The lower oil yield obtained from *S. officinalis* var *purpurea* in this experiment can be interpreted in relation to plant growth stage and geographical origin that strongly influences essential oil production (Bernath, 1986). Outcomes from various field experiments have provided evidence that sage oil yield is considerably lower at the vegetative stage and reaches a maximum during seed formation (Putievsky *et al.*, 1986; Karousou *et al.*, 2000; Santos-Gomes & Fernandes-Ferreira, 2001; Karioti *et al.*, 2003). Moreover, *S. officinalis* plants grown in Britain displayed a very low, 0.53% oil content (personal communication with Dr S. Savelev). Oil analysis by GC/MS revealed similar essential oil composition of *S. officinalis* var *purpurea* to *S. officinalis* (Langer *et al.*, 1996; Perry *et al.*, 1999a; Piccaglia *et al.*, 1997; Kintzios & Gianouli, 2000; Santos-Gomes & Fernandes-Ferreira, 2001), whereas large differences with *S. lavandulaefolia* and *S. fruticosa* were evident (Langer *et al.*, 1996; Kintzios & Gianouli, 2000; Savelev *et al.*, 2004). However, N-NO₃ treatment (100, 150 and 200 mg/L) did not significantly influence *S. officinalis* var *purpurea* essential oil production in terms of yield and composition. This finding is supported by other experiments that demonstrated no impact of N supply on essential oils. Similar concentration of nitrates (100, 150 and 200 mg/L) did not generate variation in essential oil content and quality of hydroponically grown *Origanum dictamnus* (Economakis *et al.*, 1999). Analogous outcomes have been demonstrated by other experiments (Piccaglia & Marioti, 1993; Karamanos, 1995; Baranauskiene *et al.*, 2003; Singh, *et al.*, 2005). Strong influence of genetic attributes on terpene production (Hamilton *et al.*, 2001) could provide an explanation for the consistency in oil yield and composition under various nutrient treatments. Contradictory conclusions drawn by Karioti and co-workers (2003), who reported qualitative and quantitative differences in *S. fruticosa* Mill. oil due to nitrogen supply (100,150 and 200 mg/L) and growth stage might reflect the influence of flowering and seed formation stage on essential oil production. The array of ontogenetic events that take place during transition from the

vegetative to reproductive stage depend among others on plant nutritional status (Taiz & Zeiger, 1991) and might affect secondary metabolism response to various external nutritional factors. In coherence with the previous study are Khan, *et al.* (1992) and (1999); Omidbaigi & Arjmandi, (2002) and Singh & Ganesha (2005). Terpene biosynthesis can be potentially stimulated by N because of its structural role in all enzyme proteins of secondary metabolites pathways (Marchner, 1995) and / or the well-known control that exerts over primary metabolism (see introduction), affecting in this manner interactions between primary and secondary pathways (Gershenzon, 1983). Contrasting outcomes demonstrated by various experiments investigating effects of N nutrition on essential oil production of aromatic and medicinal plants justify the conclusion drawn by Gershenzon (1983) that terpenes exhibit a variety of responses to nutrient supply with no obvious trend. More experimentation is therefore necessary to determine optimum nutrient conditions for maximum essential oil yield and quality.

4.4.3 *Salvia officinalis* var *purpurea* anti-cholinesterase activity

S. officinalis var *purpurea* anti-cholinesterase activity was examined in the present study. Essential oils from young *S. officinalis* var *purpurea* plants grown hydroponically for a period of three months under controlled nutritional conditions revealed relatively potent inhibitory activity for both cholinergic enzymes. 50% inhibition (IC₅₀) of acetylcholinesterase (AChE) (from bovine erythrocytes) occurred at 0.086 to 0.12 mg/ml oil concentration irrespective of growth conditions. The elevated IC₅₀ values of *S. officinalis* var *purpurea* for bovine in comparison to the human erythrocytes AChE (see chapter 3 and 5 of the present study and Savelev *et al.*, 2004) does not necessarily imply less potency. The apparent increase could be attributed to the membrane bound nature of bovine erythrocytes (SIGMA, Co UK) that renders this form of AChE less susceptible to inhibitors, probably by restricting access to enzyme binding sites. Other researchers employing bovine AChE in their studies demonstrated IC₅₀ values of 0.07

and 0.05 mg/ml for *S. lavandulaefolia* oil (Tildesley *et al.*, 2003 and Savelev *et al.*, 2003 respectively). However, in these other reports much lower final concentrations of bovine erythrocytes were utilized for the enzyme assays i.e. 0.006 (Tildesley *et al.*, 2003) and 0.008 (Savelev *et al.*, 2003) instead of 0.01 U/ml in the present study. The elevated amount of bovine AChE renders the enzyme less sensitive to any inhibitor. Inhibitory activity of *S. officinalis* var *purpurea* for butyryl-cholinesterase (BuChE) was time dependant as had been observed by Savelev and colleagues (2004) and increased incubation of the enzyme with the inhibitor induced significant decline in the IC50 values of the oil from 0.115 to 0.074 mg/ml, with 15 and 30 min incubation time respectively. Ogura *et al* (2000) reported that rivastigmine an alkaloid ChE inhibitor reached maximum potency after 6 h of incubation. Similar BuChE inhibitory activities have been reported before for *S. officinalis* var *purpurea* and *S. fruticosa* (Savelev *et al.*, 2004). Savelev and co-workers (2004) demonstrated that in contrast to the dual cholinergic activities of *S. officinalis* var *purpurea*, *S. lavandulaefolia* and *S. officinalis* preferentially inhibited AChE (from human erythrocytes). ChEIs used for the treatment of AD, generally exhibit a high degree of selectivity for AChE (tacrine, Enz *et al.*, 1993; donepezil, Snape *et al.*, 1999 and galanthamine, Thomsen & Kewitz, 1990), whereas rivastigmine has dual inhibitory properties (Enz *et al.*, 1991; Giacobini, 2000). Experimental evidence indicates therapeutic benefits from butyryl- along with acetylcholinesterase inhibition in the demented brain (see general introduction). Overall data revealed dual anti-cholinesterase activities of *S. officinalis* var *purpurea* plants. Nitrate growth treatment however, did not affect inhibitory properties of *S. officinalis* var *purpurea* as no significant alteration in the essential oil composition was provoked by the nitrate concentration in the nutrient solution (see paragraph above).

4.4.4 Conclusions

S. officinalis var *purpurea* an important medicinal and ornamental plant was successfully cultivated under hydroponic conditions by means of NFT utilising three concentrations of nitrate in the nutrient solution (100, 150 and 200 mg/L N-NO₃). The 150 mg/L of N-NO₃ proved to be the best level for herbage yield production while growth was suppressed under higher nitrate supply. No effect of nitrate treatment was evident on *S. officinalis* var *purpurea* essential oil content or composition. Oils from *S. officinalis* var *purpurea* have been recently reported to possess anti-cholinesterase properties. The present study corroborated this finding revealing dual cholinesterase inhibitory activity and relatively low IC₅₀ values, irrespective of growth treatment.

Chapter 5

**Effects of solution electrical conductivity on growth
and medicinal properties of *Salvia officinalis*
var *purpurea* in nutrient film culture.**

5.1 Introduction

In the last few years, herbal drugs have exhibited considerable promise as an alternative medicine for AD (Anekonda & Reddy, 2005). Cholinergic properties of *Salvia lavadulaefolia* and *S. officinalis* have been examined *in vitro* and *in vivo* for the past 10 years (see section 1.11.4), but *S. officinalis* var. *purpurea* has been considered only once (Savelev *et al.*, 2004). This study revealed important inhibitory activity of purple sage, on both ChE enzymes acetyl- and butyryl-cholinesterase, indicating the need for further investigation. The cholinergic activities of *Salvia* species have been attributed to essential oils produced by plant secondary metabolism (Perry *et al.*, 1996; Perry *et al.*, 2000, 2002, 2003; Savelev, *et al.*, 2003; Tildesley *et al.*, 2003; Savelev *et al.*, 2004 and Tildesley, *et al.*, 2005). Physiological regulation of essential oil synthesis in plants is highly susceptible to environmental influences (Sangwan, *et al.*, 2001) that include nutrient composition (see Chaps 3 and 4) as well as conditions of abiotic stress like water deficit (Bernath, 1985). Contrary to vegetable crops our knowledge on aromatic & medicinal plants cultivation is limited (Karioti *et al.*, 2003). Economakis (1992) and Economakis and colleagues (2005) demonstrated significant alterations in the essential oil content and quality of hydroponically cultivated *Origanum dictamnus* L. induced by the level of electrical conductivity (EC) in nutrient solution. Similar outcomes were reported by Udagawa (1995) for *Anethum graveolens* and *Thymus vulgaris*. Commonly, the EC regime utilised in solution cultures is approximately 2 mS/cm (Cooper, 1979; Papadopoulos 1991). It was hypothesised that elevated EC *via* the addition of extra nutrients could create water deficiency conditions as a result of an increase in the osmosis of the growth solution. Reduction in water flow through the roots at high EC / osmotic levels would be caused by the change in the water potential gradient across the root system (Herralde *et al.*, 1998). Thus EC effects should be analogous to osmotic / water stress conditions. Charles and co-workers (1990) reported that the essential oil content and composition of hydroponically-grown peppermint was

affected by growth solution potential and duration of exposure to mild and moderate osmotic stress. Whilst physiological responses of food crops to water deficiency have been extensively investigated, such studies are limited in medicinal and aromatic plants (Fatima *et al.*, 2002). Hydroponic cultivation of *Salvia officinalis* var. *purpurea* or *S. officinalis* has never been studied before and no report exists on the effects of EC on its growth and essential oil production. Secondary metabolites production is believed to be stimulated under stressful environments (Gershenzon, 1983; Sangwan *et al.*, 2001). Water stress-mediated changes in essential oil production may differ significantly depending upon the plant species. Bernath (1985) suggested that oil bearing plants can be divided into three groups according to the effects of water supply on volatile oil biosynthesis: plants in which levels of oil will increase, will not change or decrease under water limitation. Experimental outcomes have demonstrated no effect on essential oil yield by low irrigation conditions (Putievsky *et al.*, 1990; Fatima *et al.*, 1999), and some data indicates a decline in essential oil or phenolic compounds production in water-limited conditions (Hornok, 1986; Chalchat *et al.*, 1994; Solinas *et al.*, 1996; Fatima *et al.*, 1999; Nacif de Abreu and Mazzafera, 2005). However, Horonk (1986) reported increased essential oil content of coriander (*Coriandrum sativum* L.) seeds with reduced water supply; essential oil of *Origanum majorana* L. was promoted by soil moisture deficit (Rhizopoulou & Diamantoglou, 1991); plants of sweet basil (*Ocimum basilicum*) showed enhanced oil yield and altered oil composition due to mild and moderate water stress (Simon *et al.*, 1992; Omidbaigi *et al.*, 2003); and severe water stress increased essential oil content in *Satureja hortensis* L. (Baher *et al.*, 2002). Water stress increased the levels of monoterpenes in *Picea abies* (Nacif de Abreu & Mazzafera, 2005).

Stress-induced changes in oil yield and composition are considered to be the result of the effects of stress on plant growth and differentiation rather than the direct effect on oil synthesis (Gershenzon, 1983; Charles *et al.*, 1990, Simon *et al.*, 1992). Under water stress conditions increases in essential oil content partially reflect higher glandular trichome density due to reductions in leaf expansion (Gershenzon, 1983; Simon *et al.*, 1992). However, possible dehydration due to water deficiency may also directly affect the enzymes involved in the biosynthetic pathways of secondary metabolites (Fatima *et al.*, 1999; 2002); for example the activity of geraniol dehydrogenase catalysing the transformation of geraniol to another monoterpene is modulated in response to by water availability (Fatima *et al.*, 2002). There is also a strong indication that biosynthesis of IPP the primary terpene precursor, is induced by water stress (Nacif de Abreu & Mazzafera, 2005). Moreover, Gershenzon, (1983) suggested that terpenes can function to reduce transpiration under hot environments by inducing stomatal closure, an adaptive response to water limiting conditions (Herralde *et al.*, 1998), or by providing a vapour shield on the leaf surface. It is worth mentioning that the hormone abscisic acid that signals stomatal closure in response to water deficiency is a sesquiterpene (Gershenzon, 1983). Environmental stresses have been shown to generally increase sesquiterpene formation (Charles *et al.*, 1990). Finally, terpenes possess insecticidal activities (Bourgaud *et al.*, 2001) and thus could be of major importance for plant fitness under stressful conditions.

In the present chapter, *S. officinalis* var *purpurea* plants were hydroponically cultivated under three levels of electrical conductivity (EC 1.8, 3.6 and 7.4 mS/cm) in an attempt to enhance essential oil content and quality. In parallel, effects of EC on plant growth were analysed for the first time in order to define optimum EC level for high herbage yields. Analysis of pigments was conducted to indicate possible stress conditions in plants due to elevated osmosis in the nutrient solution or potential nutrient toxicity (Richardson *et al.*, 2002; Shevchenko *et al.*, 2004). Nutrient uptake

was studied with the aim of identifying plant nutritional needs and their relation to biomass accumulation. Moreover plant nutrient absorption under excessive nutrient concentrations (high EC level) was investigated.

5.2 Materials and methods

5.2.1 Plant cultivation, experimental and environmental conditions

Young plants of *Salvia officinalis* var *purpurea*, grown in 9 cm pots were obtained from a commercial supplier (Reed and Thoresby t/a Yorkstock, Yorkshire, UK) in July 2005 and cultivated under hydroponic conditions utilising Nutrient Film Technique (NFT). Six plants were randomly assigned to individual NFT subunits and established in channels at 40 cm intervals. Prior to their installation plants had been subjected to root washing under a mild stream of water to achieve removal of the compost mixture from the root environment (see section 2.2.1 for NFT system). Air temperature, PAR and pH of the nutrient solutions were recorded as described previously (see section 2.2.2, 2.2.4).

S. officinalis var. *purpurea* plants were cultivated under various electrical conductivity (EC) regimes i.e. 1.8, 3.6 and 7.4 mS/cm. Each EC treatment comprised four replicates / individual NFT sub-units (see paragraph 2.11 for experimental design). Hydroponic recipes for stock solution preparation (g of fertilisers/chemical reagents in 50 L of water) and target elemental composition of nutrient solutions in reservoir tanks are presented in Table 5.1 Target pH in all NFT sub units was 6; nitric acid was utilised for pH adjustments. For the first month of the cultivation period (20/7 – 20/8/2005) all plants received the same EC treatment (1.8 mS/cm) to allow substantial initial growth since EC of 2 mS/cm has previously been established as optimum (Cooper, 1979). Subsequently (20th August 2005), eight NFT sub-units were selected according to the experimental design and EC level was raised up to 3.6 or 7.4 mS/cm by the addition of extra stock solution (1500 ml and 3500 ml for EC 3.6 and 7.4 respectively, instead of 500 ml for EC1.8 mS/cm). In this manner, the total concentration of nutrients was significantly increased in the growth solutions of 3.6 and 7.4 mS/cm treatments, while their relative ratio was maintained the same.

Actual 10 day averages of pH and EC values of nutrient solutions before the correction with acid or stock solution are presented in Figures 5.1 and 5.2 respectively. Three EC regimes (1.8, 3.6 and 7.4 mS/cm) were imposed after one month of cultivation under the lower EC level (1.8 mS/cm). pH values fluctuated between 6.3 and 7.2. Increases in pH value of the nutrient solution are principally attributed to NO_3^- fixation into ammonia in plant roots, a process that releases OH^- in the medium (Cooper, 1979).

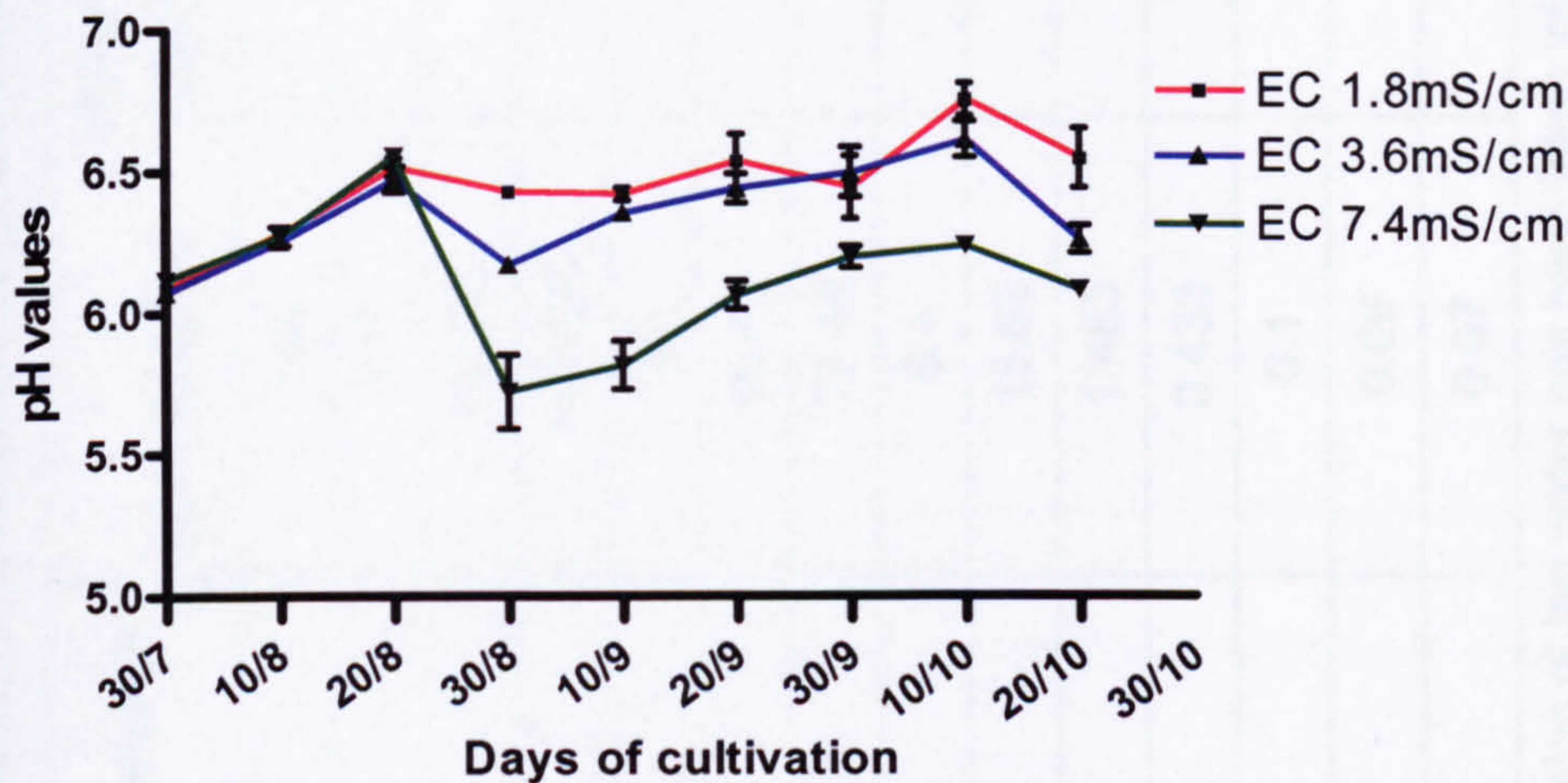


Figure 5.1 Mean pH values over ten day intervals, for the three EC treatments (1.8, 3.6 and 7.4 mS/cm), throughout the cultivation period. Differentiation in EC level commenced after the 20th of August. Target pH was 6 in all cases. Values represent the means and vertical bars indicate standard errors of the mean (n=3 NFT sub-units).

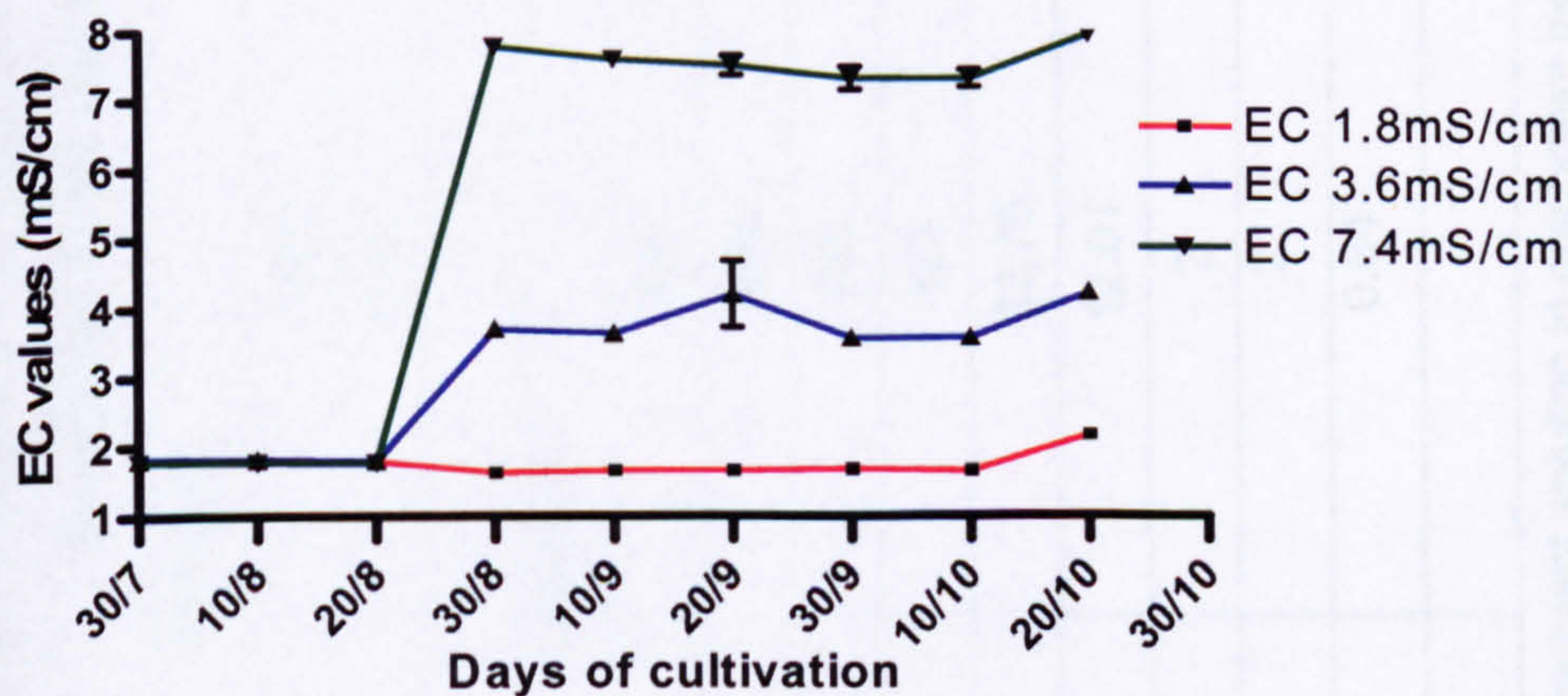


Figure 5.2 Average EC values at 10 day intervals in the nutrient solution, during the cultivation period, for the three EC treatments. Target EC was 1.8, 3.6 and 7.4 mS/cm. Differentiation in treatments started on the 20th of August, whereas before EC level was 1.8 mS/cm in all NFT sub-units. Values represent the means and vertical bars the standard errors of the mean (n=3 NFT sub-units).

Table 5.1 Stock recipe and theoretical elemental composition of nutrient solutions with three distinct electrical conductivity levels (EC) (1.8, 3.6 and 7.4 mS/cm). Stock solutions consisted of commercially obtained fertilizers (Hortifeeds) and chemical reagents (SIGMA Co, UK).

Chemical	Amount (g) in 50L stock	Elemental composition	mg/ml in solution ^a		
			EC 1.8mS/cm	EC 3.6mS/cm	EC 7.4mS/cm
Stock A					
Ca(NO₃)₂*4H₂O	2207	N-NO₃	144	420	980
KNO₃	2110	N-NH₄	4.41	13.23	30.87
		Ca	142.64	309.75	643.98
Stock B					
Mg(NO₃)₂	860	K	201.623	604.88	1411.39
MgSO₄	1955	P	32	96	224
KH₂PO₄	705	Mg	60.21	168.86	386.17
EDTA FeNa	323	S	76.44	178.44	382.44
MnSO₄*H₂O	22.75	Fe	8.4	25.2	58.8
H₃BO₃	12.01	Na	15.63	28.93	55.53
CuSO₄*5H₂O	2	Mn	1.483	4.44	10.36
ZnSO₄*7H₂O	2	B	0.433	1.26	2.94
(NH₄)₆Mo₇O₂₄	0.64	Cu	0.1	0.3	0.7
		Zn	0.09	0.27	0.63
		Mo	0.07	0.21	0.49

^a Final composition of nutrient solution in catchment tanks. Elemental contribution of tap water has been taken into account (Appendix 1, section 1.2)

Actual concentrations of nitrates (N-NO₃), potassium (K), phosphorus (P) and calcium (Ca) in nutrient solutions with the three distinct EC treatments 1.8, 3.6 and 7.4 mS/cm, as determined at the initiation of the EC differentiation, are presented in Table 5.2.

Table 5.2 Actual concentrations of N-NO₃, P, K and Ca (mg/ml) in the nutrient solutions with three electrical conductivity (EC) levels i.e. 1.8, 3.6 and 7.4 mS/cm.

Treatment	EC 1.8mS/cm^e	EC 3.6mS/cm^e	EC 7.4mS/cm^e
Element (mg/ml)^d			
N-NO₃	131.5 ± 3.9c	372.3 ± 9.5b	910.7 ± 5.6a
K	161.5 ± 2.1c	566.7 ± 7.4b	1240 ± 15.2a
P	27.22 ± 1.3c	76.88 ± 3.5b	191.5 ± 6.1a
Ca	114.6 ± 4.0c	279.5 ± 4.9b	541.6 ± 4.6a

Values within rows not followed by the same letter are significantly different at P<0.001

^d N-NO₃ was analysed by means of UV spectroscopy while K, P and Ca concentrations were determined *via* ICP.

^e Standard error of the mean (SEM) was calculated from three replicates (n=3 NFT sub-units)

Total consumption of stock solution and 5% nitric acid are presented in Table 5.3 for the first month of the experiment (20/7 - 20/8) where all plants had been cultivated under EC 1.8 mS/cm and for two months after the initiation of the three EC treatments (1.8, 3. and 7.4 mS/cm) (30/8 – 30/10). One way ANOVA revealed significant differences among treatments in stock solution consumption (P<0.001) being higher for EC 1.8 mS/cm level. Total acid utilised by plants under the same treatment was significantly increased (P<0.0001) (Table 5.3).

Table 5.3 Acid and stock solution consumption during the cultivation of *Salvia officinalis* var *purpurea* plants under three EC levels (1.8, 3.6 and 7.4 mS/cm).

Treatment		EC	EC	EC
Consumption	Dates	1.8mS/cm	3.6mS/cm	7.4mS/cm
Nitric acid (ml) ± SEM	20/7 - 20/8	90	90	90
	20/8 - 20/10	255 ± 6a	205 ± 10b	20 ± 5c
Stock solution (ml) ± SEM	20/7 - 20/8	50	50	50
	20/8 - 20/10	450 ± 50a	267 ± 44b	100 ± 12c

Plants had been cultivated under the same EC conditions (1.8 mS/cm) for one month before the differentiation of EC level on the 20th of August

Values represent the means and standard error of the mean (SEM) calculated from n= 3 NFT sub-units. Where there are no SEM values for all replicates were identical.

Stock solution values not followed by the same letter are significantly different at P<0.001

Total acid values not followed by the same letter are significantly different at P<0.0001

Min and max air temperature ranged from 15 to 18 and 25 to 33 °C respectively during the three months of cultivation (Figure 5.3).

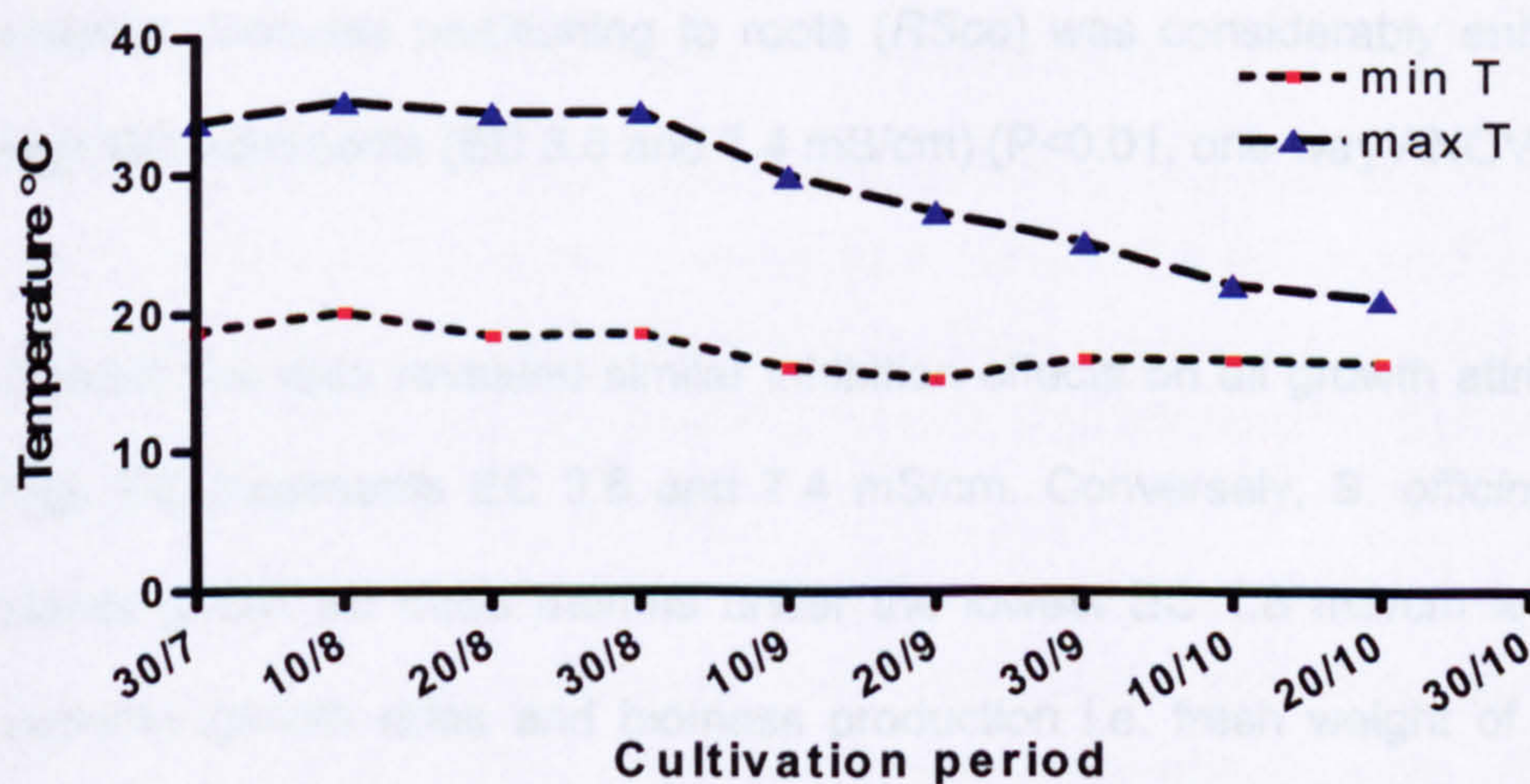


Figure 5.3 Average values over 10 day intervals for min and max air temperature throughout the cultivation period of *Salvia officinalis* var *purpurea* plants raised under EC1.8 3.6 and 7.4 mS/cm by means of a hydroponic Nutrient Film Technique (NFT) system installed in a heated glass-house.

5.3 Results

5.3.1 Growth response to electrical conductivity of the nutrient solution

The level of electrical conductivity (EC) in the hydroponic solution defined by the concentration of nutrients, profoundly affected the growth of *S. officinalis* var. *purpurea* plants (Table 5.4). Both high nutrient concentration treatments (EC 3.6 and 7.4 mS/cm) induced a significant decline in growth attributes including fresh (*FWS*) and dry (*DWS*) biomass of the shoot ($P < 0.001$ and $P < 0.01$ respectively), dry weight of the plant (*DWP*, $P < 0.01$), specific leaf area (*SLA*, $P < 0.001$) and shoot water content ($\%SWC$, $P < 0.001$) as revealed by one-way analysis of variance (Table 5.4). In addition, absolute growth rate of the shoot (*AGRs*) and plant (*AGP*) showed significant reductions in the higher EC treatments of approximately 39% and 38% of accumulated dry weight per week for shoot and plant respectively ($P < 0.001$, one-way ANOVA) (Table 5.4). On the other hand, EC level only marginally affected root biomass production (*DWR*); 6.5% decrease of root dry weight for EC 3.6 mS/cm treatment and 15% for EC 7.4 mS/cm ($P < 0.05$, one-way ANOVA), as well as the absolute growth rate of the root (*AGRr*) (see Table 5.4). Due to differing shoot and root growth responses to EC of the nutrient solution, biomass partitioning to roots (*RSco*) was considerably enhanced under the high EC treatments (EC 3.6 and 7.4 mS/cm) ($P < 0.01$, one-way ANOVA) (Table 5.4).

Overall, the data revealed similar inhibition effects on all growth attributes under both high EC treatments EC 3.6 and 7.4 mS/cm. Conversely, *S. officinalis* var. *purpurea* plants grown for three months under the lowest EC 1.8 mS/cm level demonstrated superior growth rates and biomass production i.e. fresh weight of shoot (*FWS*) the commercially important part of sage attained mean values of 380 g (Table 5.4).

Table 5.4 Impact of EC treatment on *Salvia officinalis* var *purpurea* plant growth.

Growth attributes^b	Treatment^a			P^c
	EC 1.8mS/cm	EC 3.6mS/cm	EC 7.4mS/cm	
FWS (g)	380.7 ± 14.49a	286.6 ± 14.44b	244.0 ± 19.88b	***
DWP (g)	98.20 ± 3.79a	73.14 ± 5.98b	75.09 ± 5.24b	**
DWS (g)	82.63 ± 3.44a	59.20 ± 5.67b	62.14 ± 4.57b	**
DWR (g)	15.57 ± 0.44a	14.55 ± 0.82ab	13.23 ± 0.69b	*
AGR (g/week)	6.96 ± 0.474a	4.55 ± 0.50b	4.07 ± 0.66b	***
AGRs (g/week)	5.95 ± 0.43a	3.77 ± 0.43b	3.39 ± 0.572b	***
AGRr (g/week)	1.01 ± 0.06a	0.95 ± 0.09ab	0.72 ± 0.09b	*
RSc^e	17.60a	25.41b	25.07b	**
Height (cm)	88.75 ± 3.25a	76.75 ± 2.69b	69.5 ± 2.10b	**
SLA (cm²)	135.3 ± 3.95a	124.5 ± 5.43a	101.8 ± 3.92b	***
%SWC	78.32 ± 0.25a	76.86 ± 0.47b	75.02 0.50c	***

^a *S. officinalis* var *purpurea* plants were cultivated hydroponically in an NFT unit under three EC levels in the nutrient solution (1.8, 3.6 and 7.4 mS/cm) indicating the amount of nutrients. Treatments were replicated three times (n=3 NFT sub-units). Differences of the means were tested with one-way ANOVA (one replicate/mean of 6 plants per channel and 3 replicates/channels per treatment)

^b *FWS*, *DWP*, *DWS* and *DWR* indicate the fresh weight of the shoot and the dry weight of plant, shoot and root respectively. *AGR*, *AGRs*, *AGRr* and *RSc* represent the absolute growth rate of the plant, shoot and root and the root to shoot allometric coefficient respectively, determined according to Hunt (1990). *SLA* stands for specific leaf area measured via the youngest fully expanded pairs of leaves and %SWC for the % water content of the shoots. Values represent the mean and standard errors of the mean (n=3 NFT sub units). Means followed by the same letter are not significantly different at 5% level

^c Asterisks denote level of significance * (P<0.05), ** (P<0.01) and *** (P<0.001)

^e For *RSc* statistical analysis was performed with log transformed values

5.3.2 Leaf pigment content

The highest EC level (EC 7.4 mS/cm) induced a significant decline in chlorophyll a (Chla) concentration expressed in relation to leaf fresh weight or leaf area ($P < 0.001$ and $P < 0.05$ one-way ANOVA Figure 5.4A and 5.4B respectively). Conversely, a significant increase in total carotenoid content was revealed (one-way ANOVA, $P < 0.01$ Figure 5.4A and $P < 0.001$ Figure 5.4B) for plants raised under the EC 7.4 and EC 3.6 mS/cm regimes. In addition, a significant reduction ($P < 0.001$, one-way ANOVA) in the ratio of Chla to Chlb (ratio Chla/Chlb) was evident for the high EC treatments (EC 3.6 and 7.4 S/cm) (Figure 5.4 C) due to Chla degradation as Chlb content was unaffected by the EC level ($P > 0.05$, one-way ANOVA Figure 5.4 A and B).

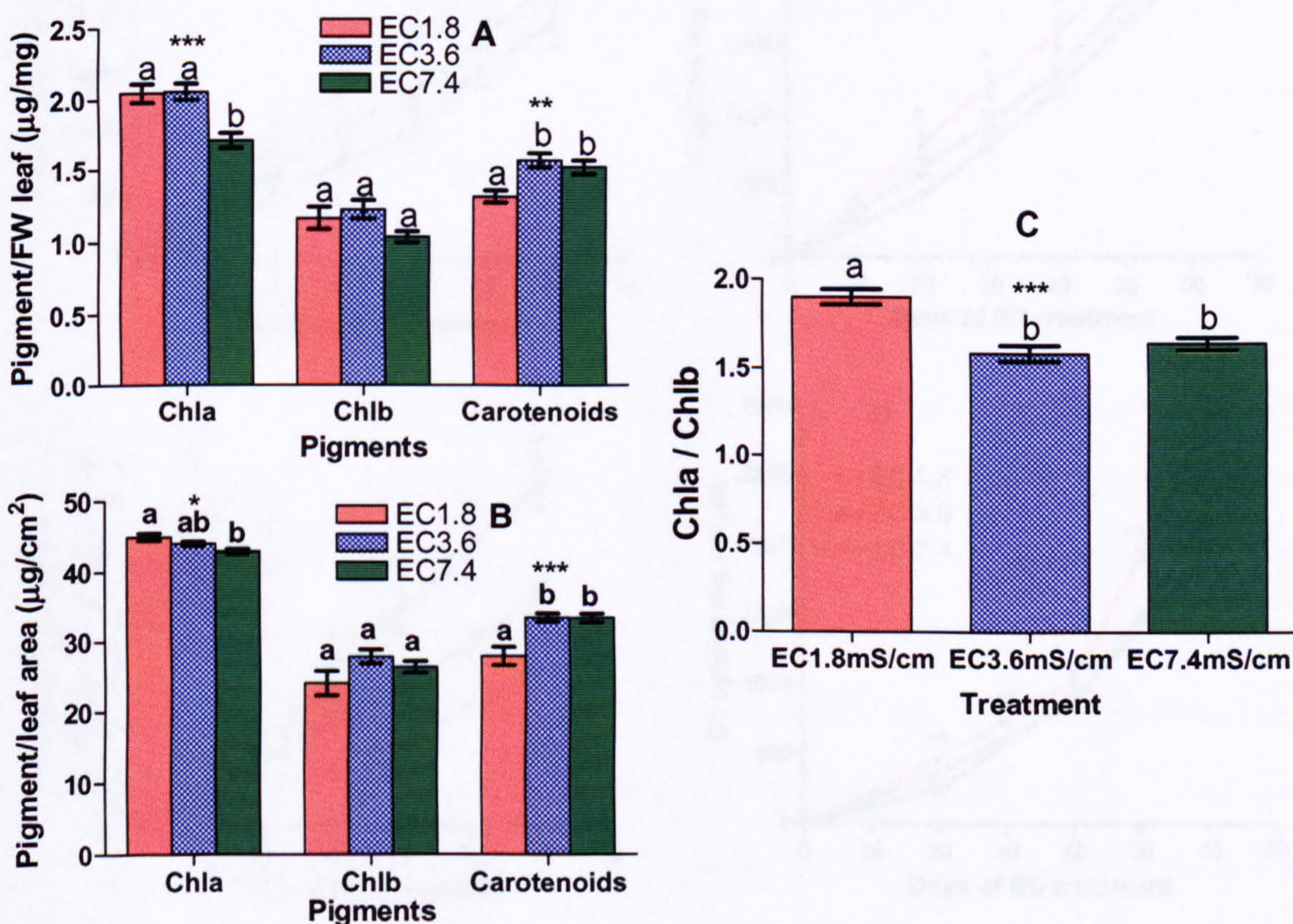


Figure 5.4 Chla, Chlb and total carotenoids content of fresh leaves expressed per leaf weight (A) or leaf area (B) and Chla to Chlb ratio (C) of *S. officinalis* var *purpurea* plants cultivated under three EC levels (1.8, 3.6 and 7.4 mS/cm). Pigment content (μg) was determined from young fully expanded sage leaves harvested one day before the end of the experiment. Vertical bars indicate standard errors of the mean ($n=3$ NFT sub-units). Columns within a group of three, followed by the same letter are not significantly different at 5% level. Asterisks denote level of significance * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$

5.3.3 Nutrient uptake as affected by EC level in the growth solution

Cumulative nutrient uptake of *S. officinalis* var *purpurea* plants for nitrates (N-NO₃), phosphorus (P), potassium (K) and calcium (Ca), and water uptake was analysed under three regimes of EC (1.8, 3.6 and 7.4 mS/cm) (Figure 5.5 A, B, C, D and E respectively). The last value on the uptake curve represents the mean total uptake per plant of the corresponding element and water for the period that followed EC differentiation.

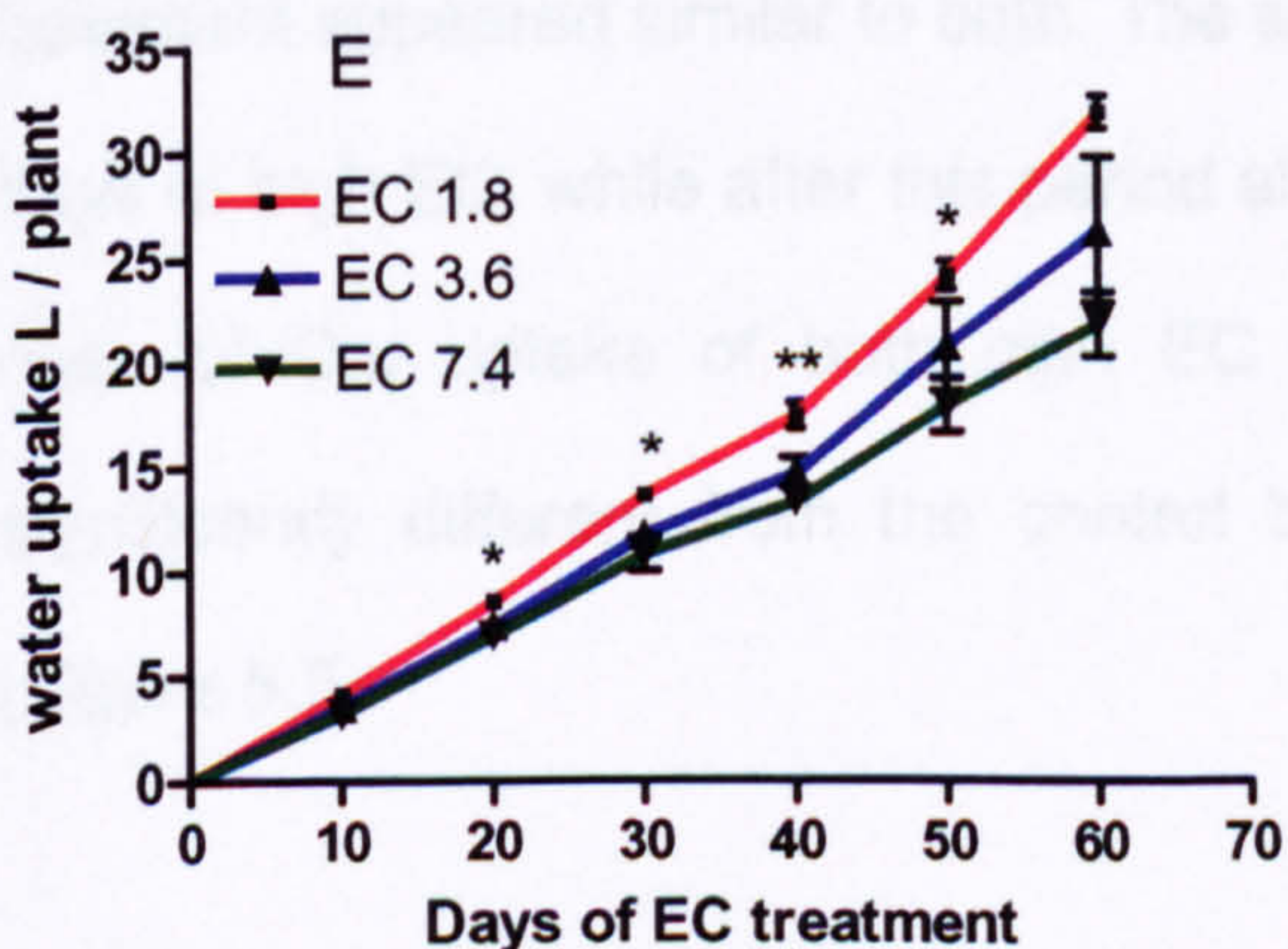
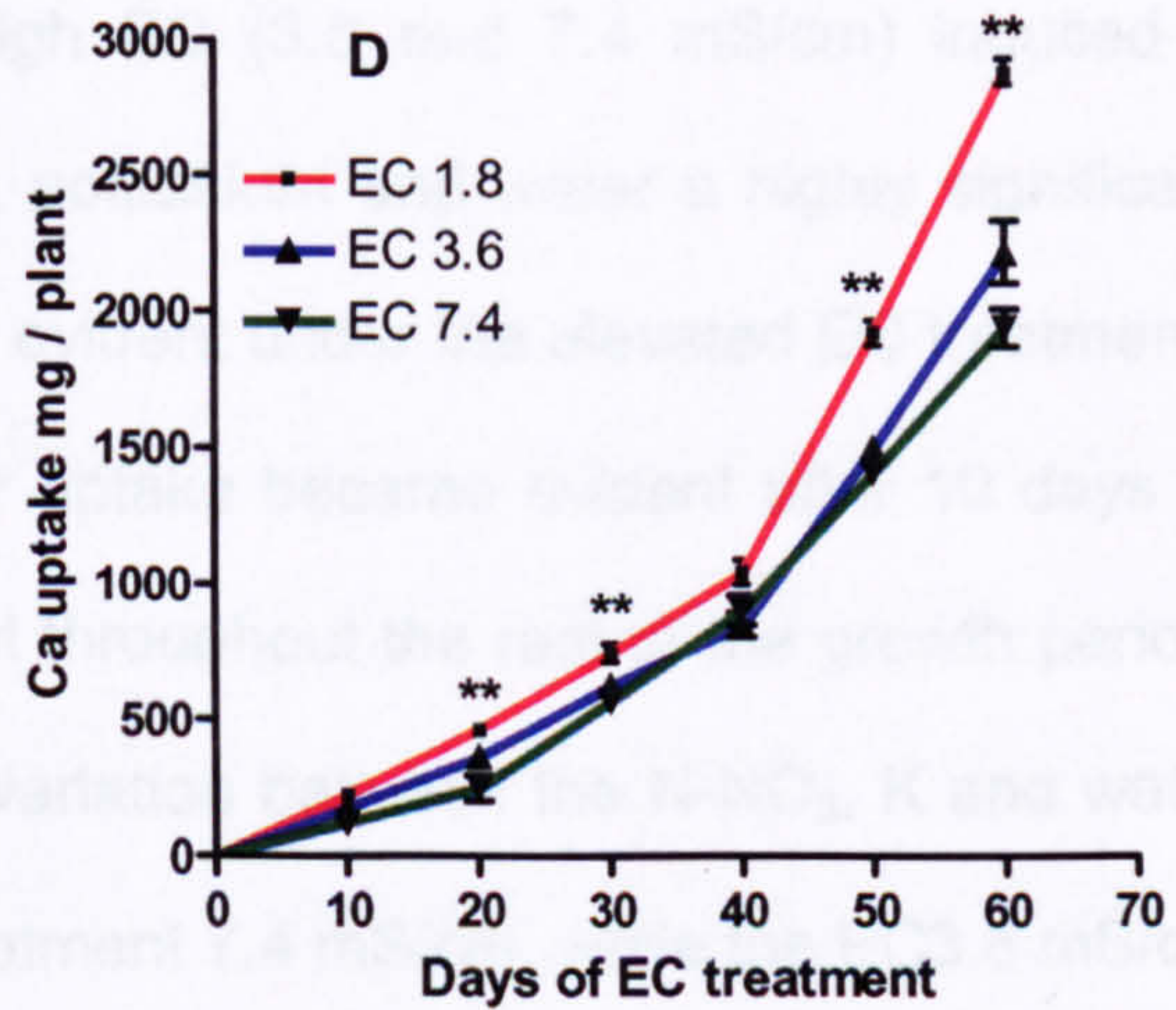
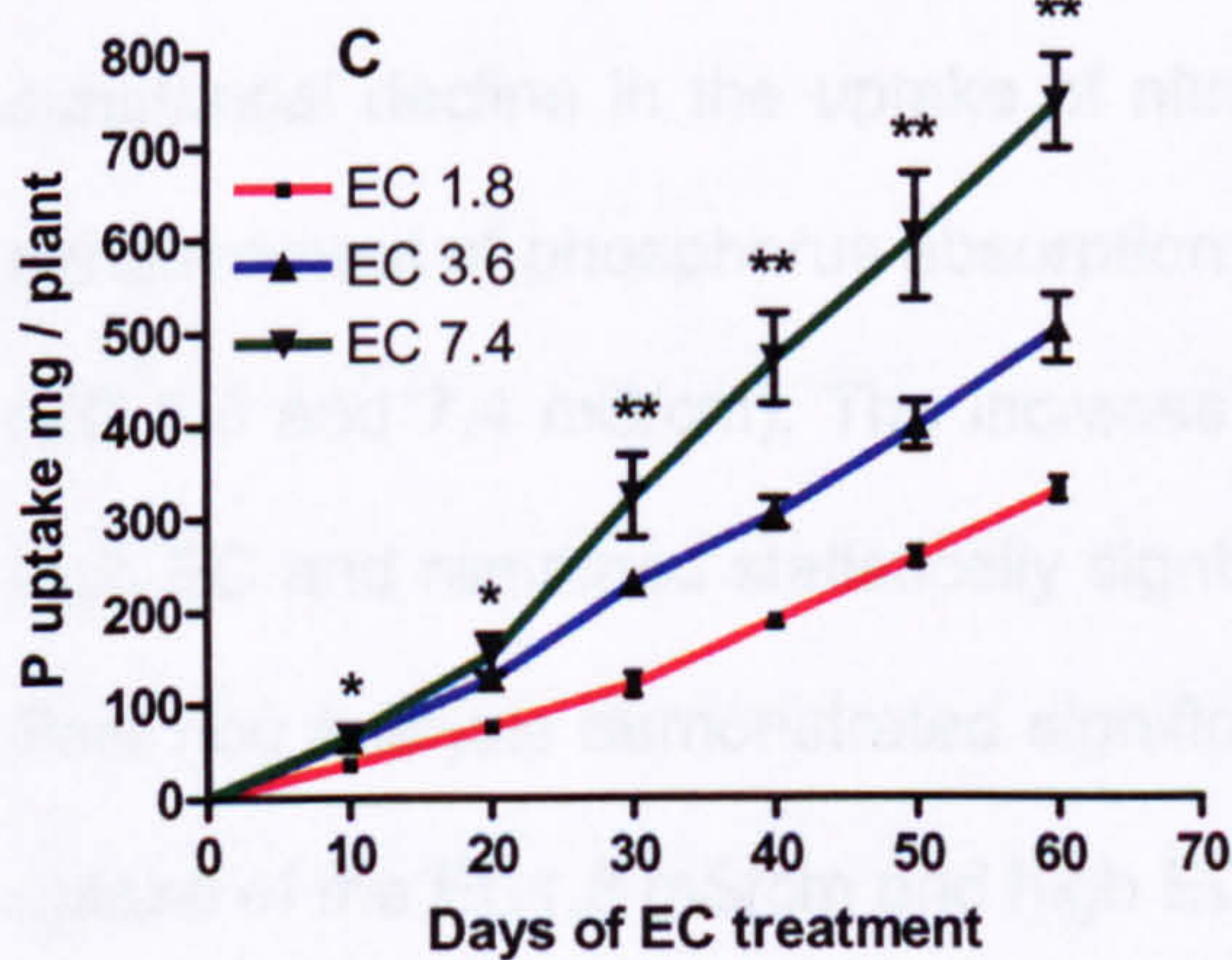
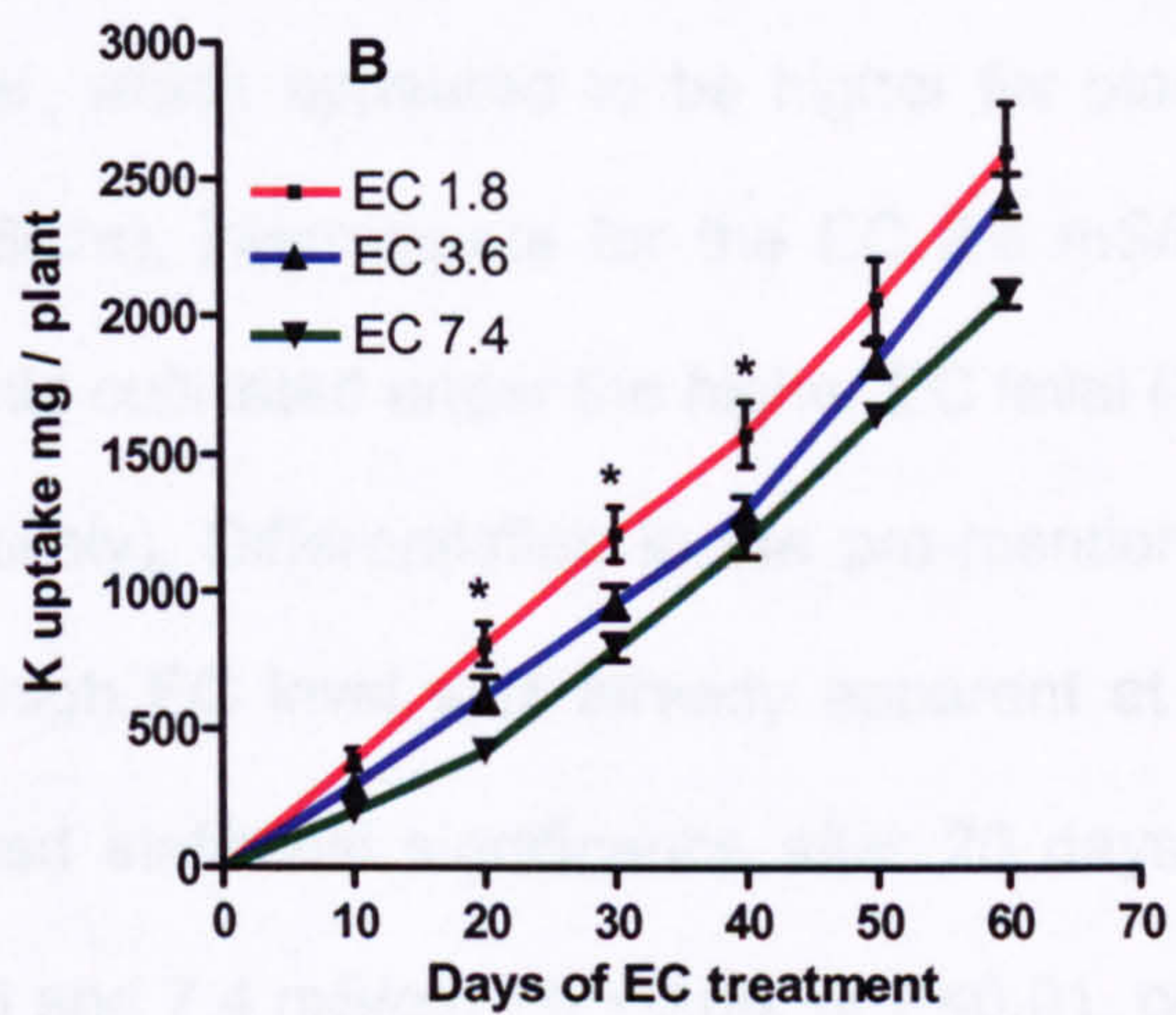
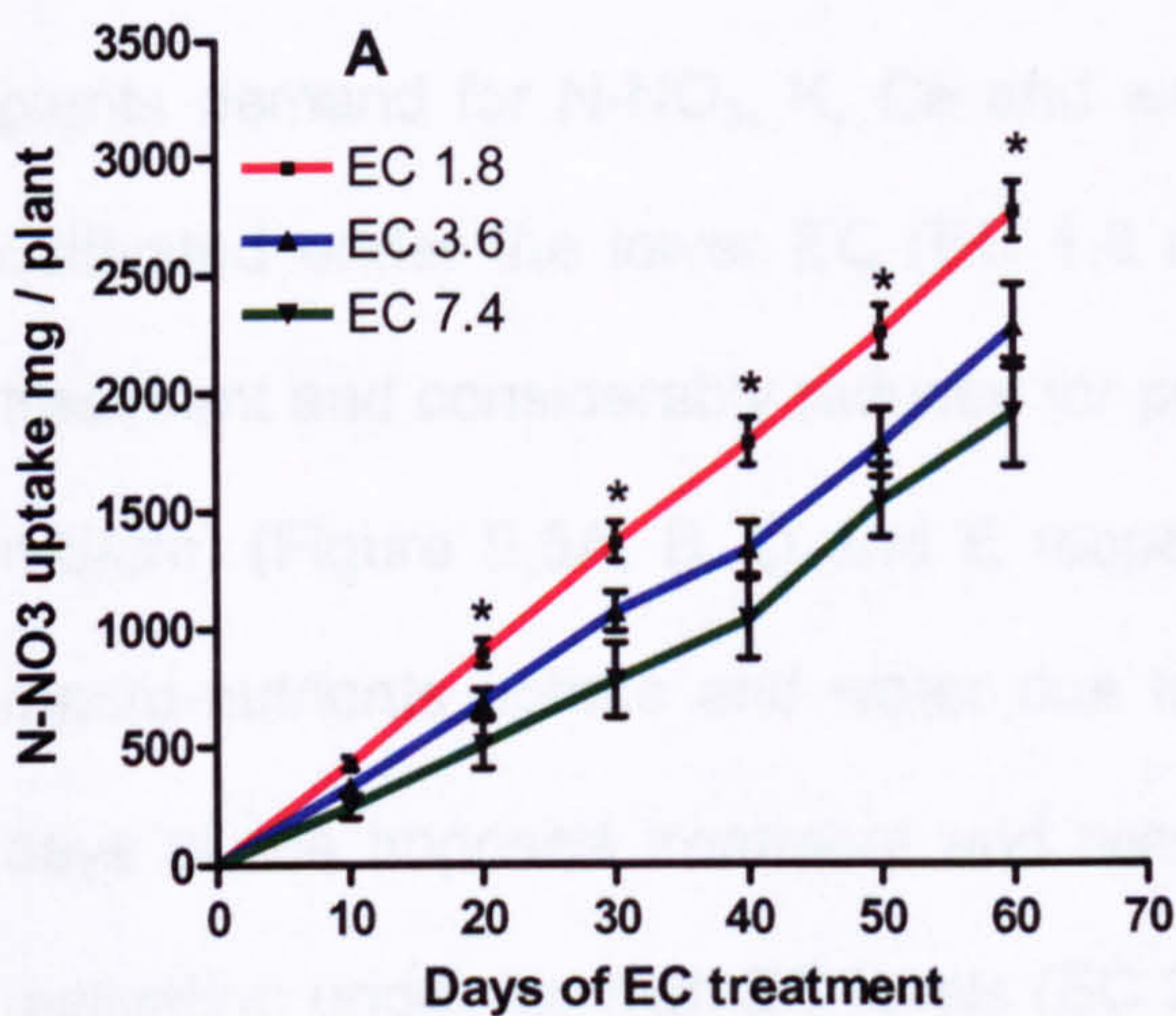


Figure 5.5 Cumulative uptake curves for nitrates (A), potassium (B), phosphorus (C) calcium (D) and water (E) of *S. officinalis* var *purpurea* grown hydroponically in an NFT unit under different EC levels (1.8, 3.6 and 7.4 mS/cm). Plants had been cultivated in the NFT for a period of one month before the differentiation of EC level. Uptake of nutrients was determined with a 10 days interval *via* solution depletion studies. The 60 days measure corresponds to the total nutrient uptake of the relevant element. Values represent the mean and vertical bars the standard errors of the mean (n=3 NFT sub-units). Asterisks denote significance level of differences among treatments at the equivalent time period * P<0.05, ** P<0.01 and ***P<0.001.

Cumulative uptake curves of *S. officinalis* var *purpurea* revealed a similar pattern in plants demand for N-NO₃, K, Ca and water, which appeared to be higher for plants cultivated under the lower EC (EC 1.8 mS/cm), intermediate for the EC 3.6 mS/cm treatment and considerably reduced for plants cultivated under the higher EC level (7.4 mS/cm) (Figure 5.5A, B, D and E respectively). Differentiation in the pre-mentioned macro-nutrients uptake and water due to high EC level was already apparent at 10 days of the imposed treatment and reached statistical significance after 20 days of cultivation under the high EC levels (EC 3.6 and 7.4 mS/cm) (P<0.05, or P<0.01, one-way ANOVA, see Figure 5.5). Whereas high EC (3.6 and 7.4 mS/cm) induced a substantial decline in the uptake of nitrates, potassium and water a highly significant enhancement of phosphorus absorption was evident under the elevated EC treatments (EC 3.6 and 7.4 mS/cm). The increase of P uptake became evident after 10 days of high EC and remained statistically significant throughout the rest of the growth period. Post hoc analysis demonstrated significant variation between the N-NO₃, K and water uptake of the EC1.8 mS/cm and high EC treatment 7.4 mS/cm, while the EC3.6 mS/cm treatment appeared similar to both. The same trend was revealed for P uptake up to 40 days of high EC, while after this period all treatments were significantly different. In the case of Ca, uptake of both high EC treatments (EC 3.6 and 7.4 mS/cm) were significantly different from the control but no variation was evident between them (Figure 5.5).

Employing the total uptake per plant at the end of the cultivation period the ratio of N-NO₃ to K (N/K), P (N/P) and Ca (N/Ca) were determined (Table 5.5). Outcomes suggested no evidence of variation in the relative uptake of N-NO₃ to K or Ca, a fact that indicates similar responses in terms of the uptake of these nutrients under different EC levels (1.8, 3.6 and 7.4 mS/cm). Conversely, the uptake of P was significantly enhanced in relation to nitrates under the high EC treatments resulting in lower N/P values (Table 5.5)

Table 5.5 Uptake ratio of N-NO₃ to K, P and Ca of hydroponically cultivated *S. officinalis* var *purpurea* under three levels of EC (1.8, 3.6 and 7.4 mS/cm).

Treatment	EC 1.8mS/cm	EC 3.6mS/cm	EC 7.4mS/cm
Uptake ratio			
N/K	1.07 ± 0.04a	0.93 ± 0.04a	1.02 ± 0.03a
N/P	8.37 ± 0.16a	4.42 ± 0.09b	2.55 ± 0.13c
N/Ca	0.96 ± 0.04a	1.03 ± 0.11a	0.95 ± 0.10a

Values represent the mean and standard errors of the mean (n=3 NFT sub-units). Values within a row followed by the same letter are not significantly different at 5% level. N/P ratio significantly differs with $P < 0.001$. Ratios complied with the assumptions of analysis of variance and hence log-transformation was not necessary.

Pearson product-moment correlation was performed to evaluate relationships among nutrients total uptake. All nutrients analysed were strongly associated in terms of total plant absorption. Significant positive relationships were revealed between N-NO₃ and K ($r=0.812$), N-NO₃ and Ca ($r=0.799$) and K and Ca ($r=0.749$). On the other hand, P uptake was significantly but negatively correlated to the rest of the nutrients (P versus N-NO₃, $r=-0.740$; P versus K, $r=-0.745$ and P versus Ca, $r=-0.894$). All pre-mentioned correlations are illustrated in Table 5.6.

Table 5.6 Correlation matrix illustrating relationships between total uptake of different nutrients (N-NO₃, P, K and Ca) of *S. officinalis* var *purpurea* plants grown hydroponically under three different regimes of EC (1.8, 3.6 and 7.4 mS/cm)

x	N-NO ₃	P	K	Ca
y				
N-NO ₃		r = -0.740 P<0.05	r = 0.812 P<0.01	r = 0.799 P< 0.01
P	r = -0.740 P<0.05		r = -0.745 P<0.05	r = -0.894 P<0.01
K	r = 0.812 P<0.01	r = -0.745 P<0.05		r = 0.749 P<0.05
Ca	r = 0.799 P<0.01	r = -0.894 P<0.01	r = 0.749 P<0.05	

r represents the Pearson product –moment correlation

P value indicates significance of the correlation

x, y represents the nutrient uptake variables of the correlation

Number of xy pairs tested was n=9 NFT sub-units/replicates

Electrical conductivity of the nutrient solution was found to be the best explanation in the uptake of N-NO₃, K, P and water as illustrated by regression analysis (Figure 5.6A, B, C, E and D respectively). Uptake of all macro nutrients except for the case of P was reduced as EC was raised from 1.8 to 7.4 mS/cm. Linear curves described best the effects exerted from EC treatments on uptake of all nutrients revealing significant r² values. EC level explained 66% of N-NO₃ uptake (P<0.01), 67% in K (P<0.01) and 76% in Ca uptake (P<0.01). A significant negative response was similarly demonstrated for water against EC treatment (r²= 0.58, P<0.05) (Figure 5.6 D). Conversely, regression analysis revealed strong positive relationship between P uptake and the EC level (r²=0.94, P<0.001) (Figure 5.6 E).

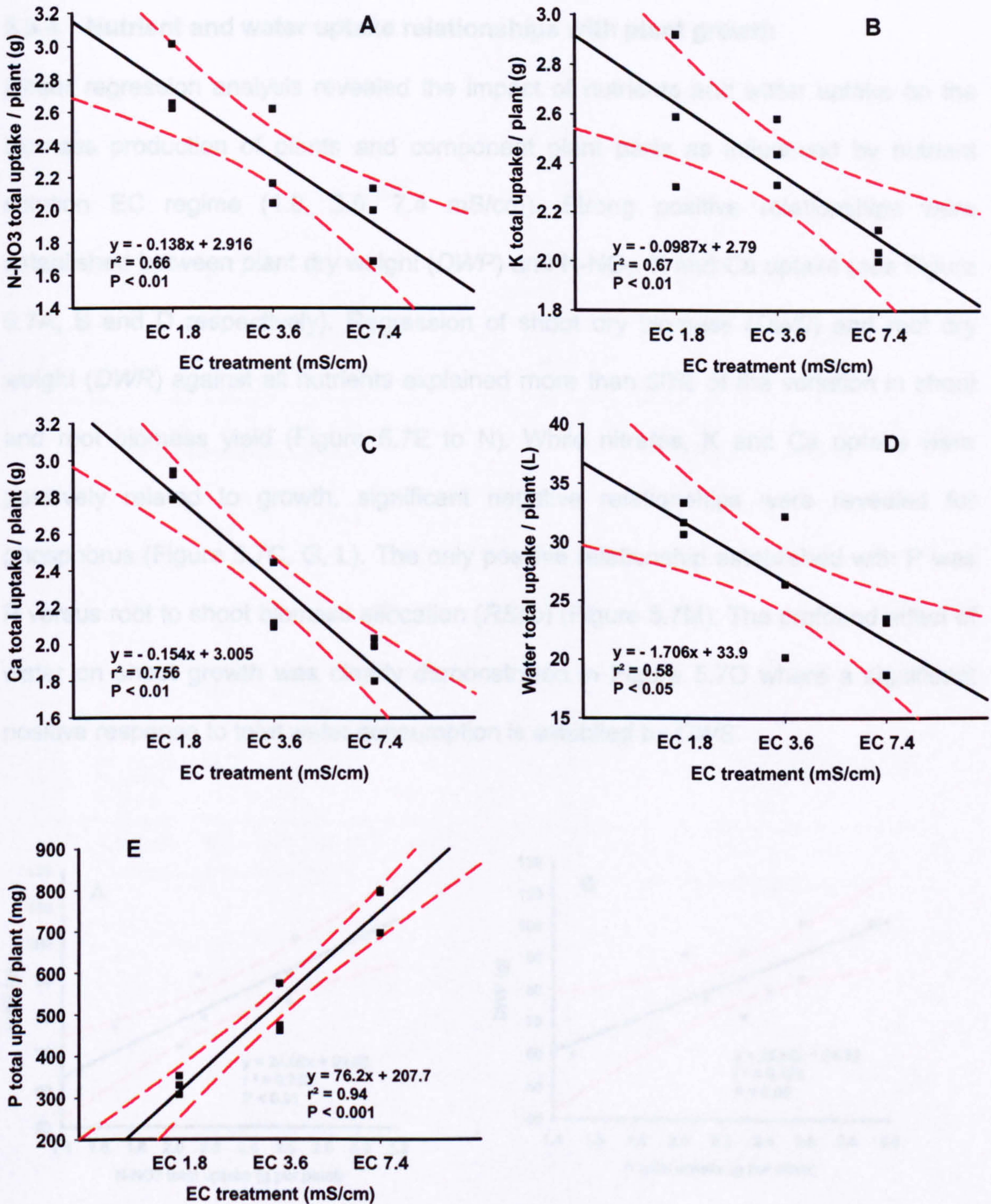


Figure 5.6 Regression analysis performed between EC treatment in the nutrient solution versus total uptake per plant of the nutrients: N-NO₃ (A), K (B), Ca (C), P (E) and water (D). *S. officinalis* var *purpurea* plants were hydroponically raised under three EC regimes (1.8, 3.6 and 7.4 mS/cm) for a period of two months.

Red dotted lines represent the 95% confidence band of the linear regressions

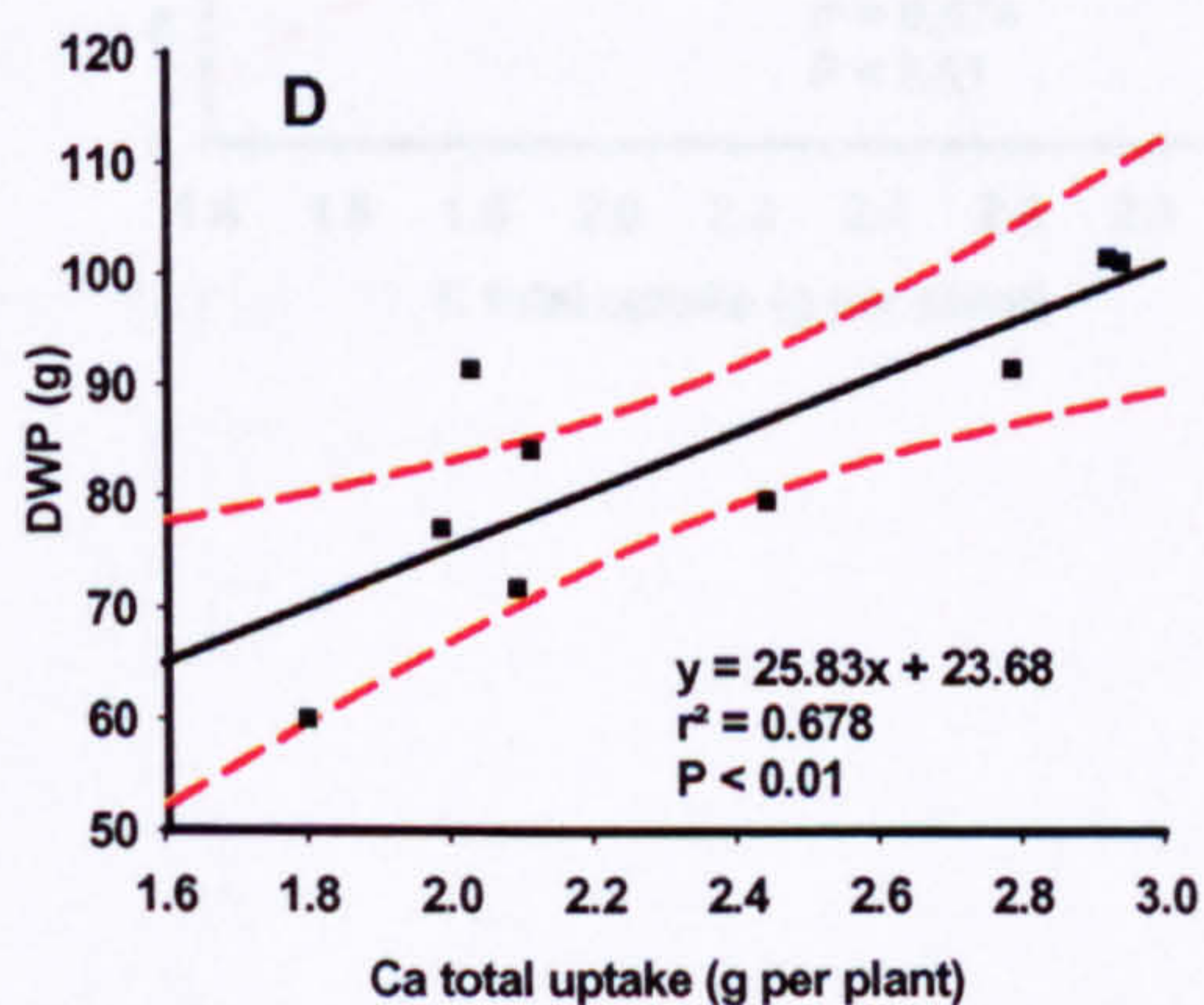
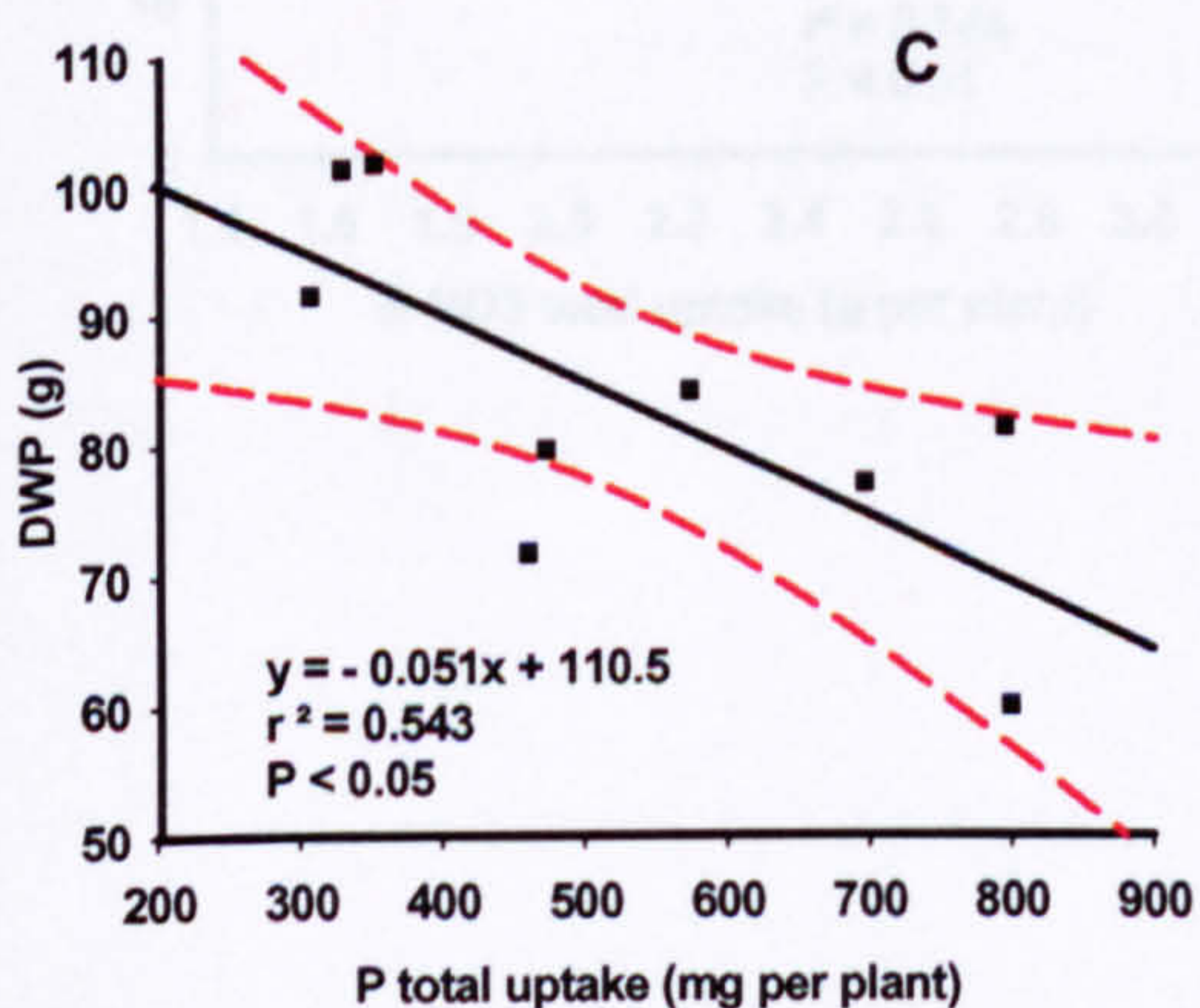
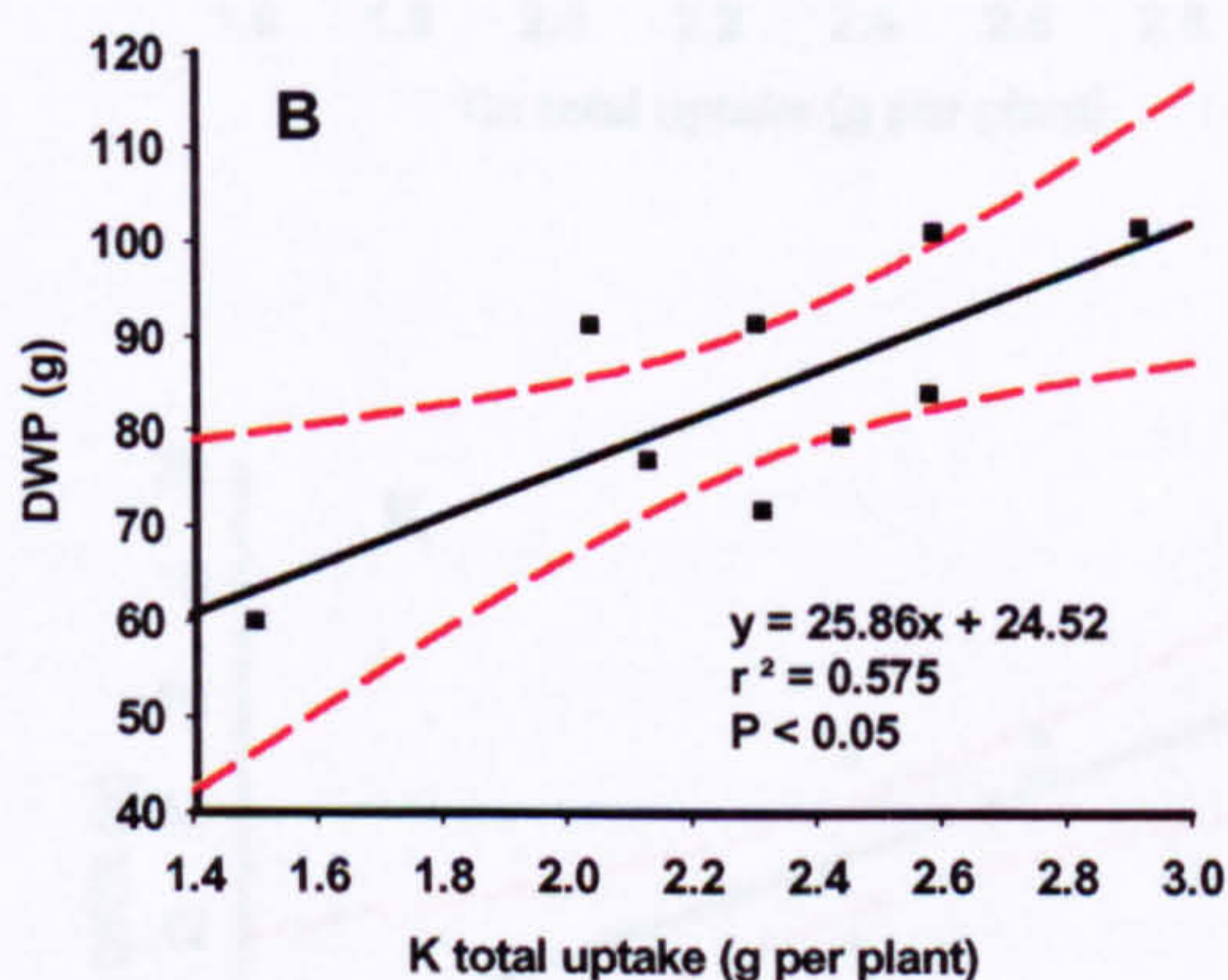
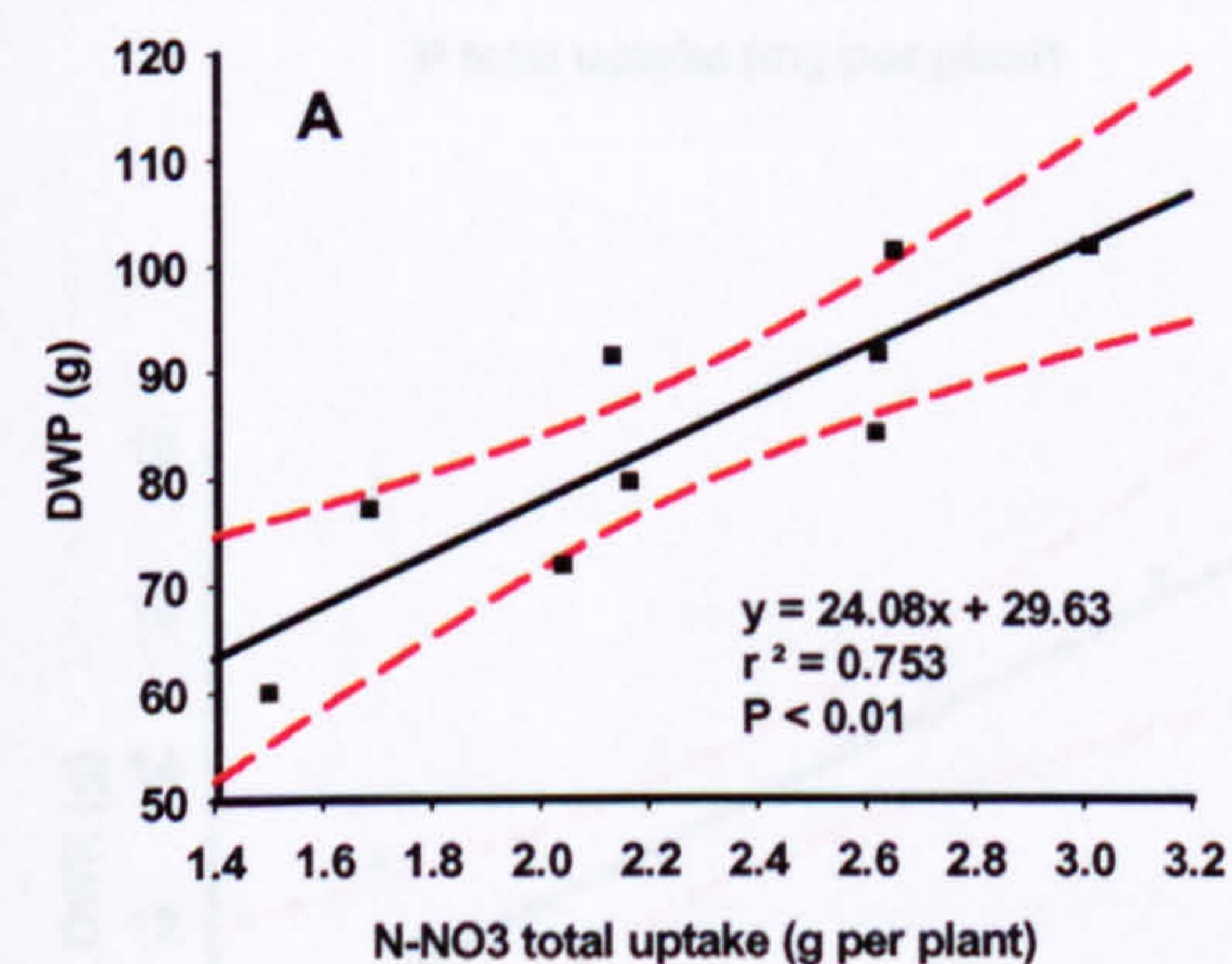
r² represents goodness of fit the linear slopes.

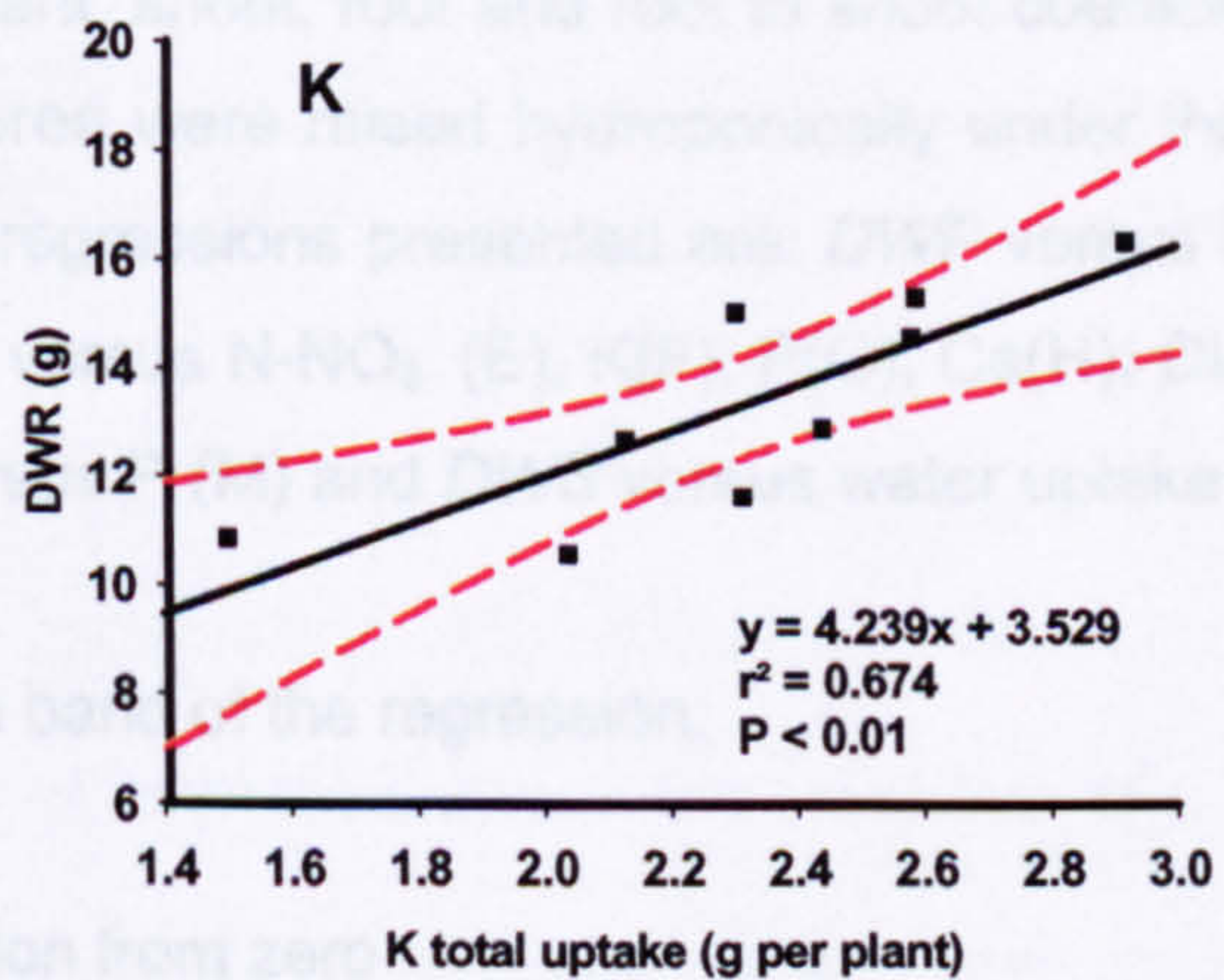
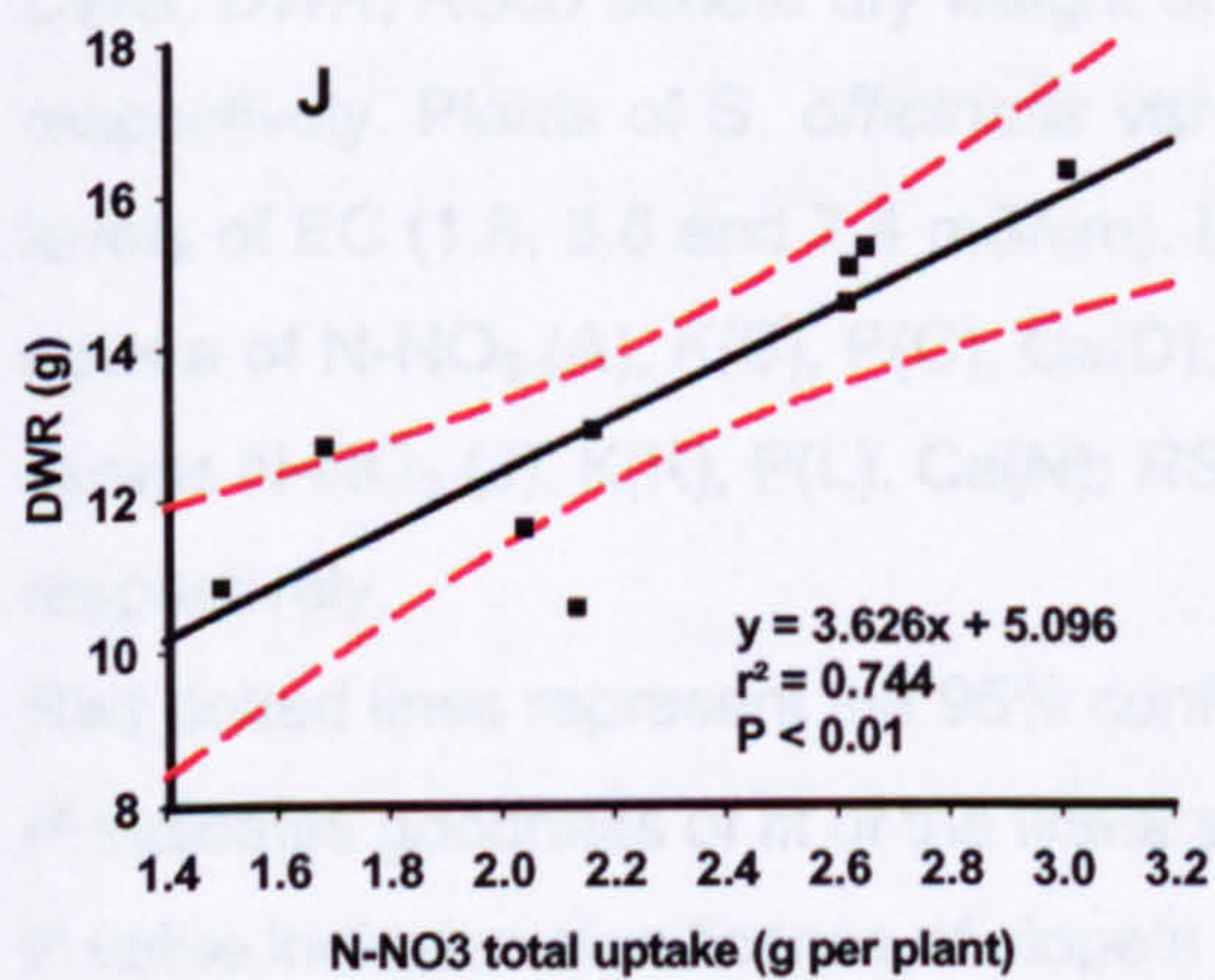
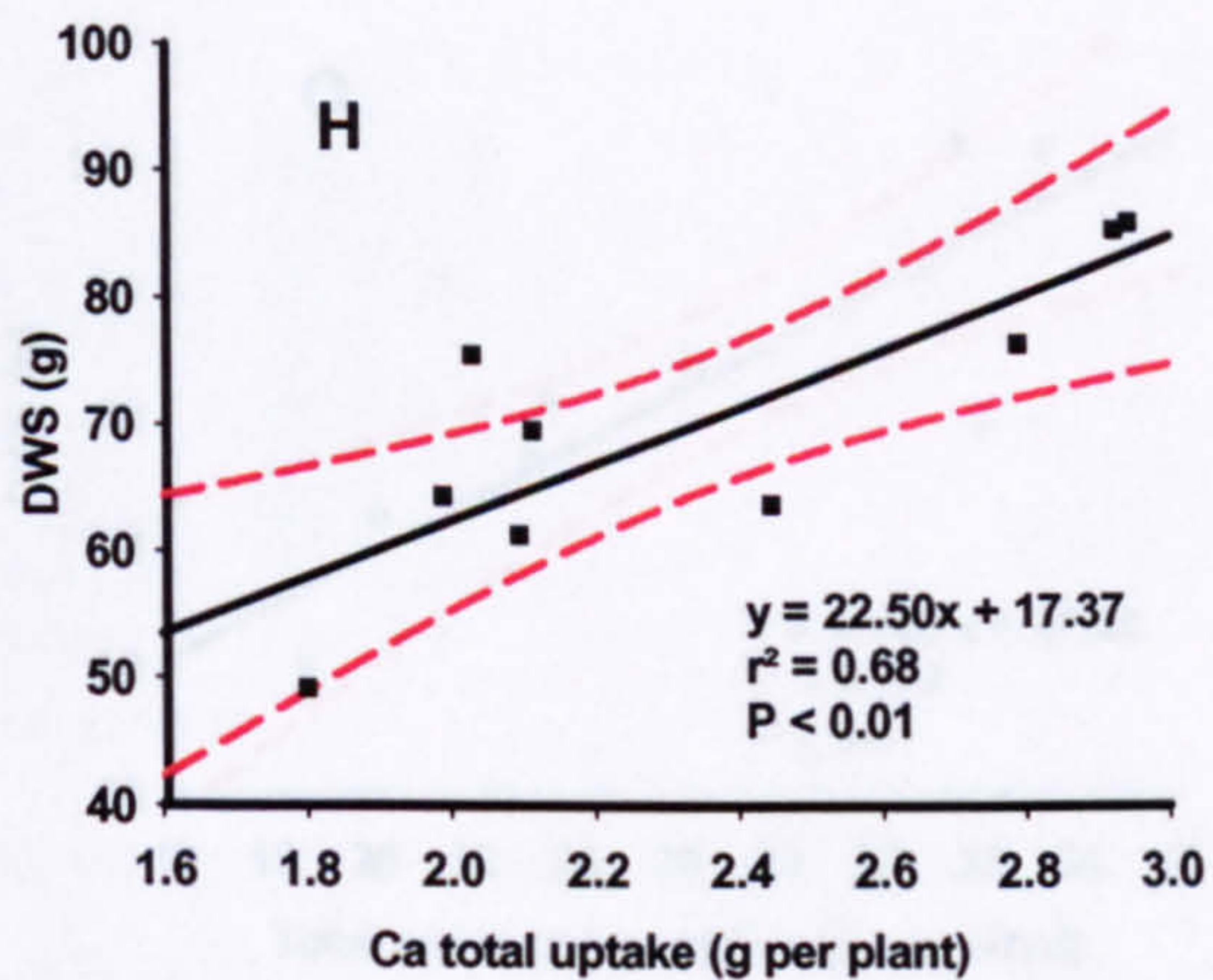
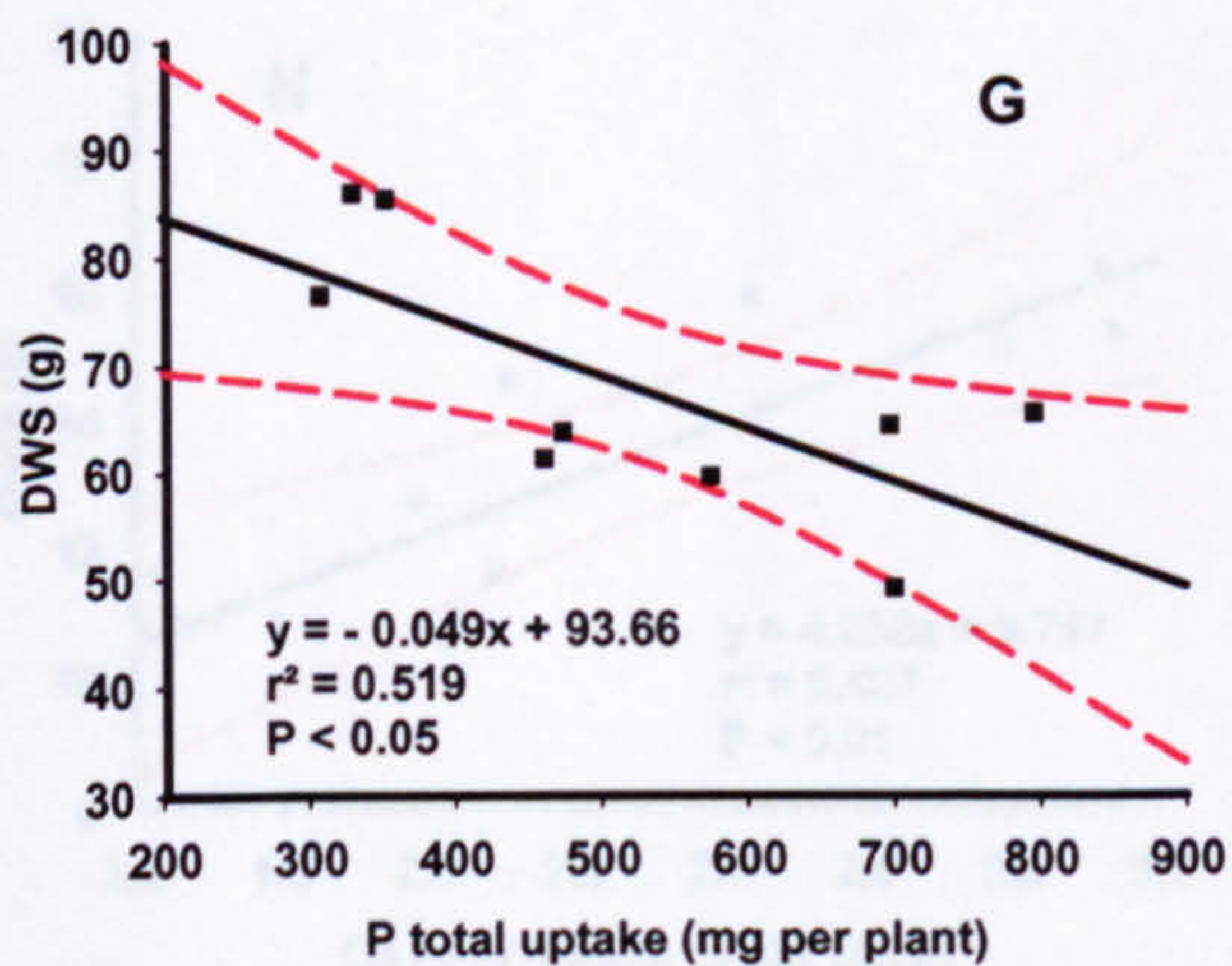
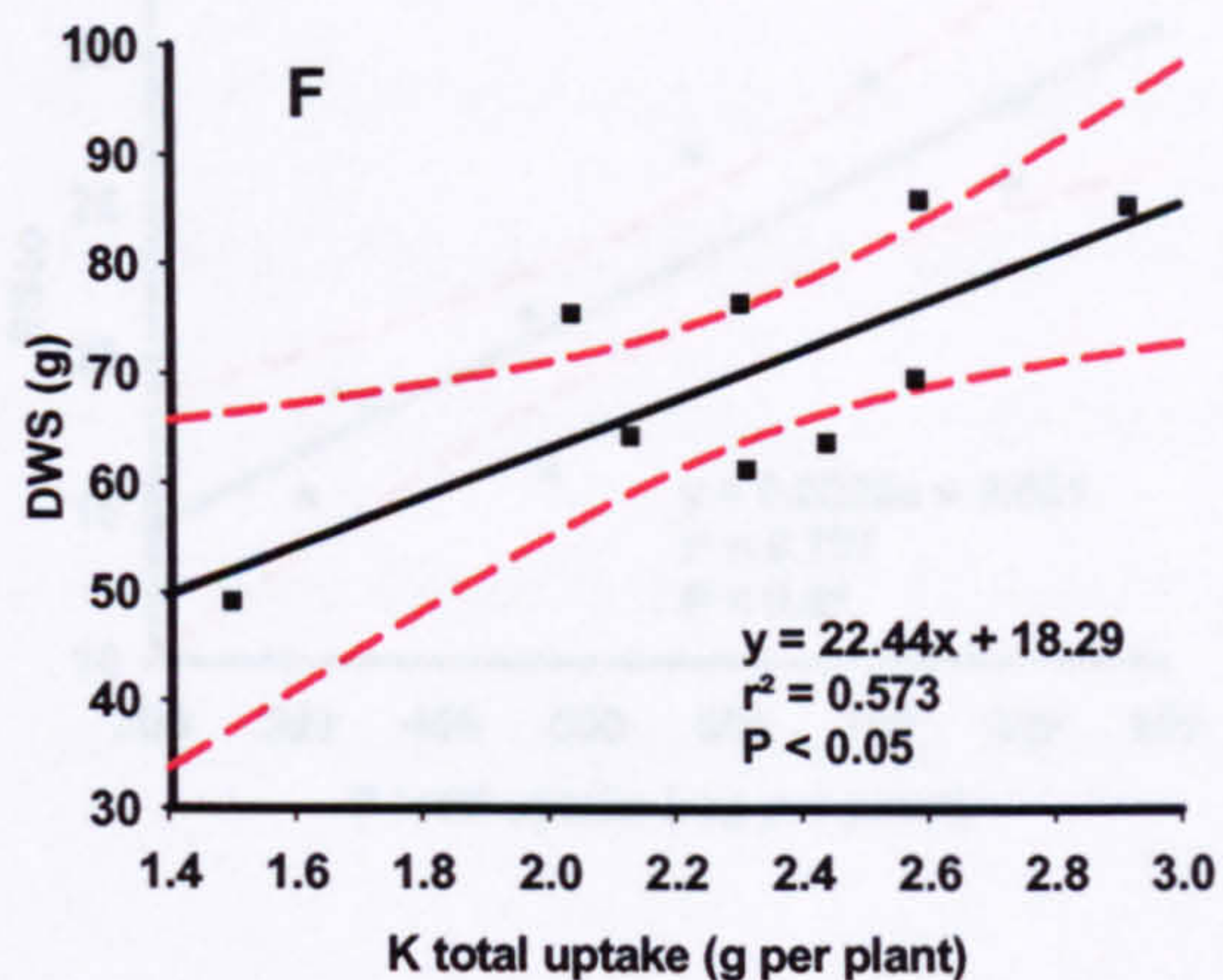
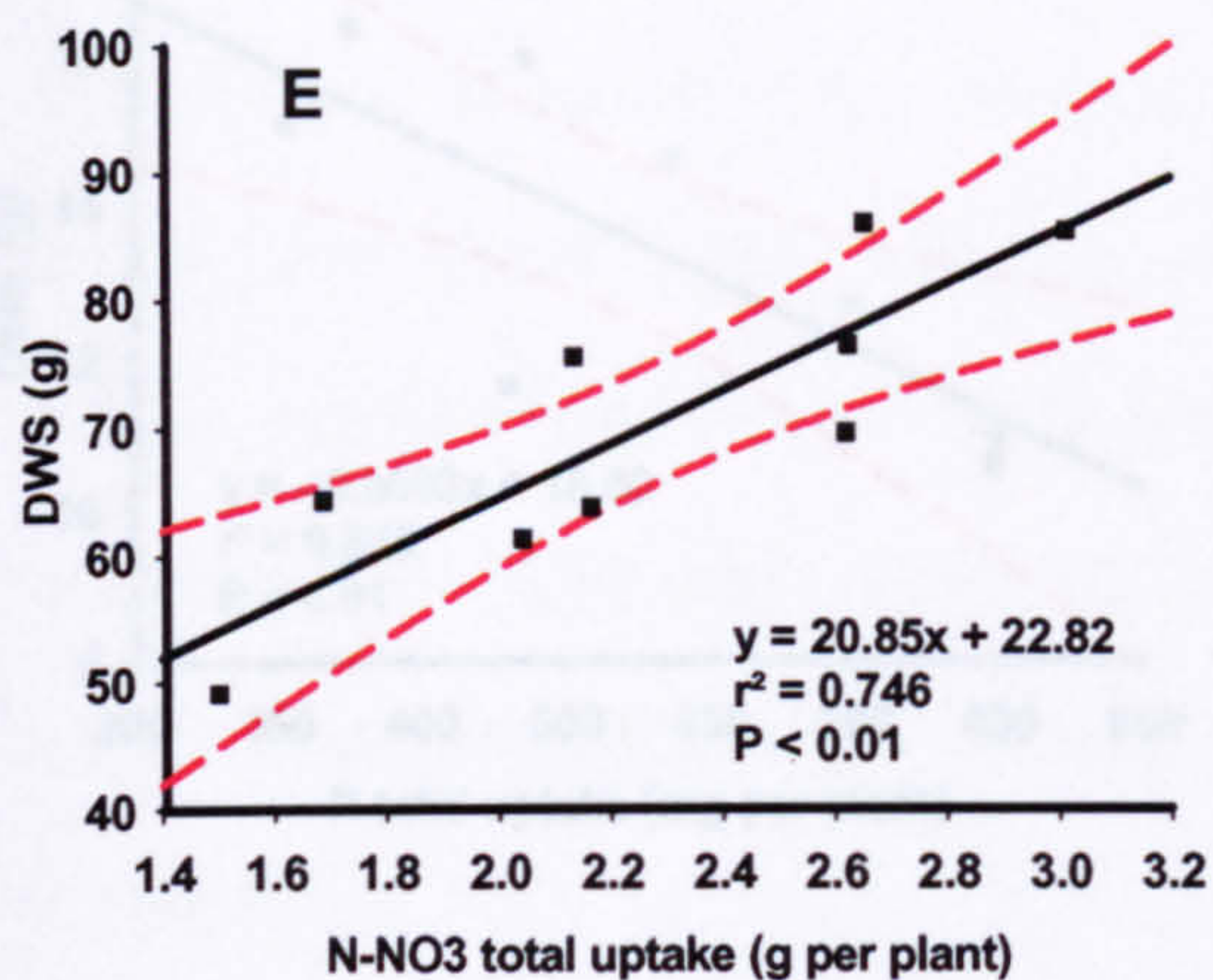
P value indicates the significance of slope's deviation from zero

The number of pairs tested was n=9 sub-units/replicates

5.3.4 Nutrient and water uptake relationships with plant growth

Linear regression analysis revealed the impact of nutrients and water uptake on the biomass production of plants and component plant parts as influenced by nutrient solution EC regime (1.8, 3.6, 7.4 mS/cm). Strong positive relationships were established between plant dry weight (*DWP*) and N-NO₃, K and Ca uptake (see Figure 5.7A, B and D respectively). Regression of shoot dry biomass (*DWS*) and root dry weight (*DWR*) against all nutrients explained more than 50% of the variation in shoot and root biomass yield (Figure 5.7E to N). While nitrates, K and Ca uptake were positively related to growth, significant negative relationships were revealed for phosphorus (Figure 5.7C, G, L). The only positive relationship established with P was P versus root to shoot biomass allocation (*RSc_o*) (Figure 5.7M). The profound effect of water on shoot growth was clearly demonstrated in Figure 5.7O where a significant positive response to total water consumption is exhibited by *DWS*.





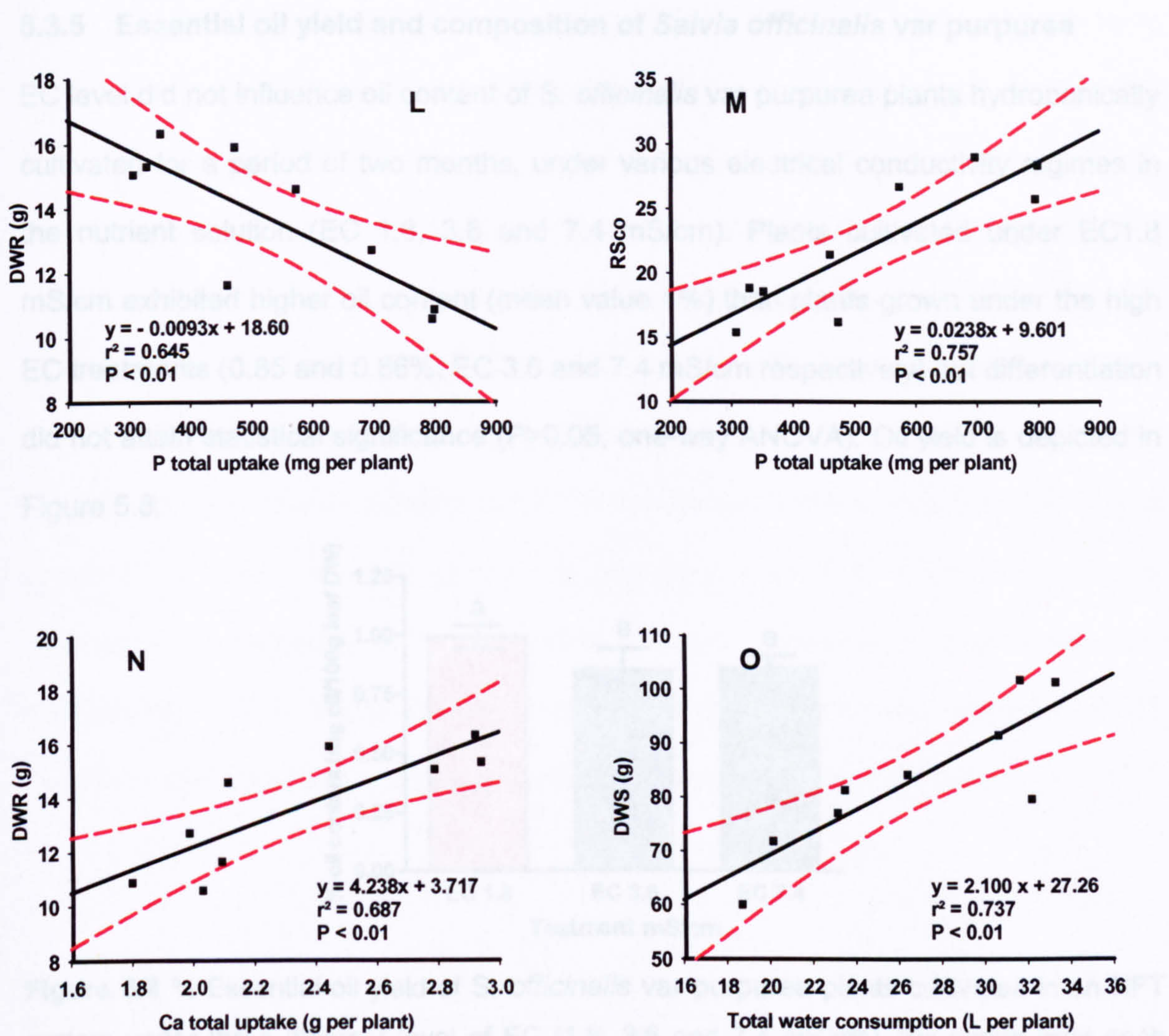


Figure 5.7 Linear regression of plants nutrient uptake with dry biomass production. *DWP*, *DWS*, *DWR*, *RSco* denote dry weight of the plant, shoot, root and root to shoot coefficient respectively. Plants of *S. officinalis* var *purpurea* were raised hydroponically under three levels of EC (1.8, 3.6 and 7.4 mS/cm). Linear regressions presented are: *DWP* versus the uptake of N-NO₃ (A), K(B), P(C), Ca(D); *DWS* versus N-NO₃ (E), K(F), P(G), Ca(H); *DWR* versus N-NO₃ (J), K(K), P(L), Ca(N); *RSco* versus P (M) and *DWS* versus water uptake (O) respectively.

Red dotted lines represent the 95% confidence band of the regression.

r^2 indicates goodness of fit of the linear slope.

P value indicates significance of slope's deviation from zero

The number of pairs tested was n=9 NFT sub-units/replicates

5.3.5 Essential oil yield and composition of *Salvia officinalis* var *purpurea*

EC level did not influence oil content of *S. officinalis* var *purpurea* plants hydroponically cultivated for a period of two months, under various electrical conductivity regimes in the nutrient solution (EC 1.8, 3.6 and 7.4 mS/cm). Plants cultivated under EC1.8 mS/cm exhibited higher oil content (mean value 1%) than plants grown under the high EC treatments (0.85 and 0.86%, EC 3.6 and 7.4 mS/cm respectively) but differentiation did not attain statistical significance ($P>0.05$, one-way ANOVA). Oil yield is depicted in Figure 5.8.

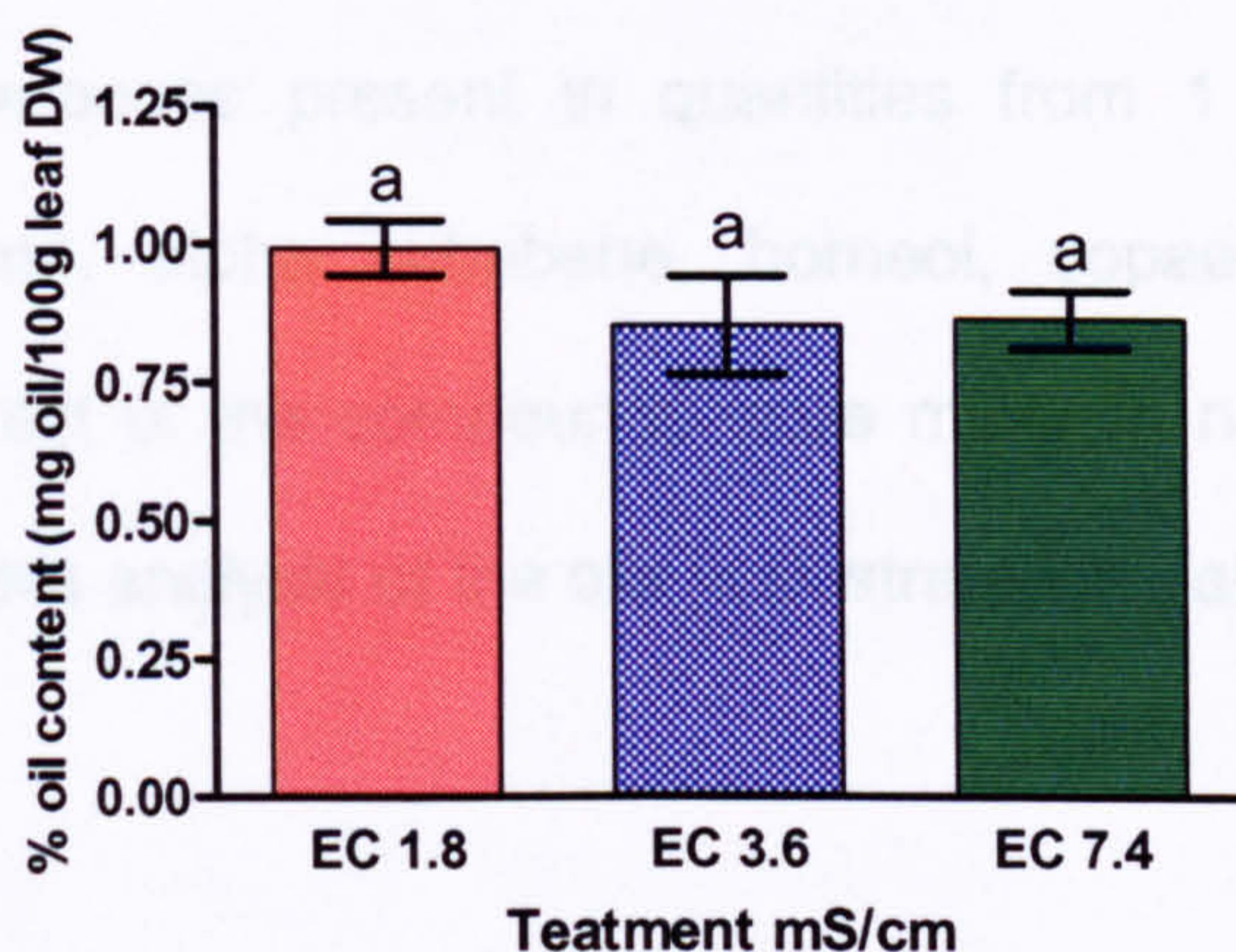


Figure 5.8 % Essential oil yield of *S. officinalis* var *purpurea* plants cultivated in an NFT system under three different level of EC (1.8, 3.6 and 7.4 mS/cm). Six plants from each replicate/channel were pooled together for every extracted oil. EC treatments were replicated three times ($n=3$ NFT sub-units)

Columns followed by the same letter are not significantly different at 0.05 level
Vertical bars indicate standard errors of the mean ($n=3$ oils).

The effects of EC treatment on essential oil composition of *S. officinalis* var *purpurea* raised in an NFT system under three levels of nutrient solution EC (1.4, 3.6 and 7.4 mS/cm) were investigated *via* GC/MS analysis. Significant differences among treatments became evident for specific constituents like 1.8 cineole, borneol, a and b thujone, isopinocampone, alpha cubebene and alpha caryophyllene (see Table 5.7). A significant decline in the concentration of 1.8 cineole, alpha cubebene and alpha caryophyllene was induced under the high EC treatments (3.6 and 7.4 mS/cm),

whereas significant increases in the content of a and b thujone as well as borneol were revealed. In addition, seven minor compounds were solely detected in high EC treatments (3.6 and 7.4 mS/cm), out of which four contained nitrogen in their structure. *S. officinalis* var *purpurea* oils overall comprised 55 compounds with 42 of them being identified. a Caryophyllene was found to be the most abundant constituent in the oil mixture contributing from 13 to 18% of the whole. a-Thujone, camphor, and ledol constituted other major compounds accounting for 10-13, 9-10 and 5% of the total respectively. 1.8 Cineole represented 3% of the mixture and b-pinene 3 to 5%. The oil consisted of many terpenes present in quantities from 1 to 2.5% like a-pinene, camphene, D-limonene, alpha cubebene, borneol, copaene, caryophyllene, and germacrene D. The rest of the compounds were minor in no case exceeding 0.5%. Qualitative and quantitative analysis of the oils is illustrated in Table 5.7 below.

Table 5.7 Chemical compounds present in essential oils analysed by GC/MS

Compound, % ^c	RT ^d (min)	% percentage of the compounds in the oil \pm SEM ^e		
		EC1.8mS/cm	EC3.6mS/cm	EC7.4mS/cm ^f
alpha Pinene 96%	11.74	1.33 \pm 0.16	1.23 \pm 0.07	0.80 \pm 0.22
camphene 98%	12.35	1.34 \pm 0.16	1.12 \pm 0.18	0.99 \pm 0.12
beta Pinene 97%	13.64	5.37 \pm 0.72	4.81 \pm 0.35	3.18 \pm 0.11
beta mycrene 83%	13.91	0.32 \pm 0.02	0.29 \pm 0.07	0.44 \pm 0.04
NI ^g	14.31	0.35 \pm 0.03	0.34 \pm 0.02	0.29 \pm 0.04
NI ^h	15.66	0.18 \pm 0.01a	0.21 \pm 0.01ab	0.23 \pm 0.00b
D-limonene 96%	15.81	0.87 \pm 0.07	0.79 \pm 0.2	0.75 \pm 0.03
eucalyptol 98% (1,8 cineole)	15.95	3.50 \pm 0.04a	3.37 \pm 0.06ab	3.13 \pm 0.04b
benzyl alcohol 95%	16.2	0.39 \pm 0.03	0.62 \pm 0.11	0.42 \pm 0.1
3 carene 91%	16.50	0.51 \pm 0.06	0.49 \pm 0.11	0.33 \pm 0.07
NI	17.45	0.23 \pm 0.01	0.24 \pm 0.02	0.24 \pm 0.03
Thujone a 96%	19.13	9.66 \pm 0.30a	9.66 \pm 0.99a	13.45 \pm 1.31b
Thujone b 98%	19.43	1.52 \pm 0.03a	1.71 \pm 0.10a	2.550. \pm 14b

Camphor 98%	20.56	9.12 ± 0.29	9.49 ± 0.75	10.71 ± 0.09
Bicyclo(3,1,1)heptan-3-one,2,6,6-trimethyl 95%	21.13	3.56 ± 0.13	3.18 ± 0.23	2.67 ± 0.01
Borneol 95%	21.33	1.32 ± 0.04a	1.52 ± 0.09b	2.15 ± 0.05b
NI	21.60	1.01 ± 0.05	0.82 ± 0.13	0.72 ± 0.03
Isopinocampone 91%	21.71	0.27 ± 0.01a	0.28 ± 0.02b	0.36 ± 0.01b
Bicyclo(3,1,1)heptan-2-one,2methanol, 6,6 95%	22.44	0.52 ± 0.01a	0.50±0.01ab	0.45 ± 0.02b
NI	22.56	-----	0.56 ± 0.2	0.27 ± 0.1
D-Verbenone 93%	22.92	0.46 ± 0.014	0.39 ± 0.12	0.55 ± 0.04
Benzofuran, 2,3-dihydro	23.35	0.24 ± 0.01	0.39 ± 0.09	0.32 ± 0.03
glycocyanidine 59%	24.10	-----	0.15 ± 0.00	0.15 ± 0.01
pyrrolidine 2,4 dione 53%	24.32	-----	0.32 ± 0.11	0.26 ± 0.00
Linalyl isobutyrate 50%	24.53	0.21 ± 0.01	0.26 ± 0.05	0.35 ± 0.03
NI	25.02	-----	0.20 ± 0.04	0.25 ± 0.01
Bornyl acetate 98%	25.59	0.53 ± 0.04	0.57 ± 0.04	0.62 ± 0.05
Indolizine 93%	25.91	-----	0.191	0.213
2-Methoxy-4-vinyphenol 91%	26.59	1.08 ± 0.12	1.77 ± 0.51	1.85 ± 0.21
alpha-cubebene 96%	27.75	0.92 ± 0.01a	0.79 ± 0.04b	0.77 ± 0.01b
eugenol 97%	28.02	0.31 ± 0.03	0.44 ± 0.05	0.50 ± 0.05
NI	28.24	-----	0.15	0.15 ± 0.01
Ylangene 99%	28.47	0.37 ± 0.03	0.47 ± 0.03	0.41 ± 0.02
Copaene 99%	28.63	1.03 ± 0.04	1.13 ± 0.09	1.01 ± 0.05
vanilin 96%	29.42	-----	0.21 ± 0.04	0.19 ± 0.02
1H-Cyclopropeazulene 1a,2,3,4,4a,5,6 99%	29.74	0.88 ± 0.03	0.87 ± 0.03	0.86 ± 0.05
caryophellene 99%	30.07	2.72 ± 0.07	1.73 ± 0.4	2.14 ± 0.08
Germacrene D 97%	30.37	0.91 ± 0.02	0.82 ± 0.05	0.79 ± 0.07
Beta panasinsene 94%	30.69	0.95 ± 0.06	0.92 ± 0.03	0.97 ± 0.04
alpha- caryophellene 97%	31.33	17.67 ± 0.4a	15.26 ±1.0ab	13.35 ± 0.2b
1H-Cyclopropeazulene decahydro-1,1,7 99%	31.42	0.41 ± 0.01	0.40 ± 0.02	0.40 ± 0.02
NI	32.17	0.32 ± 0.02	0.46 ± 0.09	0.27 ± 0.02
NI	32.42	1.24 ± 0.28	1.60 ± 0.13	1.52 ± 0.06
Butylated Hydroxytoluene 97%	32.95	2.83 ± 0.30	3.66 ± 0.38	3.48 ±0.35
Napthalene 97%	33.31	3.43 ± 0.11	3.47 ± 0.06	3.42 ± 0.03

benzofuranone tetrahydro 98%	33.53	0.32 ±0.03	0.34 ±0.05	0.38 ± 0.08
spathulenol 95%	34.91	0.33 ±0.02	0.37 ± 0.03	0.33 ±0.03
Ledol 99%	35.41	4.95 ± 0.15	5.10 ±0.19	4.74 ±0.15
NI	35.68	1.08 ± 0.03	1.11 ± 0.04	0.99 ± 0.01
NI	35.85	0.96 ± 0.07	1.08 ± 0.10	0.92 ± 0.11
NI	36.46	0.59 ± 0.16	0.80 ± 0.40	0.45 ±0.26
epiglobulol 72%	37.06	0.24 ± 0.02	0.2 ± 0.04	0.27 ± 0.01
NI	41.11	0.54 ± 0.16	0.378 ± 0.06	0.53 ± 0.06
naphthalene	47.29	4.89 ± 0.16	5.13 ± 0.75	4.04 ± 0.42

a, b, Where there is a statistically significant difference is indicated by the letters a, b. Values within a row followed by the same letter are not significantly different at 5% level (one-way ANOVA). Values in each row were tested for equality of variances and normality of distribution prior to ANOVA. Means and standard errors were determined from three replicates (n=3 oils analysed)

c, Identification was accomplished via NIST database. Percentage represents matching of the compound with library's spectrum

d, Retention time of the constituents peak in the chromatogram

e, Relative percentages of the compounds in the oil mixture, calculated from a total area of peaks (100%).

f, Plants were cultivated hydroponically under three levels of EC (1.8, 3.6, 7.4 mS/cm). Six plants from each replicate were pooled together for each oil produced. Treatments were replicated three times (n=3 NFT sub-units)

g, NI Not identified compound

h, Compounds present only in treatments EC3.6 and 7.4 mS/cm were statistically compared with Mann-Whitney test. No significant difference was detected in any case (P>0.05)

5.3.6 Acetyl and butyryl - cholinesterase inhibition by the essential oils

S. officinalis var *purpurea* oils obtained from hydroponically cultivated plants under three regimes of electrical conductivity (EC 1.8, 3.6 and 7.4 mS/cm) were investigated in terms of cholinesterase inhibitory properties. Outcomes of this experiment as in previous (chapters 3 and 4) demonstrated dual anti-cholinesterase activity for both cholinergic enzymes, acetyl- and butyryl-cholinesterase (AChE and BuChE respectively). Inhibitory activity expressed as mean IC₅₀ value ranged irrespectively of growth treatment from 0.05 to 0.06 mg/ml for human AChE and 0.07 to 0.085 mg/ml for BuChE cholinesterase enzyme (Table 5.8). Electrical conductivity treatment (EC1.8, 3.6 and 7.4 mS/cm) did not affect the potency of the oil ($P > 0.05$, one-way ANOVA). Plants cultivated under EC 1.8 mS/cm appeared to possess lower IC₅₀ values; however the difference did not attain statistical significance (Table 5.8). Comparison of IC₅₀ values for AChE and BuChE revealed preferential inhibition for AChE irrespectively of treatment ($P < 0.001$, t-test) (Figure 5.9).

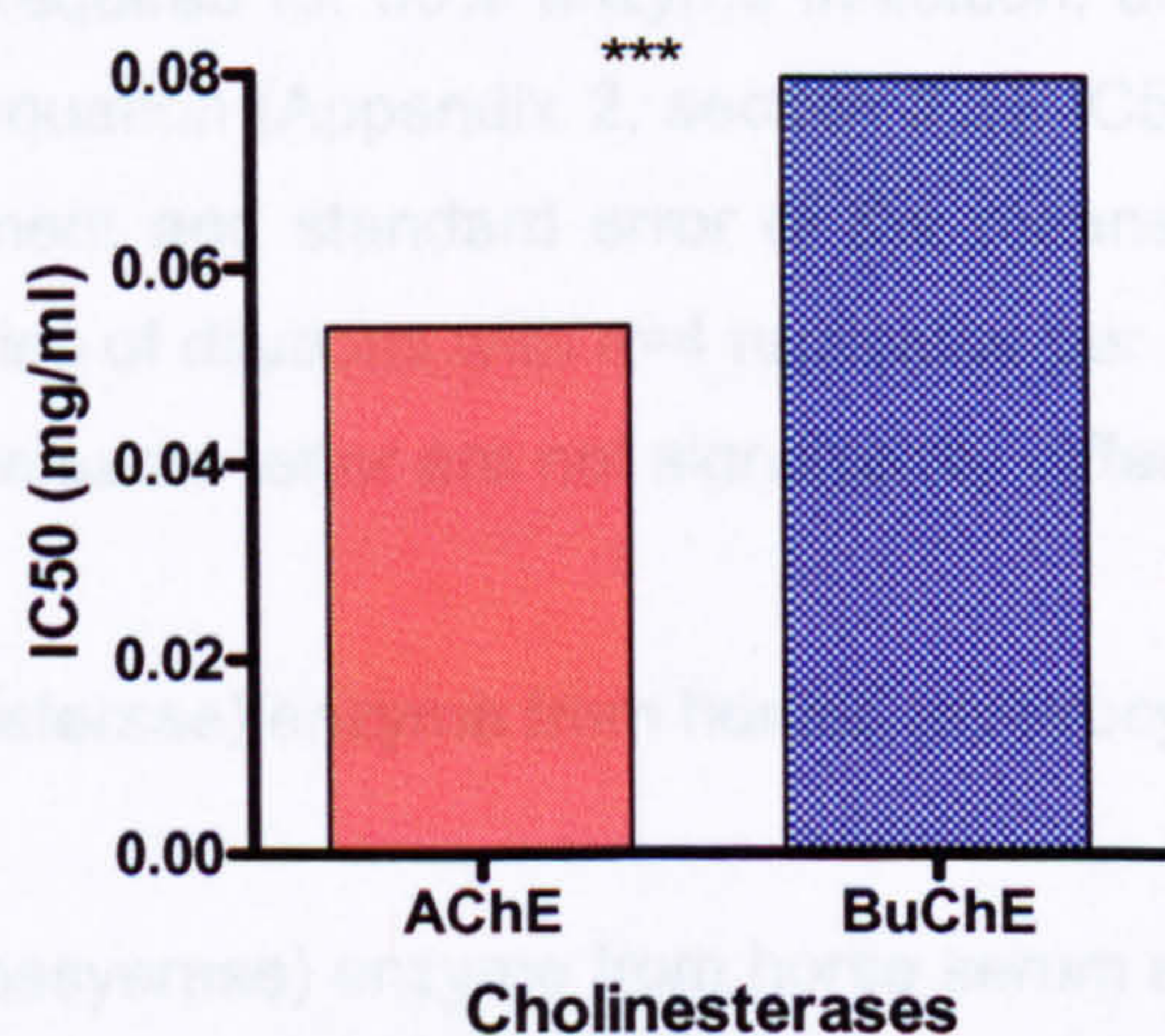


Figure 5.9 Comparison of AChE (from human erythrocytes) and BuChE (from horse serum) inhibition from *S. officinalis* var *purpurea* plants raised hydroponically in an NFT-system. IC₅₀ values indicate inhibitory activity of the oil.

Columns indicate the means ($n=9$ oils deriving from 9 NFT sub-units). Statistical analysis was performed with log transformed values; therefore standard error bars (SEM) are not presented.

Means differ significantly, $P < 0.001$.

Table 5.8 AChE and BuChE inhibition of *S. officinalis* var *purpurea* oils expressed as IC50 value

Treatment ^c	IC50 (mg/ml)^d
	AChE inhibitory activity ^e
EC 1.8 mS/cm	0.049 ± 0.003 a
EC 3.6 mS/cm	0.056 ± 0.003 a
EC 7.4 mS/cm	0.057 ± 0.008 a
	BuChE inhibitory activity ^f
EC 1.8 mS/cm	0.074 ± 0.007 b
EC 3.6 mS/cm	0.085 ± 0.012 b
EC 7.4 mS/cm	0.080 ± 0.010 b

^c Oils were extracted from *S. officinalis* var *purpurea* plants via steam distillation method. Plants had been hydroponically cultivated in an NFT system under three levels of electrical conductivity (EC 1.8, 3.6 and 7.4 mS/cm). Treatments were replicated three times, resulting in the production of equal number of oils per treatment. Six plants from each replicate were pooled together for every oil/inhibitor production

^d Concentration of oil required for 50% enzyme inhibition, as calculated from a sigmoidal dose-response curve equation (Appendix 2, section 2.3). IC50 values represent the mean of three oils per treatment and standard error of the means. Each of the three oils was tested twice after a series of dilutions with n=4 replicates per dilution per run. Values within columns followed by the same letter are not significantly different at 5% level (n=3 oils/NFT sub-units)

^e AChE (acetyl-cholinesterase) enzyme from human erythrocytes at a final concentration of 0.05 U/ml

^f BuChE (butyryl-cholinesterase) enzyme from horse serum at a final concentration of 0.02 U/ml. Enzyme inhibitory activities were determined via Ellman's method (Ellman *et al.*, 1961). Inhibitors were pre-incubated with the enzyme for a period of 30 min.

5.4 Discussion

5.4.1 *Salvia officinalis* var *purpurea* growth responses to electrical conductivity of the nutrient solution

The present study examined the effects of electrical conductivity (EC) on the growth of *Salvia officinalis* var. *purpurea*, hydroponically cultivated via Nutrient Film Technique (NFT) under three EC regimes (1.8, 3.6 and 7.4 mS/cm). Outcomes revealed that EC level in the nutrient solution substantially affected plant overall growth. Shoot biomass accumulation expressed as fresh or dry weight of the shoot (*FWS* and *DWS*) was significantly suppressed under the higher EC levels (3.6 and 7.4 mS/cm) by 36 and 24% respectively. Additionally, decreased specific leaf area (*SLA*), reduced height, total plant biomass (*FWP*, *DWP*) and greater root to shoot ratio (*RSco*) were revealed. The outcomes of this experiment are corroborated by findings of Economakis (1992) on *Origanum dictamnus* raised in nutrient film culture (NFT), who reported reduced *FWS* and *DWS* under the 3 and 4 mS/cm in comparison to lower EC level (2 mS/cm). Possible explanation for EC induced changes in plants is the increased concentration of nutrients at the higher EC treatments (3.6 and 7.4 mS/cm). Elevated solute concentration in the nutrient solution and hence increased osmotic pressure would hinder water influx in the root system (Herralde *et al.*, 1998) and potentially cause conditions analogous to water deficiency. This conclusion is strongly indicated by the fact that at EC 3.6 and 7.4 mS/cm a significant decline in the water absorption and % shoot water content (%*SWC*) was induced. Moreover, all high EC-mediated plant features of *S. officinalis* var. *purpurea* were typical of plants subjected to water stress (Taiz & Zeiger, 1991). A review of the literature confirmed that this is the first study analysing the effects of EC on growth of *Salvia officinalis*. Water stress experiments on other aromatic and medicinal plants revealed outcomes consistent with the present study. Mild and moderate water deficiency significantly reduced leaf area (*LA*), leaf and stem dry weight of *Ocimum basilicum* after 21 days of treatment (Simon *et al.*, 1992). Plant height, fresh biomass (g/m²) and *LA* of *Pelargonium graveolens* L. were

considerably decreased within one month of water deprivation (Putievsky *et al.*, 1990). Water stress of approximately 120 days on various cultivars of *Cymbopogon martini* and *C. winterianus* clearly resulted in a decline in plant height, herbage yield and LA (Fatima *et al.*, 1999 and 2002). Finally, more recent drought studies on *Satureja hortensis* L. (Baher *et al.*, 2002), *Dittricia viscosa* (Karageorgou *et al.*, 2002) *Hypericum perforatum* (Zobayed *et al.*, 2005) and *Hypericum brasiliense* Choisy (Nacif de Abreu & Mazzafera, 2005) showed significant reduction in plant biomass due to water limitation. In the present experiment growth of *S. officinalis* var *purpurea* raised hydroponically with three regimes of EC 1.8, 3.6 and 7.4 mS/cm was favoured by the lower EC treatment while under higher EC levels plants exhibited characteristics typical of water stress.

The imposition of stress by the higher EC treatment was also suggested by pigment analysis of *S. officinalis* var *purpurea* showing a significant decline in chlorophyll a (Chla) content and Chla to Chlb ratio, with concurrent enhancement of carotenoid content as a result of high EC level in the nutrient solution (3.6 and 7.4 mS/cm). Selective degradation of Chla represents potential damage to the photosynthetic apparatus whereas Chlb concentration remained unchanged (Shevchenko *et al.*, 2004). Similar alterations in pigment content have been previously associated with plant responses to abiotic stress (Richardson *et al.*, 2002; Shevchenko *et al.*, 2004).

The results obtained by nutrient uptake analysis are worth highlighting. Nitrate (N-NO₃), potassium (K) and calcium (Ca) absorption appeared considerably reduced under the high EC levels (3.6 and 7.4 mS/cm) despite the fact that concentration and thus availability of nutrients was much greater. As most of the nutrients are actively taken up by the plants (Marschner, 1995) differentiation in the concentrations absorbed could simply reflect a control mechanism of selective nutrient influx that restrains uptake of excess nutrients. Another explanation of reduced nutrient uptake at high solution EC levels might be the possible effect of the water potential gradient along the root axis on

the gradient of ion uptake *via* the obstruction of solute transport on the root surface and through the cortex (Marschner, 1995). The decrease in nutrient uptake is consistent with the observed growth decline. In contrast with the absorption of N-NO₃, K and Ca that were suppressed at EC 3.6 and 7.4 mS/cm, uptake analysis for phosphorus (P) revealed increased P influx with the increase in EC level. This is in agreement with Savvas & Lenz (1999) who reported increased P uptake in egg plants hydroponically cultivated under elevated nutrient concentrations, while there was no differentiation in the other nutrients absorption. *S. officinalis* var *purpurea* exhibited greater P uptake, as P level in the nutrient solution was elevated from 12 to 60 mg/ml (see chapter 3). Similar results for P absorption rate were obtained for *Anethum graveolens* and *Thymus vulgaris* hydroponically raised under EC 1.2, 2.4 and 3.6 mS/cm (Udagawa, 1995). This phenomenon has been known as luxury consumption (Theodorou & Plaxton, 1995; Marschner, 1995). No symptoms of P or other nutrient toxicity were observed in the course of the EC treatments. It is worth noting that Savvas & Lenz (2000) who investigated increased EC levels on hydroponically cultivated egg plants either *via* the addition of NaCl or extra nutrients demonstrated that any effects on plant growth were osmotic and not ion specific. These results strongly indicate EC treatments as a potent research tool for osmotic / water stress studies in hydroponics.

5.4.2 The influence of electrical conductivity on essential oil content and composition in *Salvia officinalis* var *purpurea*.

In parallel with growth responses, the effect of EC level in the nutrient solution on the essential oil production of hydroponically cultivated *S. officinalis* var *purpurea* was analysed for the first time. Oil content was approximately 1% (g of oil per 100 g leaf dry mass). Sage plants belonging to the same species (*S. officinalis*) cultivated in the field have exhibited oil content from 0.9 to 2.3% under different seasons and geographical origins (Putievsky *et al.*, 1986; Chalchat *et al.*, 1998; Perry *et al.*, 1999a; Karousou *et al.*, 2000; Santos-Gomes & Fernandes-Ferreira, 2001), whereas *S. officinalis* grown in

Britain displayed a very low oil production of 0.53% (personal communication with Dr S. Savelev) that could explain the relatively low yield obtained in this study. Nevertheless, *S. officinalis* var. *purpurea* oil content was not affected by EC treatment (1.8, 3.6 and 7.4 mS/cm) in the present experiment. Oil content of *Origanum dictamnus* in solution culture was enhanced under EC 2mS/cm in comparison to higher EC levels (3 and 4 mS/cm) (Economakis, 1992). Similar results were obtained by Udagawa, (1995) for *Anethum graveolens* that reached maximum oil content under EC 2.4 mS/cm in the growth solution in comparison to higher EC level (3.6mS/cm). Conversely, the same author reported that *Thymus vulgaris* oil production was favoured at the greatest EC treatment (3.6 mS/cm). However, experimental data regarding EC influence on essential oil of hydroponically cultivated aromatic and medicinal plants are scarce. If it is hypothesised that EC-mediated effects on essential oils production are exerted via osmotic effects in the nutrient solution, as in the case of growth, then a number of studies have analysed the synthesis of secondary metabolites under water limitation. Charles and co-workers (1990) reported a linear increase in oil content of *Mentha x piperita* L. and concurrent growth reduction with increasing osmotic stress in the nutrient solution.

Secondary metabolite biosynthesis and plant growth are competitive processes for photosynthate partitioning. It has been suggested that alterations in secondary metabolites of water stressed plants are associated with reallocation of the assimilated carbon from the primary pathways to secondary metabolism as plant growth is progressively reduced (Nacif de Abreu & Mazzafera, 2005). Gershenzon (1983) indicated that there is a strong association between the accumulation of secondary compounds and periods of reduced growth. In the present study however, high EC levels in the nutrient solution did not enhance essential oil yield of *S. officinalis* var *purpurea* although plant growth was retarded. The decrease in chl_a content in leaves of plants grown under the higher levels of EC indicate that photosynthetic activity may

well have declined in response to elevated EC. However, if photosynthesis was not significantly inhibited under the high EC treatments, then the assimilated carbon not utilised for extra secondary metabolites biosynthesis could have been invested for root expansion a typical strategy of water stressed plants (Sharp & Davies, 1989 in Hamlyn; Taiz & Zeiger, 1991); root biomass and root to shoot ratio were greater under the high EC treatments. Moreover, increased phosphorus uptake requires significant allocation of sugars and energy to roots (Marschner, 1985). Finally, in order to compensate for the lost turgor in cells, plants subjected to water stress tend to accumulate organic substances such as soluble carbohydrates (Sharp & Davies, 1989; Herralde *et al.*, 1998), which otherwise could have been potentially directed to secondary metabolites biosynthetic pathways.

The present study analysed the effects of EC on *S. officinalis* var. *purpurea* essential oil composition. Significant variations in the relative percentages of specific constituents were revealed. High EC treatments (3.6 and 7.4 mS/cm) induced a decline in the concentration of 1.8 cineole, alpha cubebene and alpha caryophyllene, whereas significant increases in the content of a and b thujone as well as borneol were evident. *S. officinalis* var. *purpurea* oils overall comprised 55 compounds with seven of them produced solely under the higher EC levels. Oil constituents present only at EC 3.6 and 7.4 mS/cm treatments entailed nitrogen (N) in their structure which is uncommon for *Salvia* species, and indicates that these constituents belonged to the amine group of chemicals. The enhanced present of thujones as well as the N containing compounds (amines) stimulated by the high EC levels where plants experienced stressful conditions, most probably serve defence purposes against insects and / or plant pathogens. α -Thujone is a molecule with reported toxic properties (Höld *et al.*, 2000), while amines like alkaloids are poisonous. In hydroponic thyme raised under various EC levels, carvacrol content a molecule with antibacterial activity (Baher *et al.*, 2002) was increased by the highest EC treatment (3.6 mS/cm)

(Udaqqawa, 1995). Carvacrol content was enhanced by moderately water stressed plants of *Saureja hortensis* L. In *Ocimum basilicum* oil the percentage of α -thujone was increased 1.5 fold due to water stress (Omidbaigi *et al.*, 2003). For the high EC-reduced terpenes, possible dehydration at water limiting conditions could have affected the specific enzymes involved in their biosynthetic pathways as in the case of geraniol dehydrogenase that catalyses the transformation of geraniol to another monoterpene and is moisture stress modulated (Fatima *et al.*, 2002).

5.4.3 *Salvia officinalis* var. *purpurea* cholinesterase inhibitory activity

Essential oils obtained from *S. officinalis* var. *purpurea* raised hydroponically under three EC levels were analysed in terms of their inhibitory activity on the two enzymes of human cholinergic system. Dual inhibitory activity for acetyl- and butyryl-cholinesterase (AChE and BuChE, respectively) was revealed with IC₅₀ values from 0.05 to 0.06 mg/ml for human AChE and 0.07 to 0.085 mg/ml for BuChE enzyme. Experimental data revealed outcomes consistent with the single report involving cholinergic properties of *S. officinalis* var. *purpurea* (Savelev *et al.*, 2004).

Whereas oil composition differed among growth treatments (EC 1.8, 3.6 and 7.4 mS/cm) in the relative concentration of specific compounds, anti-cholinesterase properties of the oils remained unchanged. Oils from plants cultivated under EC 1.8 mS/cm possessed lower IC₅₀ values, but differences did not attain statistical significance. However, oils produced from plants raised under the particular EC treatment appeared to have elevated concentration of 1.8 cineole; a terpene with reported anti-cholinesterase activity (Perry *et al.*, 2000; Savelev *et al.*, 2003 and 2004). Cholinergic properties of herb oils have been attributed to synergistic and antagonistic interactions among terpene constituents of the mixture and not to single compound (Miyazawa *et al.*, 1998; Savelev *et al.*, 2003 and 2004). The variation in the oil

composition observed in the present study, was apparently not sufficient to induce alteration in the inhibitory activity of the oils.

5.4.4 Conclusions

Salvia officinalis var *purpurea* an important medicinal and ornamental plant was successfully cultivated under hydroponic conditions by means of NFT. Solution electrical conductivity (EC) significantly affected all growth attributes measured. Optimum EC level for enhanced herbage yield was found to be 1.8 mS/cm, whereas overall growth was suppressed under the higher EC treatments (3.6 and 7.4 mS/cm). EC treatment had no significant influence on essential oil content. Conversely, significant variation in specific oil constituents was induced by the level of EC in the nutrient solution. When cholinergic activities of *Salvia officinalis* var *purpurea* oil were determined, it was revealed that variations in oil composition had provoked only marginal differences.

Chapter 6

**Effects of nitrogen on alkaloid content and growth of
Narcissus “Ice Follies” cultivated by means of
Nutrient Film Technique.**

6.1 Introduction

The genus *Narcissus* belongs to the Amaryllidaceae family and since ancient times has been exploited for decorative purposes (Cherkasov & Tolkachev, 2002). In the UK, *Narcissus* plants have been cultivated for more than 300 years (Hanks, 2002a); today the country constitutes the leading producer (Moraes, 2002). *Narcissus* species are very important ornamentals for Europe, US, as well as other parts of the world. Large field areas are occupied by the crop to provide bulbs and flowers, whereas in glasshouses flowers and pot-plants are produced over extended periods by means of horticultural forcing techniques (Hanks, 2002a). The Amaryllidaceae (apart from ornamentals) have been characterised as poisonous, and a number of animal poisoning incidents have been recorded in the literature (Cherkasov & Tolkachev, 2002). Poisonous properties are attributed to a wide range of alkaloid molecules, which are intrinsically synthesized by amaryllid plants and possess several important biological activities (Ingkaninan *et al.*, 2002). More than 200 alkaloids have been isolated from the Amaryllidaceae, and the family has attracted attention as a source of novel compounds with potential use in medicine (Cherkasov & Tolkachev, 2002). The most valuable pharmaceutically-active compound among the Amaryllidaceae alkaloids is galanthamine (GAL), a tertiary amine alkaloid derived from phenylalanine and tyrosine amino acids (see general introduction). Galanthamine in the form of GAL-hydrobromide has been used in medicine for the treatment of myasthenia, myopathy, neuritis, infantile paralysis, psychogenic spinal impotence, spastic pareses, muscular dystrophy, antagonistic of muscular relaxants in the case of surgical interventions and for the treatment of schizophrenia (Cherkasov & Tolkachev, 2002). In the 1980s GAL was indicated as a selective, reversible, competitive, acetyl cholinesterase (AChE) inhibitor, which can readily cross the blood brain barrier and today is one of the few licensed drugs used for the treatment of Alzheimer's disease (AD). Galanthamine is marketed under the name Reminyl® (see general introduction).

Originally GAL had been isolated from snowdrop *Galanthus nivalis* but today *Leucojum aestivum* and *Narcissus* spp are the principal natural sources for its extraction. Furthermore the total chemical synthesis of GAL has been achieved (Moraes, 2002; Heinrich & Teoh, 2004). *Narcissus* species as GAL source have the advantage over *Leucojum aestivum* for commercial exploitation. A large number of bulbs are produced from various *Narcissus* cultivars thus facilitating the establishment of large-scale cultivation for medicinal purposes, while extensive published information exists on *Narcissus* physiology, breeding and cultivation for flower production (Moraes, 2002). Screening of 20 *Narcissus* cultivars for bulb GAL content among other Amaryllidaceae, suggested that *Narcissus* “Ice Follies” was one of the four richest species / cultivars tested in terms of total alkaloids and galanthamine (Kreh, 2002). Moraes (2002) proposed “Ice Follies” as a prospective source for GAL extractions due to its fast growth rate, availability and sufficient content of the corresponding alkaloid (Moraes-Cerdeira *et al.*, 1997a and 1997b). Additionally, *Narcissus* “Ice Follies” contains lycorine (LYC) (Moraes-Cerdeira *et al.*, 1997a), another alkaloid with very important pharmacological activities that include anti-tumor, anti-arrhythmic, broncholytic and inhibitory activity on herpes virus (Cherkasov & Tolkachev, 2002).

Constant supply of the active compound at an affordable price can only be achieved by systematic cultivation of the medicinal plants (Moraes, 2002). Alzheimer's disease (AD) is the most common cause of dementia in the elderly and according to the World Health Organization (WHO), around 35 million people in industrialised countries will suffer from AD by 2010 (Heinrich & Teoh, 2004). Therefore, stable production and augmentation of GAL yields have become essential to cover the needs of the pharmaceutical industries.

Alkaloid biosynthesis is highly susceptible to various environmental factors such as light intensity (including quality and duration), water availability, temperature and

nutrition (Waller & Nowacki, 1978; Bernath, 1986). There have been a number of endeavours to improve alkaloid yield by altering nutrient conditions (Waller & Nowacki, 1978; Kennedy & Bush, 1983; Gershenzon, 1985; Khan *et al.*, 1995; Höld *et al.*, 1996; Lata & Sadowska, 1996; Demeyer & Dejaegere, 1997; Baricevic *et al.*, 1999; Baricevic & Zupancic, 2002; Kumar *et al.*, 2004; Sreevalli, *et al.*, 2004; Abdolzadeh *et al.*, 2006; Misra & Gupta; 2006). Since alkaloids are nitrogen (N) containing compounds the availability of N can potentially affect the amount of alkaloids synthesised by plants. While promotion of alkaloid production by additional N is expected (Gershenzon, 1985), augmentation of alkaloids due to N does not constitute a universal response, but depends upon the plant species and / or specific variety (Waller & Nowacki, 1978; Gershenzon, 1985). As a general trend, alkaloids that react consistently positively to added N are those rich in N molecules (low carbon to nitrogen ratio; Gershenzon, 1985). Nevertheless, Kreh, (2002) reported a 70% increase in GAL yield of field-grown *Narcissus* "Carlton" bulbs over the control as a result of N fertilisation. The profound effects of N nutrition on plants overall growth have been described before (see chapter 4, section 4.1).

As found for a number of species, alkaloid content in *Narcissus* is known to fluctuate depending upon ontogenetic stage. Dhar & Bhat (1982), in a study on five *Atropa belladonna* L. genotypes provided strong evidence that plant ontogeny can significantly influence alkaloid biosynthesis (Dhar & Bhat, 1982). Total alkaloids of periwinkle (*Catharanthus roseus*) were negatively correlated with the dry mass of flowers indicating the influence of ontogeny (Abdolzadeh *et al.*, 2006). Likewise, Kreh (2002), reported that GAL levels in both bulbs and leaves of *Narcissus* "Carlton" were maximum before the stage of anthesis. Galanthamine content of *Narcissus* "Inglescombe" was higher during the growing period between emergence and anthesis, whereas a decrease was observed in the stages after flowering characterised by starch accumulation in bulb scales and leaf senescence (Moraes, 2002). Analysis of 81

Narcissus cultivars for alkaloid content revealed that GAL biosynthesis was maximum at bud formation stage when plants growth rates were the highest (Cherkasov & Tolkachev, 2002).

Alkaloid levels differ among different plant parts. In the case of *Narcissus* "Carlton" all plant parts including bulbs, roots, leaves, flowers, flowering stems and seeds contained alkaloids and in particular galanthamine. However, bulbs exhibited the highest GAL concentrations being thus the most interesting narcissus part for GAL extractions (Kreh, 2002). Galanthamine content in leaves of the pre-mentioned *Narcissus* cultivar appeared to be significantly lower but within a range suitable for commercial exploitation (Kreh, 2002). When 81 *Narcissus* cultivars were analysed as potential sources for GAL production, 72 appeared to contain GAL in both bulbs and leaves and interestingly the highest levels of GAL were found in the leaves of eight populations (Cherkasov & Tolkachev, 2002).

Cultivation of *Narcissus* and generally bulbous species by means of Nutrient Film Technique (NFT) has never been exploited before. Cooper (1979) suggested after preliminary trials that *Narcissus* bulbs could potentially grow in an NFT system. *Narcissus* cultivars have been raised in static solutions *via* aeration in order to study effects of nutrient deficiencies on growth (Ruamrungsri *et al.*, 1996a, 1996b). In an attempt to compare hydroponic and conventional / soil cultivation of medicinal plants for the production of bioactive compounds, Manukyan (2005) reported enhancement of alkaloid levels in celandine poppy (*Chelidonium majus* L.) which constituted up to 1.53% (on a dry raw material basis) under outside hydroponic conditions in contrast with 1.22% in the concurrently soil grown plants.

Narcissus "Ice Follies" is a popular ornamental plant that additionally contains important pharmaceutical alkaloid compounds with various medical applications. Bulbs

of “Ice Follies” were hydroponically cultivated by means of NFT under two nitrogen levels, with the aim to increase alkaloid content and specifically the levels of galanthamine, a licensed agent for Alzheimer’s disease as well as lycorine. Possible alkaloid yield fluctuations due to ontogenetic effects were investigated. In parallel, because of *Narcissus* species importance for floriculture, the effect of nitrogen nutrition on plant growth was studied.

6.2 Materials and methods

6.2.1 *Narcissus* “Ice Follies” cultivation by Nutrient Film Technique (NFT), experimental and environmental conditions

Narcissus “Ice Follies” dormant bulbs were purchased from R.A. Scamp Quality Daffodils (see section 2.1.2) early October 2004 and weighed utilising a Mettler AC 100 balance prior to the onset of the hydroponic experiment (25/10/2004). Bulbs of similar size i.e. 35 to 45 g ($P > 0.05$, unpaired t-test; Figure 6.1) were labelled and randomly assigned to eight NFT sub-units; 16 bulbs per channel, in 3 cm net pots, at approximately 12 cm intervals. The NFT system was set up in a growth room with controlled light and air temperature conditions. Standard floodlights were fixed 1 m above the bench level and light emission was accomplished via two 400 Watt (SON-T NAV) high pressure sodium and four 250 Watt (MHN-MHW double ended, Philips) metal halide discharge lamps. Photoperiod, was regulated by a central electric system with a timer incorporated and was arranged at 8 h per day and increased to 12 h after 90 days of cultivation to promote plant flowering. Photosynthetic active radiation (PAR) measured at plant-level (Skye, Q. light meter) was found to be $250 \pm 20 \mu\text{mol m}^{-2} \text{s}^{-1}$. Controlled air temperature was fixed at 8 °C (dark period) and 15 °C (light period) and was recorded daily via a min-max thermometer. The NFT unit comprised 1.8 m length channels, plastic dark coloured reservoir tanks of 30 L capacity (Wilkinson’s stores, UK) with one submersible water pump attached to black plastic tubing to serve nutrient solution circulation. A detailed description of an NFT unit set up at Moorbank is given in the general Materials and Methods (see section 2.2.1). Channels were supported by metal bench constructions of 1 m height.

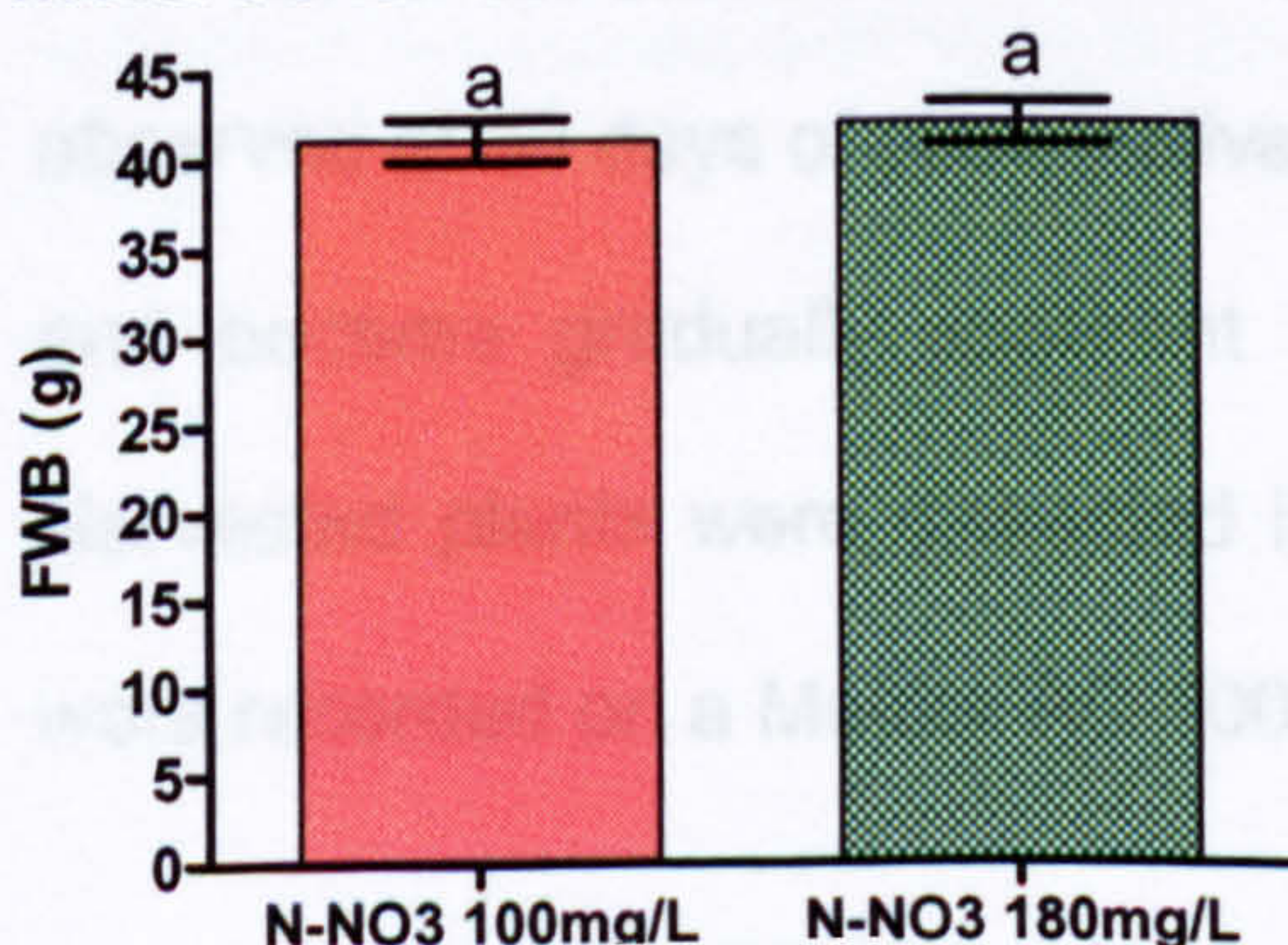


Figure 6.1 Weight of dormant bulbs (FWB) that were subsequently utilised in the experiment for nitrate effects on *Narcissus* “Ice Follies”. Columns indicate means & vertical bars SEM. Means did not differ statistically at 5% level (unpaired t-test).

Subsequently *Narcissus* bulbs establishment in the channels, tap water (electrical conductivity EC=0.5 mS/cm and pH=7) with no nutrients added was circulated in the system to start bulbs rooting (personal communication with Dr Economakis, Ruamrungsri *et al.*, 1996a). Calcium (Ca) concentration in the tap water at a level of 59 mg/L (see Appendix 1, section 1.2) was reported to promote root growth (Ruamrungsri *et al.*, 1996a and 1996b). After a period of 15 days, bulbs were supplied with complete nutrient solutions in the reservoir tanks, to give two distinct nitrate (N-NO₃) concentrations, specifically 100 and 180 mg/L N-NO₃. Hydroponic recipes for stock solution preparation (g of fertilisers/chemical reagents in 30 L of water) and target elemental composition of nutrient solutions in reservoir tanks are presented in table 6.1. Variation of SO₄²⁻ constitutes a common practice in hydroponics as the sulphate anion is well known not to interfere in the uptake of other ions (Alloush 2002, Savvas & Passam, 2002). Target electrical conductivity (EC) of nutrient solutions was 1.8 and 1.9 mS/cm for the 100 and 180 mg/L N-NO₃ treatments. pH was arranged at 7; 5% nitric acid (HNO₃) was utilised for pH corrections (see paragraph 2.2.4 for EC and pH measurements). The nitrate treatment was comprised of four independent NFT subunits organised according to the complete randomised design (CRD) (Gomez & Gomez, 1984).

Half of the *Narcissus* "Ice Follies plants (8 plants per channel) were harvested for the first time at the vegetative growth stage after 75 days of treatment initiation (25/1/05). A second harvest was conducted when all plants per channel were at the anthesis stage, 105 days since the onset of the nitrate treatments (25/2/05). Flowering was originally observed at 80 days of plant cultivation under the two levels of N-NO₃ (100 & 180 mg/L) and became gradually apparent for all *Narcissus* plants within the next fortnight. Harvested plants were dissected into shoot, bulbs, roots and flowers. Fresh weights were recorded on a Mettler AC 100 and all plant parts were afterwards dried in an oven

at 60 °C, inside various sized paper bags. Growth measurements included plant height, fresh and dry weights and the ratio of dry weight of the shoot to dry weight of the bulb (DWS / DWB), fresh weight of bulb to bulb dry weight (FWB / DWD) and % shoot water content (%SWC).

Actual mean pH and EC values (15 days average) of nutrient solutions in reservoir tanks, before the correction with acid or stock solution, are presented in Figures 6.2 and 6.3 respectively. pH values varied from 7.3 to 7.6. A rise in pH of the nutrient solutions is principally attributed to nitrate (NO_3^-) fixation into ammonia (NH_4^+) in plant roots, a process that releases OH^- into the growth medium (Cooper, 1979). EC ranged from 1.68 to 1.92 mS/cm and appeared to be lower for the 180 mg/L nitrate treatment (Figure 6.3).

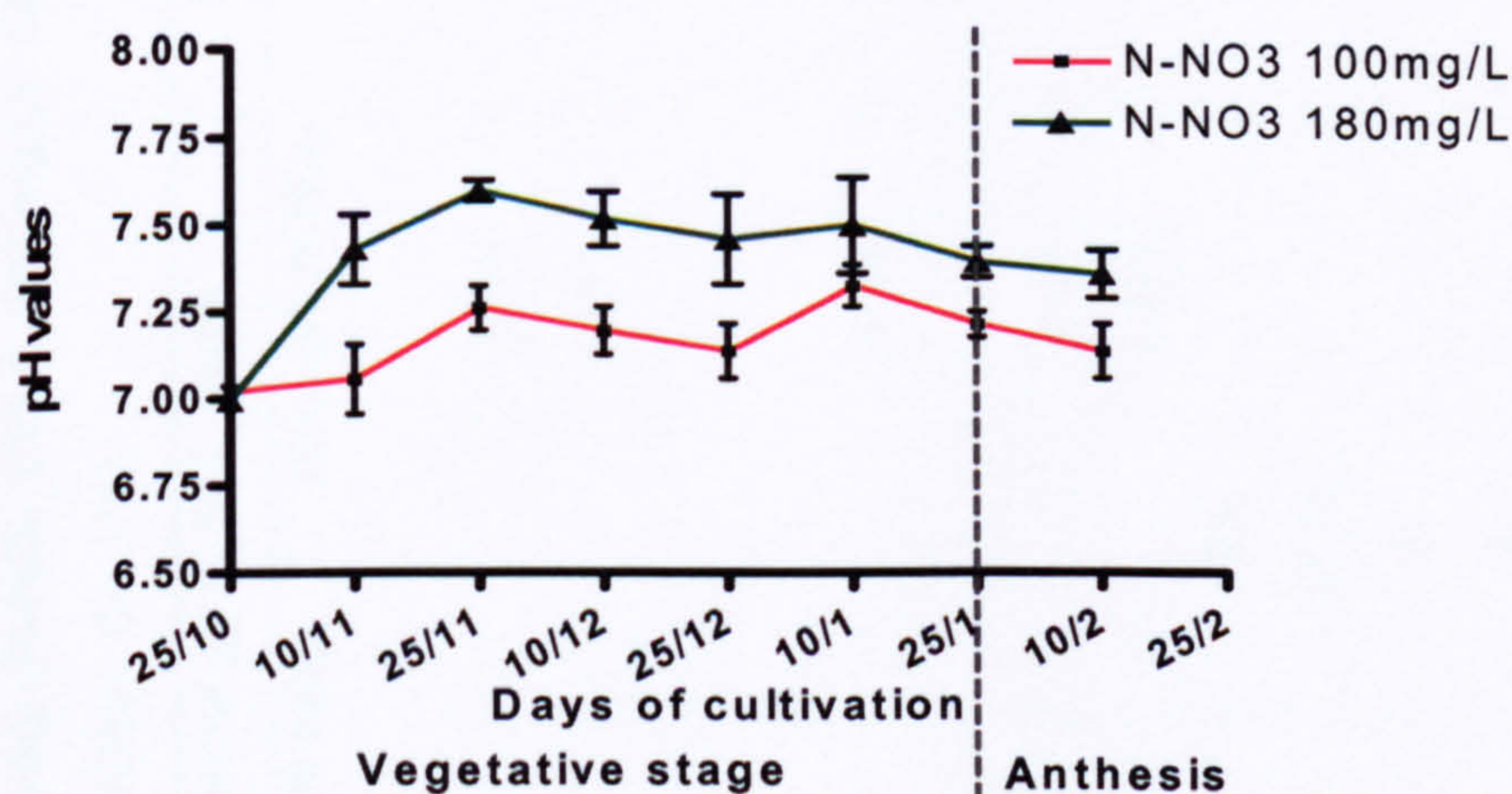


Figure 6.2 Mean pH of nutrient solutions over 10 day intervals for the two nitrate treatments (N-NO₃ 100 & 180 mg/L), throughout the cultivation period. Treatment commenced after 15 days of bulbs supplied only with tap water. Target pH was 7. Values represent the means and vertical bars indicate standard error of the mean (n=4 NFT sub-units).

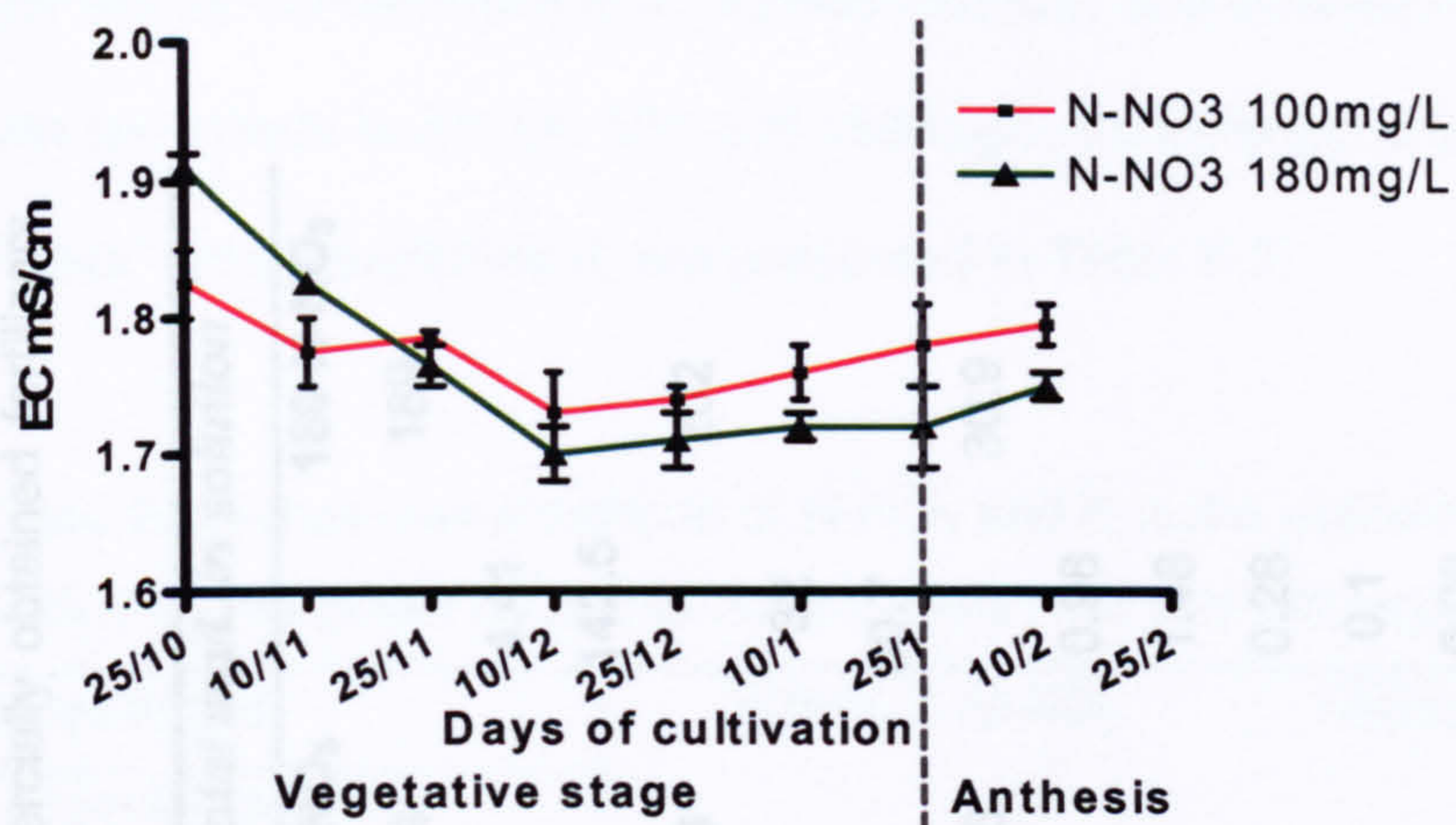


Figure 6.3 15 day average EC values in the nutrient solutions during the cultivation period, for the two N-NO₃ treatments (100 and 180 mg/L). Target EC was 1.8 and 1.82 mS/cm for the 100 and 180 mg/L N-NO₃ treatments. Treatment commenced after 15 days of bulbs supplied only with tap water. Values represent the means and vertical bars indicate standard error of the mean (n=4 NFT sub-units).

Table 6.1 Hydroponic recipe for stock solutions preparation and theoretical elemental composition of nutrient solutions in reservoir tanks with two concentrations of nitrates (N-NO₃ 100 & 180 mg/L). Stock solutions consisted of commercially obtained fertilizers (Hortifeeds) and chemical reagents (SIGMA, Co, UK)

Chemical	Amount (g) in 50 L stock		Elemental composition			Total mg/L in solution	
	100mg/L N-NO ₃	180mg/L N-NO ₃	N-NO ₃	100 N-NO ₃	180 N-NO ₃		
Stock A							
Ca(NO ₃) ₂ *4H ₂ O	2.21	2.21	N-NH ₄	99.64	4.41		189
KNO ₃	1.32	2.11	Ca		142.5		
Stock B			K	199			202
Mg(NO ₃) ₂			P		32		
K ₂ SO ₄	0.7	2.64	Mg		60.7		
MgSO ₄	2.74	0.21	S	122.6			30.9
KH ₂ PO ₄	0.705	0.705	Na		0.98		
EDTA FeNa		323	Mn		1.48		
MnSO ₄ *H ₂ O		22.75	B		0.28		
H ₃ BO ₃		8	Cu		0.1		
CuSO ₄ *5H ₂ O		2	Zn		0.09		
ZnSO ₄ *7H ₂ O		2	Fe		8.4		
(NH ₄) ₆ Mo ₇ O ₂₄		0.64	Mo		0.07		

^a Final composition of nutrient solution in catchment tanks. Elemental contribution of tap water has been taken into account (Appendix 1, section 1.2)

The actual concentrations of nitrates (N-NO₃) and potassium (K) in nutrient solutions with two nitrate levels i.e. 100 and 180 mg/L, determined at the initiation *Narcissus* "Ice Follies" nitrate experiment, are presented in Table 6.2.

Table 6.2 Actual concentrations of N-NO₃ and K in the nutrient solutions of *Narcissus* "Ice Follies" plants grown under two nitrate levels (100 and 180 mg/L).

<i>Treatment</i>	<i>100mg/L N-NO₃</i>	<i>180mg/L N-NO₃</i>	<i>P</i>
<i>Element (mg/L)^d</i>			
<i>N-NO₃</i>	101.0 ± 0.67b	181.4 ± 1.40a	***
<i>K</i>	208.5 ± 1.09a	212.7 ± 1.56a	

Values represent the means and standard error of the mean (N=4 NFT sub-units). Values within rows not followed by the same letter are significantly different at P<0.0001 (***)

^d N-NO₃ was analysed by means of UV spectroscopy, while K concentration was determined utilising a flame photometer.

Total consumption of stock solution and 5% nitric acid are shown in table 6.3. Statistical analysis (un-paired t-test) revealed significant differences among treatments. Stock solution utilised by plants (P<0.001) was higher for EC 1.8 mS/cm level. Total acid utilised by plants under the same treatment was significantly increased (P<0.0001; Table 6.3).

Table 6.3 Total acid and stock solution consumption during the cultivation of *Narcissus* "Ice Follies" plants raised under two nitrate levels (100 and 180 mg/L) in the nutrient solutions.

<i>Treatment</i>	<i>100mg/L N-NO₃</i>	<i>180mg/L N-NO₃</i>	<i>P</i>
<i>Consumption</i>			
<i>Nitric acid (ml) ± SEM</i>	30.0 ± 2.0b	45.0 ± 2.4a	**
<i>Stock solution (ml) ± SEM</i>	43.8 ± 2.3b	56.3 ± 2.2a	*

Plants had been cultivated with running tap water for 15 days before the initiation of nitrate treatment. Values represent the means and standard error of the mean (SEM) calculated from n= 4 NFT sub-units. Values not followed by the same letter are significantly different P<0.05 (*) or P<0.01(**)

Controlled min and max air temperature in the growth room ranged from 8 to 9 and 15 to 16 °C respectively during the cultivation period (Figure 6.4).

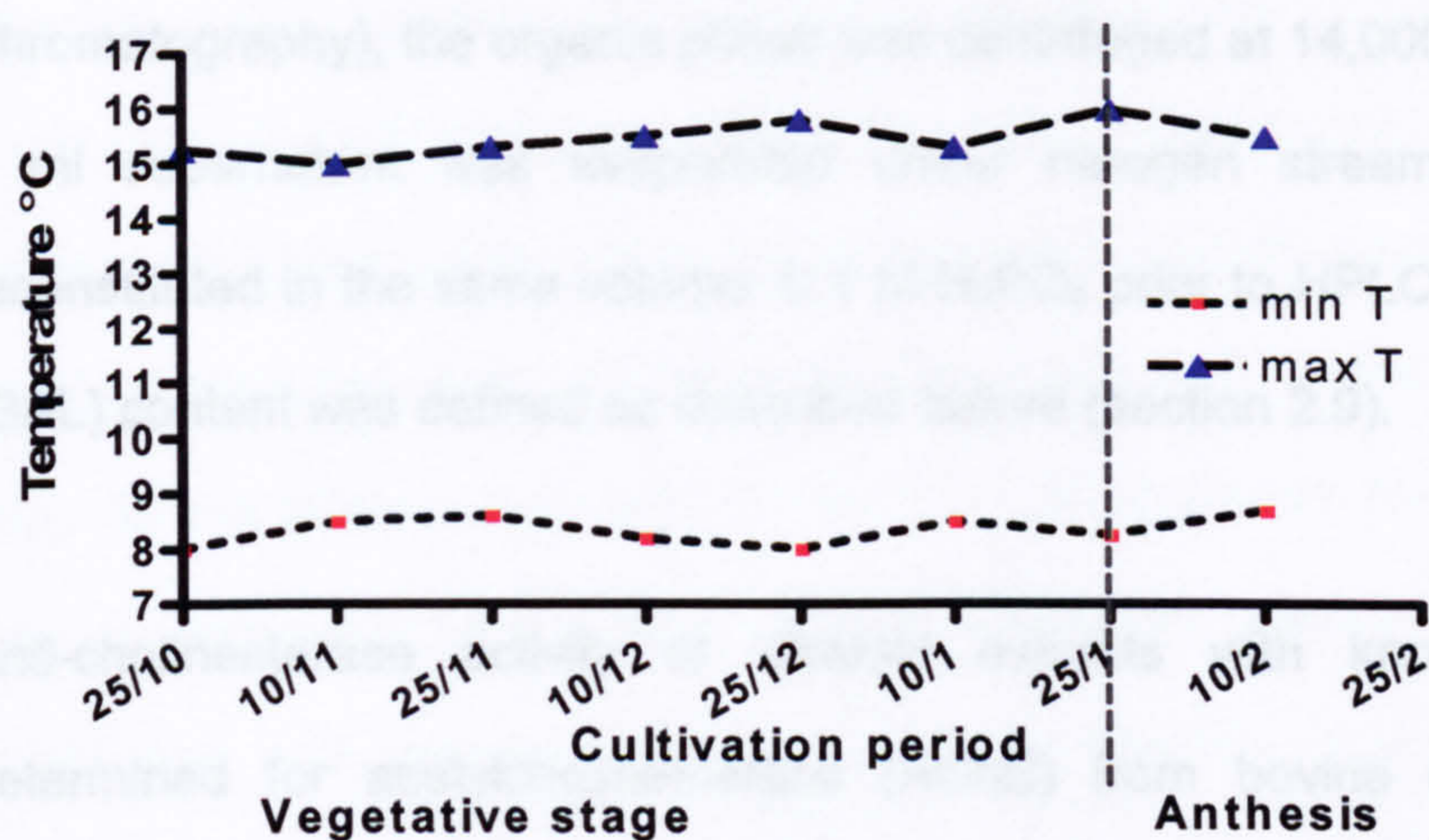


Figure 6.4 Average values measured at 15 day intervals for min and max air temperature throughout the cultivation period of *Narcissus* “Ice Follies” plants raised under two nitrate concentrations (100 and 180 mg/L) in a hydroponic (NFT) system installed in a controlled light and air temperature growth room. Values represent the means of 15 observations.

6.2.2 Anti-cholinesterase activity of *Narcissus* bulb extracts in relation to their galanthamine content

Alkaloids from *Narcissus* “Ice Follies” bulbs were obtained adopting an acid/base extraction procedure (Kreh and Matusch, 1994; Selles et. al., 1997; Machocho et. al., 1999; Mustafa *et al.*, 2003) a common approach for the isolation of amines from plant tissue. One ground bulb sample was randomly picked per channel (see section 2.8). The method was finalised as: 1 g powdered bulb material was macerated in 30 ml aqueous solution of phosphoric acid (H_3PO_4 85%, Analar, BDH chemicals Ltd, Poole, England). Extracts were shaken for 8 hours, at room temperature and sonicated for 30 min twice with a 4 hour interval, utilising an ultrasonic bath (Decon Ultrasonics Ltd, UK). Sediment was separated by pouring off the aqueous phase, which was then subjected to 15 min centrifugation at 14,000 rpm and 4 °C. The acidic supernatant (pH

value approximately 2) was subsequently basified with 35% ammonia solution (NH₄OH, Sigma Co, UK) up to pH 9 and extracted once with dichloromethane (extract: CH₂Cl₂ 1:1 v/v, Sigma Co, UK). Prior to HPLC injection (High Performance Liquid Chromatography), the organic phase was centrifuged at 14,000 rpm for 15 min. Exactly 1 ml supernatant was evaporated under nitrogen stream and the extract was reconstituted in the same volume, 0.1 M H₃PO₄ prior to HPLC analysis. Galanthamine (GAL) content was defined as described before (section 2.9).

Anti-cholinesterase activity of alkaloid extracts with known GAL content was determined for acetylcholinesterase (AChE) from bovine erythrocytes, employing Ellman's method. Final volume of the alkaloid extract in the assay was 10 µl (see section 2.10.1). The % percentage of inhibition of the alkaloid extracts was compared with the % inhibition of the same amount of pure GAL for bovine AChE (SIGMA Co, UK) obtained from a standard sigmoidal curve (Figure 6.5).

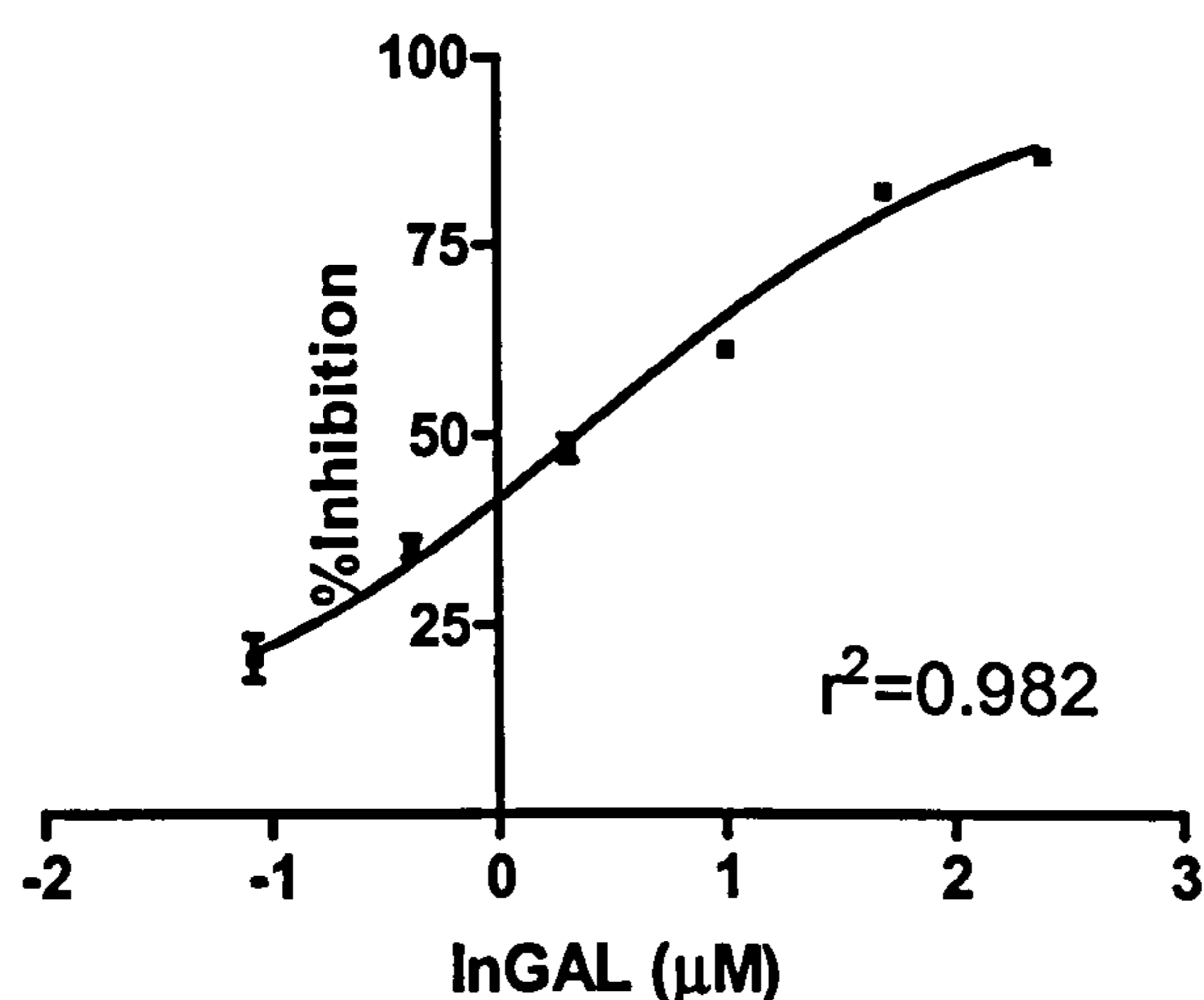


Figure 6.5 Sigmoidal standard curve of galanthamine inhibition for bovine acetylcholinesterase (see section 2.13), plotted with Prism graph pad (version 4). GAL dilutions employed were: 15, 30, 60, 120, 240 and 480 µM. Values represent the means and vertical bars the SEM (n=2). Each GAL concentration was replicated four times on a single run. The assay was run twice. Mean values from each assay were used for the construction of the curve. r^2 denotes goodness of fit of the sigmoidal curve.

6.3 Results

6.3.1 Growth responses to nitrate level in the nutrient solution

Narcissus “Ice Follies” plants were successfully cultivated by means of nutrient film technique (NFT) applying two different concentrations of nitrates (N-NO₃) in the nutrient solution (100 mg/L and 180 mg/L N-NO₃). Biomass accumulation expressed as fresh and dry weight of the shoot (*FWS* and *DWS*) and dry weight of the roots (*DWR*), as well as plant height are presented in Figure 6.6 A, B, C and D respectively. Nitrate supply did not affect growth and development of *Narcissus* “Ice Follies” at any growth stage i.e. vegetative or anthesis, while no interaction between nutrient treatment and harvest time was detected ($P>0.05$, two-way ANOVA; Figure 6.6). Shoot biomass accumulation and plant height were marginally higher under the 100 mg/L N-NO₃ level but differences did not attain statistical significance ($P>0.05$, two-way ANOVA; Figure 6.6 A, B and D respectively). In order to eliminate any possible effect of the initial bulb size on shoot growth the ratio of dry weight of the shoot to bulb dry weight (*DWS/DWB*) was determined (Figure 6.6 E). The ratio of bulb fresh weight to bulb final dry weight (*FWB/DWB*) was measured as an indication of differentiation in biomass partitioning to the bulb between N-NO₃ treatments. Statistical analysis by two-way ANOVA revealed similar patterns in biomass allocation to the bulb during the cultivation period under both N-NO₃ treatments ($P>0.05$ in all cases; Figure 6.6 F). All *Narcissus* “Ice Follies” plants reached full-bloom three and a half months after bulbs were first established in the NFT system. Daily observation suggested no evidence of anthesis promotion by any of the treatments. Total fresh weight of the flowers produced per bulb (*FWF*) is shown in Figure 6.6 G ($P>0.05$, un-paired t-test). Water content of the shoots (%*SWC*) appeared also to be unaffected by N-NO₃ level in the nutrient solutions at both growth stages ($P>0.05$, two-way ANOVA; Figure 6.6 H).

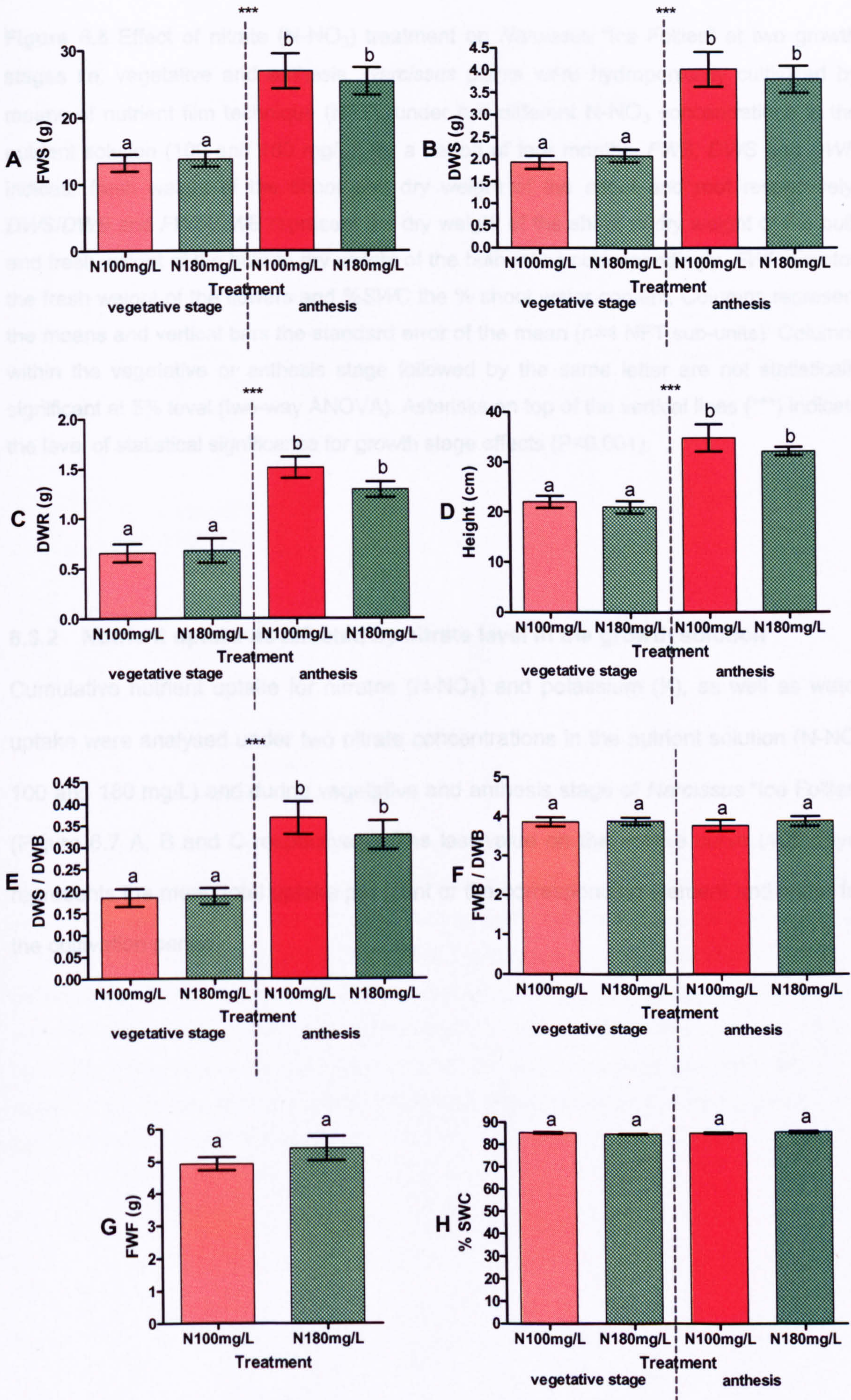


Figure 6.6 Effect of nitrate (N-NO₃) treatment on *Narcissus* “Ice Follies” at two growth stages i.e. vegetative and anthesis. *Narcissus* plants were hydroponically cultivated by means of nutrient film technique (NFT), under two different N-NO₃ concentrations in the nutrient solution (100 and 180 mg/L), for a period of four months. *FWS*, *DWS* and *DWR* indicate fresh weight of the shoot and dry weight of the shoot and root respectively. *DWS/DWB* and *FWB/DWB* represent the dry weight of the shoot to dry weight of the bulb and fresh weight of the bulb to dry weight of the bulb ratios correspondingly. *FWF* denotes the fresh weight of the flowers and %*SWC* the % shoot water content. Columns represent the means and vertical bars the standard error of the mean (n=4 NFT sub-units). Columns within the vegetative or anthesis stage followed by the same letter are not statistically significant at 5% level (two-way ANOVA). Asterisks on top of the vertical lines (***) indicate the level of statistical significance for growth stage effects (P<0.001).

6.3.2 Nutrient uptake as affected by nitrate level in the growth solution

Cumulative nutrient uptake for nitrates (N-NO₃) and potassium (K), as well as water uptake were analysed under two nitrate concentrations in the nutrient solution (N-NO₃ 100 and 180 mg/L) and during vegetative and anthesis stage of *Narcissus* “Ice Follies” (Figure 6.7 A, B and C respectively). The last value on the uptake curve (105 days) represents the mean total uptake per plant of the corresponding element and water for the cultivation period.

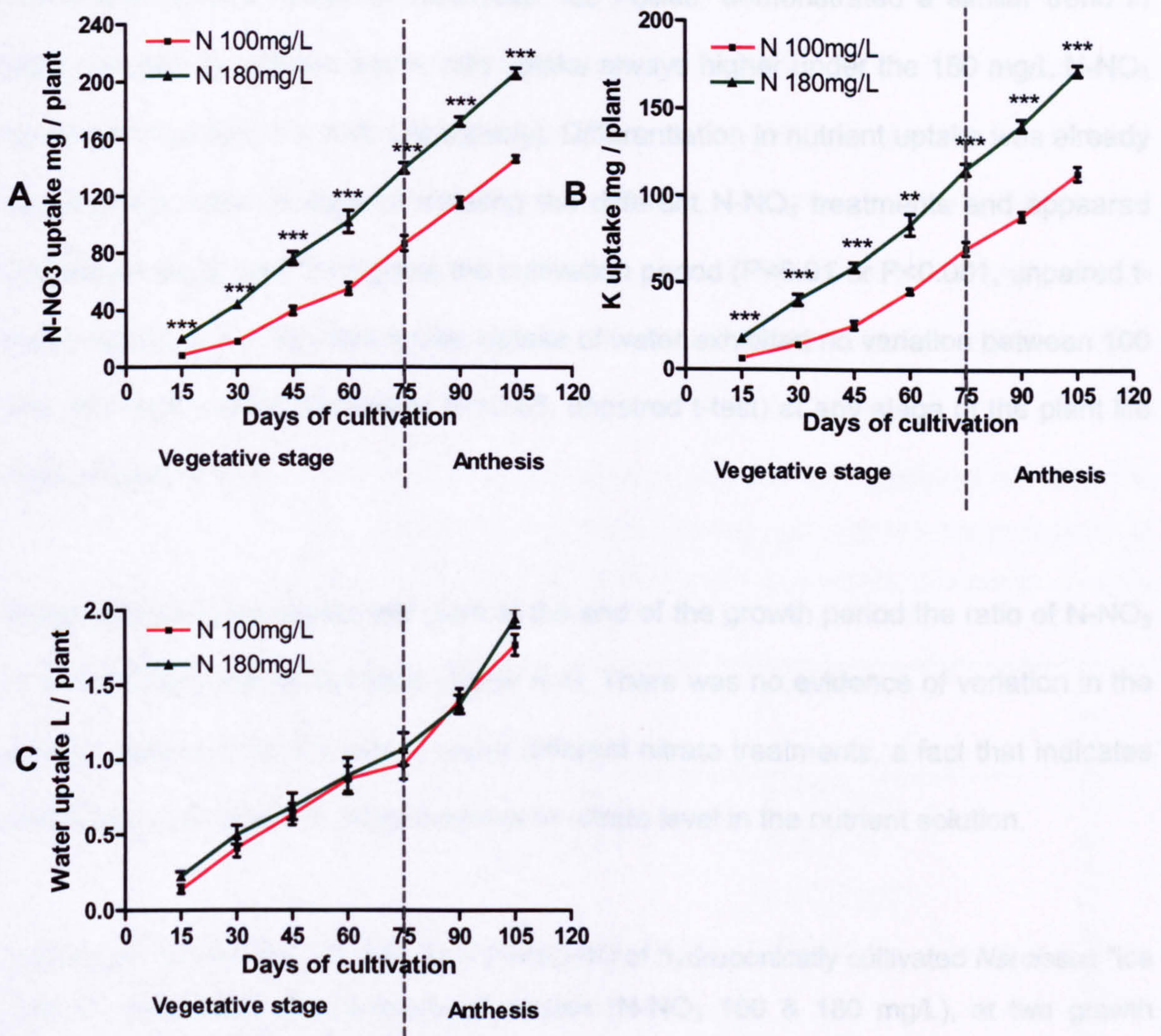


Figure 6.7 Cumulative uptake curves for nitrates (A), potassium (B) and water (C) of *Narcissus* "Ice Follies" grown hydroponically in an NFT system under different N-NO₃ levels (100 and 180 mg/L). *Narcissus* plants had been cultivated in the NFT with only tap water circulated for a period of 15 days before the initiation of N-NO₃ treatments. Uptake of nutrients was determined over 15 day intervals *via* solution depletion studies. The 105 day measure corresponds to the total nutrient uptake of the relevant element or water. Values represent the mean and vertical bars the standard errors of the mean (n=4 NFT sub-units). Asterisks denote significance level of differences among treatments at the equivalent time period ** P<0.01 and ***P<0.001.

Cumulative uptake curves of *Narcissus* "Ice Follies" demonstrated a similar trend in plant demand for nitrates and K with uptake always higher under the 180 mg/L N-NO₃ treatment (Figure 6.7 A & B respectively). Differentiation in nutrient uptake was already considerable after 15 days of initiating the different N-NO₃ treatments and appeared statistically significant throughout the cultivation period ($P < 0.01$ or $P < 0.001$, unpaired t-test; Figure 6.7 A & B). Conversely, uptake of water exhibited no variation between 100 and 180 mg/L nitrate treatments ($P > 0.05$, unpaired t-test) at any stage of the plant life cycle (Figure 6.7 C).

Employing the total uptake per plant at the end of the growth period the ratio of N-NO₃ to K (N-NO₃/K) was determined (Table 6.4). There was no evidence of variation in the relative uptake of N-NO₃ and K under different nitrate treatments, a fact that indicates analogous responses of these nutrients to nitrate level in the nutrient solution.

Table 6.4 Uptake ratio of N-NO₃ to K (N-NO₃/K) of hydroponically cultivated *Narcissus* "Ice Follies" under two concentrations of nitrates (N-NO₃ 100 & 180 mg/L), at two growth stages i.e. vegetative and anthesis.

<i>Treatment</i>	<i>N-NO₃ 100 mg/L</i>	<i>N-NO₃ 180 mg/L</i>
	<i>Vegetative stage</i>	
<i>N-NO₃/K uptake ratio</i>	1.27 ± 1.13a	1.21 ± 0.04a
	<i>Anthesis stage</i>	
	1.30 ± 0.04a	1.23 ± 0.03a

Values represent the mean and standard errors of the mean (n=4 NFT sub-units). Values within a row followed by the same letter are not significantly different at 5% level. Ratios values complied with the assumptions of analysis of variance and hence log-transformation was not necessary.

Pearson product-moment correlation was performed to evaluate relationships among nutrients and water total uptake at vegetative (Table 6.5 above) and anthesis stage (Table 6.5 below) of *Narcissus* "Ice Follies" plants. A significant positive relationship was revealed between N-NO₃ and K uptake ($r=0.91$, $P<0.01$ and $r=0.97$, $P<0.001$ for the vegetative and anthesis stage respectively; Table 6.5). On the other hand, uptake of water was poorly correlated to N-NO₃ and K at both growth stages (see Table 6.5).

Table 6.5 Correlation matrix illustrating relationships between total uptake of nutrients (N-NO₃ and K) and total water uptake at vegetative (above) and anthesis stage (below) of *Narcissus* "Ice Follies" plants hydroponically raised under two nitrate treatments (N-NO₃ 100 & 180 mg/L).

x	N-NO₃	K	Water	Vegetative stage
y				
N-NO₃		$r = 0.909$ $P < 0.01$	$r = 0.345$ $P > 0.05$	
K	$r = 0.909$ $P < 0.01$		$r = 0.3607$ $P > 0.05$	
Water	$r = 0.345$ $P > 0.05$	$r = 0.3607$ $P > 0.05$		

x	N-NO₃	K	Water	Anthesis stage
y				
N-NO₃		$r = 0.963$ $P < 0.001$	$r = 0.589$ $P > 0.05$	
K	$r = 0.963$ $P < 0.001$		$r = 0.517$ $P > 0.05$	
Water	$r = 0.589$ $P > 0.05$	$r = 0.517$ $P > 0.05$		

r represents the Pearson product –moment correlation

P value indicates significance of the correlation

x, y represents the nutrient uptake variables of the correlation

Number of xy pairs tested was n=8 NFT sub-units/replicates

6.3.3 Nutrient and water uptake relationships with plant growth attributes

Linear regression analysis was performed in an attempt to explain the variation in *Narcissus* "Ice Follies" growth characteristics as a result of nutrients and water absorption. There was no evidence of a relationship between nutrient uptake and the measured growth parameters (Table 6.6). Regression of fresh and dry weight of the shoot (*FWS*, *DWS*), root dry weight (*DWR*), flowers fresh weight (*FWF*), the ratio of dry weight of the shoot to bulb dry weight (*DWS/DWB*) and height against N-NO₃ or K uptake revealed very low r^2 values at vegetative and anthesis stages (Table 6.6). On the other hand, a significant positive relationship was established between total water uptake and *FWS*, *DWS*, *DWS/DWB* ratio and height at both growth stages (Table 6.6).

Table 6.6 Linear regressions of growth attributes versus nutrients and water total uptake at two growth stages i.e. vegetative and anthesis of *Narcissus* "Ice Follies" hydroponically cultivated in an NFT system under 100 and 180 mg/L nitrate treatment for a period of four months.

	Vegetative stage				Anthesis stage			
	<i>N-NO₃</i> uptake	<i>K</i> uptake	Water uptake		<i>N-NO₃</i> uptake	<i>K</i> uptake	Water uptake	
<i>FWS</i>	$r^2 = 0.21, P > 0.05$	$r^2 = 0.11, P > 0.05$	$r^2 = 0.62, P < 0.05$		$r^2 = 0.174, P > 0.05$	$r^2 = 0.11, P > 0.05$	$r^2 = 0.55, P < 0.05$	
<i>DWS</i>	$r^2 = 0.49, P > 0.05$	$r^2 = 0.22, P > 0.05$	$r^2 = 0.61, P < 0.05$		$r^2 = 0.12, P > 0.05$	$r^2 = 0.12, P > 0.05$	$r^2 = 0.52, P < 0.05$	
<i>DWR</i>	$r^2 = 0.13, P > 0.05$	$r^2 = 0.12, P > 0.05$	$r^2 = 0.18, P > 0.05$		$r^2 = 0.28, P > 0.05$	$r^2 = 0.20, P > 0.05$	$r^2 = 0.38, P > 0.05$	
<i>Height</i>	$r^2 = 0.180, P > 0.05$	$r^2 = 0.13, P > 0.05$	$r^2 = 0.52, P < 0.05$		$r^2 = 0.10, P > 0.05$	$r^2 = 0.14, P > 0.05$	$r^2 = 0.61, P < 0.05$	
<i>DWS / DWB</i>	$r^2 = 0.24, P > 0.05$	$r^2 = 0.21, P > 0.05$	$r^2 = 0.68, P < 0.05$		$r^2 = 0.24, P > 0.05$	$r^2 = 0.17, P > 0.05$	$r^2 = 0.84, P < 0.01$	
<i>FWF</i>	_____	_____	_____		$r^2 = 0.20, P > 0.05$	$r^2 = 0.21, P > 0.05$	$r^2 = 0.18, P > 0.05$	

FWS, *DWS* and *DWR* indicate fresh weight of the shoot and dry weight of the shoot and root respectively. *DWS/DWB* represents the dry weight of the shoot to dry weight of the bulb ratio. *FWF* denotes the fresh weight of the flowers.

r^2 indicates goodness of fit of the linear slope.

P value indicates significance of slope's deviation from zero

The number of pairs tested was n=8 NFT sub-units/replicates

6.3.4 Effect of nitrate level and growth stage on alkaloid content of bulbs and leaves of *Narcissus* “Ice Follies”.

Statistical analysis by two-way ANOVA revealed a significant impact of nitrate treatment and ontogenetic plant stage on the content of galanthamine (GAL) and lycorine (LYC), two major alkaloids from *Narcissus* “Ice Follies”. Concentrations of both GAL and LYC were significantly enhanced under the higher level of nitrates (180 mg/L N-NO₃), in bulbs and leaves of *Narcissus* plants ($P < 0.001$, two-way ANOVA; Figure 6.8 A and B for GAL and LYC in bulbs and 6.8, C and D in leaves respectively). A 26% increase in GAL was recorded in bulbs due to nitrate concentration in the nutrient solutions i.e. from 1.41 ± 0.05 to 1.77 ± 0.08 mg of GAL per g of bulb dry weight (Figure 6.8 A). Analysis of GAL and LYC content performed at vegetative and anthesis stage of *Narcissus* “Ice Follies”, strongly suggested ontogenetic influences on alkaloid production. GAL and LYC appeared to be significantly reduced during anthesis in comparison with the vegetative stage ($P < 0.001$, two-way ANOVA; Figure 6.8 A and C for GAL, B and D for LYC). However, no interaction between nutrient treatment and ontogenetic stage became evident ($P > 0.05$, two-way ANOVA). Nitrate level (100 and 180 mg/L) or plant growth stage did not significantly affect the relative content of GAL and LYC in bulbs and leaves of *Narcissus* plants, as indicated by the ratios GAL in bulbs / GAL in leaves and the equivalent ratio for LYC ($P > 0.05$, two-way ANOVA; Figure 6.8 E and F respectively). *Narcissus* “Ice Follies” bulbs and leaves appear to possess similar GAL content as ratio values are approximately 1 (Figure 6.8 E). Conversely, LYC yield is higher in leaves ratio values < 1 (Figure 6.8 F). Finally, the relative concentration of GAL to LYC (GAL/LYC) was maintained the same under different nitrate treatments and growth stages ($P > 0.05$, two-way ANOVA; Figure 6.8 G and H respectively)

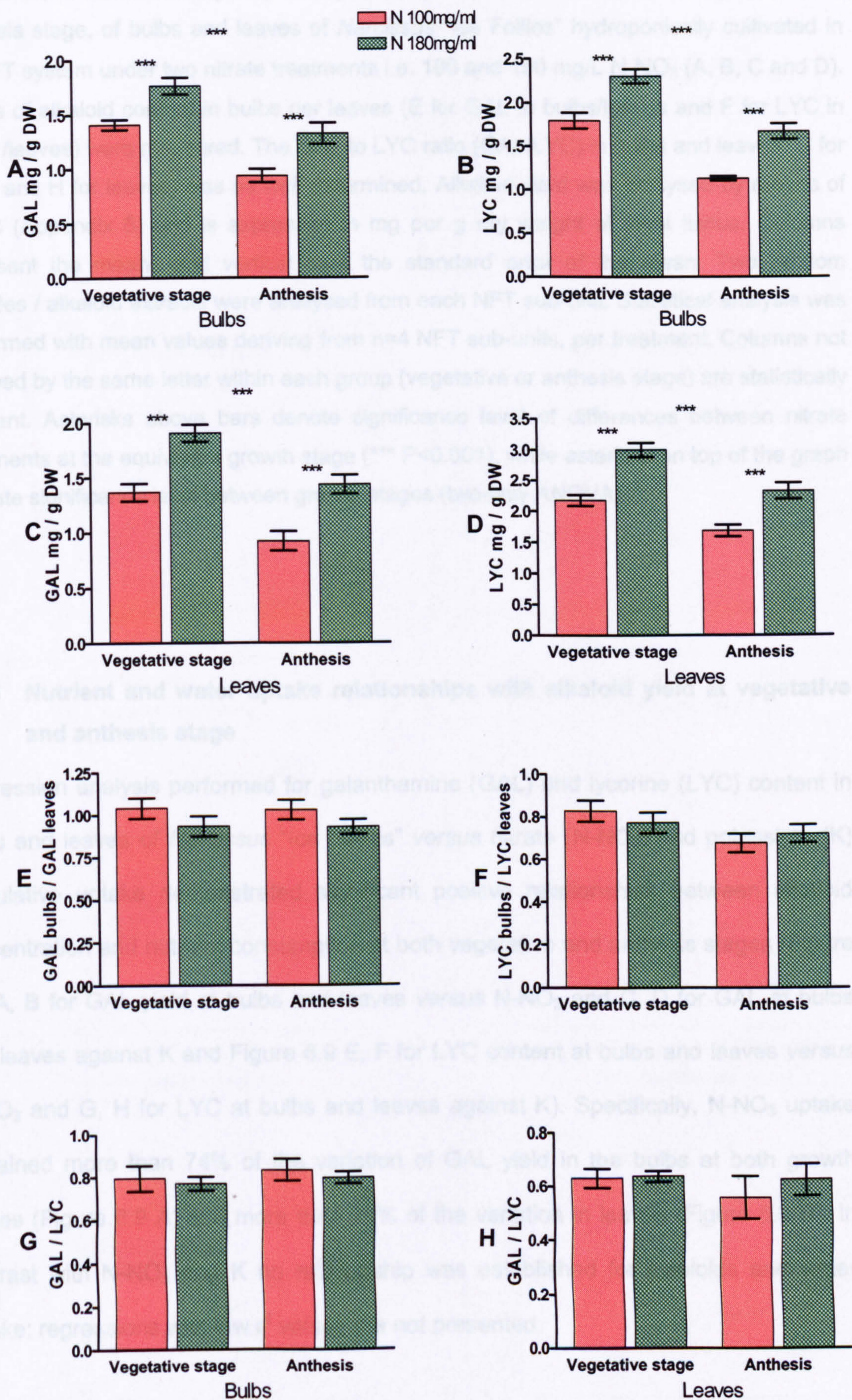


Figure 6.8 Galanthamine (GAL) and lycorine (LYC) alkaloid content at vegetative and anthesis stage, of bulbs and leaves of *Narcissus* “Ice Follies” hydroponically cultivated in an NFT system under two nitrate treatments i.e. 100 and 180 mg/L N-NO₃ (A, B, C and D). Ratios of alkaloid content in bulbs per leaves (E for GAL in bulbs/leaves and F for LYC in bulbs /leaves) were measured. The GAL to LYC ratio (GAL/LYC) in bulbs and leaves (G for bulbs and H for leaves) was as well determined. Alkaloid yield was analysed by means of HPLC (Appendix 5) and is expressed in mg per g dry weight of plant tissue. Columns represent the means and vertical bars the standard error of the mean. Two random samples / alkaloid extracts were analysed from each NFT sub-unit. Statistical analysis was performed with mean values deriving from n=4 NFT sub-units, per treatment. Columns not followed by the same letter within each group (vegetative or anthesis stage) are statistically different. Asterisks above bars denote significance level of differences between nitrate treatments at the equivalent growth stage (***) P<0.001), while asterisks on top of the graph indicate significance level between growth stages (two-way ANOVA).

6.3.5 Nutrient and water uptake relationships with alkaloid yield at vegetative and anthesis stage

Regression analysis performed for galanthamine (GAL) and lycorine (LYC) content in bulbs and leaves of *Narcissus* “Ice Follies” versus nitrate (N-NO₃) and potassium (K) cumulative uptake demonstrated significant positive relationships between alkaloid concentration and nutrient consumption at both vegetative and anthesis stages (Figure 6.9 A, B for GAL yield at bulbs and leaves versus N-NO₃ and C, D for GAL at bulbs and leaves against K and Figure 6.9 E, F for LYC content at bulbs and leaves versus N-NO₃ and G, H for LYC at bulbs and leaves against K). Specifically, N-NO₃ uptake explained more than 74% of the variation of GAL yield in the bulbs at both growth stages (Figure 6.9 A) and more than 71% of the variation in leaves (Figure 6.9 B). In contrast with N-NO₃ and K no relationship was established for alkaloids and water uptake; regressions with low r^2 values are not presented.

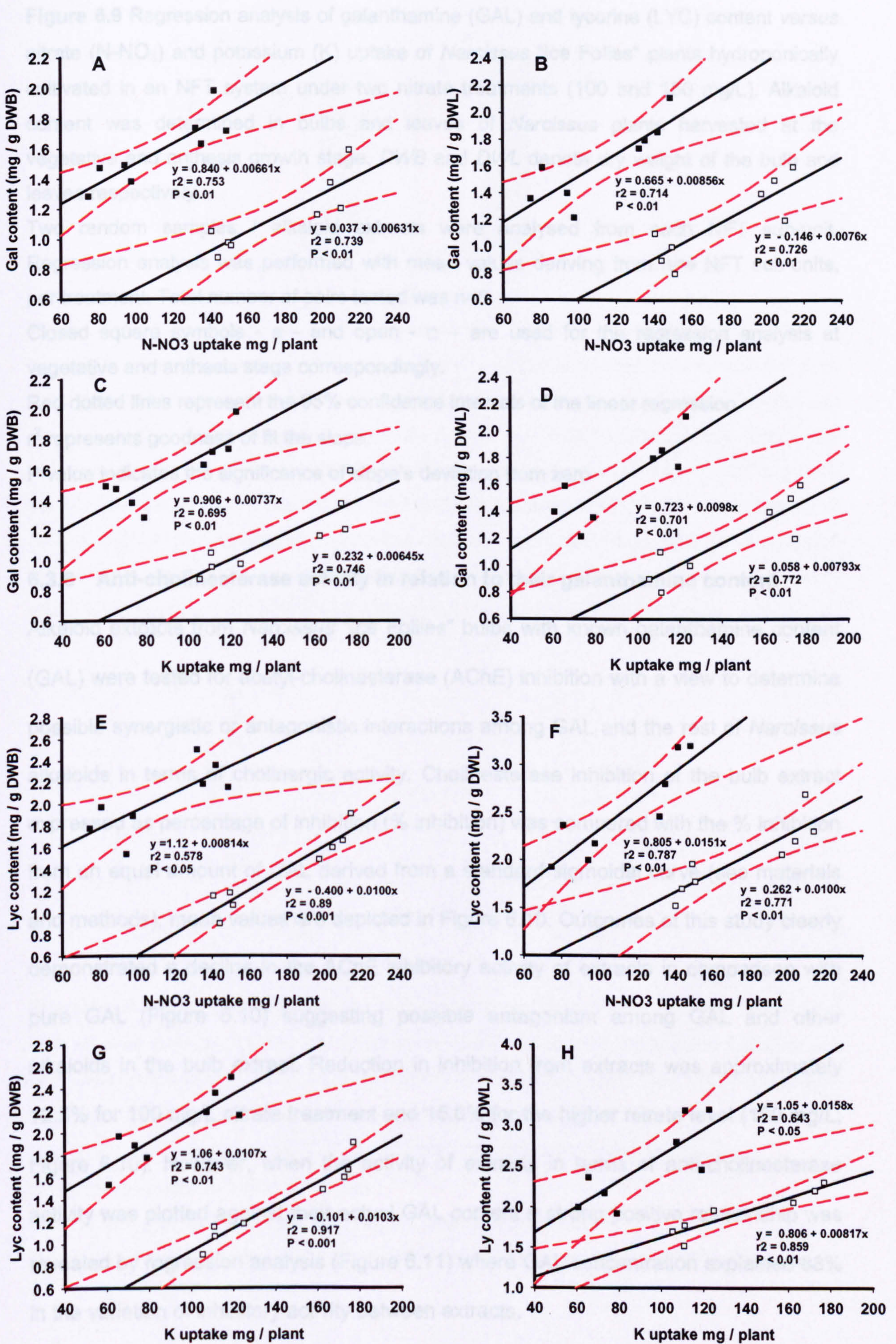


Figure 6.9 Regression analysis of galanthamine (GAL) and lycorine (LYC) content *versus* nitrate (N-NO₃) and potassium (K) uptake of *Narcissus* “Ice Folies” plants hydroponically cultivated in an NFT system under two nitrate treatments (100 and 180 mg/L). Alkaloid content was determined in bulbs and leaves of *Narcissus* plants harvested at the vegetative and anthesis growth stage. *DWB* and *DWL* denote dry weight of the bulb and leaves respectively.

Two random samples / alkaloid extracts were analysed from each NFT sub-unit. Regression analysis was performed with mean values deriving from n=4 NFT sub-units, per treatment. Total number of pairs tested was n=8

Closed square symbols - ■ - and open - □ – are used for the regression analysis at vegetative and anthesis stage correspondingly.

Red dotted lines represent the 95% confidence intervals of the linear regression

r^2 represents goodness of fit the slope.

P value indicates the significance of slope's deviation from zero

6.3.6 Anti-cholinesterase activity in relation to their galanthamine content

Alkaloid extracts from *Narcissus* “Ice Folies” bulbs with known galanthamine content (GAL) were tested for acetyl-cholinesterase (AChE) inhibition with a view to determine possible synergistic or antagonistic interactions among GAL and the rest of *Narcissus* alkaloids in terms of cholinergic activity. Cholinesterase inhibition of the bulb extract expressed as percentage of inhibition (% inhibition) was compared with the % inhibition from an equal amount of GAL derived from a standard sigmoidal curve (see materials and methods); mean values are depicted in Figure 6.10. Outcomes of this study clearly demonstrated a decline in the AChE inhibitory activity of extracts in comparison with pure GAL (Figure 6.10) suggesting possible antagonism among GAL and other alkaloids in the bulb extract. Reduction in inhibition from extracts was approximately 12.5% for 100 mg/L nitrate treatment and 15.6% for the higher nitrate level (180 mg/L; Figure 6.10). However, when the activity of extracts in terms of anti-cholinesterase activity was plotted against their actual GAL content a strong positive relationship was revealed by regression analysis (Figure 6.11) where GAL concentration explained 88% in the variation of inhibitory activity between extracts.

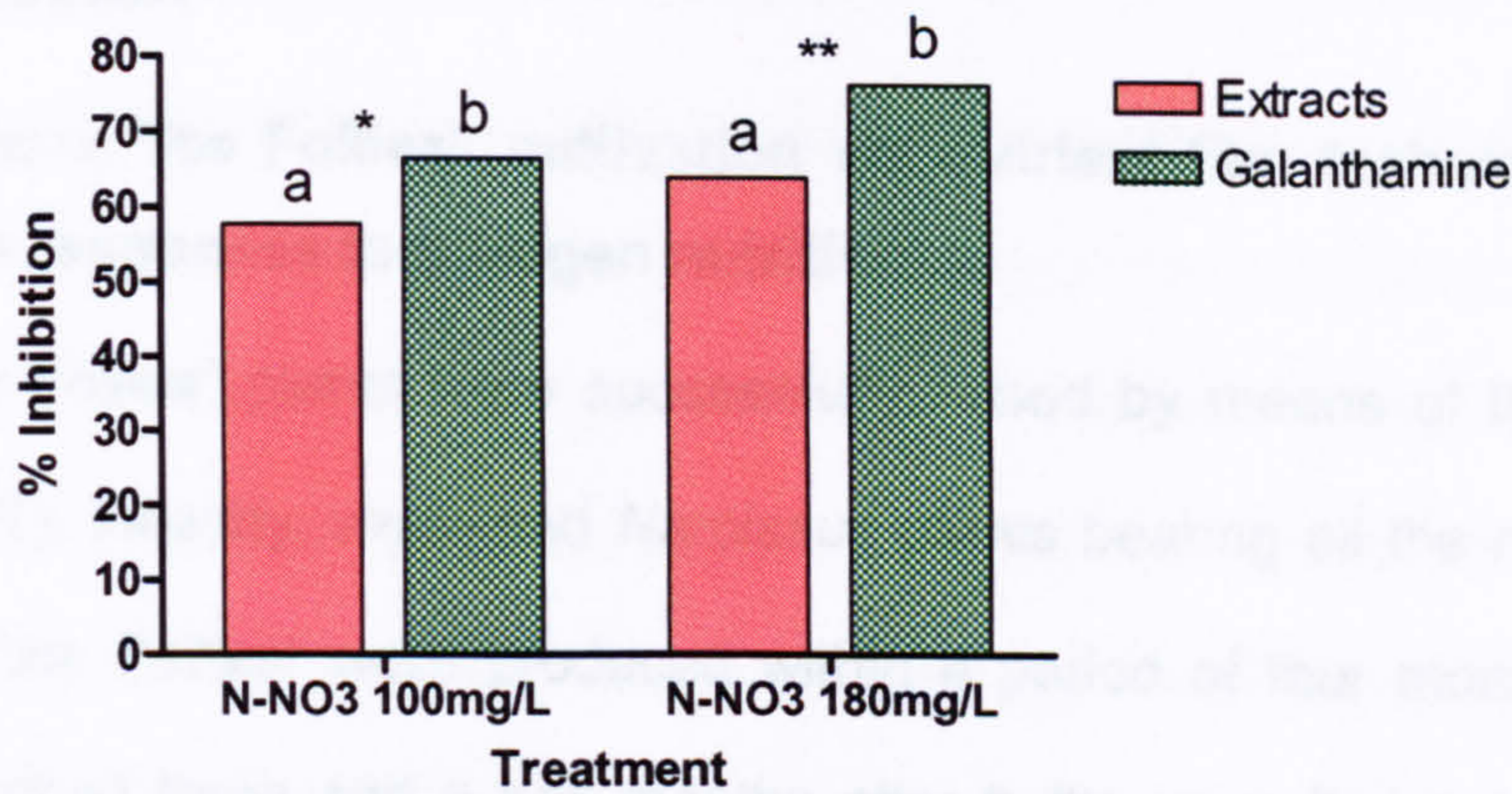


Figure 6.10 Comparison of acetyl-cholinesterase (AChE) % inhibition from alkaloid extracts (known GAL content by HPLC) with the % inhibition of equivalent amount of pure GAL calculated from a dose-response sigmoidal standard curve. Alkaloids were extracted *via* an acid-base method from *Narcissus* “Ice Follies” bulbs hydroponically cultivated under two levels of nitrates (100 and 180 mg/L). Plants were harvested at the vegetative growth stage. Columns represent the means (n=4 alkaloid extracts deriving from equal number of NFT sub-units per treatment). Statistical analysis was performed with ln-transformed values thus standard errors are not presented. Columns not followed by the same letter within each treatment are statistically different at $P < 0.05$ (*) or $P < 0.01$ (**) (un-paired t-test).

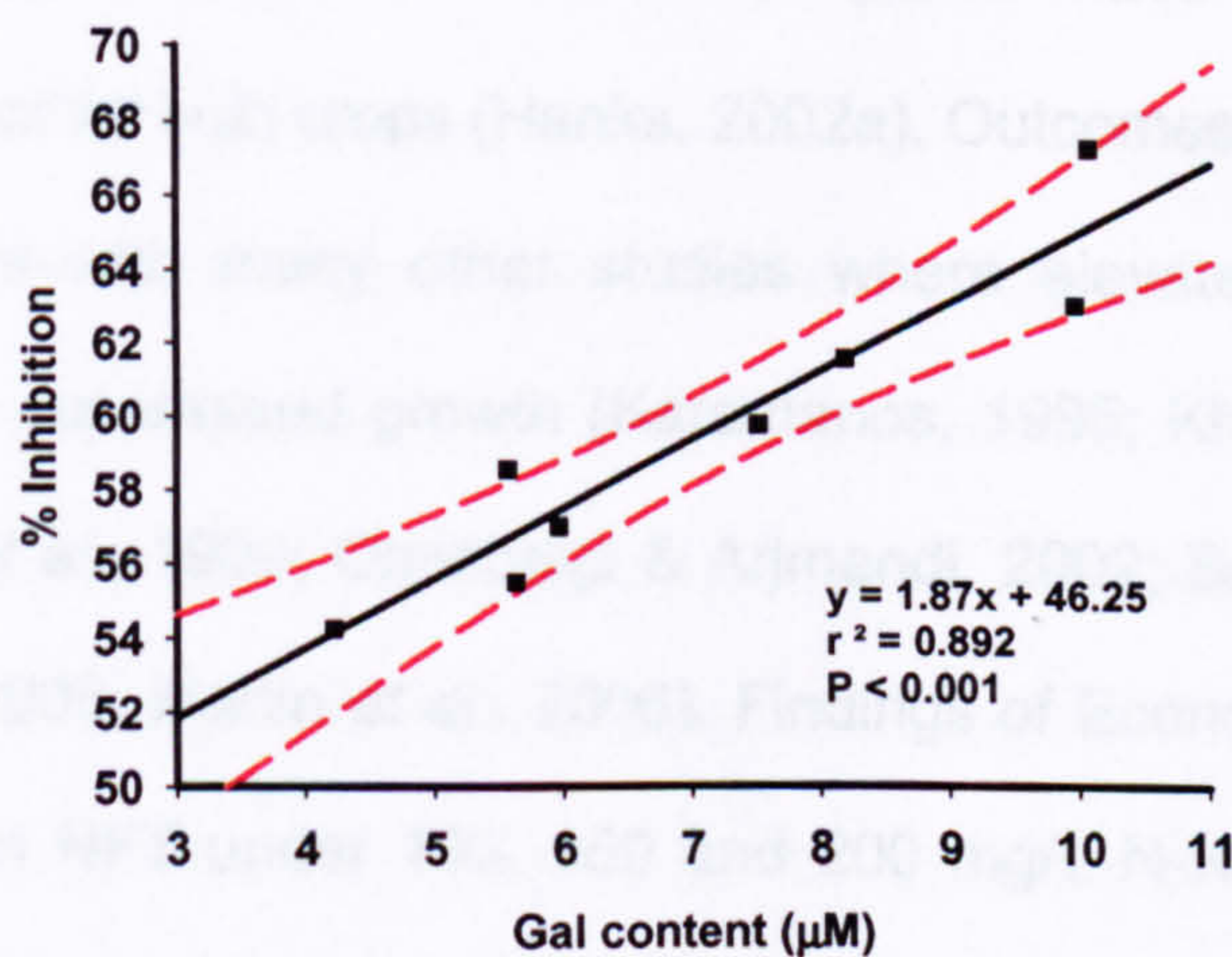


Figure 6.11 Linear regression of % cholinesterase inhibition from *Narcissus* “Ice Follies” bulb alkaloid extracts against the determined concentration of galanthamine (GAL) in the bulb extract. *Narcissus* plants were hydroponically cultivated under two nitrate treatments (100 and 180 mg/L N-NO₃) and harvested at the vegetative growth stage.

Red dotted lines represent the 95% confidence intervals of the linear regression

r^2 represents goodness of fit the slope.

P value indicates the significance of slope's deviation from zero

The number of pairs tested was n=8 alkaloid extracts deriving from eight NFT sub-units

6.4 Discussion

6.4.1 *Narcissus* “Ice Follies” cultivation via nutrient film technique (NFT) and growth responses to nitrogen nutrition

Narcissus “Ice Follies” plants were successfully raised by means of the nutrient film technique (NFT). Healthy, well-sized *Narcissus* plants bearing all the normal features of soil-grown “Ice Follies” were produced within a period of four months. Full-bloom stage was reached three and a half months after bulbs were first established in the NFT system, whereas plants grown under field conditions necessitate a period of several months before anthesis occurs. In glasshouse conditions, forcing at 9 °C for a 9 week period sets off flowering after 35 to 65 days (Hanks, 1996). The two nitrate nutrient treatments (100 & 180 mg/L N-NO₃) used in the NFT did not result in significant variations in plant biomass production. Similarly, nutritional soil experiments with nitrogen (N) fertilisers have demonstrated poor responses of *Narcissus* species to increased N and it seems that *Narcissus* plants have lower N-requirements in comparison with other bulb crops (Hanks, 2002a). Outcomes of the present experiment are in agreement with many other studies where elevated amounts of N did not promote or even suppressed growth (Karamanos, 1995; Khan *et al.*, 1995a; Schenk, 1996; Andrews *et al.*, 1999; Omidbaigi & Arjmandi, 2002; Baranauskiene *et al.*, 2003; de Groot *et al.*, 2003; Martin *et al.*, 2006). Findings of Economakis (1993b), for *Salvia fruticosa* raised in NFT under 100, 150 and 200 mg/L N-NO₃ strongly indicated that growth was considerably enhanced at 150 mg/L N-NO₃, while higher concentrations of N-NO₃ (200 mg/L) did not induce further biomass accumulation. Similar results have been produced in this study for *Salvia officinalis* var *purpurea* (see chapter 4). Alkaloid bearing plants of *Catharanthus roseus* L. grown in pots did benefit from N up to 200 mg/L although additional N supply (N 300 & 450 mg/L) did not increase biomass yield (Lata & Sadowska, 1996).

On the other hand, nutrient uptake for nitrates (N-NO₃) and potassium (K) of *Narcissus* “Ice Follies” was significantly influenced by nitrate concentration in the growth solutions and appeared to be constantly higher under the higher N-NO₃ level (180 mg/L). The lack of correlation between nutrient uptake and plant biomass production is consistent with suggestions from Andrews and colleagues, (1999) and de Groot *et al.*, (2003) that accumulated nitrates in plants that do not improve growth rates, represent the plant trend of “luxury consumption” (Marschner, 1995). Daily observations suggested no evidence of anthesis promotion or delay by the N-NO₃ level in the nutrient solution while no variation in flower yield was detected. In plants of *Solanum nigrum* L. nitrogen fertilization was demonstrated to boost the number of flowers and consequently flower biomass per plant at a N supply optimum for vegetative growth, whereas additional N did not enhance flower production (Khan, 1995a). A review of the literature revealed no references regarding N effects on *Narcissus* flowers.

6.4.2 Nitrogen effect on *Narcissus* “Ice Follies” alkaloid yield

In contrast to the lack of response shown by plant biomass, nitrate concentration in the nutrient solution exerted substantial changes in the alkaloid yield of *Narcissus* “Ice Follies”. The content of galanthamine (GAL) and lycorine (LYC), two major alkaloids of “Ice Follies”, was significantly enhanced by the higher nitrate treatment (180 mg/ml N-NO₃). Augmentation of GAL and LYC was demonstrated in both bulbs and leaves. Accumulating evidence has strongly suggested that N nutrition can potentially increase alkaloid content in medicinal and non-medicinal species like tobacco, lupines, barley, *Datura*, *Atropa*, *Papaver* (Waller & Nowacki, 1978). For example, nitrogen supply in the form of urea significantly increased solasodine yield in black nightshade (*Solanum nigrum* L.) at all growth stages studied (Khan *et al.*, 1995a). In leaves of *Tabernaemontana pachysiphon* Stapf (Apocynaceae) indole alkaloid accumulation was enhanced with increased N application. Moderate fertilization was more efficient than

excessive amounts of N that did not alter the level of alkaloids (Höft *et al.*, 1996). Kumar and co-workers (2004) reported enhanced berberine content in *Tinospora cordifolia* Miers by the application of urea. Although a number of N nutrient experiments with *Catharanthus roseus* L. (periwinkle) have demonstrated no effect on alkaloid concentration, improved alkaloid content due to N levels up to 300 mg/L was confirmed by Lata & Sadowska, (1996). In more recent studies, urea supply (100 & 150 kg/ha) significantly increased alkaloid content in leaves and roots of high alkaloid containing mutants of periwinkle over the control (no urea added; Sreevalli *et al.*, 2004). In agreement with that, Abdolzadeh and colleagues, (2006) reported an increase in alkaloid content of periwinkle in hydroponic culture with N supply from 2.75 up to 11 mM in the nutrient solution. Manukyan, (2005) in an outside hydroponic experiment reported augmentation of total alkaloid level in celandine employing a nutrient solution with higher N ratio. Finally, Kreh, (2002) reported a 70% increase in GAL content of field-grown *Narcissus* "Carlton" bulbs as a result of N addition. Since alkaloids entail nitrogen (N) in their structure, availability of the nutrient in the growth medium is expected to play an important role in biosynthesis and accumulation of alkaloids in plant tissues (Waller & Nowacki, 1979; Gershenzon, 1985). Nitrogen supply promotes the production of soluble N compounds like glutamine and asparagine molecules (Gershenzon, 1985) employed by the enzymatic machinery for the synthesis of amino acids (Diamandithis, 1994). The latter constitute important alkaloid biosynthetic precursors (see general introduction). Furthermore, alkaloid production can be enhanced by N through its stimulating effects on the activity of enzymes involved in alkaloid pathways (Waller & Nowacki, 1978).

Gal and LYC yield in bulbs and leaves were significantly correlated with N-NO₃ and K uptake at both vegetative and anthesis stages. Given that an essential role of alkaloids in plants is secure N storage (Waller & Nowacki, 1978; Gershenzon, 1985), it is concluded that surplus N-NO₃ in the nutrient solution after being absorbed by

Narcissus "Ice Follies" plants is directed to secondary / alkaloid biosynthetic pathways, under optimal growth conditions, where carbon supply is not therefore a limiting factor (Gershenzon, 1985). Interestingly, a previous experiment of Lata & Sadowska, (1996) revealed increments in *Catharanthus* plants biomass with N supply up to 200 mg/L, while the highest alkaloid content was observed at 300 mg/L of nitrogen.

In the present study mean GAL concentration in *Narcissus* "Ice Follies" from whole powdered bulbs was found to be 1.41 and 1.77 mg/g dry weight for the 100 and 180 mg/L nitrate treatments respectively. In another field experiment GAL content in different bulb parts of the same cultivar ranged from 0.59 to 1.68 mg/g dry weight with the higher value representing GAL concentration in the bulb basal plates (Moraes *et al.*, 1997a). Moraes and co-workers (1997b) employing various agronomic practices measured GAL contents in "Ice Follies" bulbs ranging from 0.6 to 0.79 mg/g dry weight. Screening of 20 *Narcissus* cultivars (Kreh, 2002) revealed 1.88 mg GAL/g in bulbs of *Narcissus* "Carlton" and 0.74mg/g in "Ice Follies". In all cases, the *Narcissus* "Ice Follies" plants in the present experiment exhibited considerably higher levels of GAL that those reported above (in some cases more than 100%) even under the lower nitrate treatment, a fact that strongly indicates NFT as a potent technique for boosting the yield of GAL and alkaloid production generally in *Narcissus* species. Manukyan, (2005) investigating the production of bioactive compounds from hydroponically raised medicinal plants, reported a 25% enhancement of alkaloid levels in celandine under outside hydroponic conditions in comparison with plants raised in the field. The balanced nutrient supply and the greater control over plant growth exerted by hydroponics (see general introduction) renders this method of cultivation an important tool for secondary metabolite production.

6.4.3 Ontogenetic variability in alkaloid content in *Narcissus* “Ice Follies” and variability in different plant parts

Alkaloids levels were determined at two different growth stages i.e. vegetative and anthesis. Plant ontogeny induced significant variation in both GAL and LYC content, which appeared to decline substantially during the flowering period in bulbs and leaves of *Narcissus* “Ice Follies” (approximately 30% decrease). Bernath, (1986) suggested that the levels of alkaloids as well as other secondary metabolites naturally fluctuates depending upon the ontogenetic stage of plant. Dhar & Bhat, (1982) also reported variations in alkaloid content throughout the cultivation period of five belladonna genotypes, at different ontogenetic stages. The maximum content of total alkaloids (0.67%) was observed at the stage of young foliage production, whereas lowest alkaloid synthesis occurred at the stage of 50% flowering (only 0.38%). In another experiment, hyoscyamine one of the main belladonna alkaloids exhibited similarly high concentrations prior to the stage of anthesis (Dhar & Bhat, 1982). Abdolzadeh and colleagues (2006) reported that total alkaloids of periwinkle (*Catharanthus roseus*) were negatively correlated with flower dry mass. Galanthamine content of *Narcissus* “Inglescombe” appeared to be elevated during the growing period (between emergence and anthesis), whereas a decline was observed after flowering (Moraes, 2002). Corroborating data from Kreh, (2002) revealed that alkaloid levels in bulbs and leaves of *Narcissus* “Carlton” were at a maximum before the stage of anthesis. Moraes and co-workers, (1997a) in agreement with the previous outcomes indicated that bulb meristematic tissue of *Narcissus* “Ice Follies” had greater alkaloid content than storage tissue and hence treatments that stimulate active growth, like the case of nitrogen addition could lead to increased biosynthesis of GAL and LYC alkaloids (Waller and Nowacki, 1978). Supporting evidence is also provided by the analysis for alkaloid content of 81 *Narcissus* cultivars that demonstrated maximum yields at bud formation stage, when plant growth rates were at their highest (Cherkasov & Tolkachev, 2002). In the case of belladonna species (*Atropa belladonna*) extensive research has shown

that young metabolically active leaves contain more alkaloids than the old ones (Dhar and Bhat, 1982). During the stage of anthesis flowers act as a strong sink for C, N and other nutrients. Possibly, greater translocation of N compounds and carbon skeletons to flowers results in a decline in alkaloids and other N compounds in leaves (Marschner, 1995).

6.4.4 Alkaloid synergy in terms of cholinesterase inhibitory activity

Alkaloid extracts from bulbs of *Narcissus* “Ice Follies” of known galanthamine content were tested for acetyl-cholinesterase (AChE) % inhibition. Inhibitory activity of the alkaloid mixture was subsequently compared with the inhibition from equal amounts of pure GAL. Data demonstrated marginally lower % inhibition for “Ice Follies” extracts suggesting a kind of antagonism in the alkaloid mixture. Three other alkaloids of “Ice Follies” lycorine, lycoramine and haemanthamine (Moraes *et al.*, 1997a) have shown very weak or no cholinesterase inhibition at all when tested in previous experiments (Lopez *et al.*, 2002; Houghton *et al.*, 2006). However, decline in % inhibition values was only in the order of 12% on average, which could have been generated due to interference deriving from any solvents and / or non alkaloid compounds possibly remaining in the bulb extract after the acid / base procedure. Regression analysis of % AChE inhibition of the extracts against their actual GAL concentration revealed a strong linear relationship illustrating the dependence of the inhibitory activity of extracts on the concentration of GAL present. It was assumed that without the phenomenon of antagonism r^2 would have attained even higher values.

6.4.5 Conclusions

Narcissus “Ice Follies” an important ornamental and medicinal plant was successfully cultivated by means of NFT a hydroponic method that could be employed in glasshouses for the fast and efficient production of cut-flowers. Moreover, bulbs and leaves of “Ice Follies” which are known to contain relatively high amounts of galanthamine (GAL) in comparison to other *Narcissus* species could be exploited for the commercial production of GAL, an essential alkaloid for the pharmaceutical industry. Finally, under the influence of the specific hydroponic recipe utilised in the present study (180 mg/L N-NO₃), the concentrations of galanthamine and lycorine were significantly augmented, strongly indicating NFT as a suitable method for the production of bioactive compounds.

Chapter 7
General Discussion

7.1 Effect of nutritional factors and solution electrical conductivity on primary and secondary metabolism of medicinal plant species.

The growing interest of the pharmaceutical and food industry in exploiting natural products has encouraged various scientific studies on aromatic & medicinal plants. Controlled cultivation of aromatic & medicinal species is a potential way to satisfy the needs of an expanding market protecting in parallel the diminishing wild stock (Lange, 1998). The fact that in Europe only 10% of the commercially used medicinal species are cultivated (Vines, 2004) underlined the necessity to investigate the growth requirements of aromatic & medicinal plants, for which available information is still limited (Economakis *et al.*, 2002; 2005). Environmental variables significantly affect plant growth by controlling physiological processes that underpin biomass accumulation and partitioning to shoots and roots, onset of the reproductive cycle, flowering intensity, absorption and mobilization of nutrients as well as secondary metabolite biosynthesis. The production of bioactive phytochemicals is highly regulated by the environment (Sangwan *et al.*, 2001; Raskin *et al.*, 2002) so optimization and careful control of plant cultivation is essential in order to guarantee a cost-effective, standardized and high-quality medicinal product (Raskin *et al.*, 2002).

In the present study, hydroponic cultivation of two medicinal plant species i.e. *Salvia officinalis* var *purpurea* and *Narcissus* "Ice Follies" was investigated for the first time, under the effect of nitrate (N-NO₃) and phosphorus (P) concentrations as well as electrical conductivity (EC) levels, employing the nutrient film technique (NFT). Hydroponics is a powerful tool in plant/nutrient studies (Wilcox, 1982) and is an efficient method for commercial production of herbs and ornamentals (Economakis *et al.*, 2005; Savvas & Passam, 2002). This thesis reported the influence of the NFT for biomass production as well as implications for the quantity and quality of important bioactive compounds in these two medicinal plant species.

7.1.1 Plant growth responses to nutrition.

By definition, mineral nutrients have specific and vital roles in plant metabolism (Marshner, 1995). Primary metabolic pathways are highly susceptible to nitrate (N-NO₃) and phosphorus (P) nutrition (Smirnoff, 1995). Findings in chapters 3 and 4 of the present study on *S. officinalis* var *purpurea* corroborate the well documented impact of N and P on plant growth. Data from the hydroponic experiments indicated that a N-NO₃ concentration of 150 mg/L and 12 mg/L P were the most appropriate for high shoot biomass production, the commercially important part of the sage plant. Experimental outcomes in this investigation are in agreement with the conclusions drawn for *S. fruticosa* Mill. raised in the NFT under various N-NO₃ and P regimes (Economakis, 1993b and 1995 respectively). Increasing the supply of N-NO₃ (200 mg/L) and P (60 mg/L) induced a decline in shoot biomass, while plants exhibited contrasting strategies for the uptake of the equivalent nutrients. Interestingly, in the treatment of 60 mg/L P, uptake of the corresponding nutrient was significantly enhanced, a finding that was most probably associated with P-mediated augmentation of root biomass. Increased root to shoot ratio under this range of P is in agreement with experimental outcomes for *S. fruticosa* Mill. (Economakis, 1995). The adaptive response to capture excess phosphorus apparently occurs at the expense of shoot production and is possibly controlled by changes in the levels of plant cytokinins (CYT), negative regulators of root growth. P starvation has been shown to reduce CYT content (Raghothama & Karthikeyan, 2005; Vance *et al.*, 2003; de Groot *et al.*, 2003; Lopez-Buccio *et al.*, 2002; Martin *et al.*, 2000; Neumann *et al.*, 2000; Gilbert *et al.*, 2000). Moreover, an earlier study on the effect of P on hydroponically cultivated *Platanus* seedlings (Dhillon, 1978) revealed decreased CYT at P concentration exceeding the 60 mg/L, which could explain the improved root biomass of *S. officinalis* var *purpurea* at this treatment. Nevertheless, P-sufficient plants persist in taking up phosphorus, even when growth and metabolic demands for the nutrient have been satisfied (Theodorou & Plaxton, 1995) and store it in the vacuoles as inorganic Pi. Stored vacuolar Pi can be utilised by

the cell in times of P deficiency and thus avoid the need to invoke rescue mechanisms that are energetically expensive (Theodorou & Plaxton, 1995). Phosphorus influx is mediated by a very efficient system of low- or high- affinity P transporters present on the cell plasma membrane (Raghothama & Karthikeyan, 2005; Ticconi & Abel, 2004; Abel *et al.*, 2002; Bucher *et al.*, 2001; Smith *et al.*, 2003). Such transporters facilitate P absorption even under high electrical conductivity treatments (e.g. EC 7.4 mS/cm; chapter 5) when uptake of other nutrients i.e. nitrates, potassium and calcium was significantly suppressed due to elevated osmotic pressure in the nutrient solutions.

Contrary to phosphorus, nitrate uptake at the higher N-NO₃ supply (200 mg/L) appeared to be down-regulated, possibly *via* a negative feedback mechanism based on downstream nitrogenous compounds (e.g. glutamine, Cardenas-Navarro *et al.*, 1998; Glass *et al.*, 2001). Down-regulation of nitrate influx at higher concentrations constitutes a protective strategy against osmotic damage or potential ion toxicity as a result of excessive accumulation (Glass *et al.*, 2001) and can also occur through effects exerted by nitrate itself on nitrate transporters i.e. allosteric effects or post translational modification of transporters *via* phosphorylation (Glass *et al.*, 2001). Effects of N on plants are also mediated by the action of CYT that is enhanced due to the nitrate supply (Kuiper *et al.*, 1988; Van der Werf & Nagel, 1996), and thereby resulting in greater shoot to root ratios. In both cases of elevated N-NO₃ and P supply, the induction of the corresponding mechanism i.e. inhibition of nitrate influx and increased uptake of phosphorus inflicted a significant decline on shoot biomass accumulation.

In chapter 6, the similar shoot biomass production of *Narcissus* "Ice Follies" under the 100 and 180 mg/L N-NO₃ strongly suggested that the utilisation of nitrogen (N) for primary metabolic processes was saturated at the lower nitrate supply (100 mg/L N-NO₃) in contrast to *S. officinalis* species where optimum nitrate concentration was

found to be the 150 mg/L. Some of the excess nitrates absorbed by *Narcissus* plants under the 180 mg/L treatment appeared to be diverted towards the biosynthesis and accumulation of alkaloids. Plants are able to store alkaloids in relatively large quantities, whereas amino acid storage may be more limited (Bernath, 1986). The alkaloid yield of "Ice Follies" was significantly augmented at 180 mg/L nitrate supply and the present study demonstrated considerably higher values i.e. 1.77 ± 0.08 mg/g compared to 0.59 - 1.68 mg/g that had been reported in the existing literature. Differentiation in optimum N-NO₃ for growth could simply reflect variations in plant size and general plant morphology/physiology. However, inconsistency in light conditions at the two hydroponic experiments (e.g. lower PAR in the growth room), might have influenced nitrate uptake of *Narcissus* as differences in N demand are expected at different irradiance levels mediated by the impact of light on photosynthesis (De Pinheiro Henriques & Marcelis, 2000).

The growth of *S. officinalis* var *purpurea* raised hydroponically under three regimes of EC 1.8, 3.6 and 7.4 mS/cm was favoured by the lower EC treatment while under higher EC levels plants exhibited characteristics typical of water stress. Elevated solute concentration in the nutrient solution at the 3.6 and 7.4 mS/cm treatments increased osmotic pressure, which hindered water influx in the root system (Herralde *et al.*, 1998) and caused conditions analogous to water deficiency such as suppressed biomass accumulation, lower specific leaf area and greater investment in root biomass. As a general rule for all species, a balanced nutrient solution should have an electrical conductivity about 1.5 mS/cm plus the EC of the water (ca 0.5 mS/cm, Papadopoulos, 1991). It is worth noting that Savvas & Lenz (2000) who investigated increased EC levels on hydroponically cultivated egg plants *via* the addition of extra nutrients, demonstrated that effects on plant growth were osmotic and not ion specific. These results strongly indicate EC application as a potent research tool for osmotic / water stress studies in hydroponics.

7.1.2 Essential oil responses to nutrition and electrical conductivity treatments

Production of plant secondary metabolites (SM) or natural products has traditionally been achieved through harvesting field grown aromatic & medicinal plants (Bourgaud *et al.*, 2001). Canter and colleagues (2005) suggested that cultivation of aromatic & medicinal plants under controlled environments involving hydroponic systems could be a promising way for efficient production of bioactive compounds on a commercial scale. Economakis (1992) and Economakis *et al.* (2005) reported that plants of *Origanum dictamnus* L. raised *via* the nutrient film technique (NFT) exhibited higher oil content than plants from the same population grown in the wild. In the present study, *Salvia officinalis* var *purpurea* was cultivated by means of NFT under different nitrate (N-NO₃), phosphorus (P) and electrical conductivity (EC) regimes. Although the various nutrient treatments exerted evident impacts on plant growth, there was little influence on essential oil content or quality. However, EC was found to significantly affect the essential oil composition of this species. Interestingly, the high EC treatment induced a significant increase in the concentration of toxic molecules like α -thujone, perhaps reflecting a requirement for enhanced defence in plants under conditions that cause osmotic stress. In all cases, oil yield appeared to be approximately 1% (mg of oil per g dry matter). To date, oil yield of the *purpurea* variety has never been reported and as expected it fell within the same range of *S. officinalis* (Karousou *et al.*, 2000). However, *S. officinalis* plants grown in the field in Britain possessed a very low oil content of 0.53% (personal communication with Dr S Savelev), which indicates to some extent the superiority of hydroponics over conventional soil culture for increasing oil yield in *Salvia*. GC/MS analysis revealed that α -caryophyllene and camphor were abundant, comprising approximately 16-23% and 10-17% respectively of the oil mixture. α -Thujone the third major compound contributed 9-10% in the mixture, while the rest of the constituents e.g. α and β pinene, 1,8 cineole, ledol, borneol and bornyl acetate made up less than 4% of the oil. Experimental data from other relevant hydroponic studies have demonstrated considerable variation in the yield and

composition of essential oils extracted from aromatic & medicinal species belonging to the Labiateae in response to N-NO₃, P and EC application (Economakis *et al.*, 2002; Karioti *et al.*, 2003; Economakis *et al.*, 2005). However, none of the above studies analysed essential oil yield or composition at the vegetative growth stage; plants were harvested during flowering or seed formation stage. The strong influence of ontogeny on secondary metabolite (SM) composition and accumulation has been well documented (Bernath 1986; Sangwan *et al.*, 2001). It is possible that ontogenetic events i.e. mobilisation of carbohydrates, changes in hormonal balance and mineral composition/allocation that take place during development influence the response of secondary metabolism to external nutritional factors. Experimental outcomes from the hydroponic cultivation of *S. fruticosa* corroborated previous statement that the demand for N-NO₃ and P is significantly amplified during anthesis and the seed formation stage (Economakis 1993b and 1995, respectively), thereby coinciding with the boosted oil yield of sage at that time in comparison with the vegetative phase. At the vegetative stage under controlled environmental conditions, it is possible that growth and primary metabolism take priority over secondary metabolite production in terms of responses to nutrient input. The assumption that elevated levels of secondary metabolites enhance plant defence may be particularly relevant to the protection of the reproductive organs. This hypothesis is supported by findings that the yield of essential oils in *Salvia* species increases at anthesis and during seed formation (Karousou *et al.*, 2000; Putievsky *et al.*, 1986; Karioti *et al.*, 2003; Santos-Gomes & Fernandes-Ferreira, 2001).

Plant secondary metabolism is also known to respond to abiotic stressors that include nutrient or water deficiency. Biochemical and molecular changes that constitute physiological adaptations to stress, allow plants to survive unfavourable environmental conditions. During stress, plant survival is often more important than growth or development (Gershenzon, 1983). Ultimately, the chemical profile of plants is regulated by the combined action of external/environmental influences and genetic factors. The

lack of response of essential oil yield and composition to nitrate and phosphorus nutrition at the vegetative stage might be due to an overriding genetic regulation of terpene production (Hamilton *et al.*, 2001). Gershenzon (1983) illustrated great discrepancies between various species in terms of the influence of nutrition on terpene biosynthesis.

In order to elucidate the mechanisms that underpin the impact of nutrients and EC conditions on SM biosynthesis at the cellular level, modern technologies that include proteomics, micro arrays, metabolomics and recombinant DNA technology will be indispensable in identifying the genes, enzymes and metabolites that regulate the production of targeted bioactive compounds. However, the more modern approaches in SM production such as plant cell culture technologies often encounter problems that do not allow commercialisation i.e. low yield of the desirable compound as well as the high cost of these techniques (Jacobs *et al.*, 2000; Bourgaud *et al.*, 2001; Gontier *et al.*, 2002). For example, cell cultures have been found incapable of synthesising commercially important alkaloids i.e. morphine, codeine, scopolamine, vincristine and vinblastine (Hughes & Shanks, 2002). Recent advances in biotechnology have initiated metabolic engineering and molecular breeding of medicinal plants with the view to enhance active compounds and select for desirable agronomic traits (Canter *et al.*, 2005). Metabolic engineering has shown promise for the future (Bourgaud *et al.*, 2001; Broun & Somerville, 2001; Canter *et al.*, 2005), but a number of important constraints that include poor characterization of SM biosynthetic pathways and the relative lack of genome information for important medicinal plant species still challenge the genetic control of natural product biosynthesis. One of the most successful genetic engineering approaches for secondary metabolism improvement was the 50% augmentation of peppermint essential oil (Mahmoud & Croteau, 2001). Nevertheless, genetically modified plants are considered “not natural” and public acceptance of transgenic herbs is still uncertain.

7.1.3 Anti-cholinesterase activity of *Salvia officinalis* var *purpurea*

Since ancient times, sage has had the reputation of possessing memory enhancing properties. *In vitro* investigations of the cholinergic activities of oils extracted from *S. lavandulaefolia* and *S. officinalis* revealed preferential inhibition of the enzyme AChE. In contrast, in the present study, *S. officinalis* var *purpurea* exhibited dual anti-cholinesterase activity with relatively low IC₅₀ values ca 0.055 to 0.07 mg/ml for human AChE and 0.07 mg/ml for BuChE enzyme from horse serum. These findings are in agreement with the only other report involving the *purpurea* variety (Savelev *et al.*, 2004). Existing *in vivo* studies strongly suggest that the IC₅₀ values obtained in this investigation are probably within the therapeutic dose range. Tildesley *et al.* (2003; 2005) reported enhanced mood and mnemonic performance in young participants who received *S. lavandulaefolia* oil that possessed IC₅₀ values similar to *S. officinalis* var *purpurea* (i.e. 0.07 mg/ml, brain autopsy tissue, Perry, 1996 and 0.053 to 0.12 mg/ml, human erythrocyte AChE, Savelev *et al.*, 2004). Corroborating evidence was presented by Kennedy and colleagues (2005) who demonstrated beneficial effects on memory and mood of young volunteers in a double-blind, placebo study, following consumption of *S. officinalis* leaves (80% ethanolic extract IC₅₀ values: 0.056 and 0.398 mg/ml for BuChE and AChE respectively). Another pilot tolerability clinical trial with *S. lavandulaefolia* suggested significant improvement in patients' condition after 6 weeks, while tolerability of the oil was proved to be excellent (Perry *et al.*, 2003). Perry and colleagues (2002) confirmed that *S. lavandulaefolia* oil or its metabolites can cross the blood brain barriers and induce a decline in AChE activity of selected brain areas in rats. If positive effects on the CNS have been evident from inhibitors that exhibit *in vitro* 50% inhibition at concentrations of 0.05 to 0.12 mg/ml, then *S. officinalis* var *purpurea* oil can be considered a potent *in vivo* inhibitor with the condition that the oil or its constituents reach the brain like the pre-mentioned sage oils presumably do.

Cholinergic properties of *Salvia* species are due to phytochemicals (Perry *et al.*, 1996; Perry *et al.*, 2000, 2002, 2003; Savelev, *et al.*, 2003; Tildesley *et al.*, 2003; Savelev *et al.*, 2004 and Tildesley, *et al.*, 2005) constitutively produced *via* secondary metabolic pathways. Cholinesterase inhibition by *Salvia* essential oils is determined by synergistic and antagonistic interactions among the terpene compounds of the mixture (Miyazawa *et al.*, 1998; Savelev *et al.*, 2003 and 2004). Interestingly, no single terpene or combination of them has ever demonstrated higher or the same potency in comparison with the whole oil (Miyazawa *et al.*, 1998; Savelev *et al.*, 2003). For example, combinations of 8 and 4 terpenes in the ratios that they naturally occur in the oils of *S. lavandulaefolia* and *Mentha piperita* (Savelev *et al.*, 2003 and Miyazawa *et al.*, 1998 respectively) were not as potent as the oil itself. *S. officinalis* var *purpurea* essential oil composition was closely related to that of *S. officinalis*, but much less to the oil from *S. lavandulaefolia* and *S. fruticosa*, which are known to contain cholinesterase inhibitors. The abundant α -caryophellene (16-23%) of *S. officinalis* var *purpurea* oil and camphor (10-17%) when tested alone showed no cholinesterase activity (Savelev *et al.*, 2004). Moreover, other oil constituents that demonstrated *in vitro* AChE inhibition (Perry *et al.*, 2000; Savelev *et al.*, 2003, 2004) 1.8 cineole, α - and β -pinene, 3-carene and β -caryophyllene, were present in minor concentrations. Terpenes from *Salvia* species with dual cholinergic activity, when tested individually for BuChE inhibition appeared to be almost or completely inactive (Savelev *et al.*, 2004). Therefore, it is suggested that the anti-cholinesterase properties of *S. officinalis* var *purpurea* oil depend upon unknown interactions in the unique mixture. The competitive type of inhibition exerted by the oil in combination with the low concentrations of camphor (10-17%) a non-competitive antagonist to nicotinic ACh receptors (Park *et al.*, 2001) and α -thujone (9%) a molecule with reported toxic properties (Höld, 2000), indicate that *S. officinalis* var *purpurea* is a new potential ChE inhibitor relevant to the treatment of AD.

Apart from the oils, 80% ethanol extracts prepared from hydroponically grown *S. officinalis* var *purpurea* revealed selected inhibition for BuChE with significantly lower IC₅₀ values than the oil. For the first time, the present thesis demonstrated BuChE inhibitory activity from ethanolic extracts of *S. officinalis* var *purpurea* with IC₅₀ values (0.054 mg/ml) that are consistent with those of *S. officinalis* (Kennedy *et al.*, 2005) and very weak inhibitory activity for AChE. The contrasting cholinergic activities exhibited by distilled and organic extracts from the same species is possible considering the likely differences in chemical composition between such extracts (Langer *et al.*, 1996) and reflects structural differences between the two enzymes (Silman & Sussman, 2000). It is possible that AChE is less susceptible to inhibition from relatively polar mixtures (see section 3.4.5; chapter 3). Nevertheless, a growing body of evidence has suggested that selective inhibition of BuChE enzyme or dual inhibition of ChEs is relevant for the treatment of dementia. Cholinergic activities of sage on BuChE has been reported twice (Savelev *et al.*, 2004; Kenedy *et al.*, 2005), despite positive indications for the beneficial inhibition of this enzyme in the progress of AD (Greig, 2001; Giacobini, 2004; Lane *et al.*, 2005; Tasker *et al.*, 2005).

7.1.4 Recommendations for future research

1) The optimum potassium (K) concentration for the growth of aromatic herbs needs to be determined as experimental outcomes have strongly indicated quantitative and qualitative variations in the essential oils due to K application. Increasing the K concentration from 150 to 450 mg/L in the nutrient solution induced a significant decline in the essential oil content of leaves of *Origanum dictamnus* L. raised in NFT (Economakis, 1993a). High K supply reduced oil content in field grown peppermint and sweet basil, whereas menthol and linalool constituents in peppermint oil were enhanced (Hornok, 1986). Galanthamine content in *Narcissus* bulbs was impressively augmented (113%) by applying potassium/magnesium fertilisers (Kreh, 2002).

- 2) The impact of EC on the cultivation of *Narcissus* in hydroponics is of great interest. Imposed water stress on alkaloid-containing species can augment alkaloid yield (Gershenzon, 1983; Höft et al., 1996) and water deficit in combination with various nitrogen levels significantly affected the yield of hyoscyamine and scopolamine in the roots of *Atropa belladonna* (Baricevic et al., 1999).
- 3) The optimum nutrient combination of N, P, K and EC levels for *Salvia officinalis* var *purpurea* should be tested under conditions of high light intensity, a common factor in the natural habitats of sage. Stimulation of terpene production and light-induced changes in the yield and quality of essential oils have been well documented in a number of herbs (Bernath, 1986).
- 4) Given the fact that growth of *Narcissus* plants saturated at 100 mg/L nitrate, it is possible that higher light intensity / PAR would enhance nitrate absorption and consequently N storage in the form of alkaloids. Conditions of increased light intensity in combination with relatively high nutrient supply promoted alkaloid biosynthesis in alkaloid-containing plants (Bernath, 1986).
- 5) Additionally, hydroponically-grown plants could be raised under UV radiation that has been shown to enhance the essential oil content in *Mentha spicata* (karousou et al., 1998). Light quality has been shown to influence essential oil production in other species (Sangwan et al., 2001).
- 6) Further investigations on how various combinations of nutrients and water treatments administered at different ontogenetic stages influence essential oils in *S. officinalis* var *purpurea* are required. *Salvia* species are known to exhibit ontogenetic variation in oil yield and composition (Putievsky et al., 1986; Karousou et al., 2000; Santos-Gomes & Fernandes-Ferreira, 2001; Karioti et al., 2003).
- 7) Ultimately, optimisation, standardisation and full control of the growth media under hydroponic conditions is an important goal that will guarantee a high quality and standardised herbal product especially for species like *Salvia* that show significant genetic (Skoula et al., 2000) and seasonal variation in essential oil yield and

composition (Putievsky *et al.*, 1986; Karousou *et al.*, 2000; Santos-Gomes & Fernandes-Ferreira, 2001; Karioti *et al.*, 2003).

8) Outcomes of this study suggest that continued screening for activities relevant to AD within medicinal herbs that have been traditionally consumed is a priority.

9) Screening for anti-cholinesterase activity of a *S. fruticosa* population from the eastern part of Crete (Greece) that has been reported to contain considerably high 1.8 cineole (ca 60%) (Karousou *et al.*, 2000) could reveal promising outcomes.

10) As already established for *S. officinalis*, further testing of *S. officinalis* var *purpurea* for anti-inflammatory, antioxidant, receptor binding properties as well as inhibition of β -amyloid formation, will be necessary to emphasise the significance of this variety in the symptomatic treatment of AD, according to the new more holistic approach to treating this condition.

11) *In vivo* studies will also be required to investigate pharmacokinetics and confirm the ability of oil constituents to cross into the brain tissue whilst human volunteers will be needed to further assess the effectiveness and tolerability of extracts prepared from *S. officinalis* var *purpurea*.

In conclusion, ChE inhibitors have wide medical applications and new potential roles under investigation, include their use in the treatment of dementia with Lewy bodies, vascular dementia, Downs syndrome, traumatic brain injury and other cases (Giacobini, 2004). Although the main strategy so far approved for the treatment of AD is cholinesterase inhibition (Heinrich & Teoh, 2004; Ellis, 2005), the use of ChE inhibitor drugs has been questioned by certain researchers (Shen, 2004). New guidelines have objected to their application for mild cases of AD (www.nice.org.uk; 2006-052 Lanch of dementia guideline 1). Such opinions provide strong support for the use of herbs like *Salvia* that by exerting a weaker effect on brain cholinesterases could be more relevant for alleviating the symptoms of mild to moderate Alzheimer's disease, and strongly suggest the need for further research in this direction.

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Appendices

Appendix 1 Hydroponics

1.1 Calculations for the estimation of fertilizer / chemical reagent amount in the stock solution

Element in stock mg/L = % Element X (Weight g X 1000) / 50 L or

Weight g = Element in stock mg/L X 50 L / (% Element X 1000)

Where:

Weight g represents the desirable amount of the chemical in the stock solution in g.

% Element denotes the percentage of the nutrient in the fertilizer / reagent calculated as the ratio of its atomic weight divided by the formula weight of the fertilizer/ reagent.

1.2 Elemental analysis of tap water

Elemental composition of the tap water in the Moor Bank greenhouse utilised for the hydroponic cultivation of *Salvia* was determined via ICP (see section 2.3.1). Tap water was analysed for Mn, Na, Mg, Ca, S and Bo concentration.

Table 1 Elemental composition of tap water in the Moor Bank greenhouse

<i>Element</i>		<i>Concentration mg/L</i>
Maganium	Mn	0.003
Sodium	Na	8.982
Magnesium	Mg	5.883
Calcium	Ca	59.08
Sulphur	S	25.44
Boron	Bo	0.013

1.3 Spectrophotometric method for nitrate determination

1.3.1 Principle

Nitrate can be directly determined in aqueous solution by its strong absorbance at about 200 nm. Because organic constituents of natural water also absorb at the same wave length it is necessary to make a correction by relating their absorbance at this wavelength with that at higher wavelength. The correction wavelength 275 nm is chosen as the absorbance of organic constituents is then approximately one quarter that of the principal wavelength. The specific absorption coefficient for 1 mg/L of nitrogen in nitrate is 0.55 at 210 nm. This figure may be applied directly as an alternative to the calibration procedure described below.

1.3.2 Reagents

1. Deionised water (DIW water). DIW must be used for the preparation of all standard solutions and the dilutions and blanks.
2. Stock nitrate solution. Dissolve 722.1 mg of potassium nitrate (KNO_3) in twice distilled water, and make up to 1 L. This is a solution of 100 mg/L N.
3. Standard nitrate solution. Dilute 100 ml of stock nitrate solution to 1 L TD water. This is a 1:10 dilution of the stock which will give a solution of 10 mg/L N. (100 ml stock add 900 ml water)

1.3.3 Calibration

Prepare N-NO₃ standards in the range 0-10 mg/L N by transferring 0-10 ml volumes of standard nitrate solution of 10 mg/L to 10 ml volumetric flasks. Make up to the mark with DIW water.

Procedure

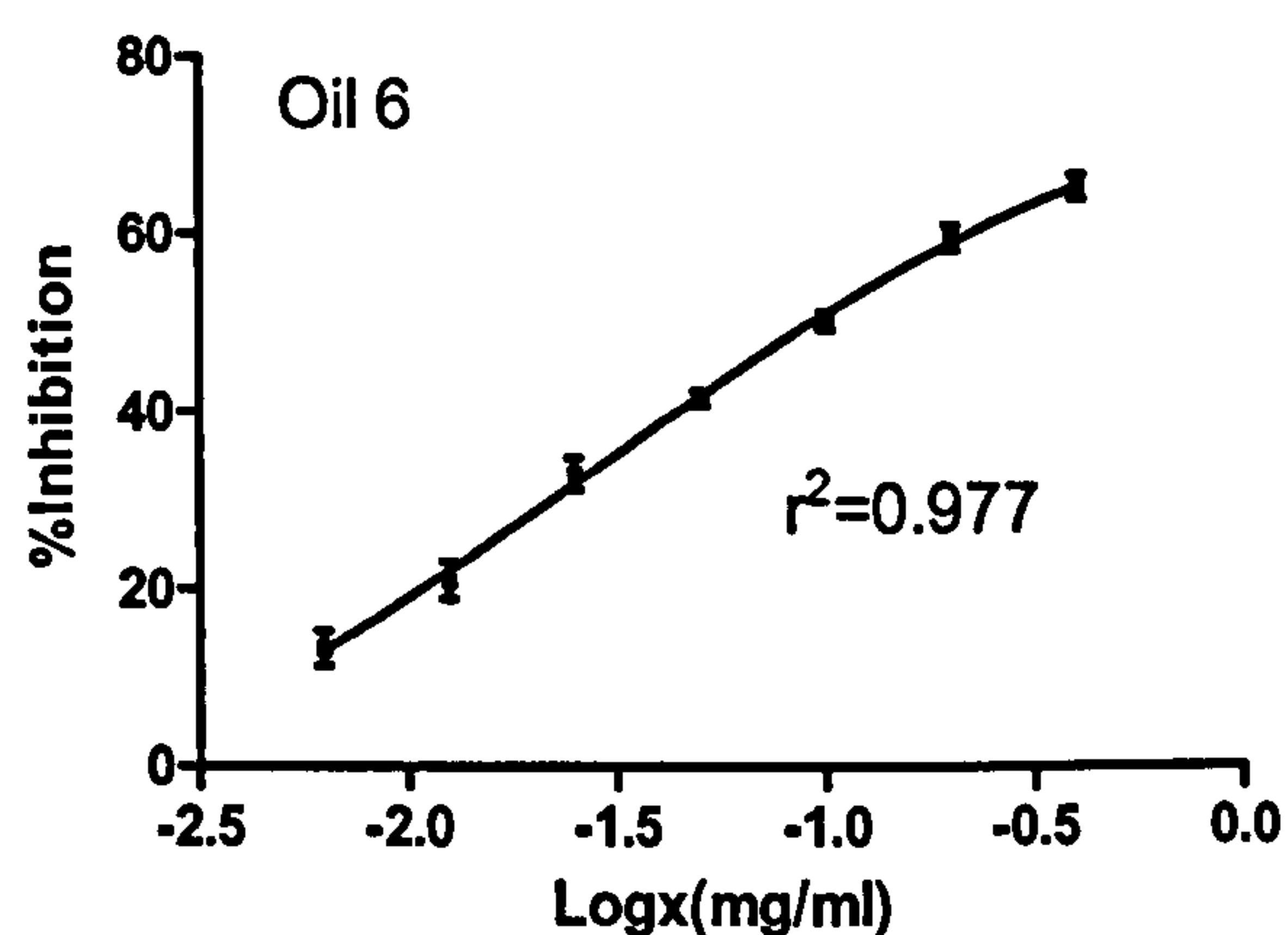
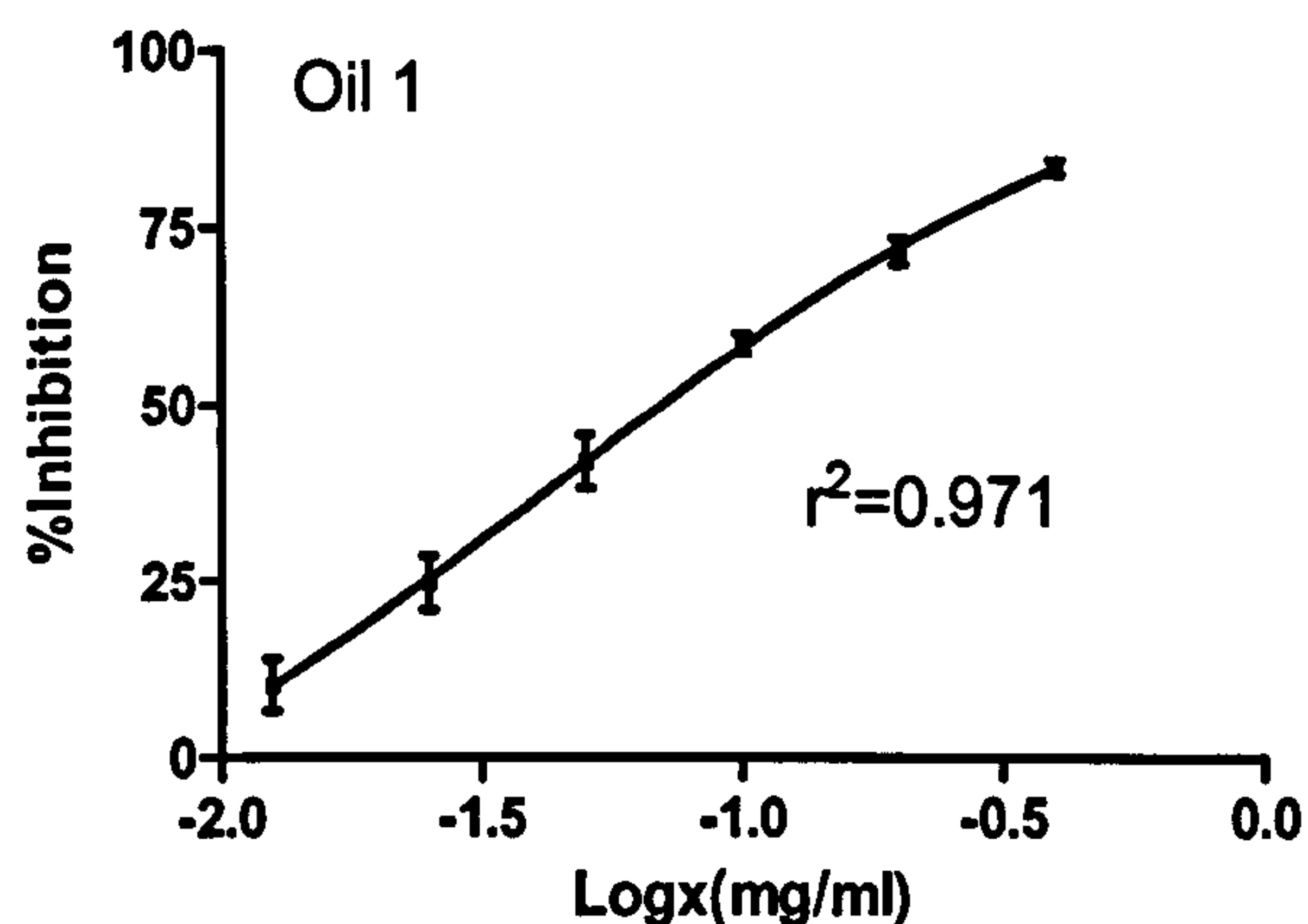
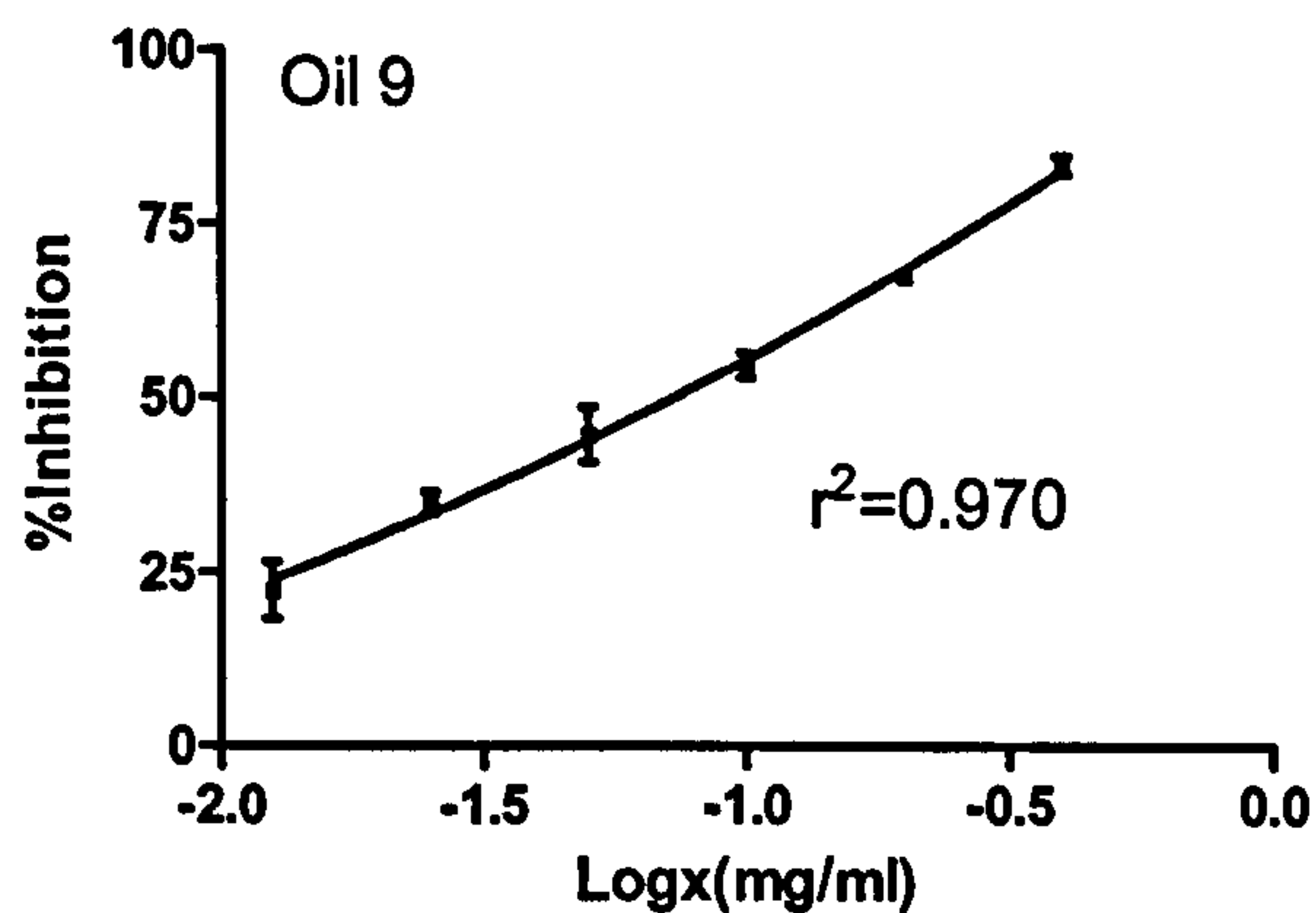
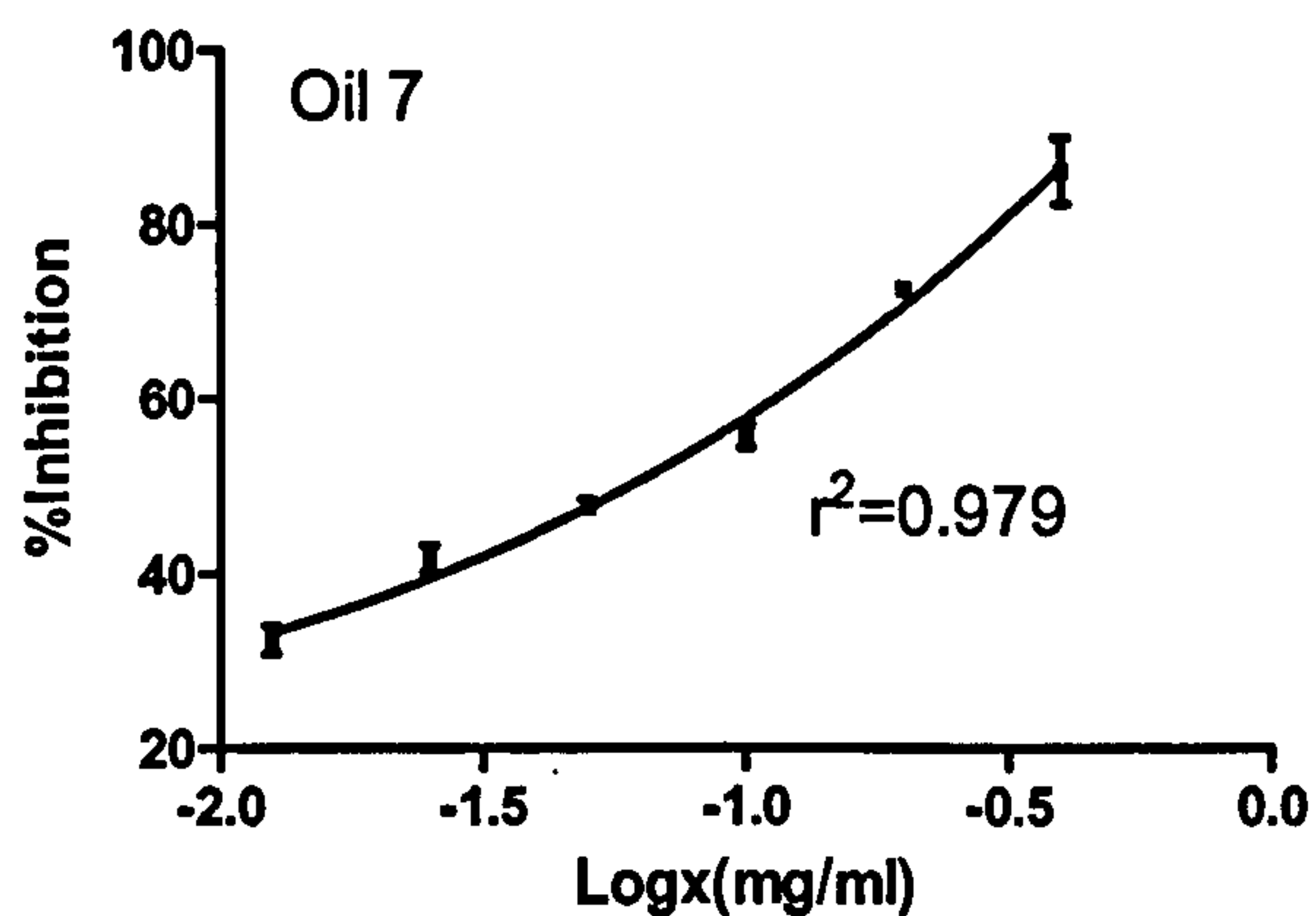
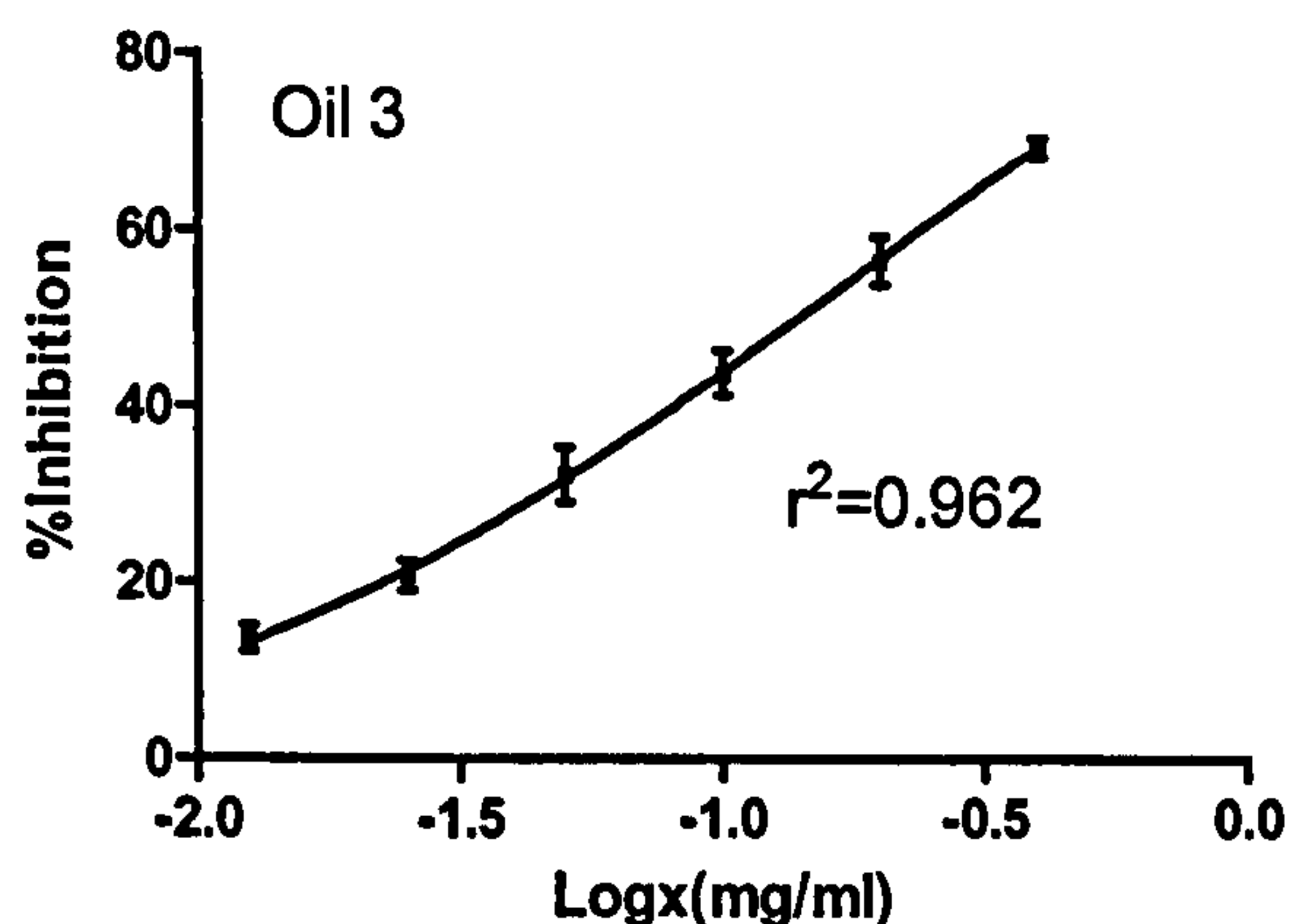
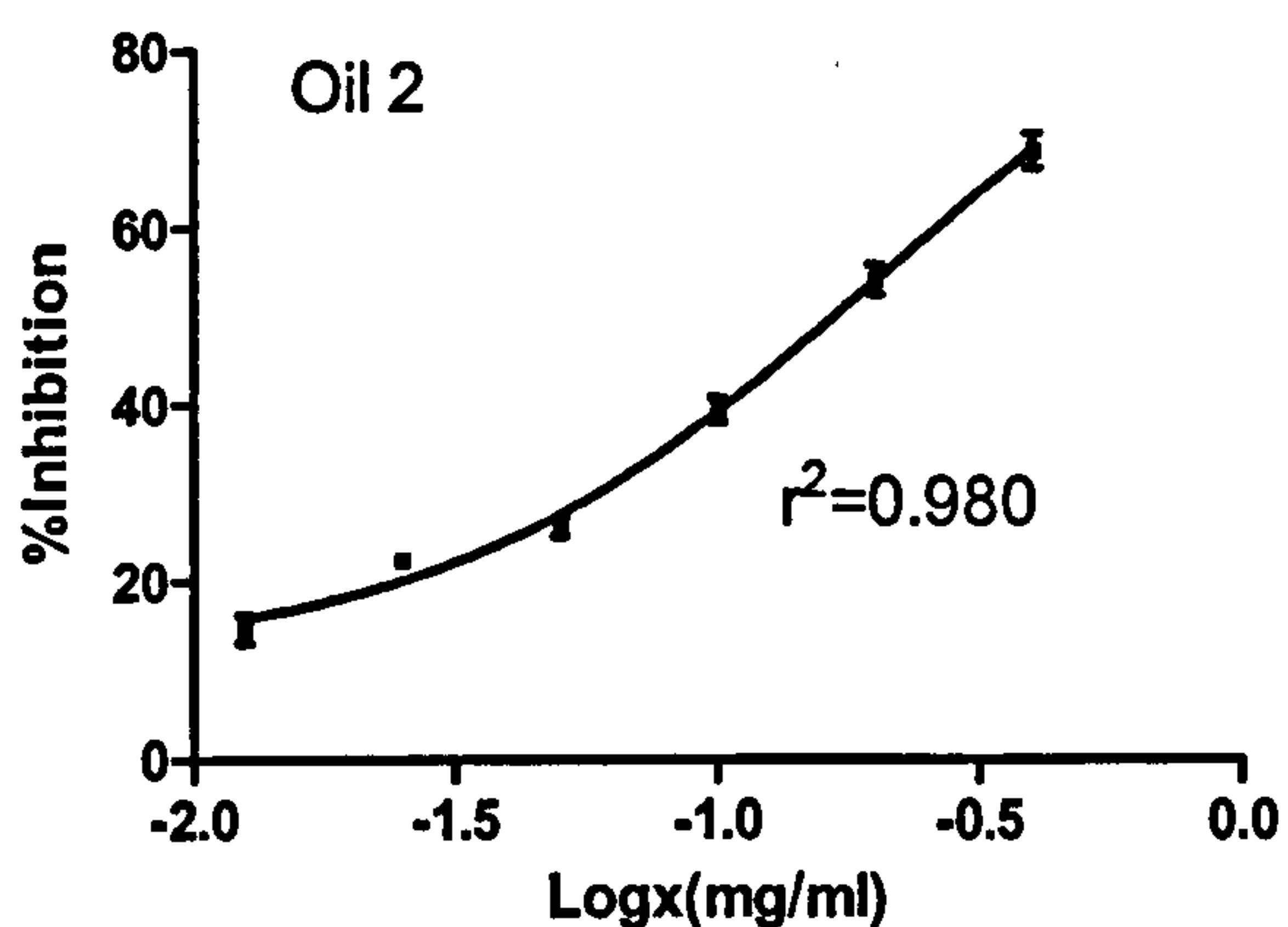
Read the absorbance of standards and samples at 210 nm and 275 nm. Zero the machine with DIW every 10 samples. Draw a calibration graph relating absorbance at 210 nm with concentration of nitrogen in mg/L in the standards. Correct sample readings for organic content by subtracting four times the 275nm value from the 210 nm absorbance value: $A=A_{210} - 4 \cdot A_{275}$

This concentration for organic matter is satisfactory when it corresponds to only 5% or 10% on the nitrate. This applies to practically all underground waters and many others. If the correction is greater then it is better to remove the organic matter by coagulating with about 50 mg/L of aluminium sulphate, settling and decanting or filtering (the correction is always applied).

Appendix 2 Enzyme inhibition (IC₅₀ values)

Inhibitory activity of plant extracts for cholinesterase enzymes, expressed as an IC₅₀ value (extract concentration required for 50% inhibition) was determined via the construction of sigmoidal dose response curves (four parameters logistic equation), where percentage of inhibition from a series of dilutions of the plant extract is plotted against the logarithm of plant extract concentration (Prism graph pad, version 4). Each plant extract was run twice. One data set for AChE and BuChE is presented here.

2.1 *Salvia officinalis* var *purpurea* anti-cholinesterase activity data from Chapter 3



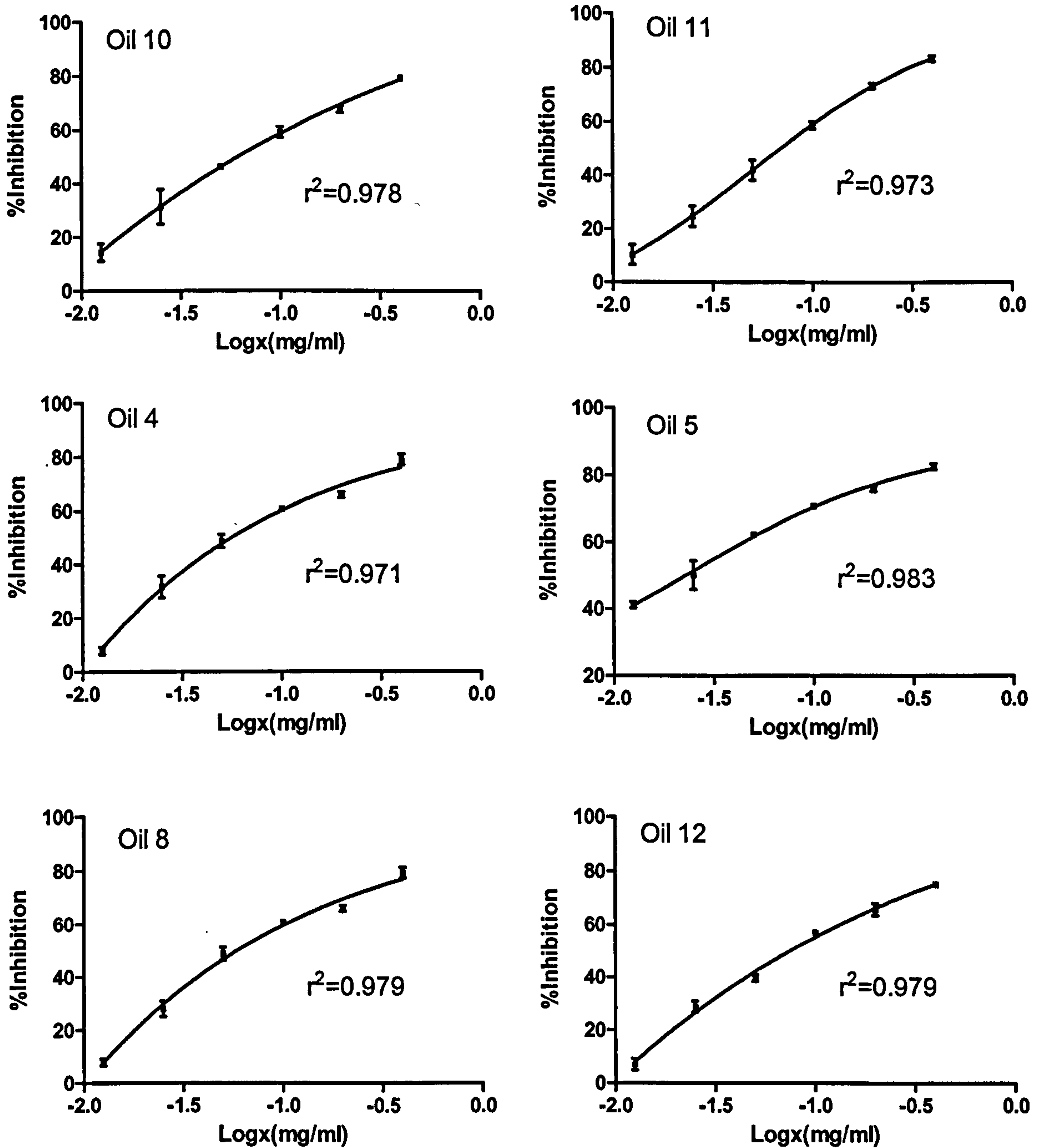
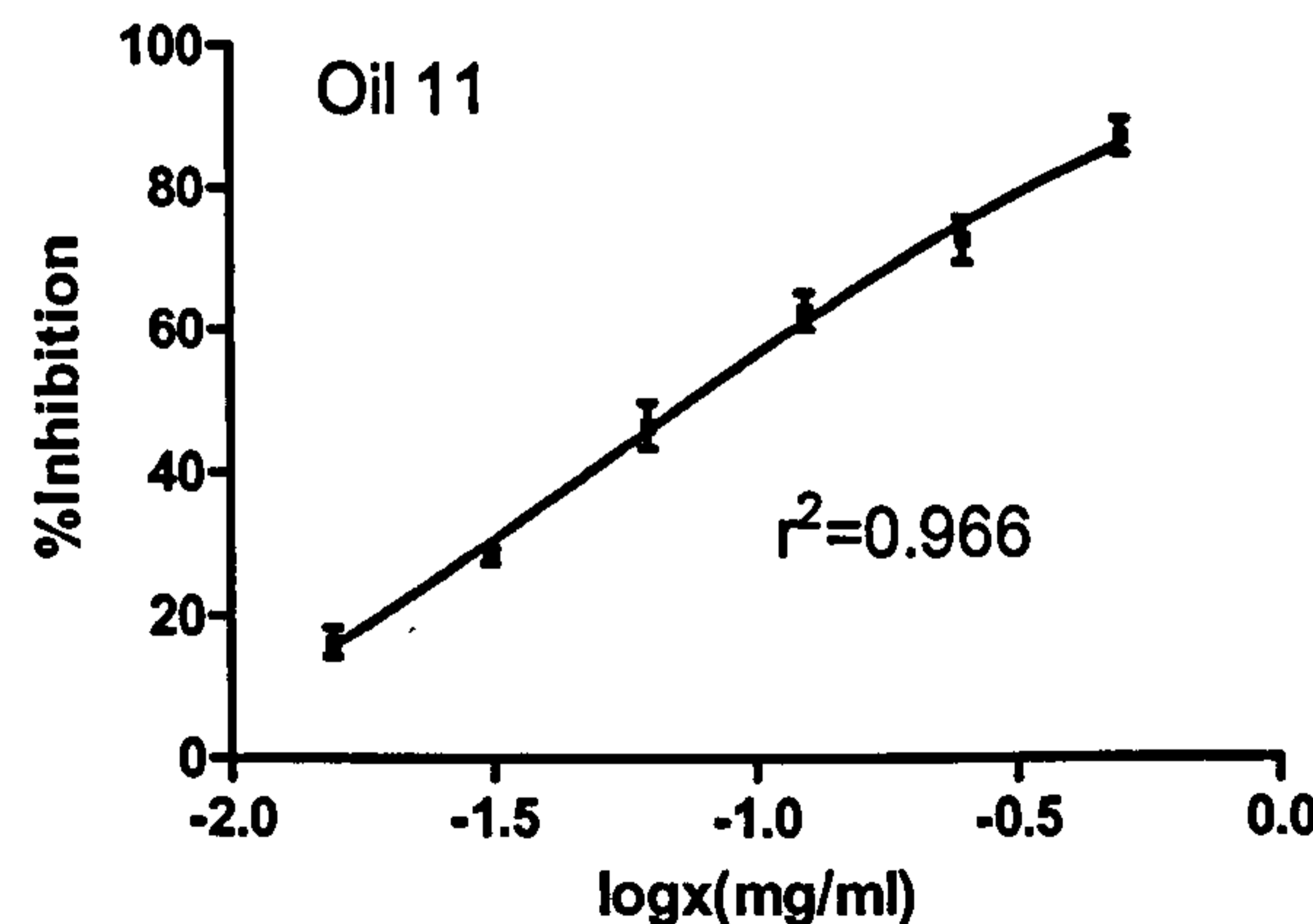
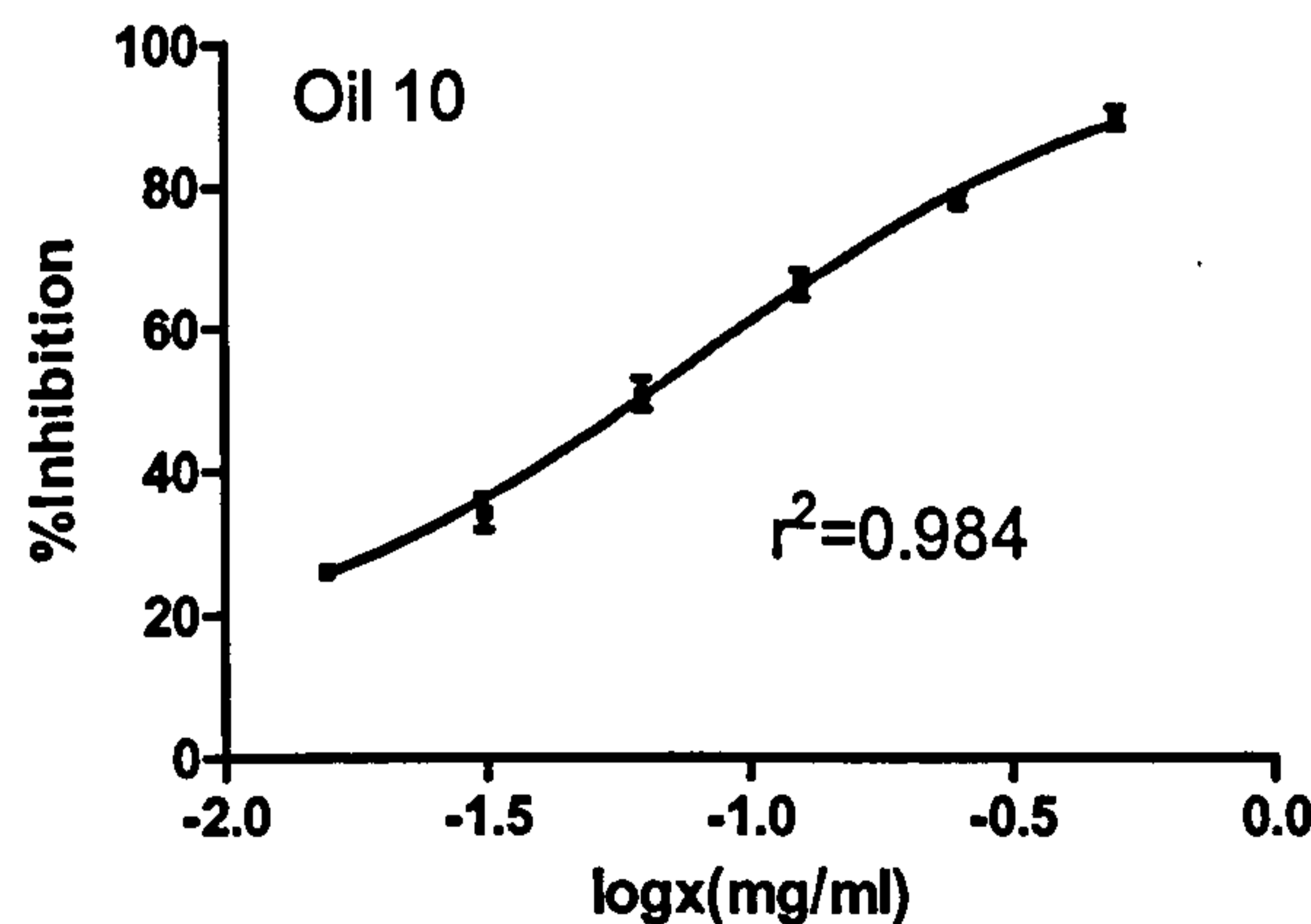
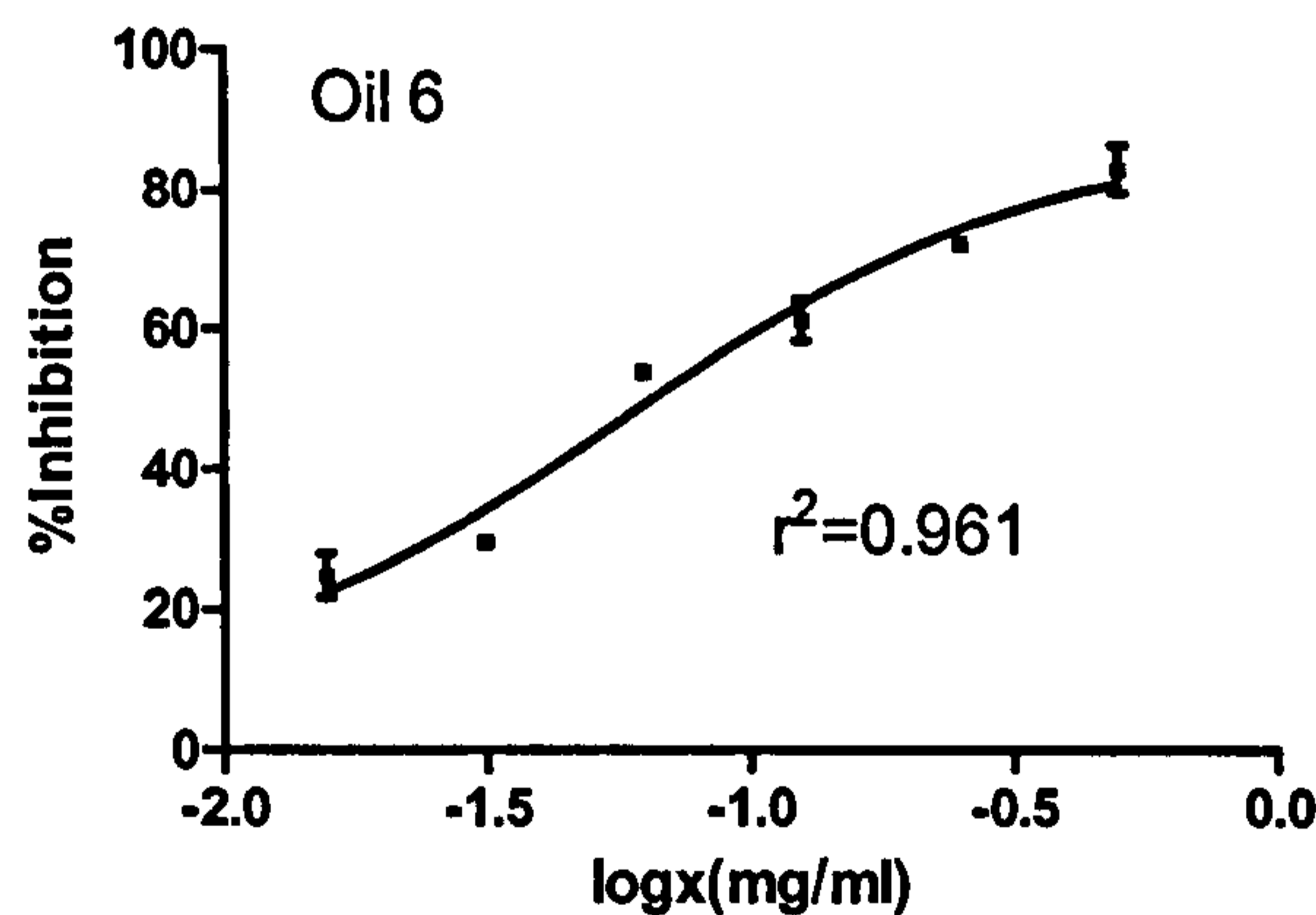
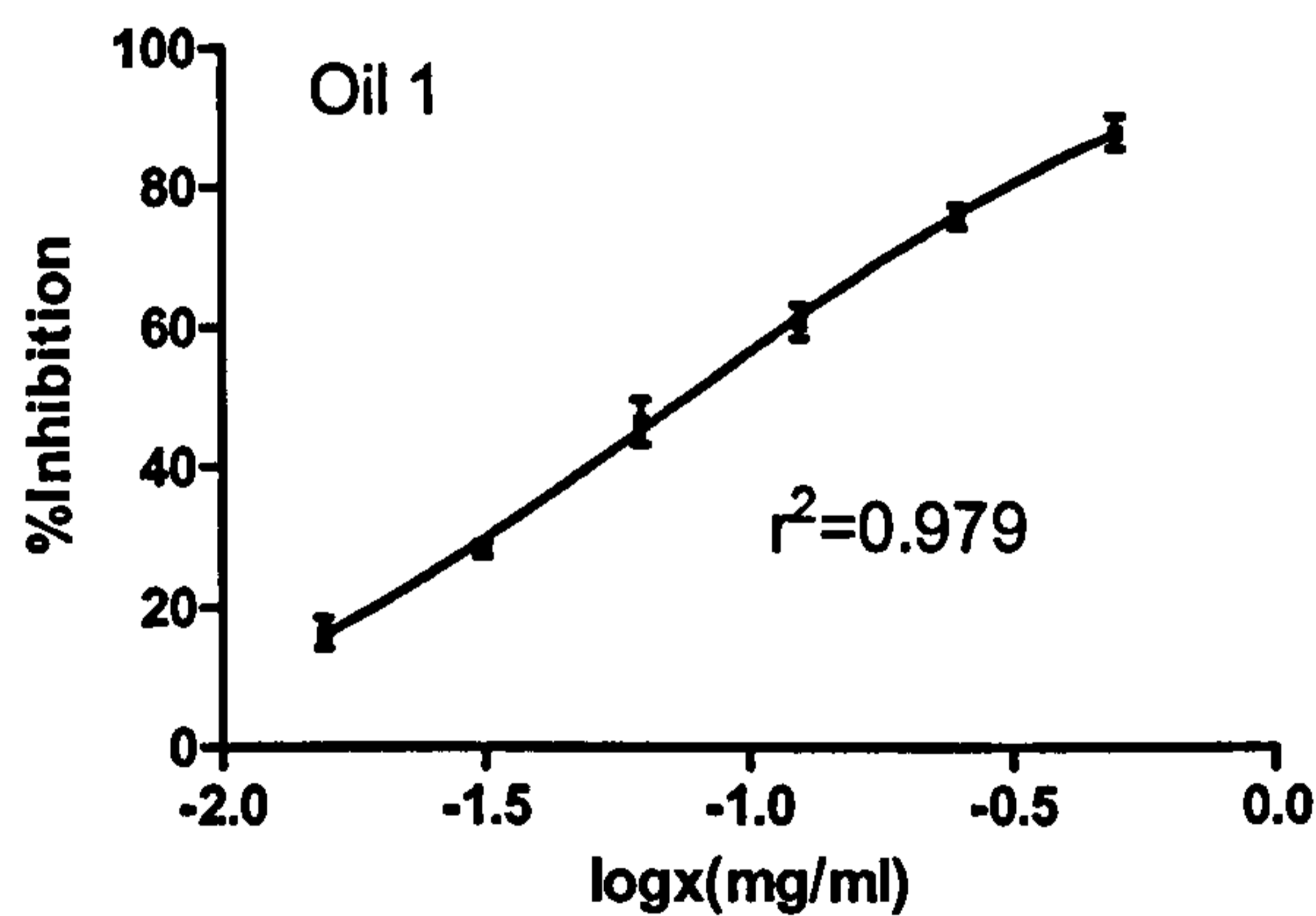
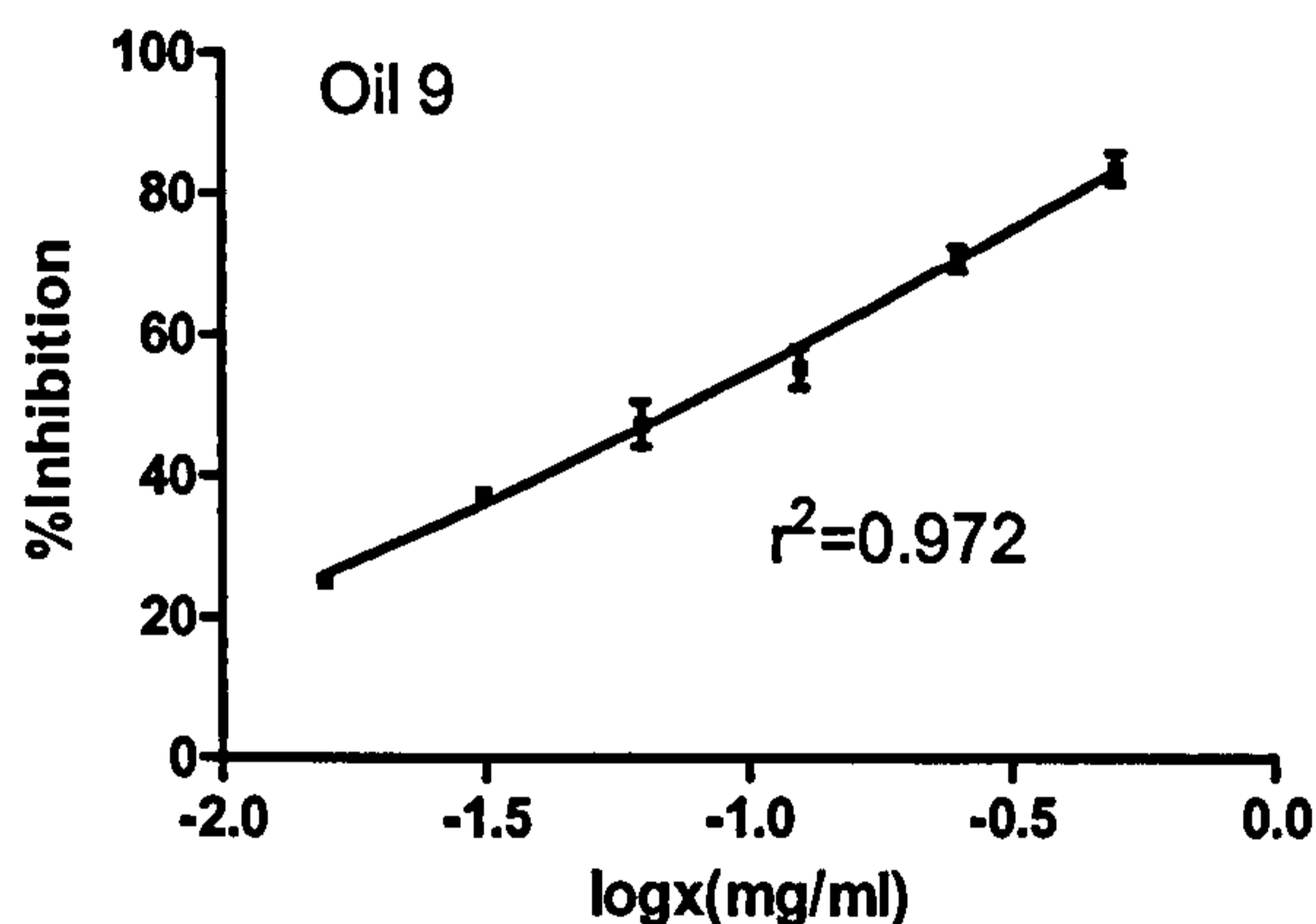
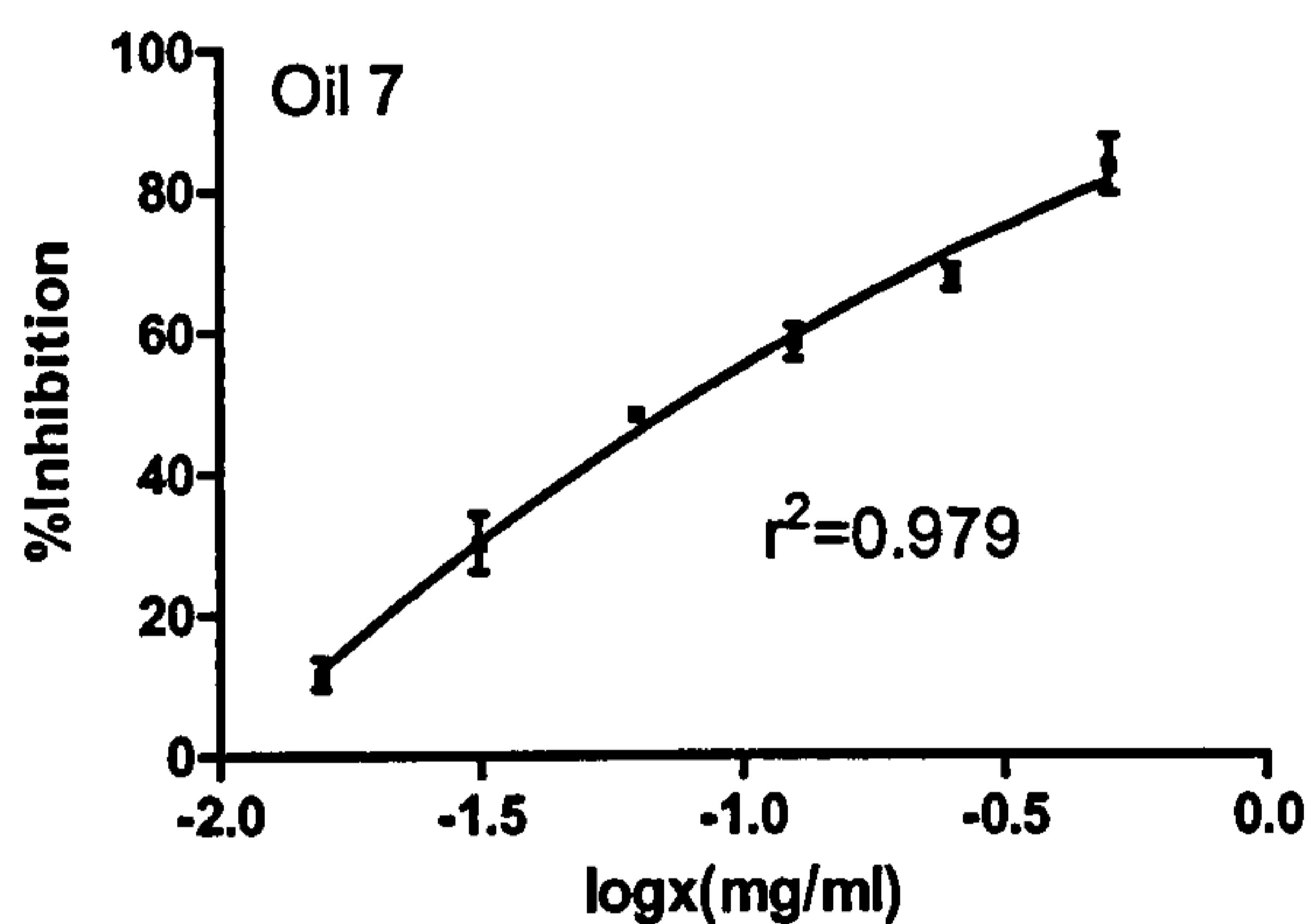
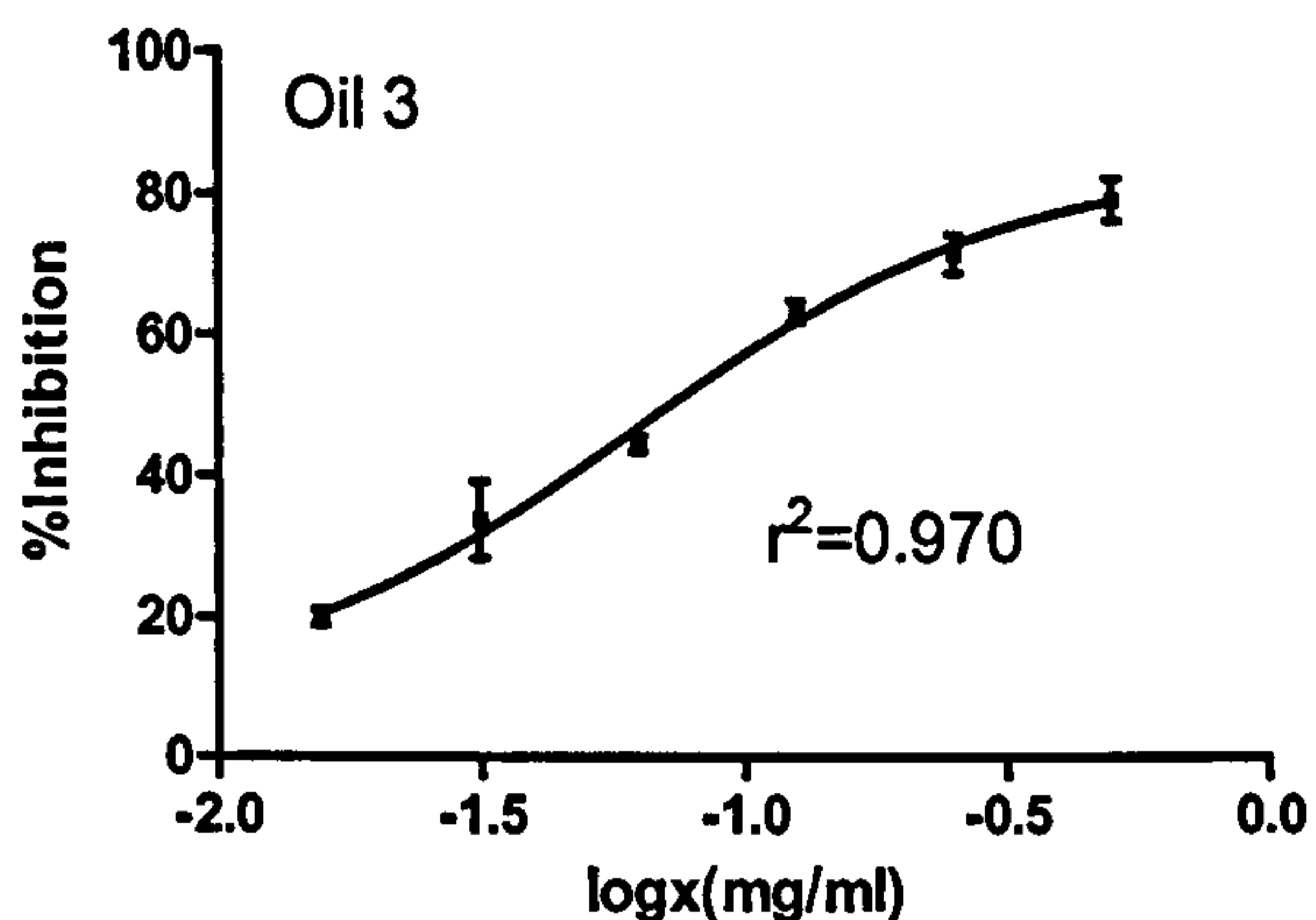
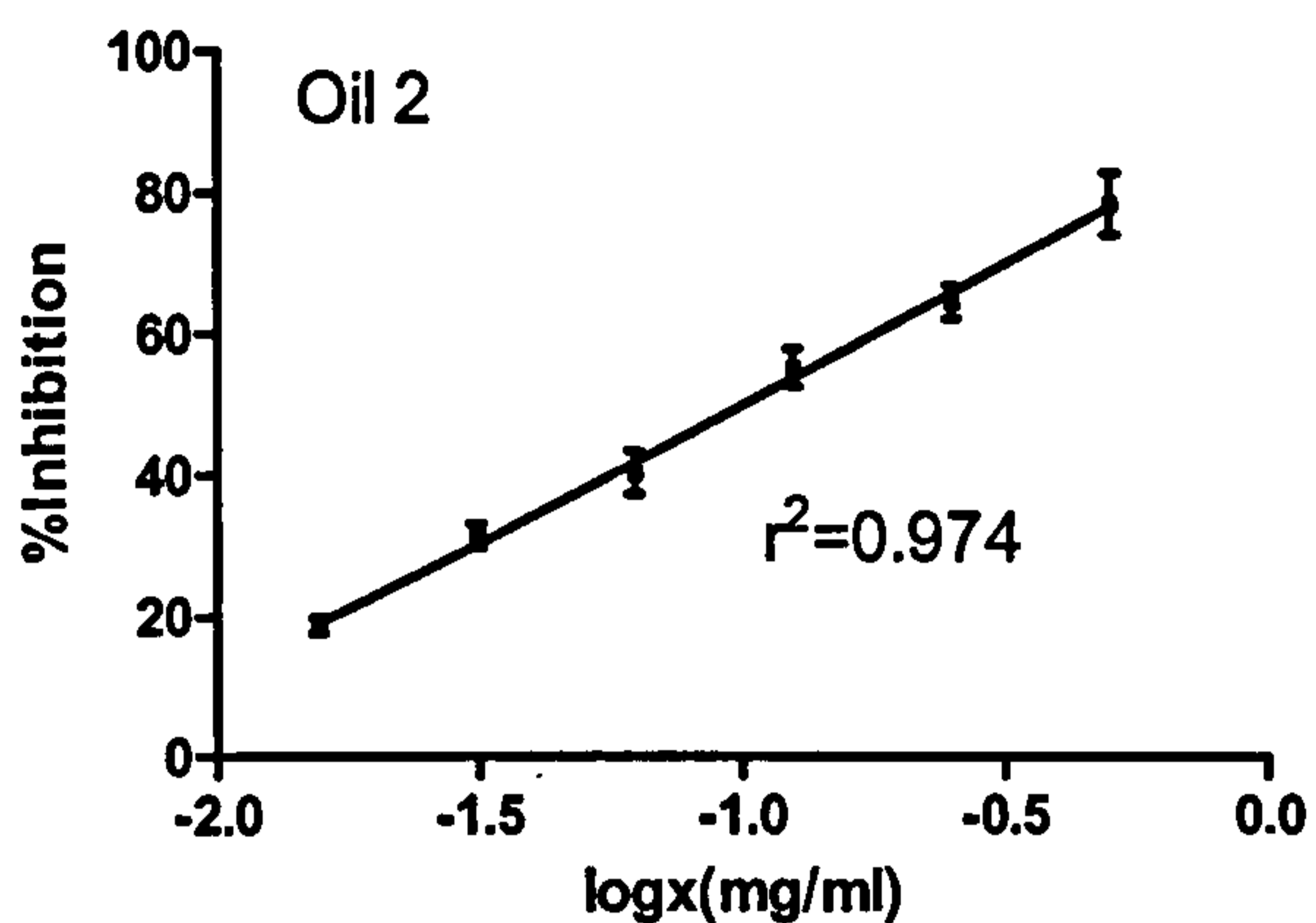


Figure 2.1 Sigmoidal dose response curves for acetylcholinesterase (AChE; human erythrocytes) inhibitory activity of *Salvia officinalis* var *purpuea* oils from plants raised hydroponically under three levels of phosphorus (P 12, 34 and 60 mg/L).



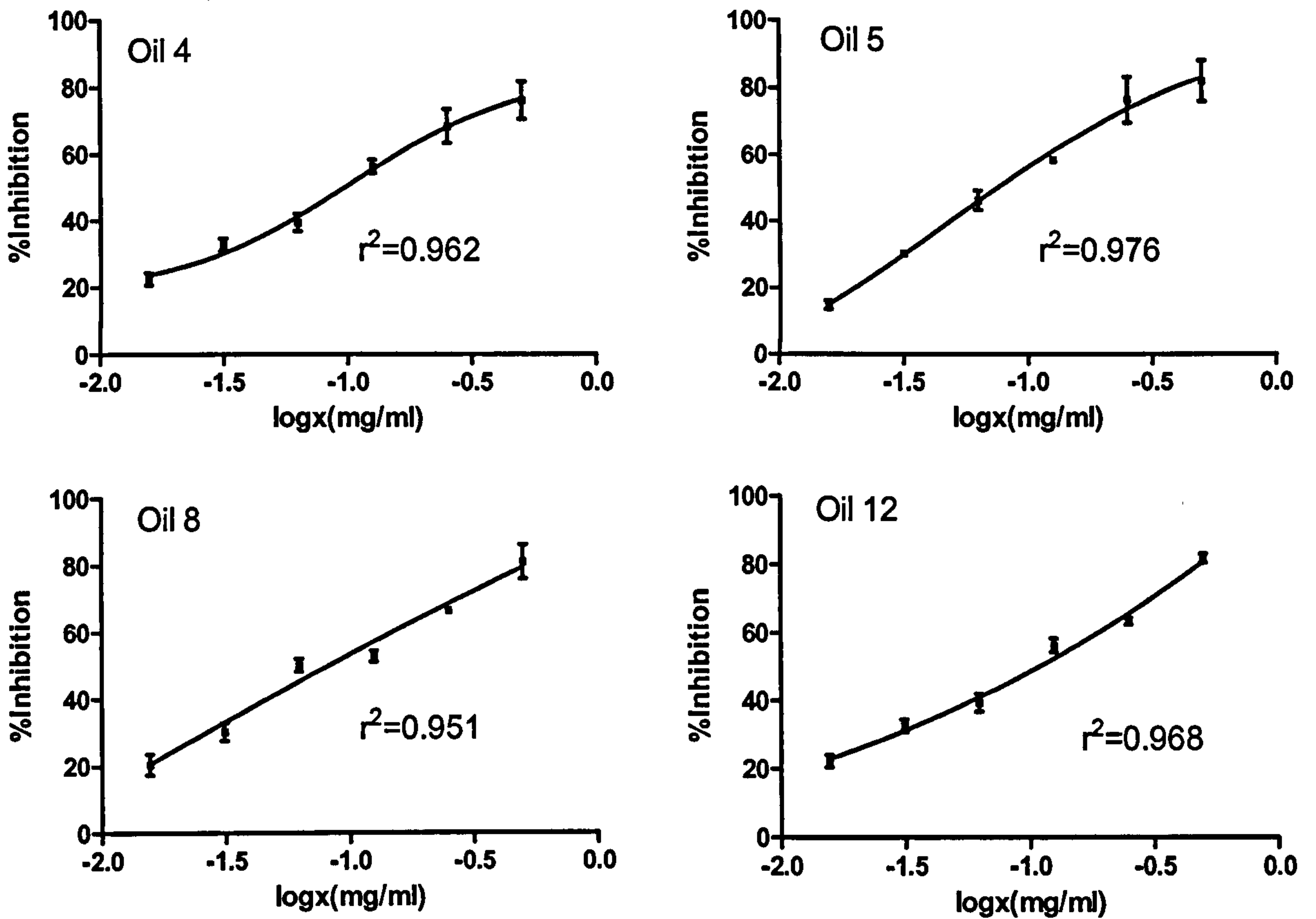
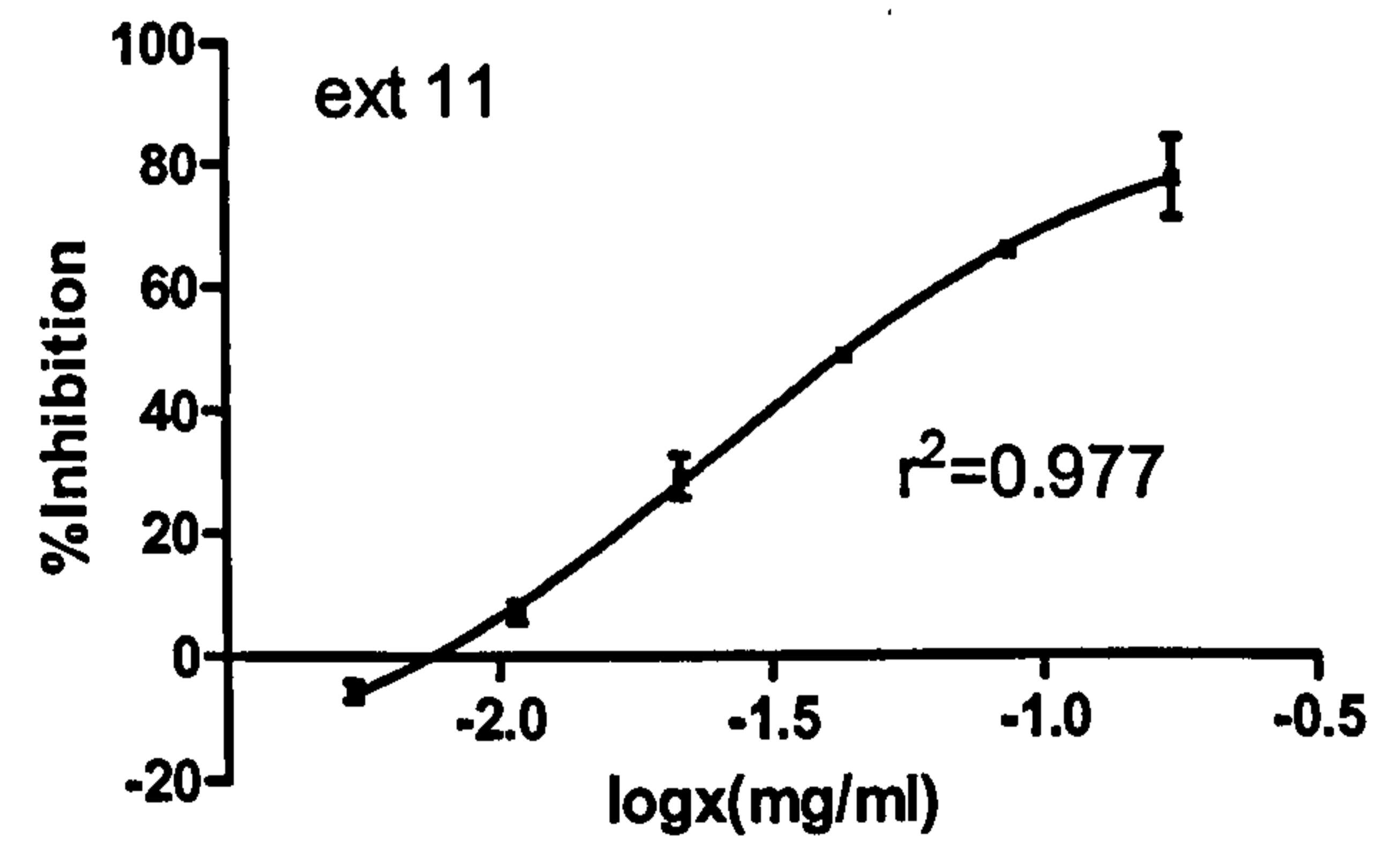
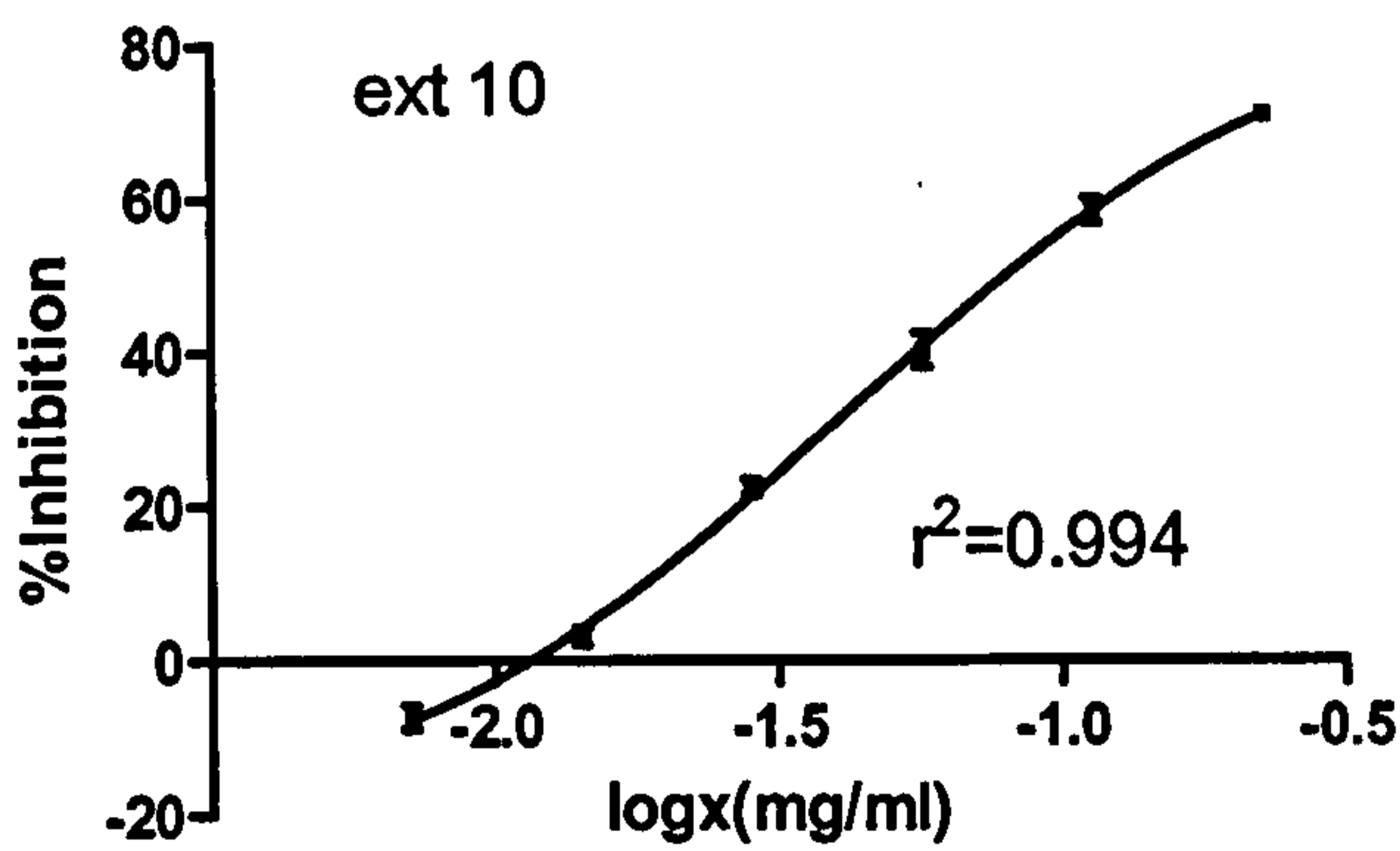
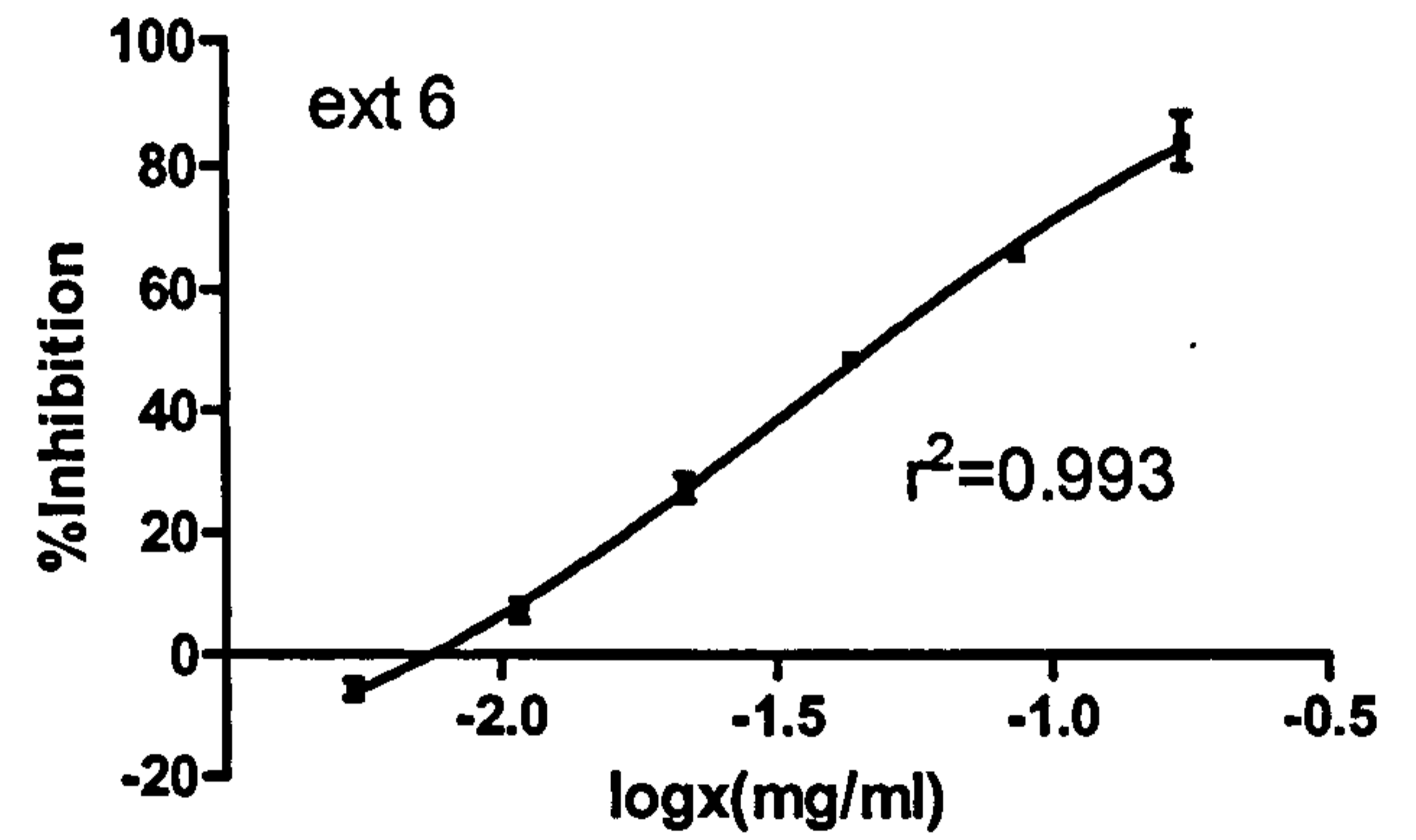
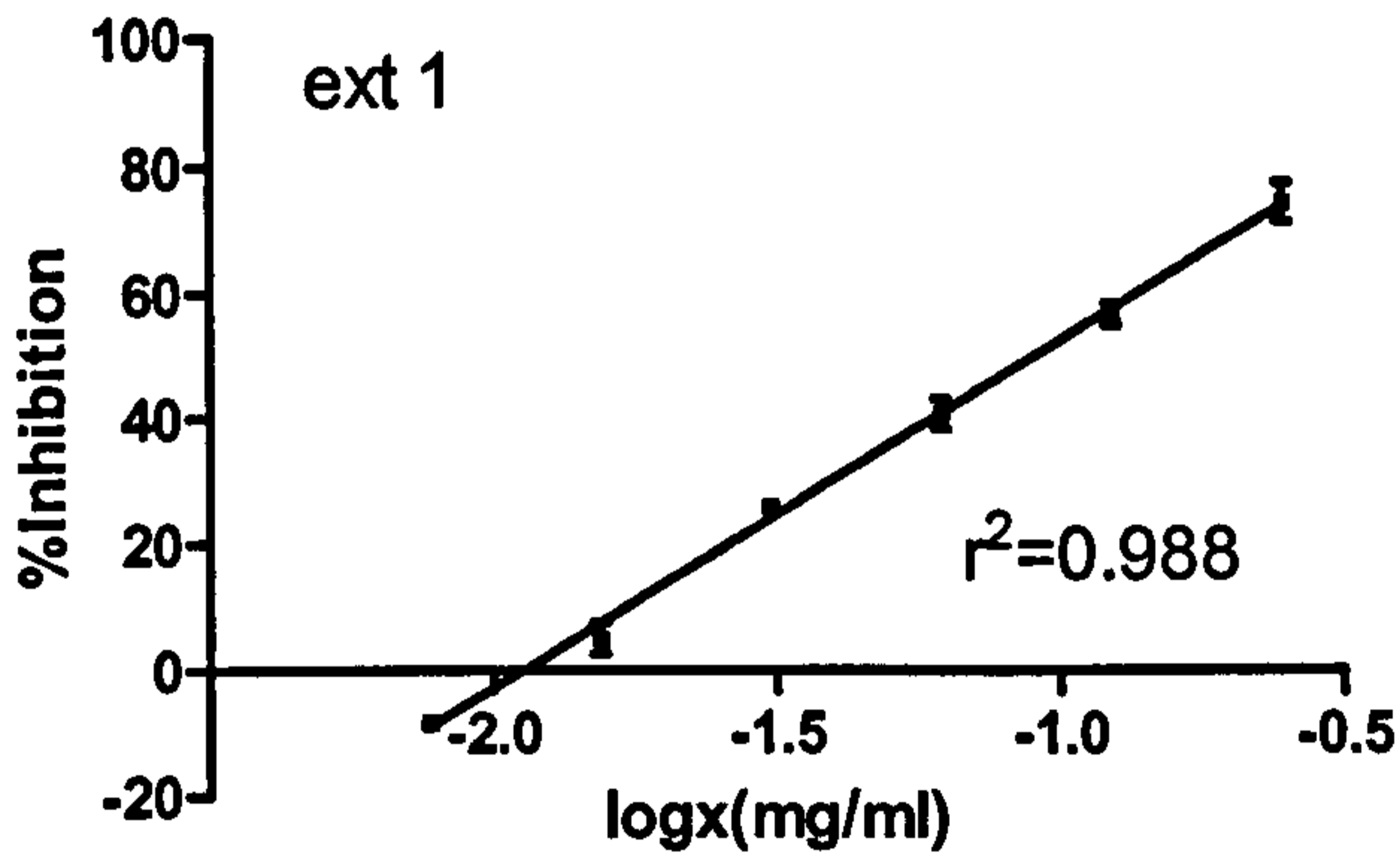
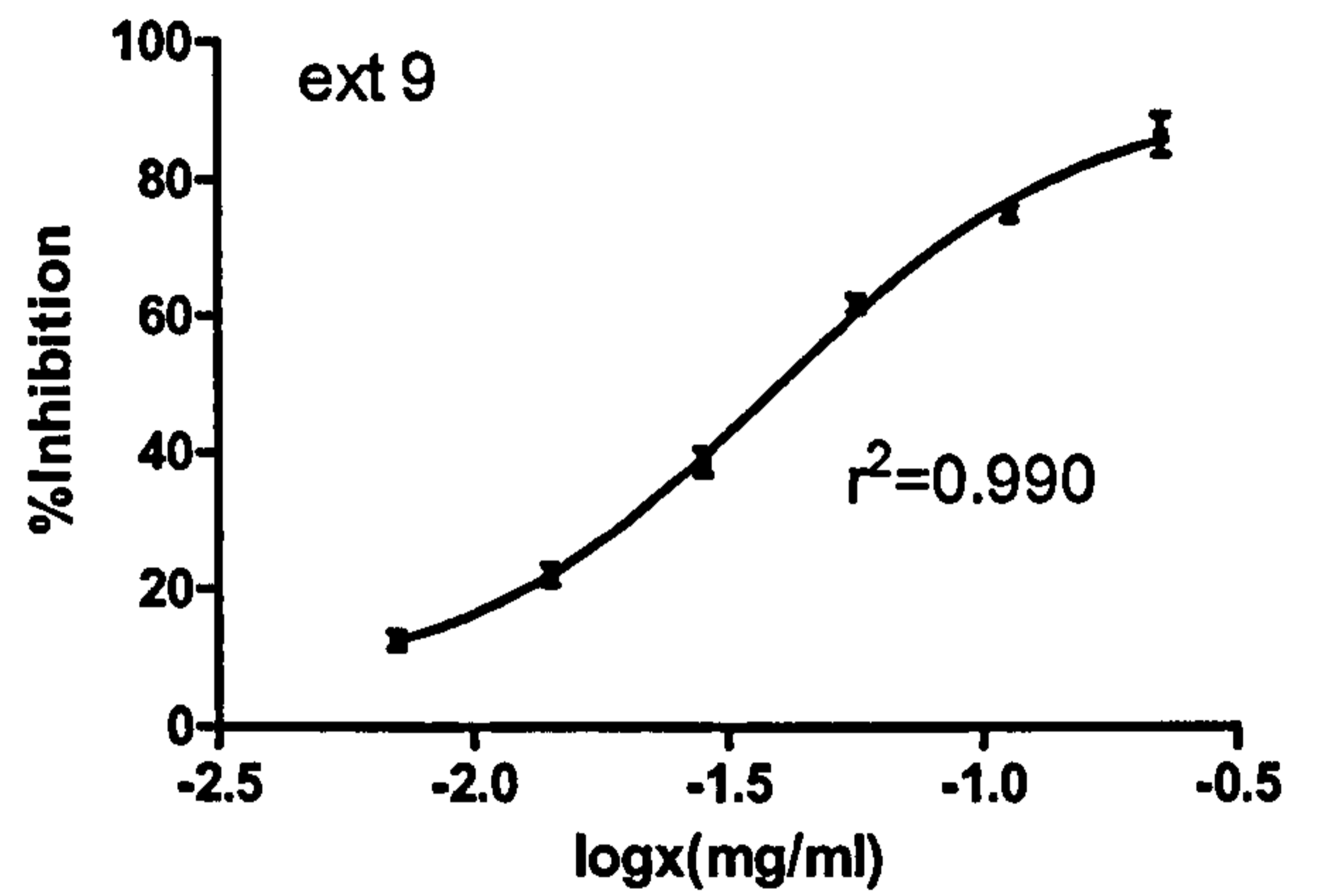
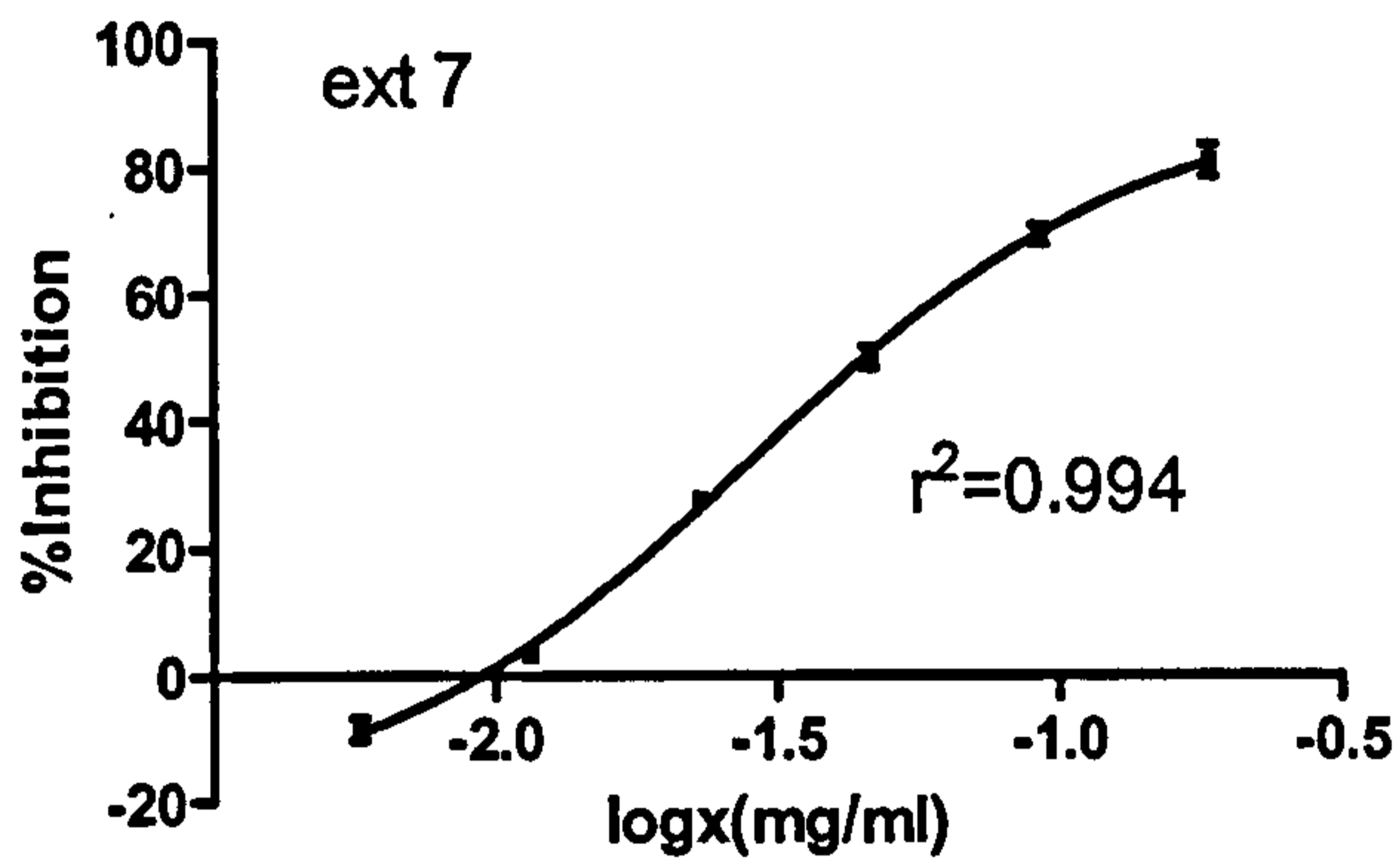
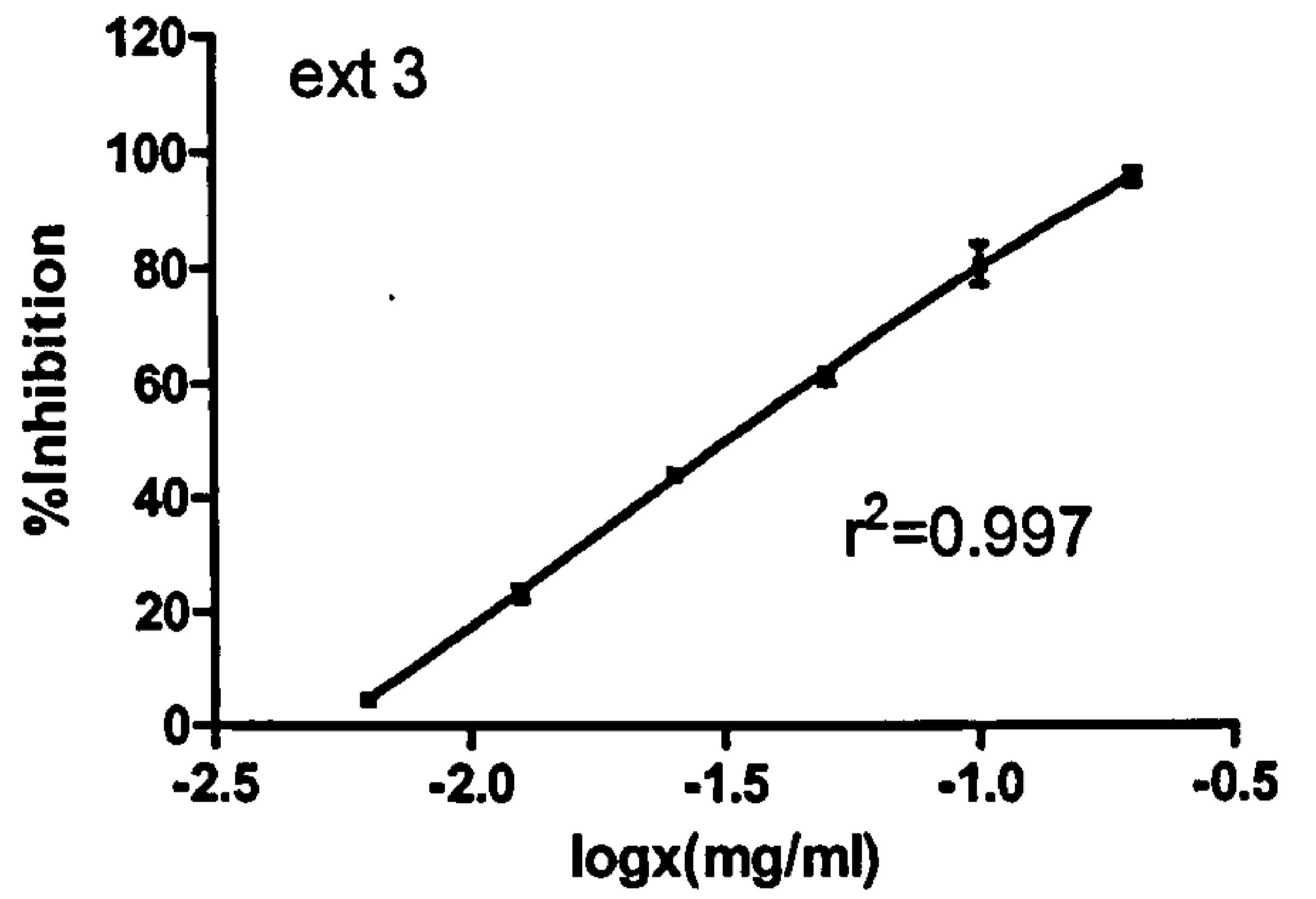
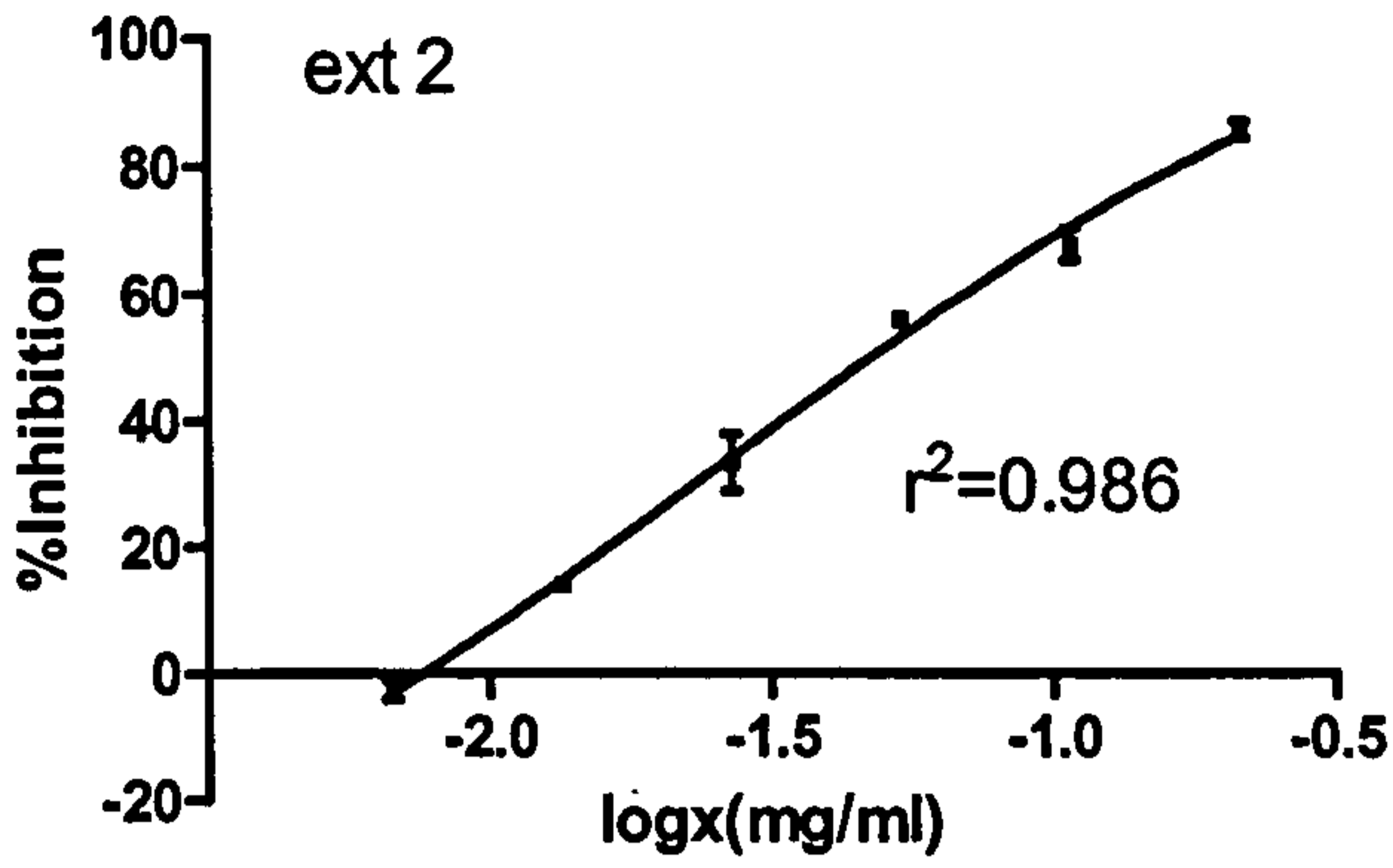


Figure 2.2 Sigmoidal dose response curves for butyrylcholinesterase (BuChE from horse serum) inhibition by *Salvia officinalis* var *purpurea* oils from plants raised hydroponically under three levels of phosphorus (P 12, 34 and 60 mg/L). BuChE enzyme was pre incubated with sage oils for a period 30 min.



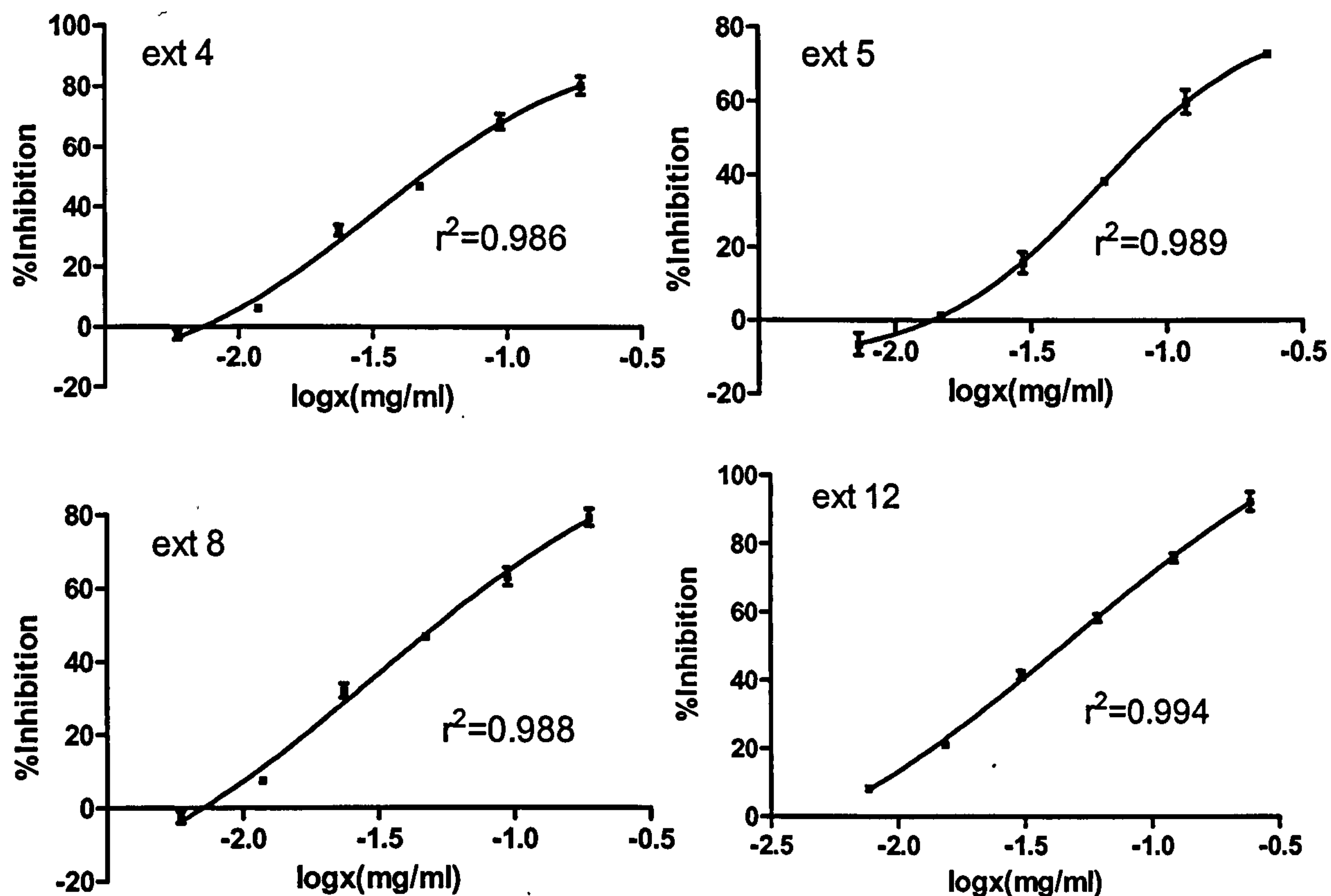
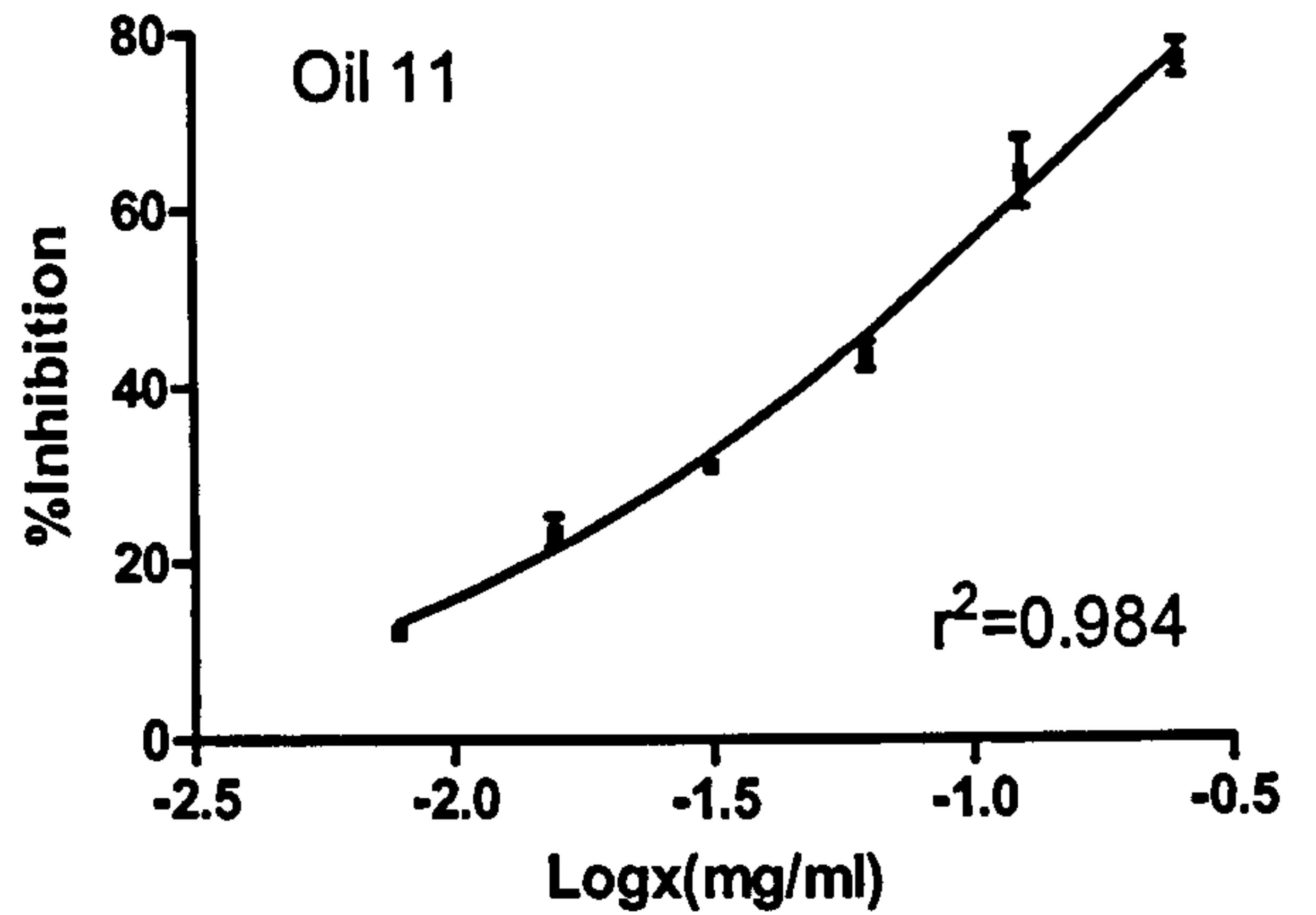
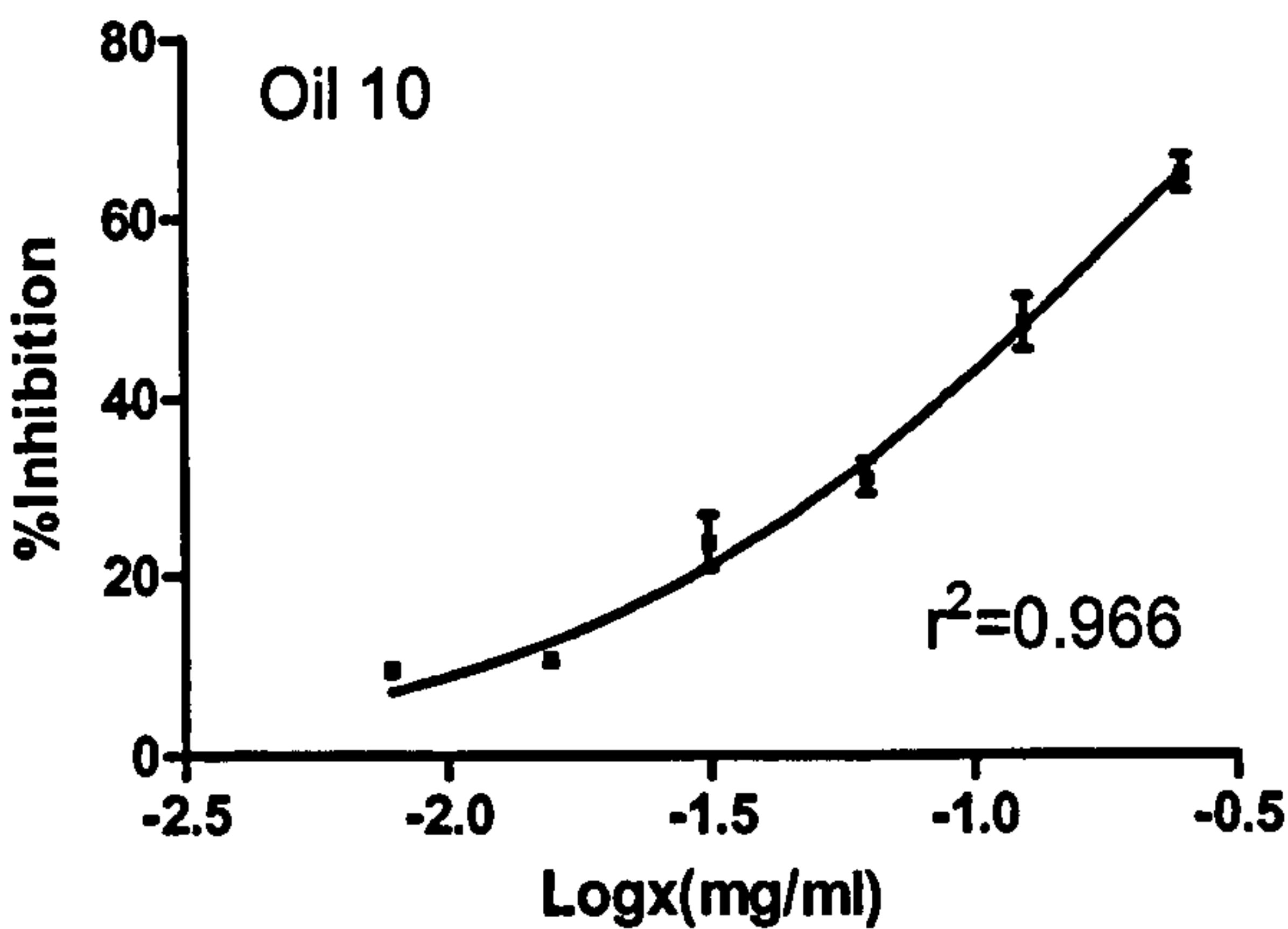
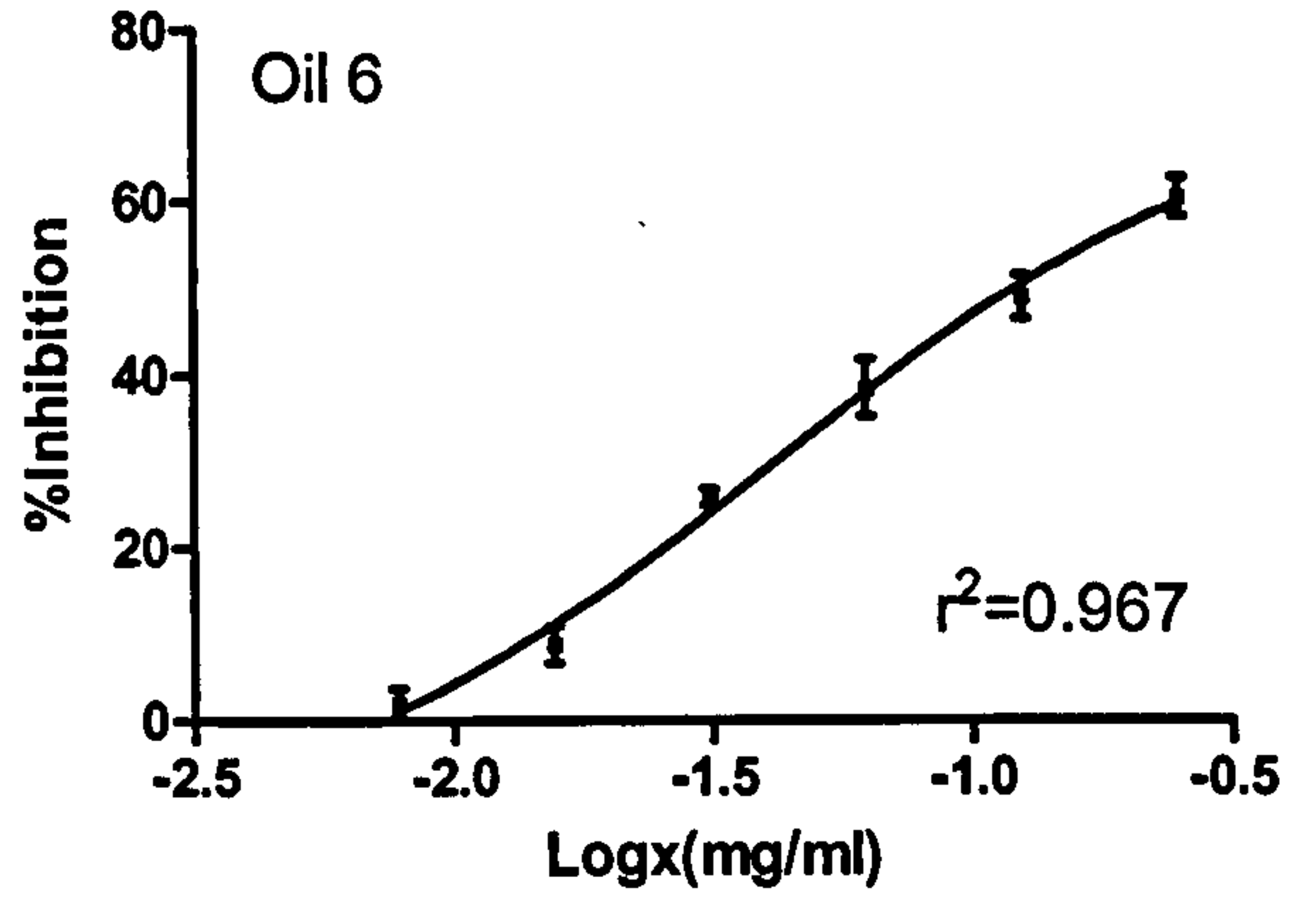
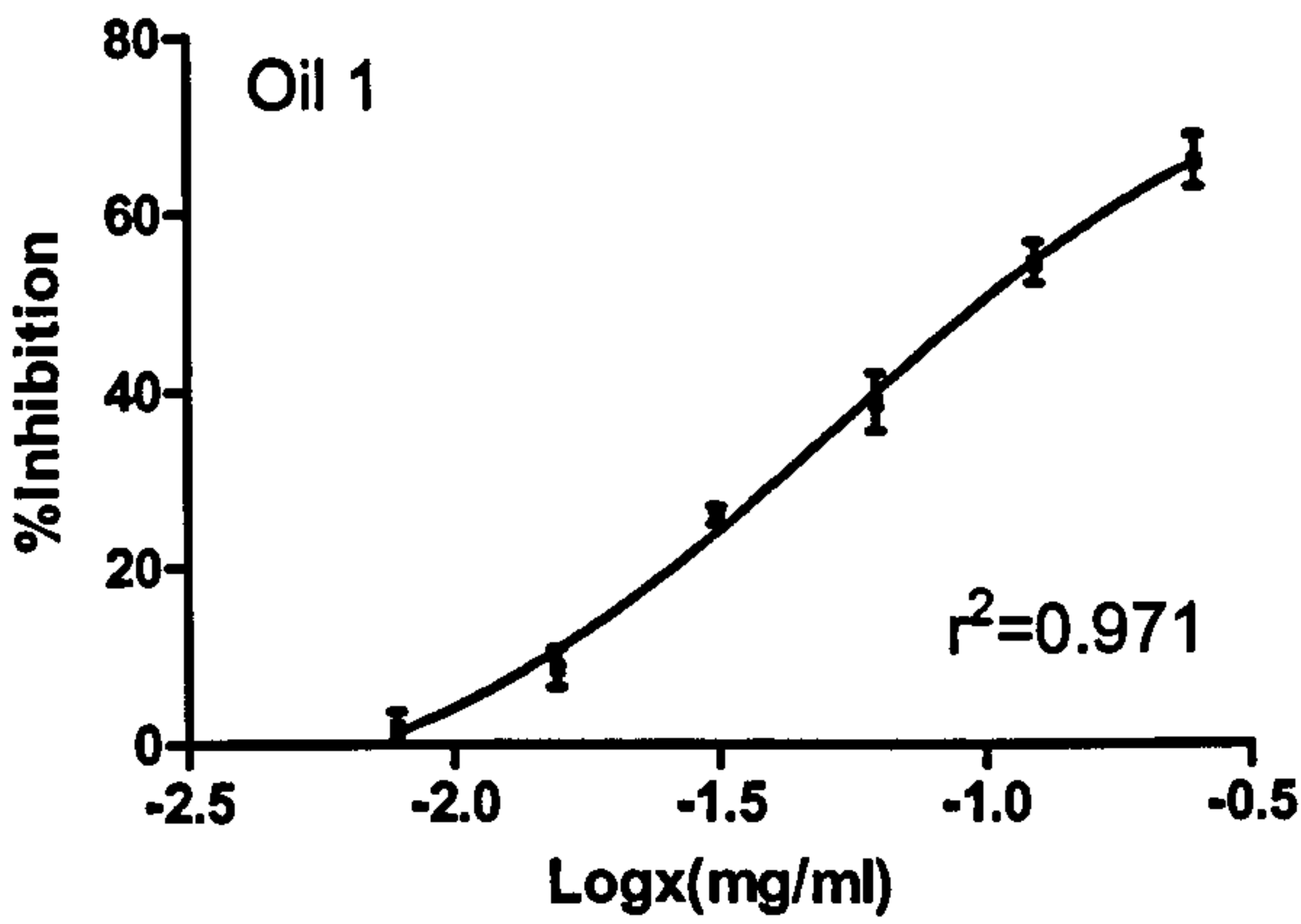
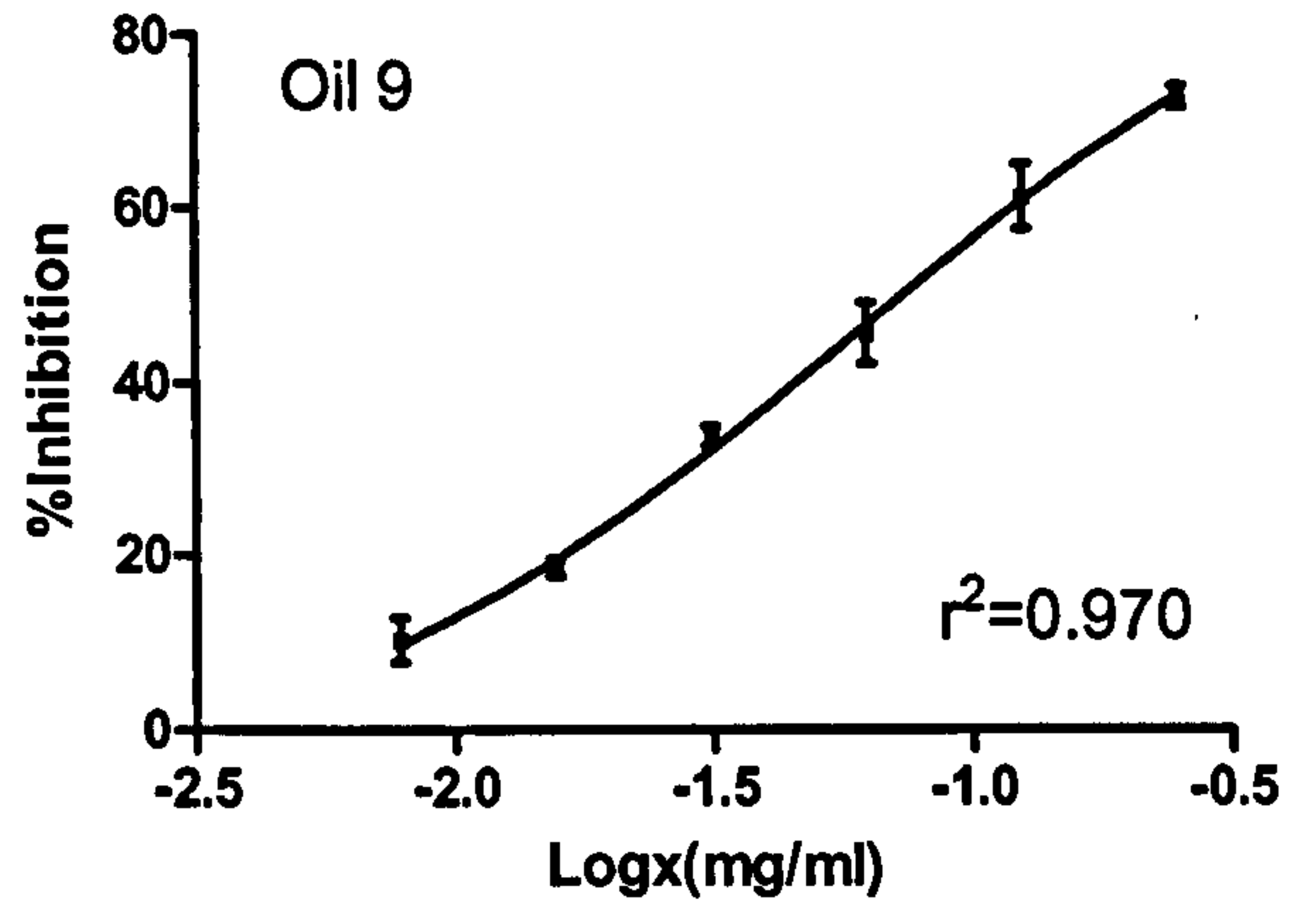
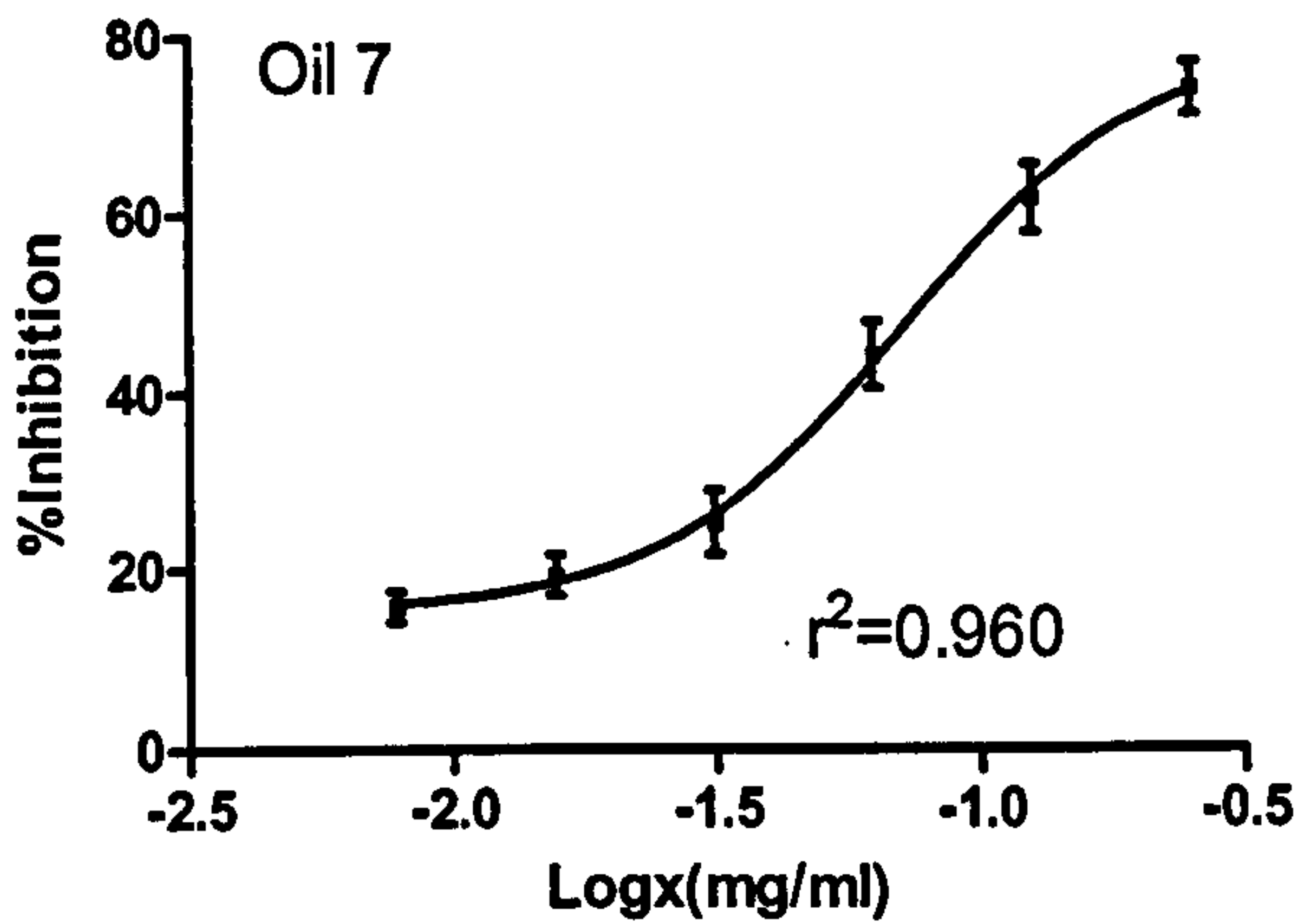
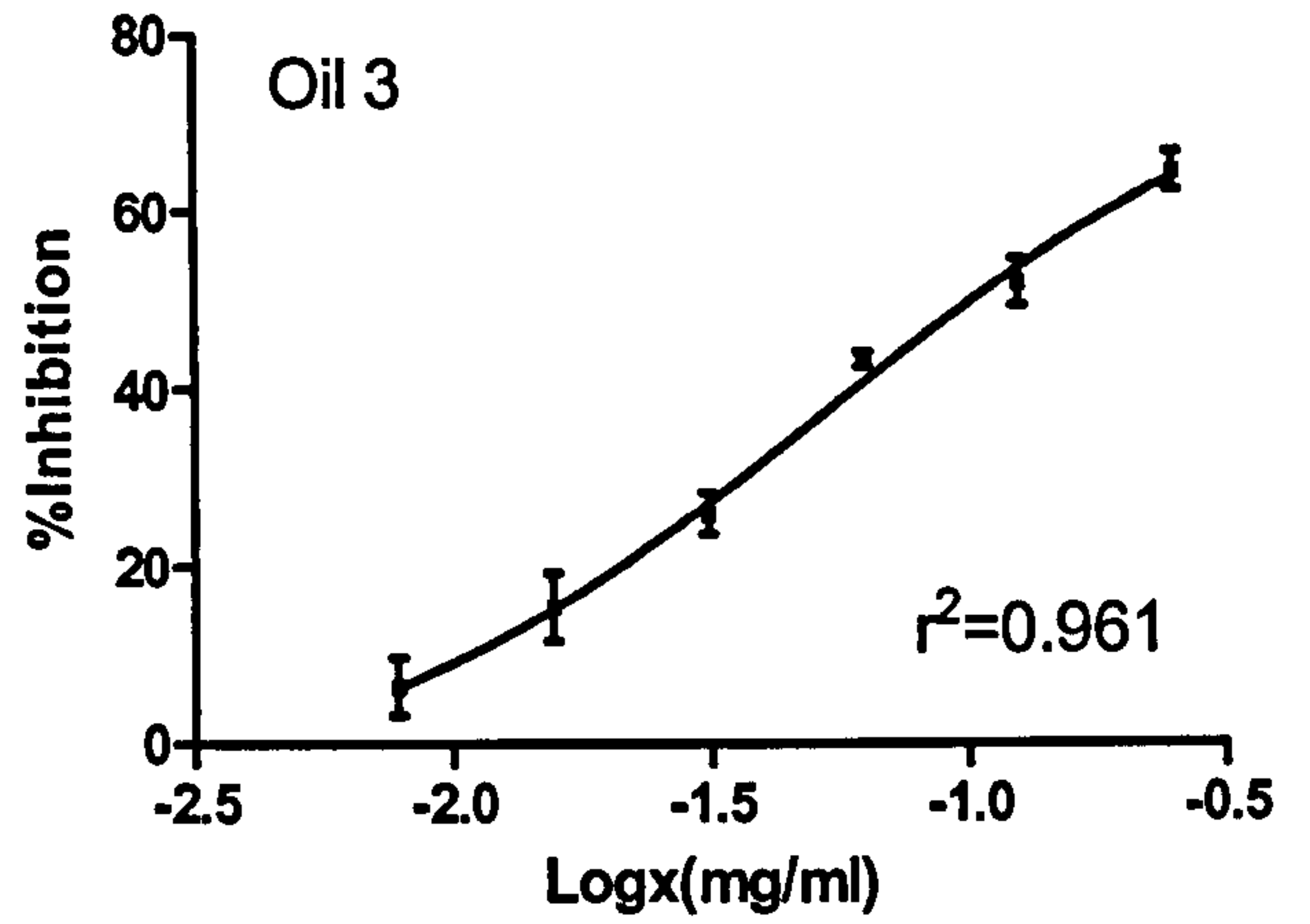
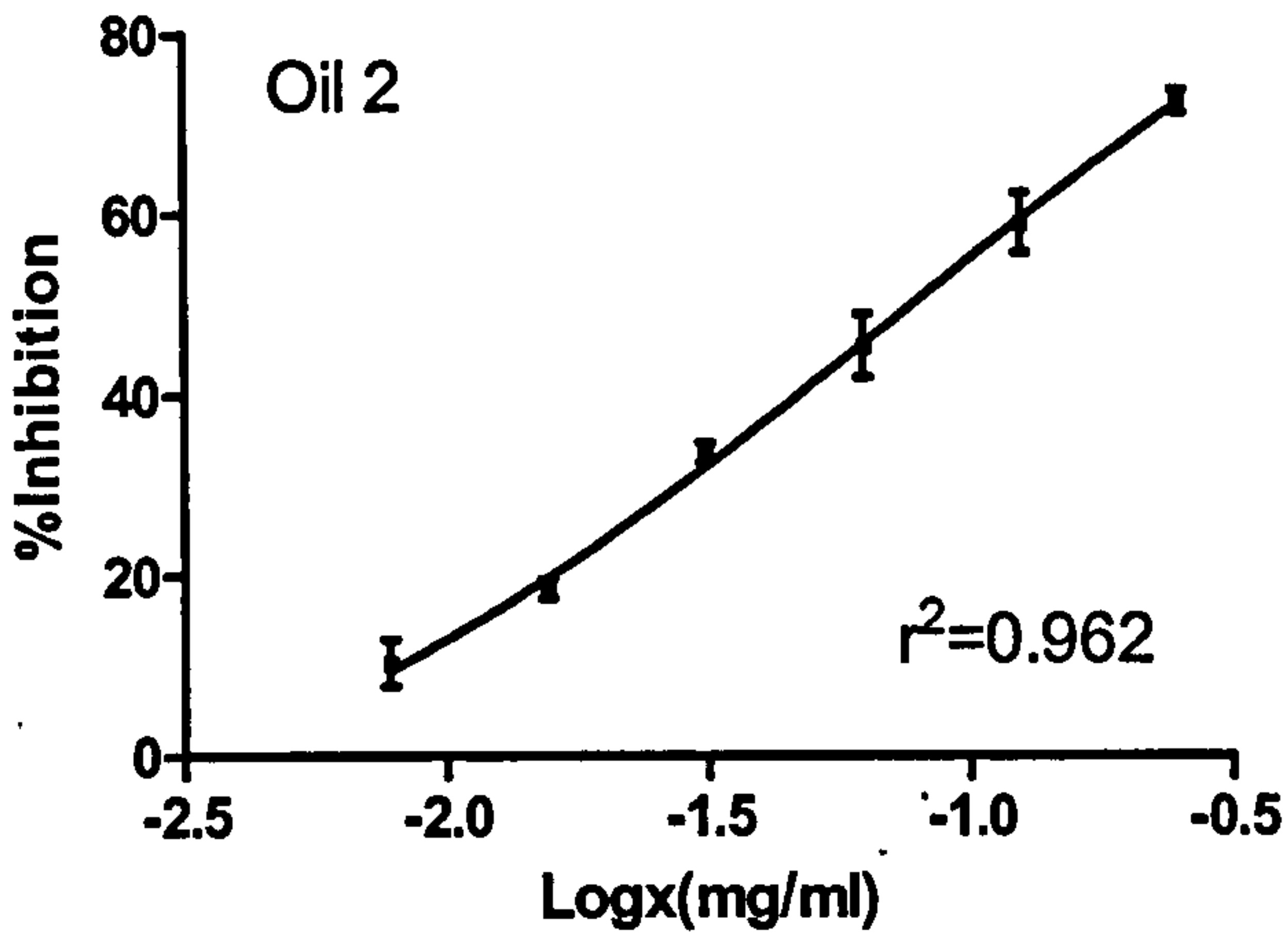


Figure 2.3 Sigmoidal dose response curves for butyrylcholinesterase (BuChE from horse serum) inhibition by *Salvia officinalis* var *purpuea* 80% ethanolic leaf extracts from plants raised hydroponically under three levels of phosphorus (P 12, 34 and 60 mg/L). BuChE enzyme was pre incubated with sage extracts for a period 30 min.

2.2 *Salvia officinalis* var *purpurea* anti-cholinesterase activity data from Chapter 4



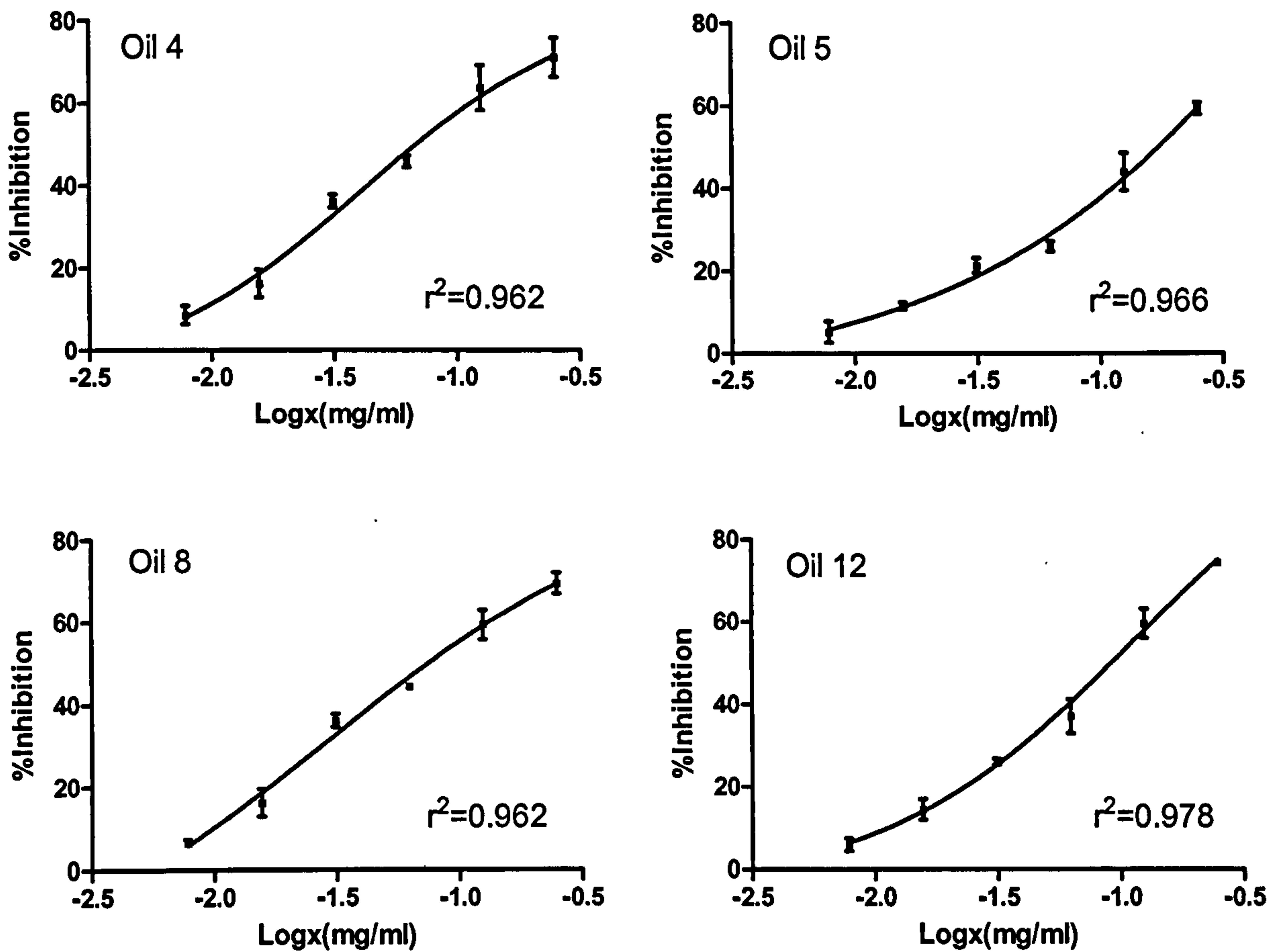
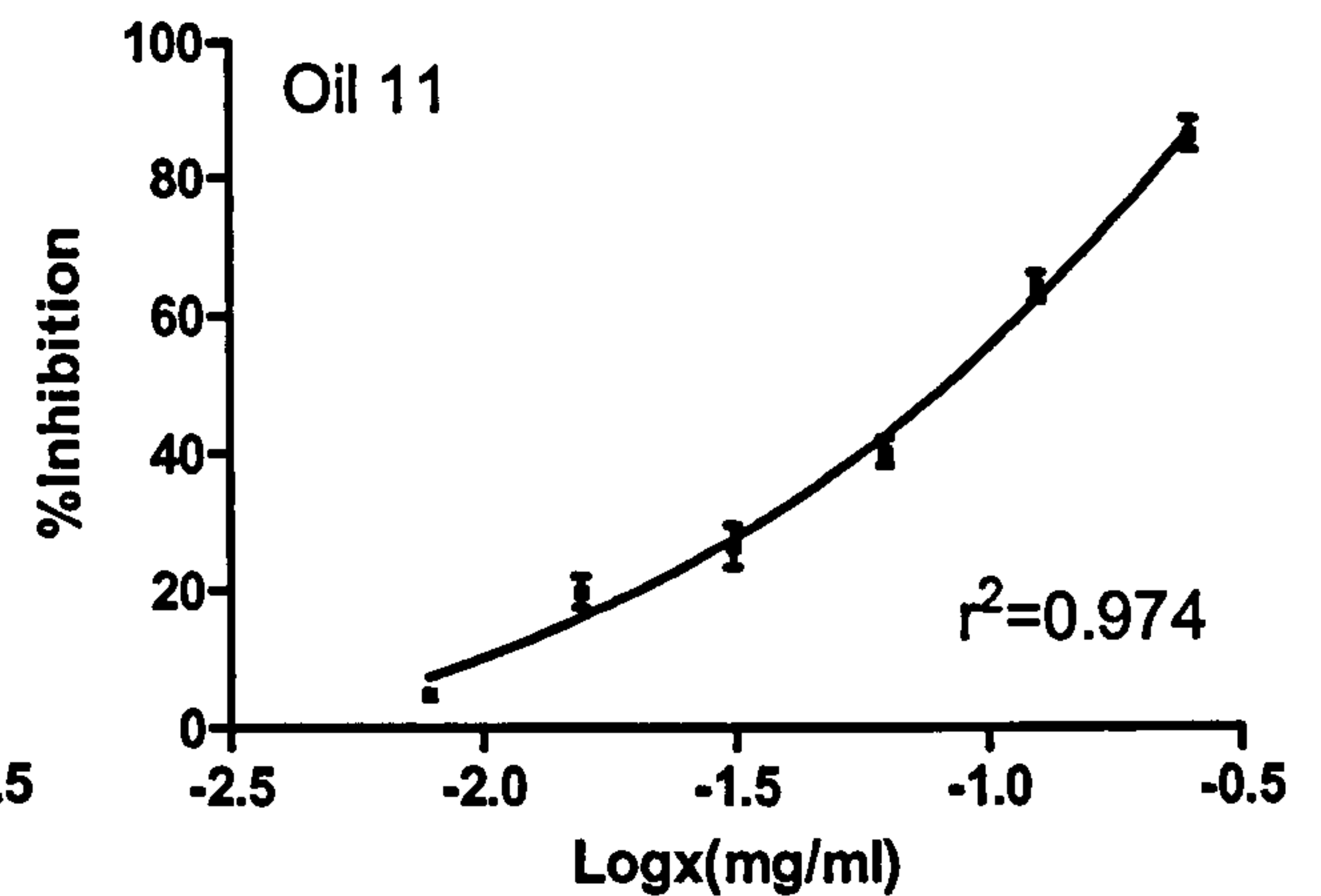
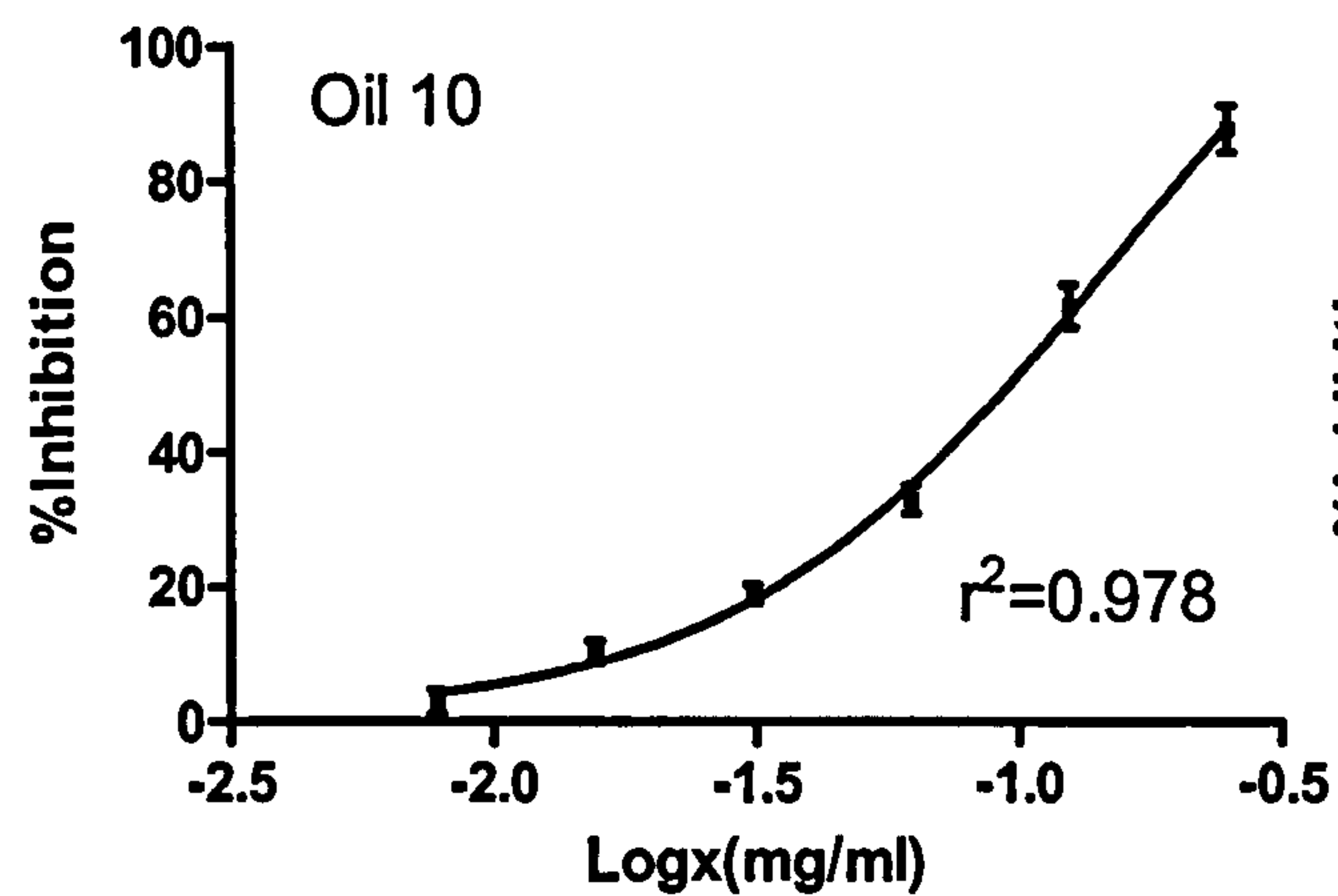
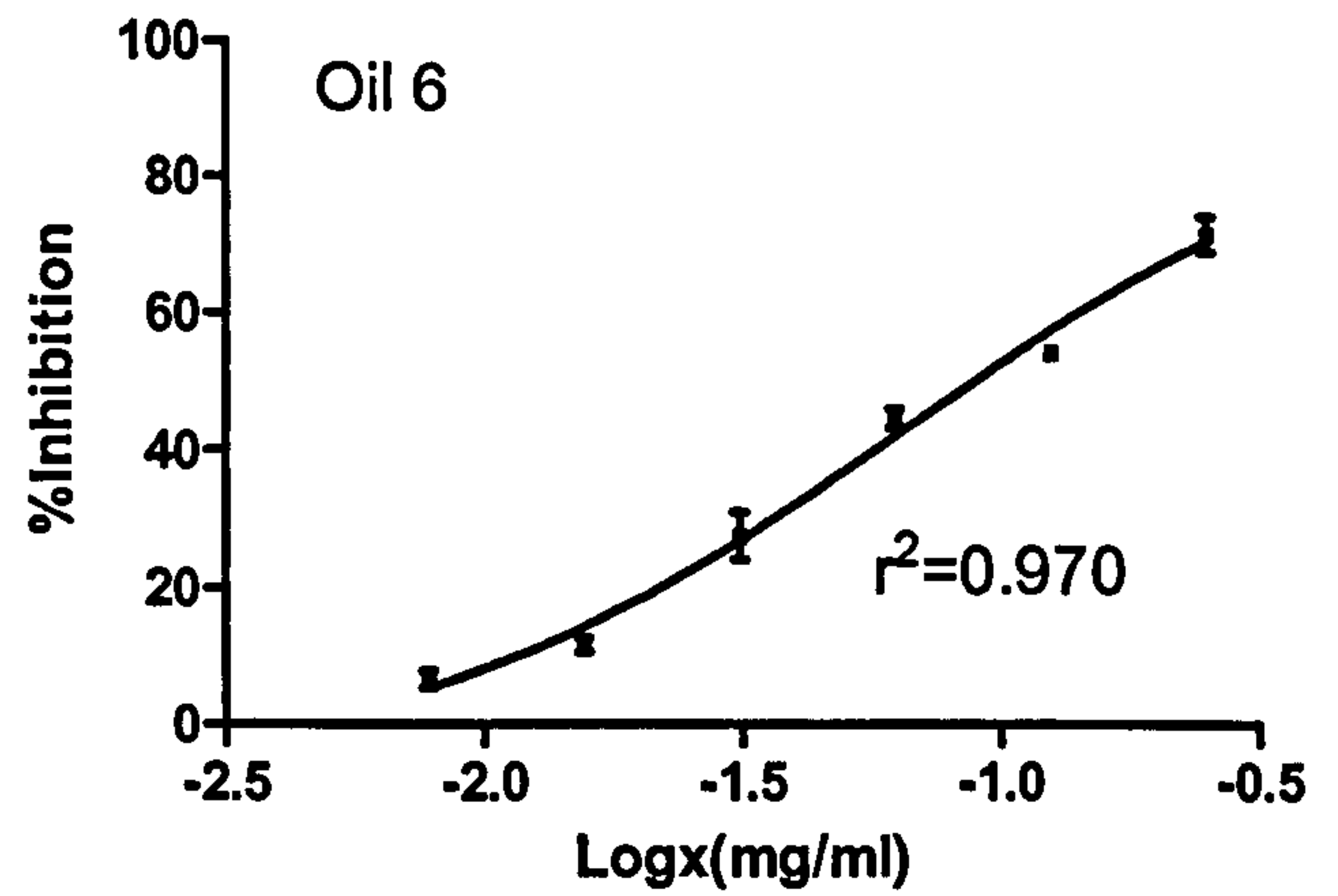
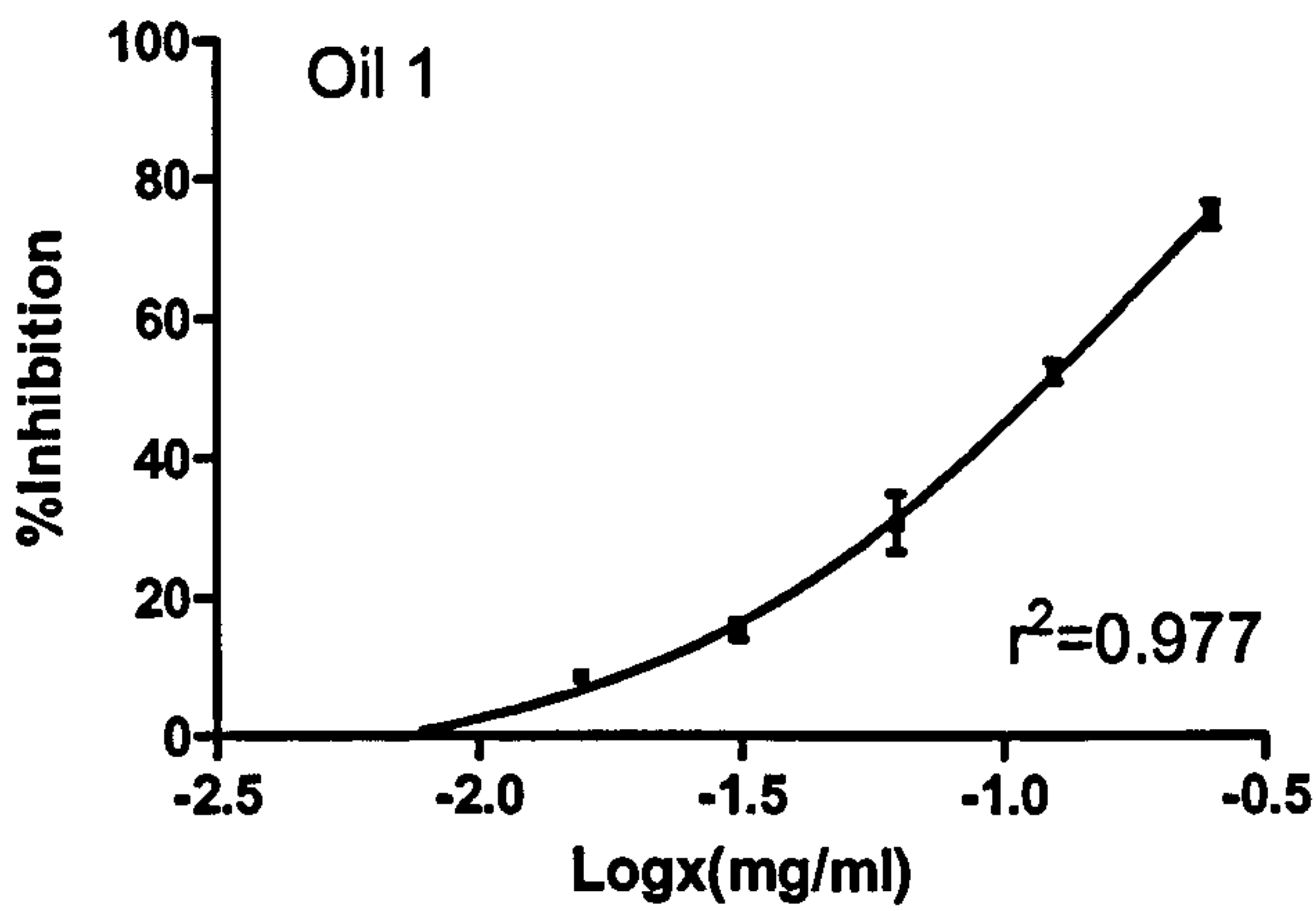
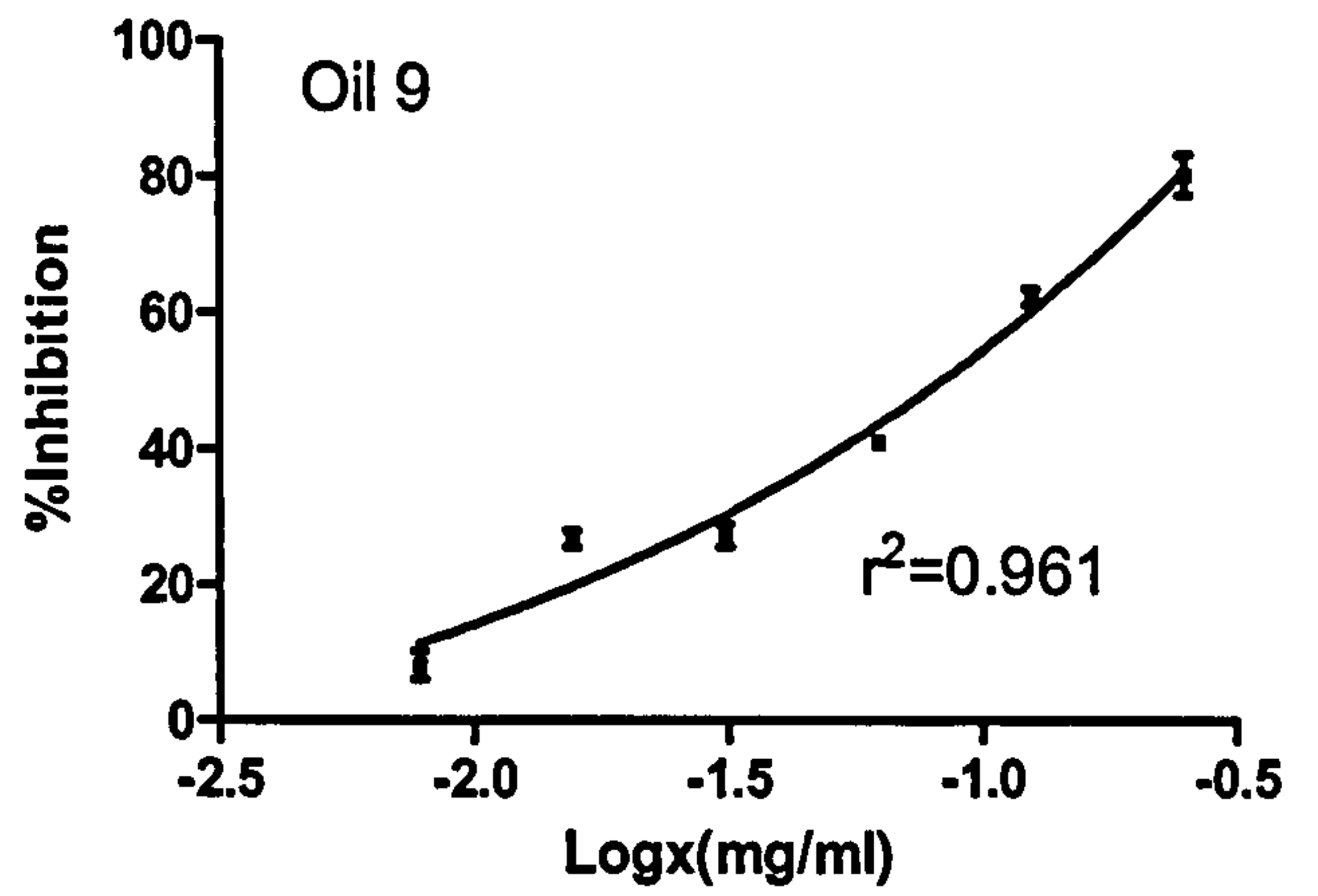
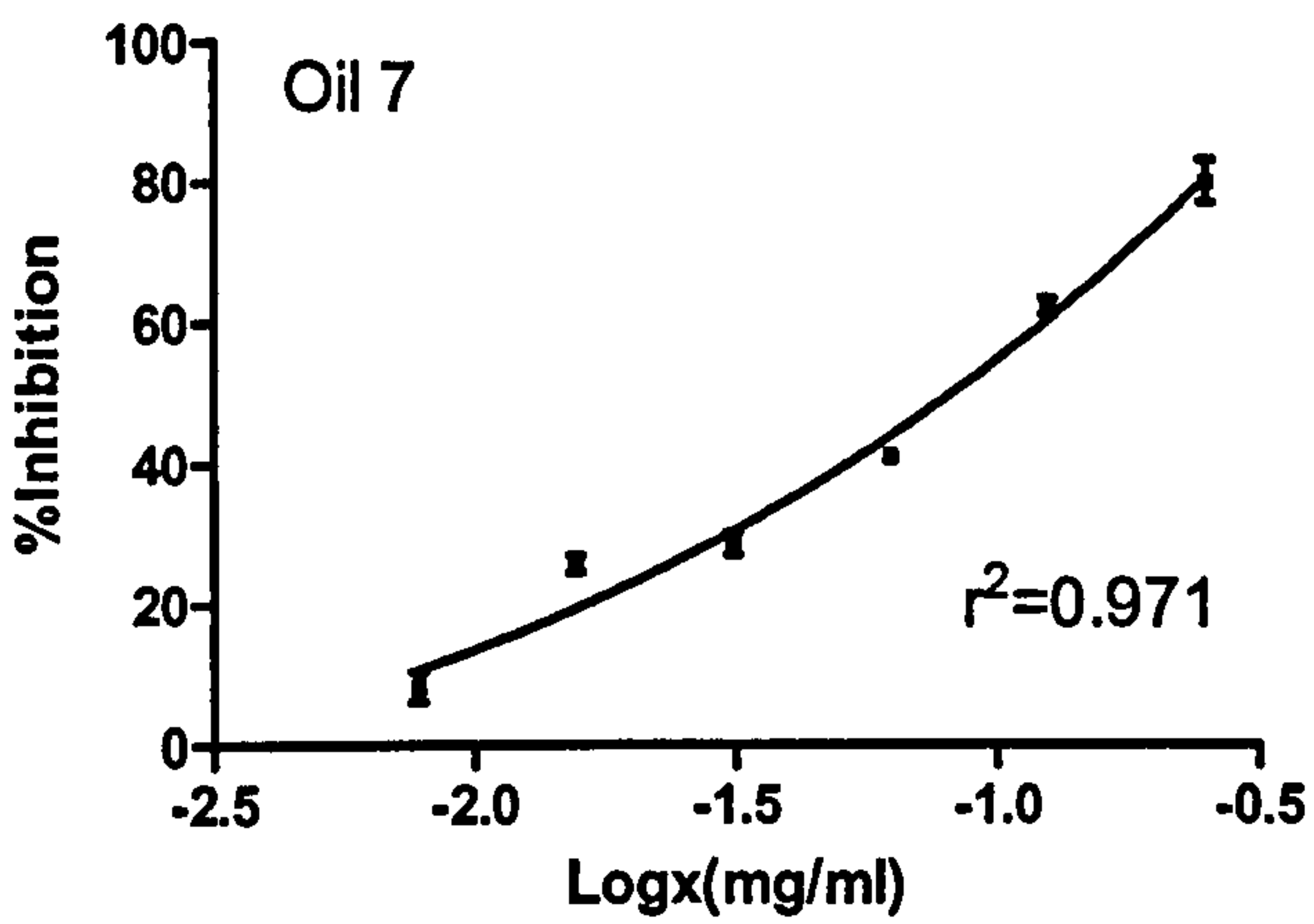
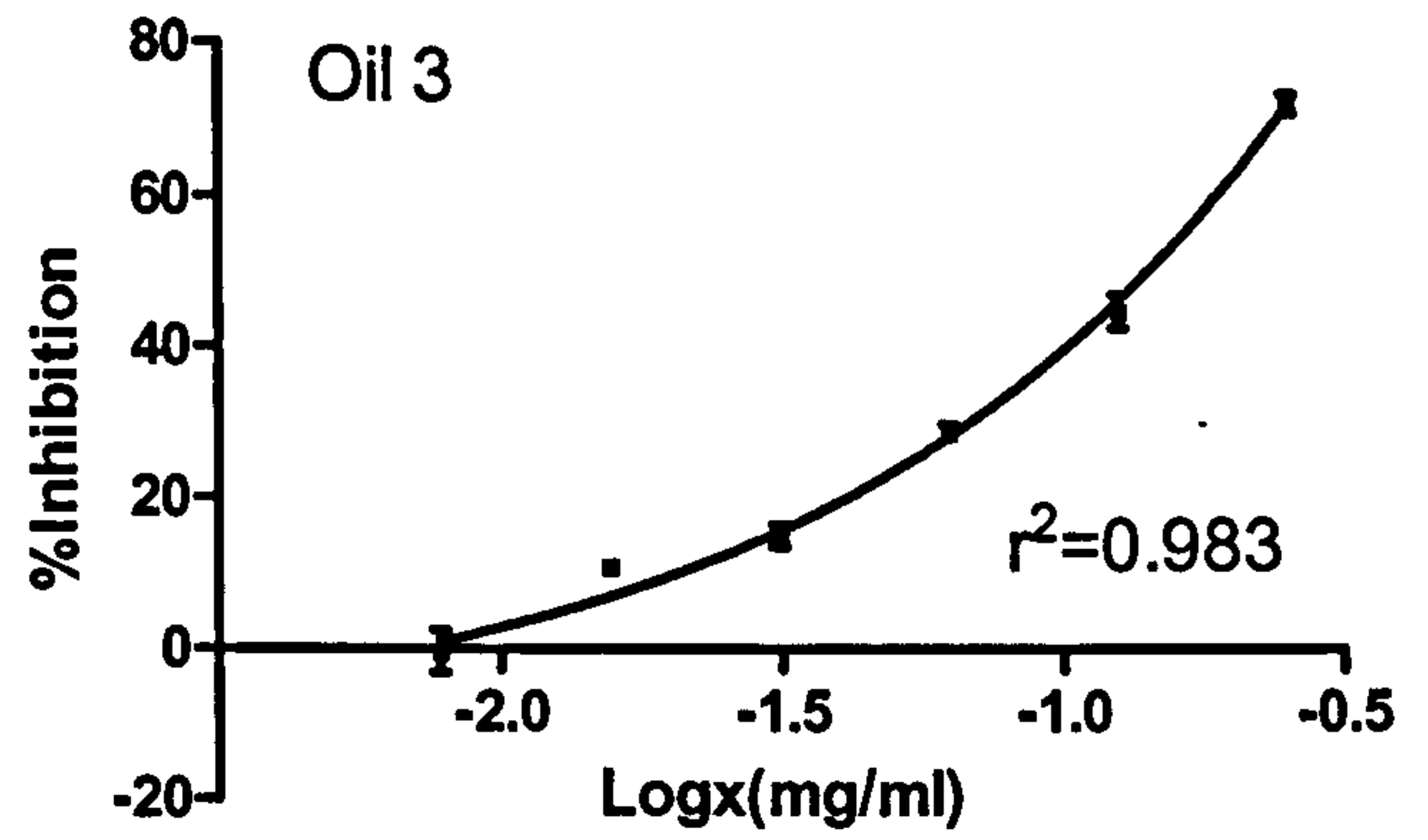
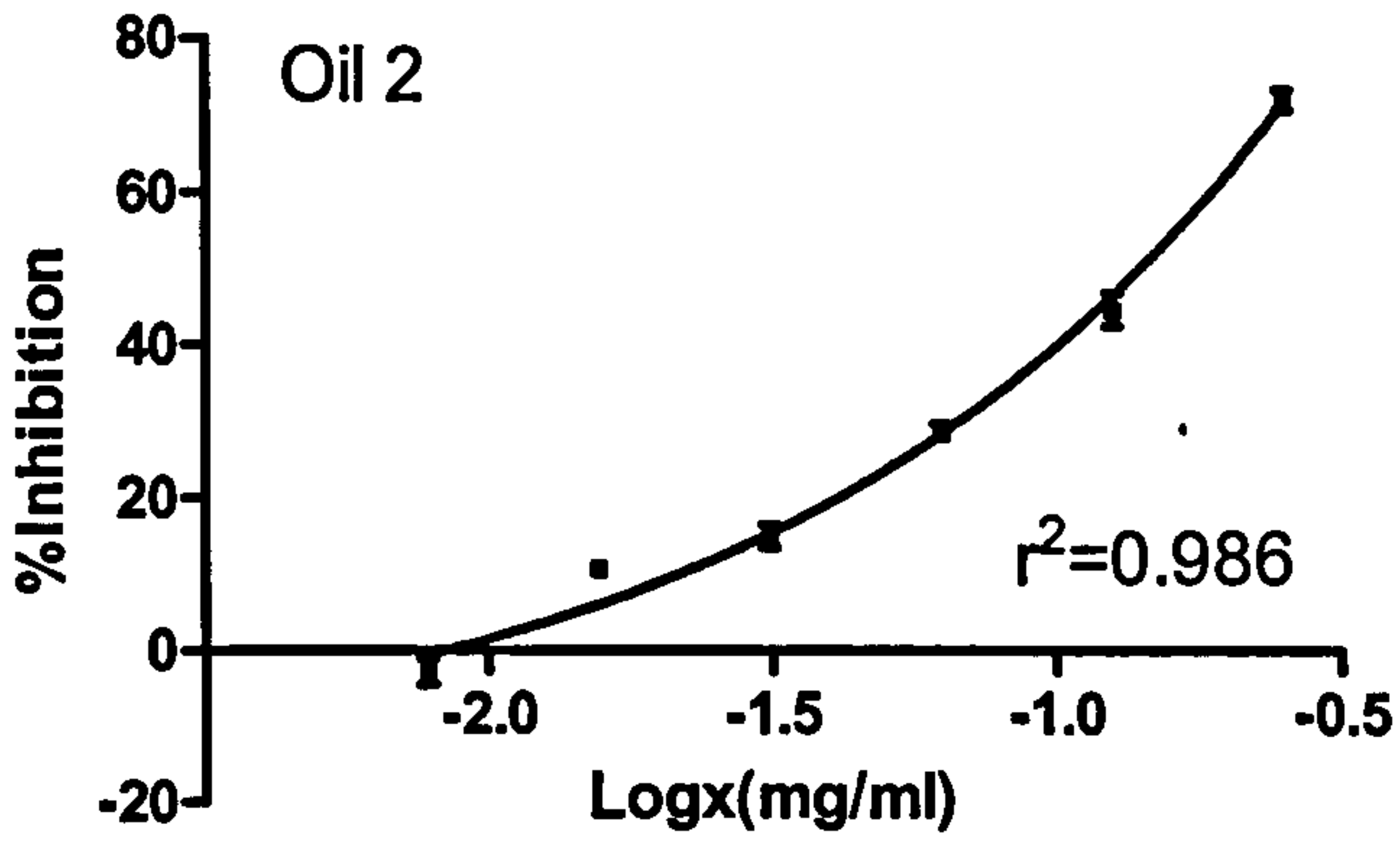


Figure 2.4 Sigmoidal dose response curves for bovine acetylcholinesterase (AChE) inhibition by *Salvia officinalis* var *purpuea* oils from plants raised hydroponically under three levels of nitrates (N-NO₃ 100, 150 and 200 mg/L).



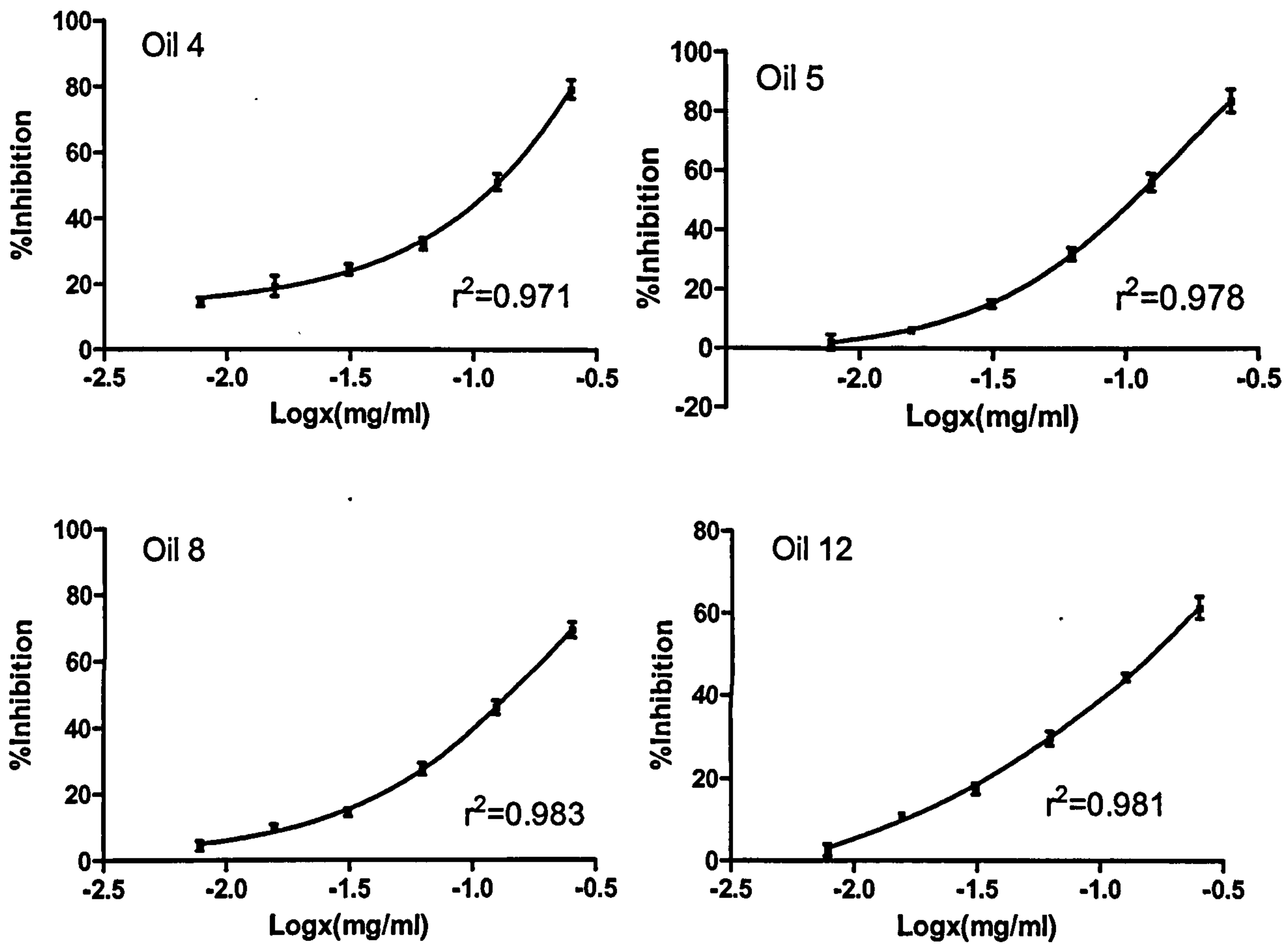
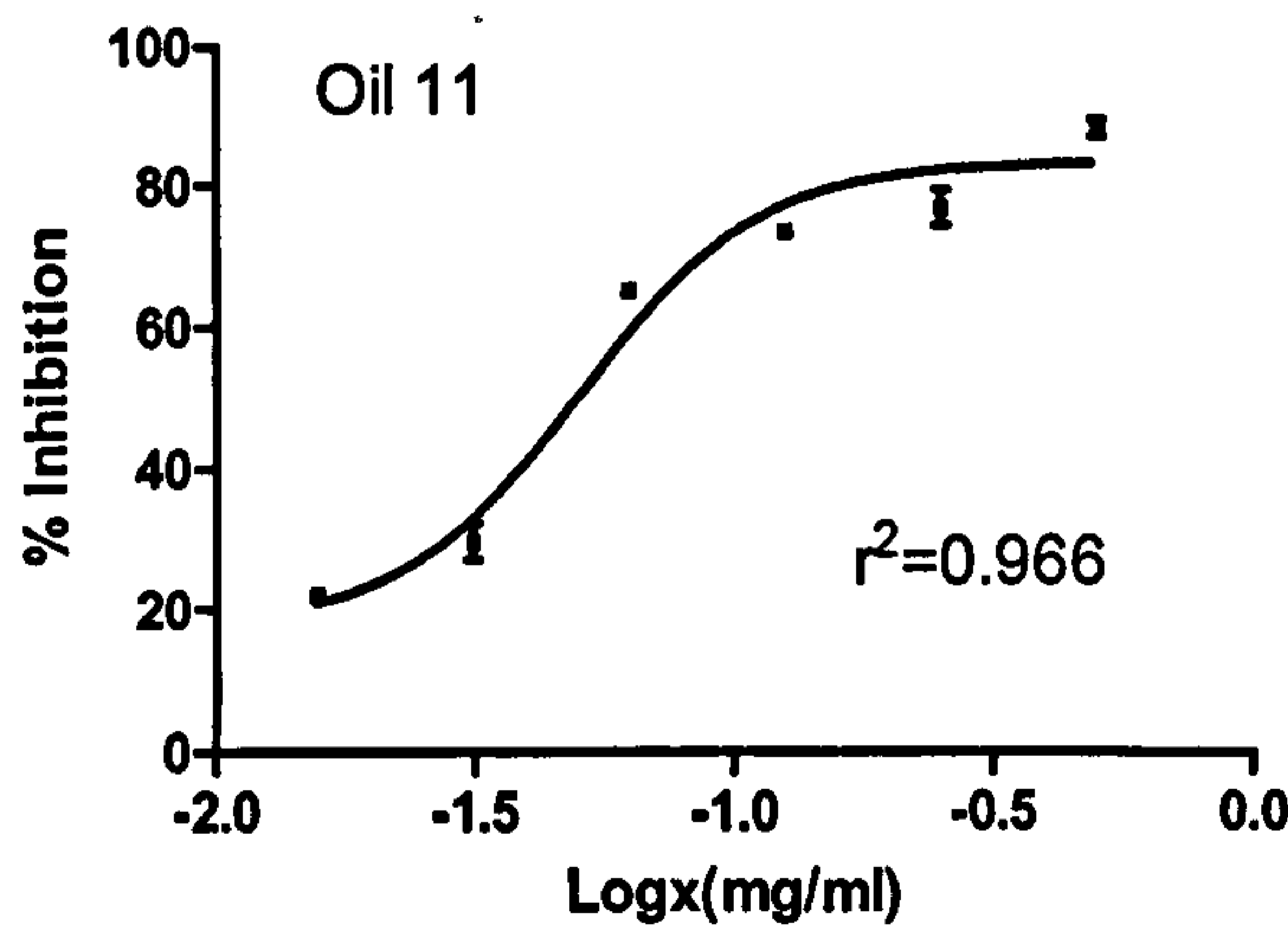
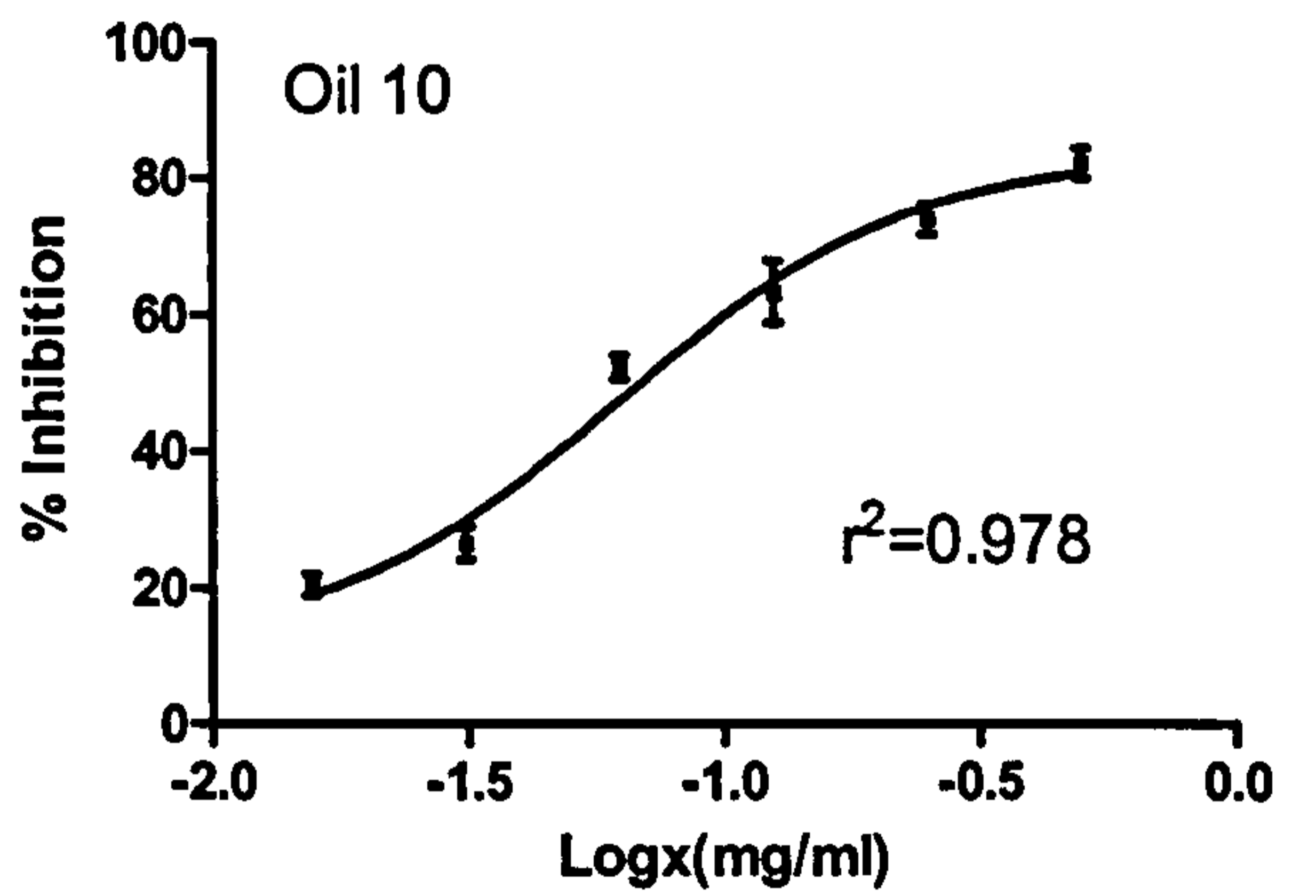
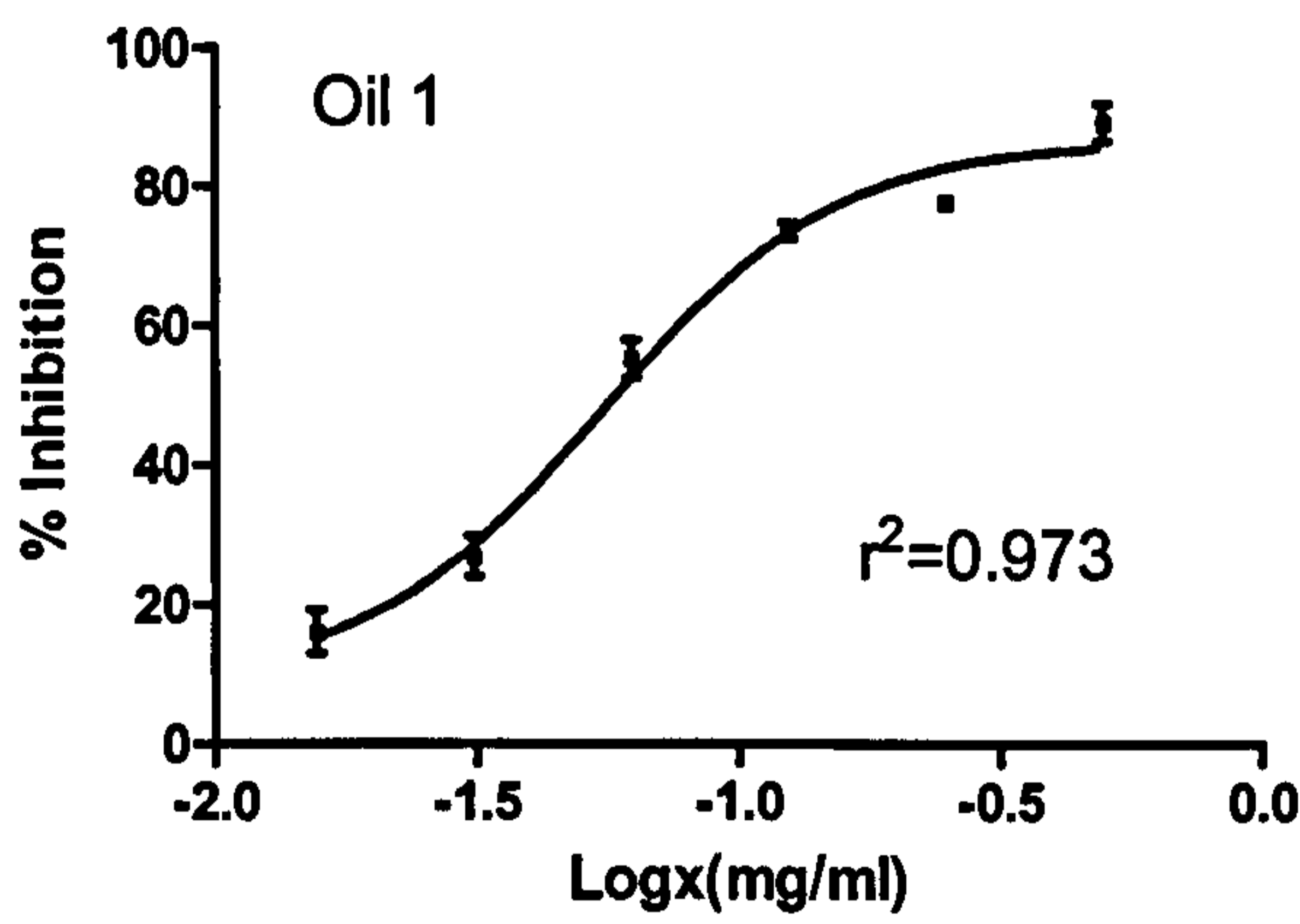
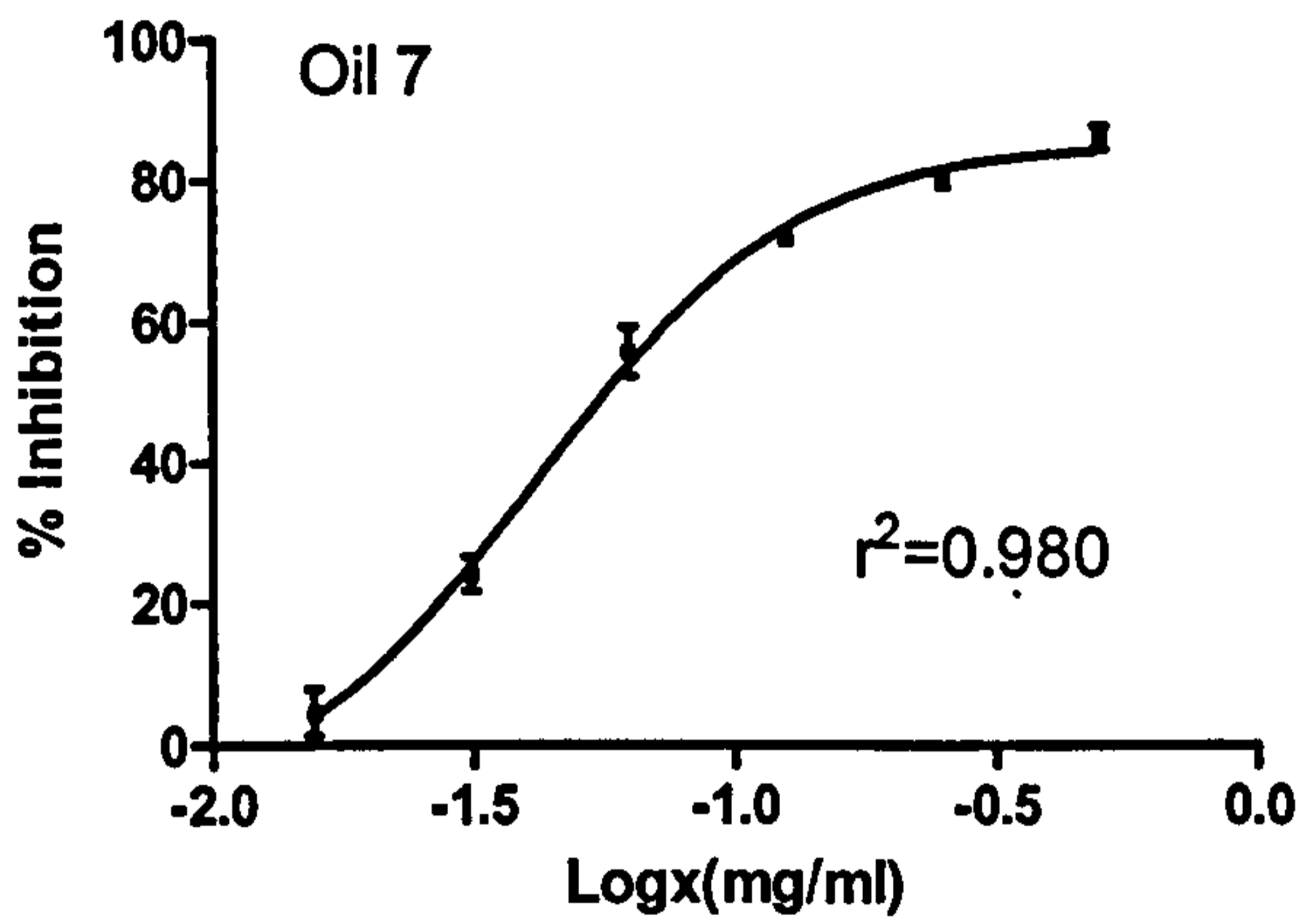
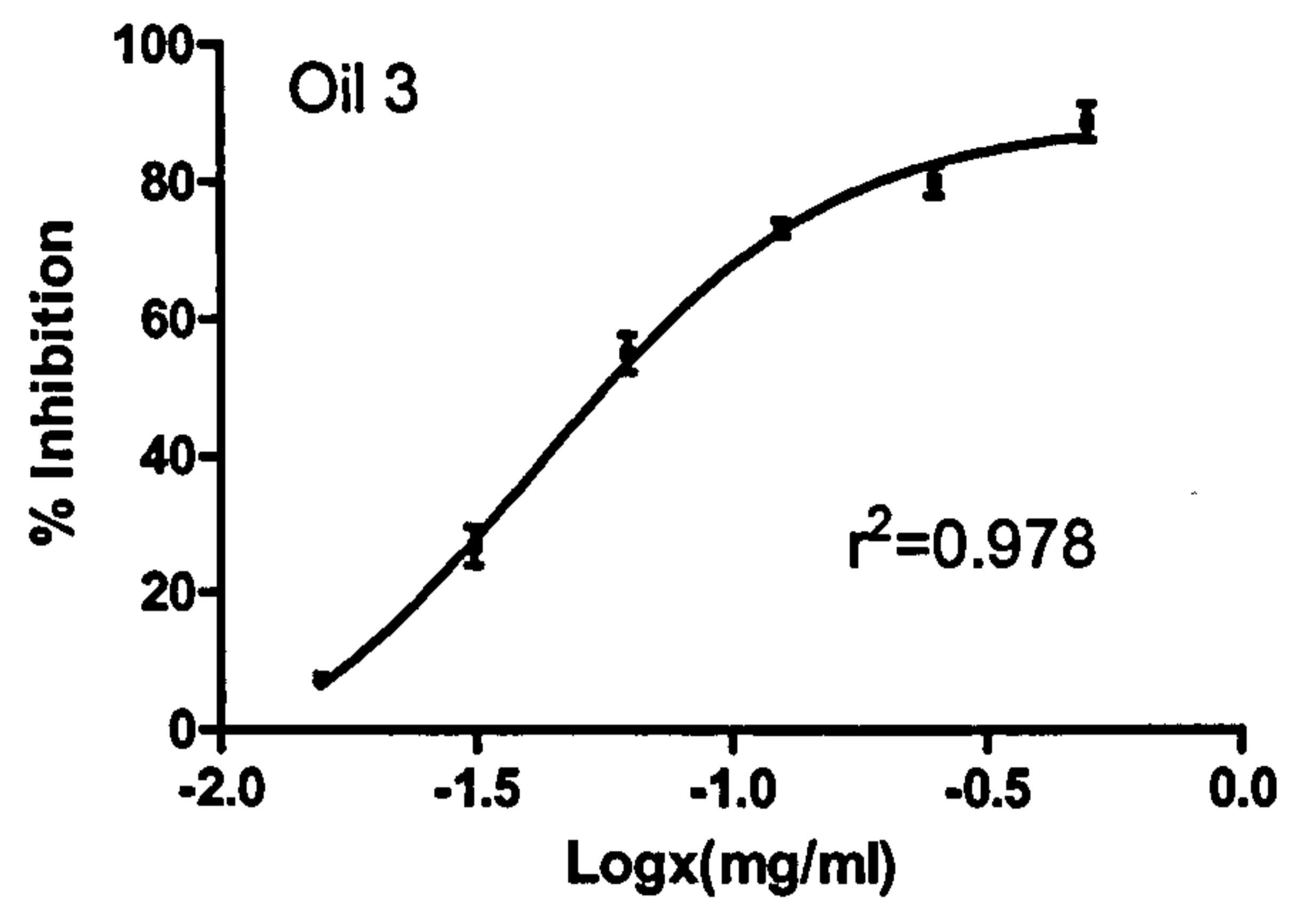
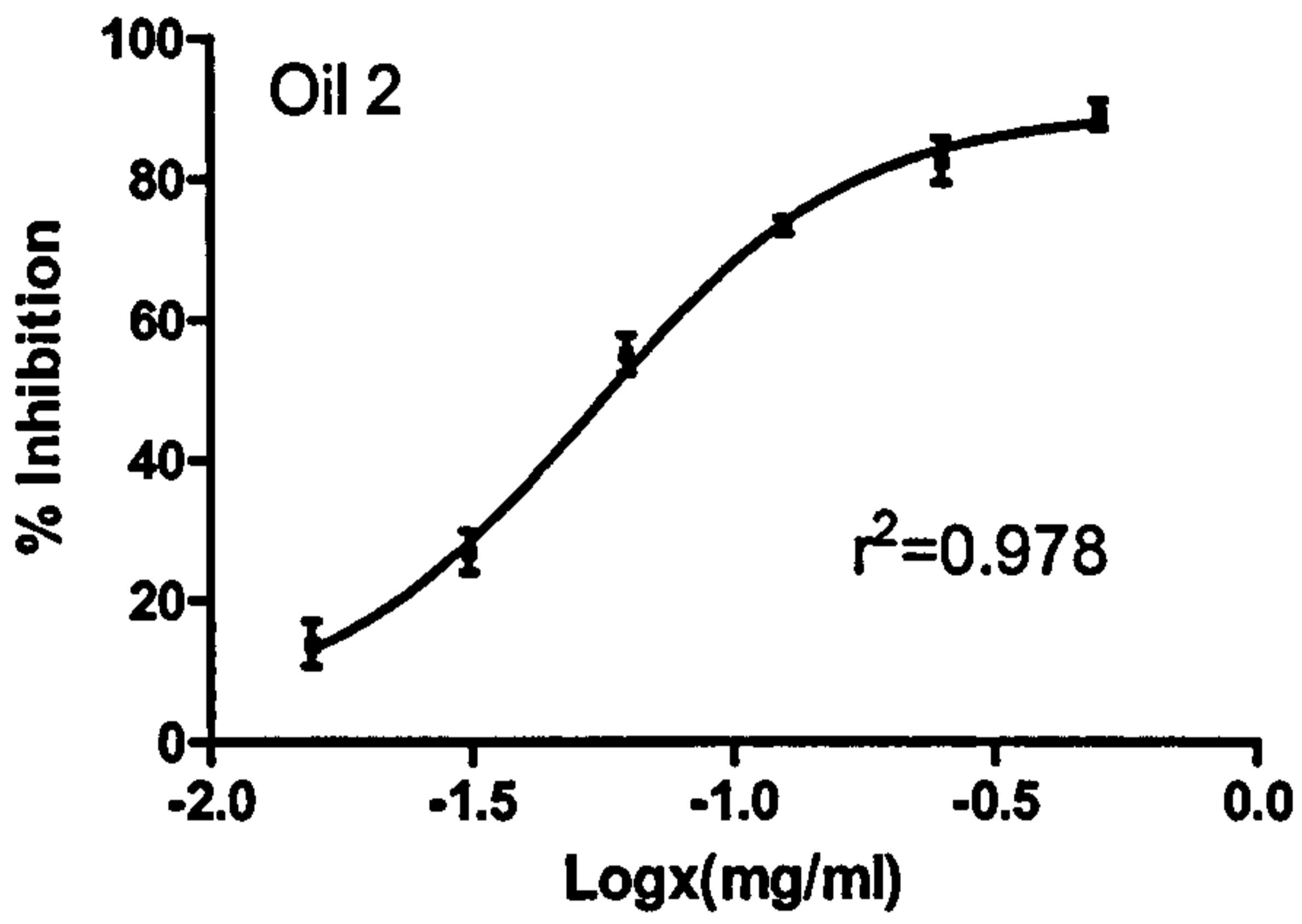


Figure 2.5 Sigmoidal dose response curves for butyrylcholinesterase (BuChE from horse serum) inhibition by *Salvia officinalis* var *purpuea* oils from plants raised hydroponically under three levels of nitrates (100, 150 and 200 mg/L). BuChE enzyme was pre incubated with sage extracts for a period 15 min.

2.3 *Salvia officinalis* var *purpurea* anti-cholinesterase activity data from Chapter 5



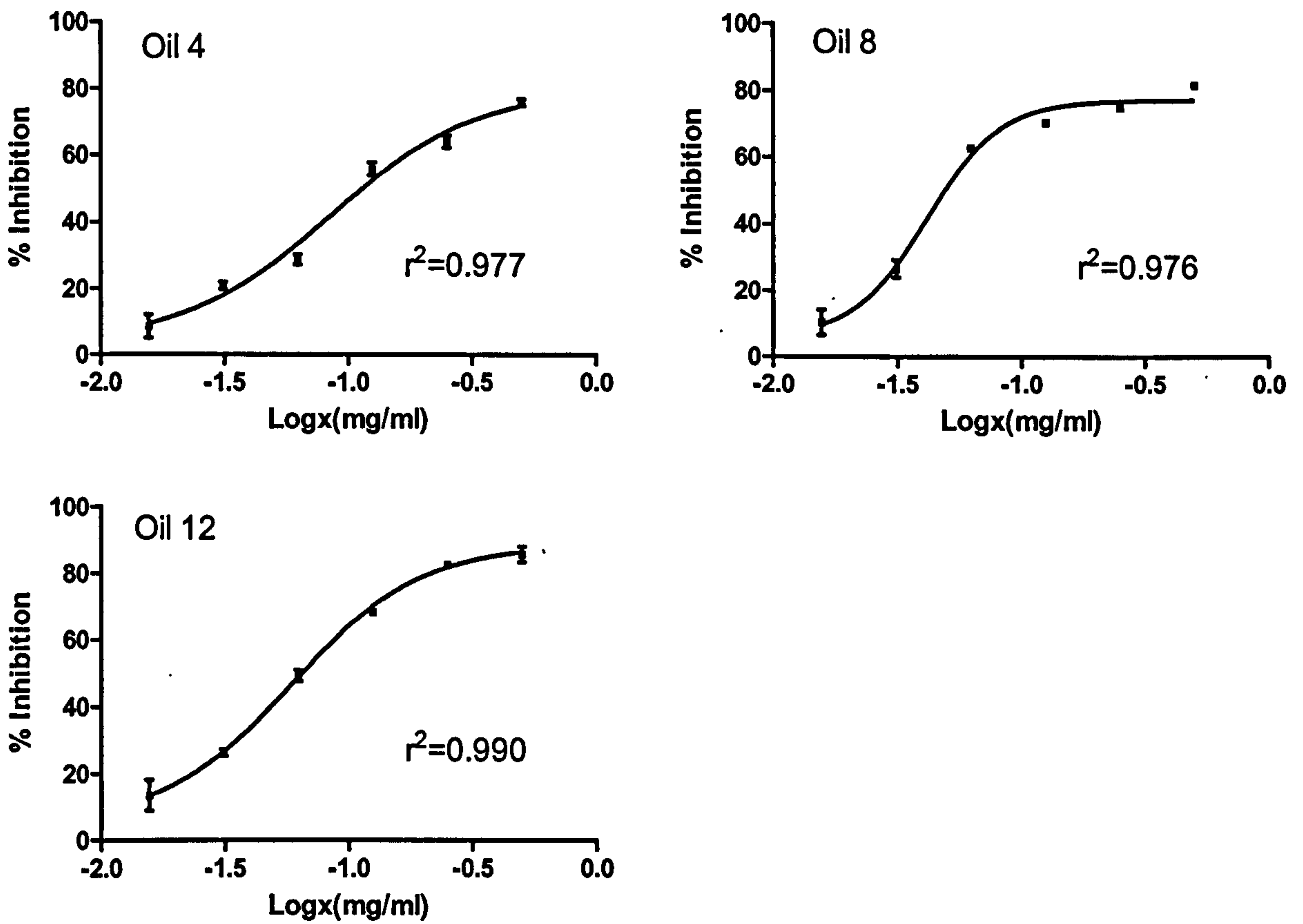
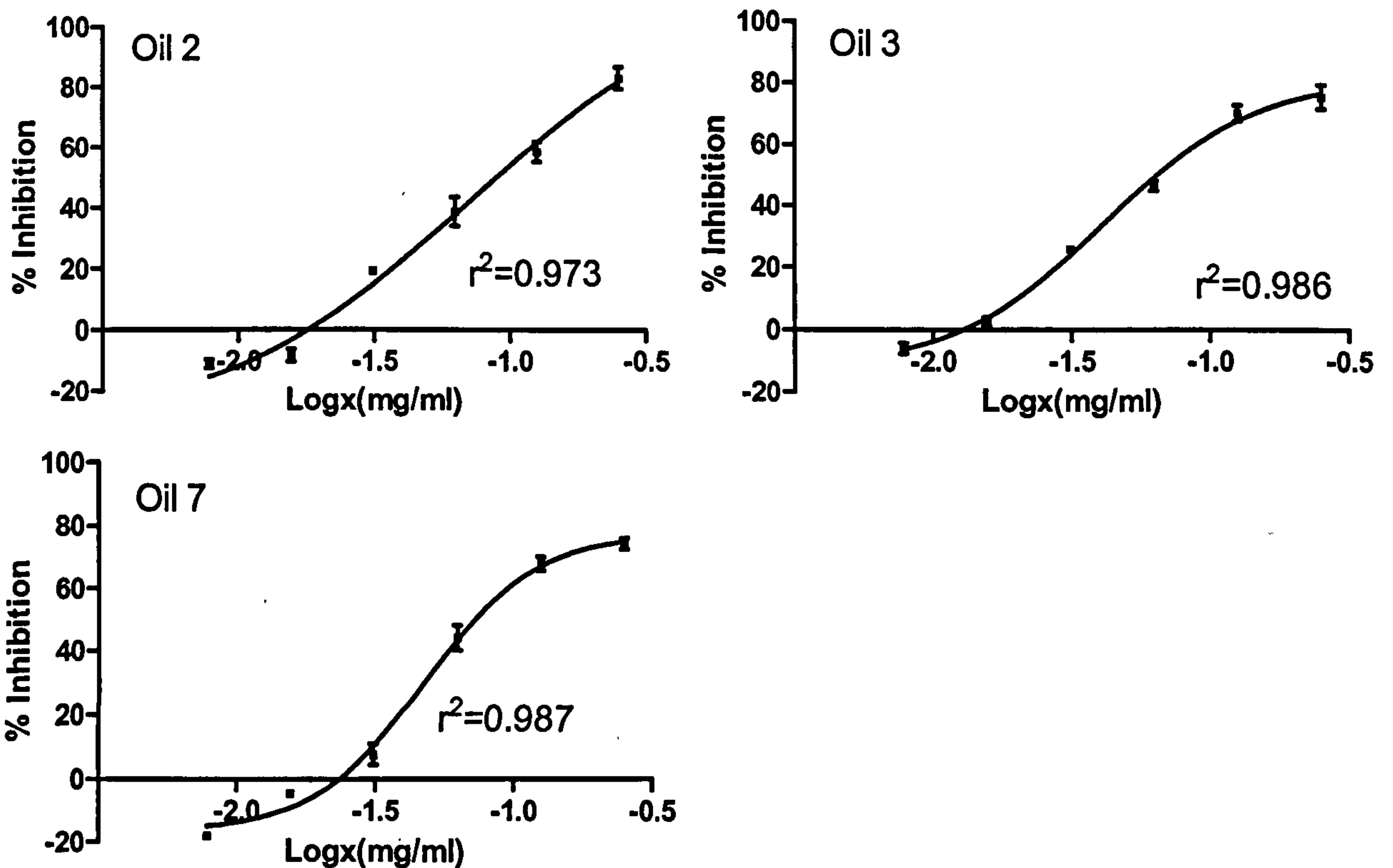


Figure 2.6 Sigmoidal dose response curves for acetylcholinesterase (AChE; human erythrocytes) inhibitory activity of *Salvia officinalis* var *purpurea* oils from plants raised hydroponically under three electrical conductivity levels (EC 1.8, 3.6 and 7.4 mS/cm).



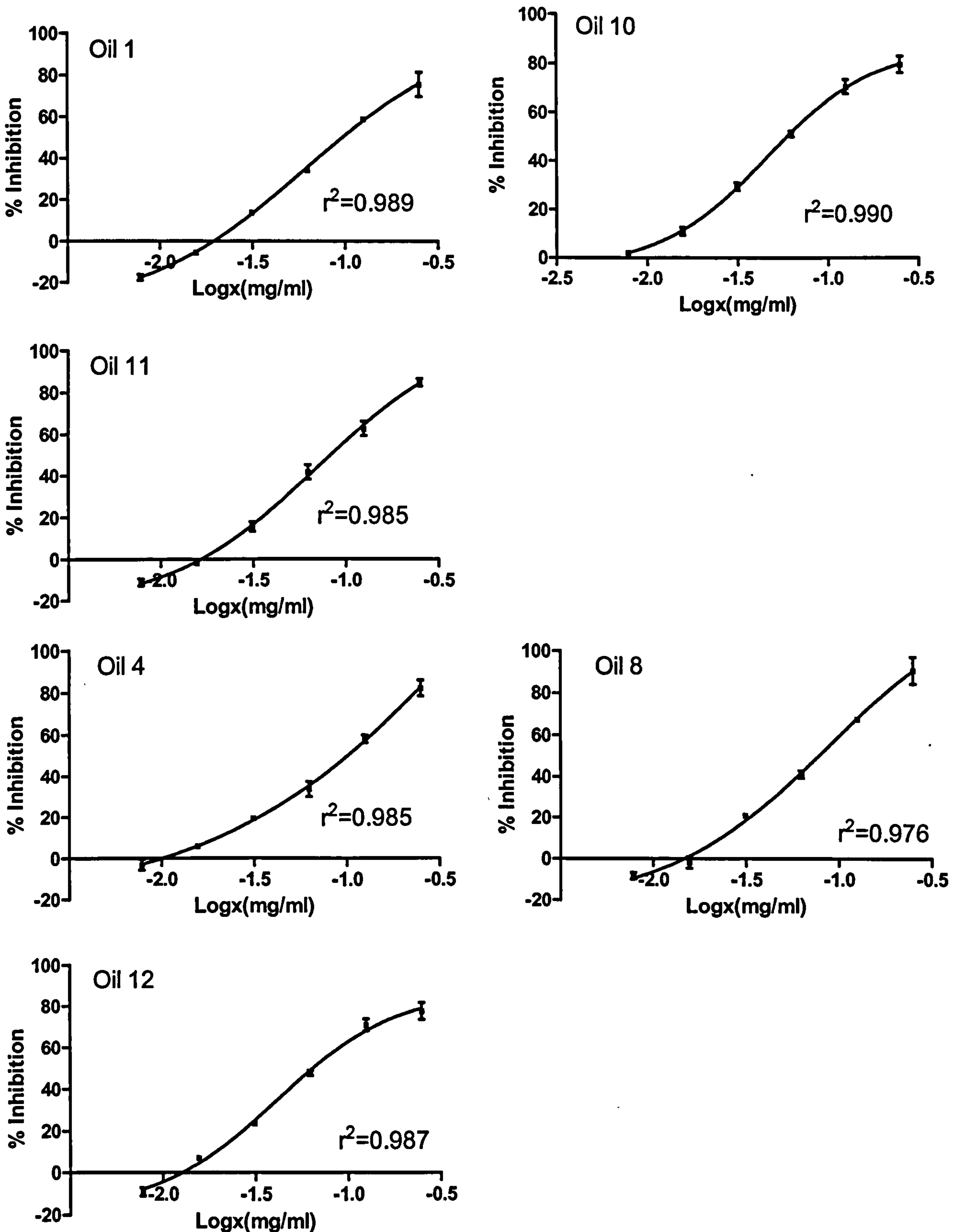


Figure 2.7 Sigmoidal dose response curves for butyrylcholinesterase from horse serum inhibitory activity of *Salvia officinalis* var *purpurea* oils from plants raised hydroponically under three electrical conductivity levels (EC 1.8, 3.6 and 7.4 mS/cm).

Appendix 3 Enzyme kinetics

Type of inhibition of *Salvia officinalis* var *purpurea* for BuChE enzyme from horse serum and human erythrocytes AChE was investigated in phosphorus experiment (Table 3.1, Figure 3.1 and Table 3.2, Figure 3.2 respectively). Michaelis constant (K_m) and initial enzyme velocity (V_{max}) were directly determined employing the Lineweaver – Burk plot, from the intercepts of the linear regression line with the axes. K_m value equals to $-1/S$ and V_{max} to $1/V$, where S and V are the intercepts on the abscissa and ordinate respectively (Cornish-Bowden, 1995; Figure 3.1 for BuChE and Figure 3.2 for AChE). Absorbance per min values that indicated enzyme velocity were transformed into moles/min using the Beer-Lambert law deriving equation below (Equation 1; Ellman *et al.*, 1961).

$$\text{Rate (mole min}^{-1}\text{)} = \frac{\Delta A \text{ min}^{-1} \times V}{e \times \ell} \quad \text{Equation 1}$$

Rate (enzyme velocity) = moles of substrate hydrolysed per minute,

$\Delta A \text{ min}^{-1}$ = absorbance per minute of TNB determined *via* a microplate reader

V = total volume of reaction mixture in the wale, 0.00022 L

e = extinction coefficient of TNB = $1.36 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ (Ellman *et al.*, 1961)

ℓ = path length, 0.848 cm (the distance that light travels through the reaction mixture in the wale with a total volume of 0.00022 L)

The average K_m and V_{max} values of *S. officinalis* var *purpurea* inhibition for human erythrocyte AChE were 0.06 ± 0.004 mM and 0.66 ± 0.033 nmoles/min and for BuChE (horse serum) 0.22 ± 0.006 mM and 0.80 ± 0.013 nmoles/min respectively (without the presence of an inhibitor). Each oil was run twice. Non-competitive type of inhibition was revealed for BuChE enzyme and competitive for human AChE (Table 3.1 for BuChE and Table 3.2 for AChE).

Table 3.1 Kinetic parameters for inhibition of BuChE from horse serum.

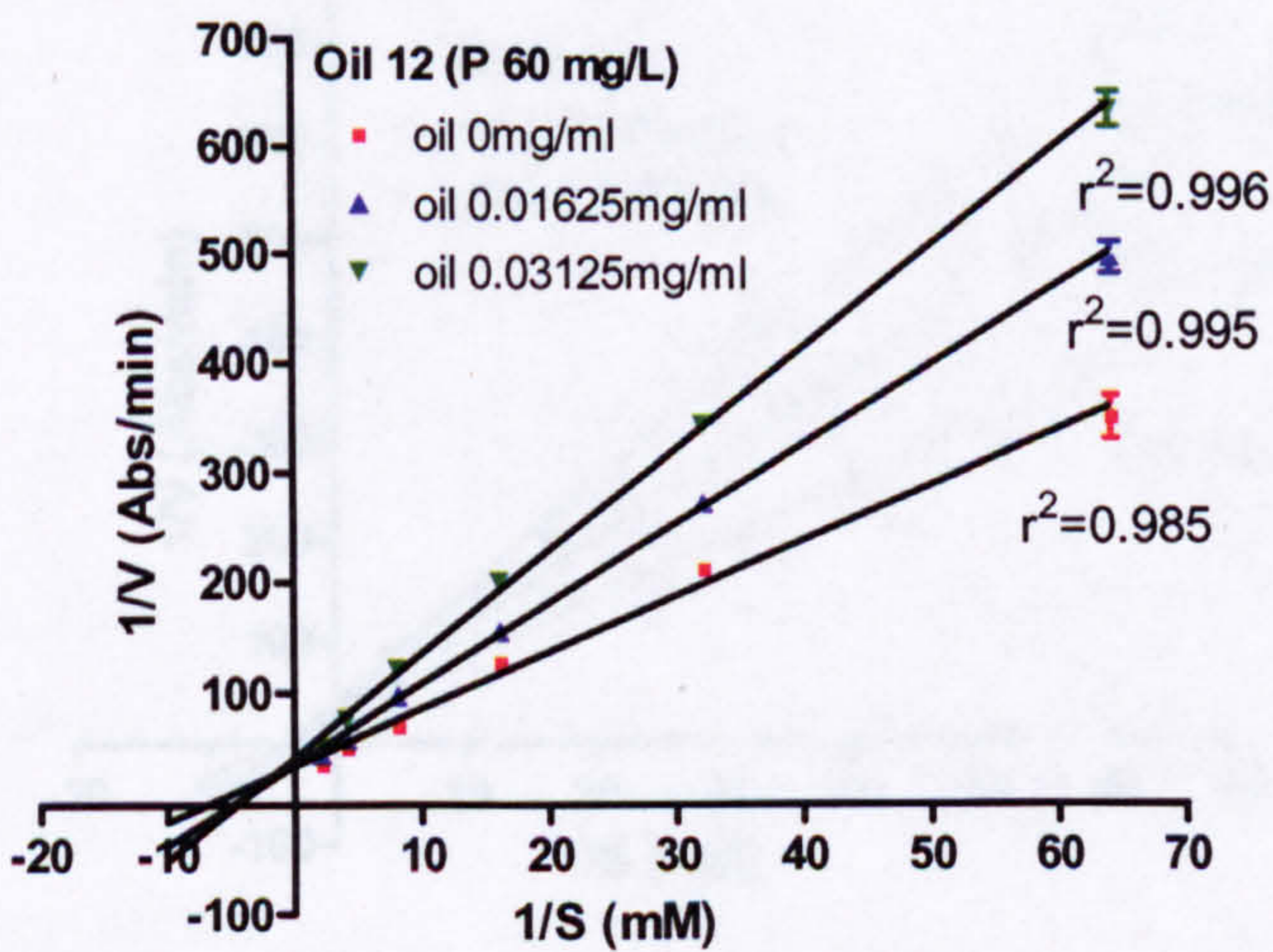
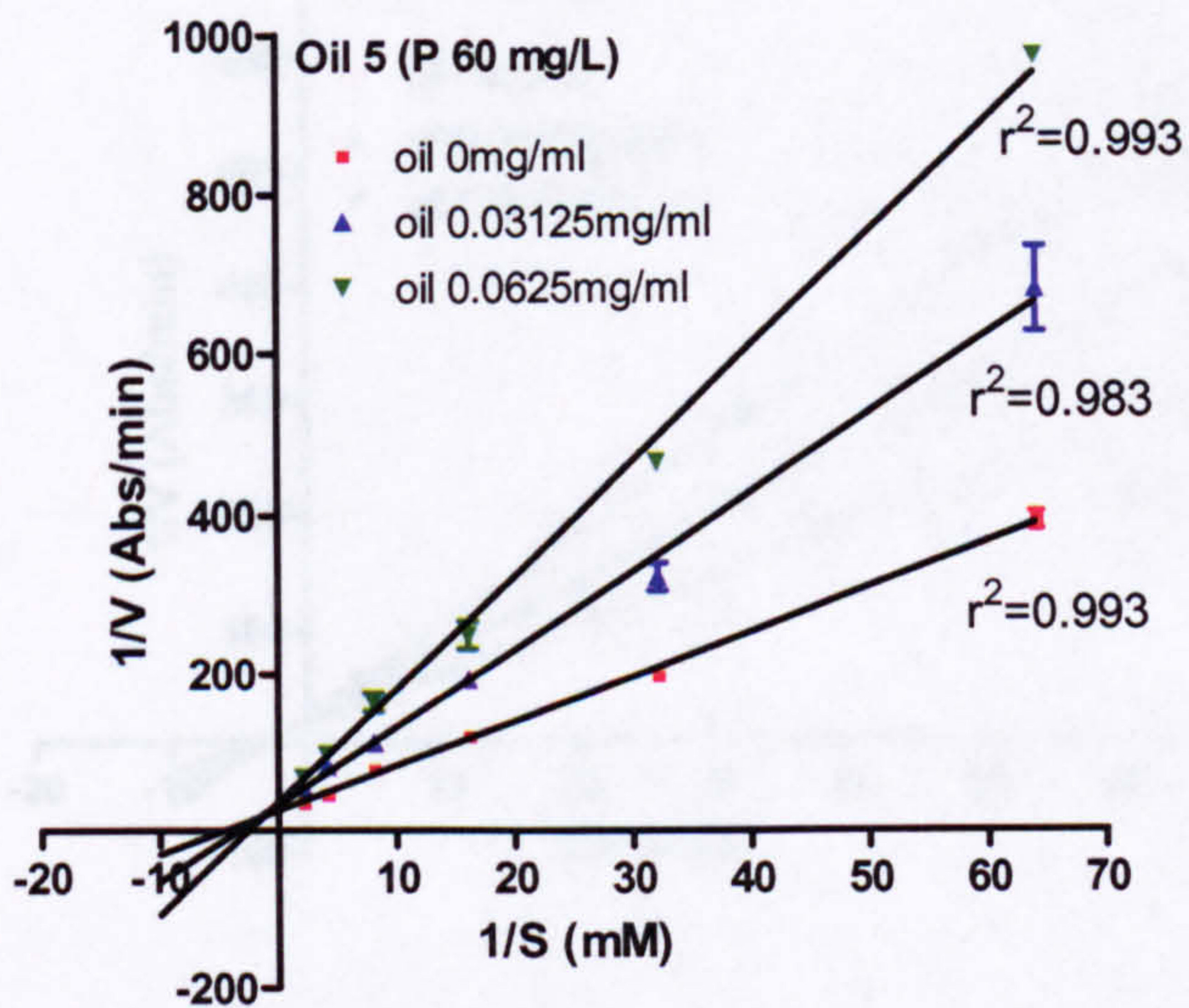
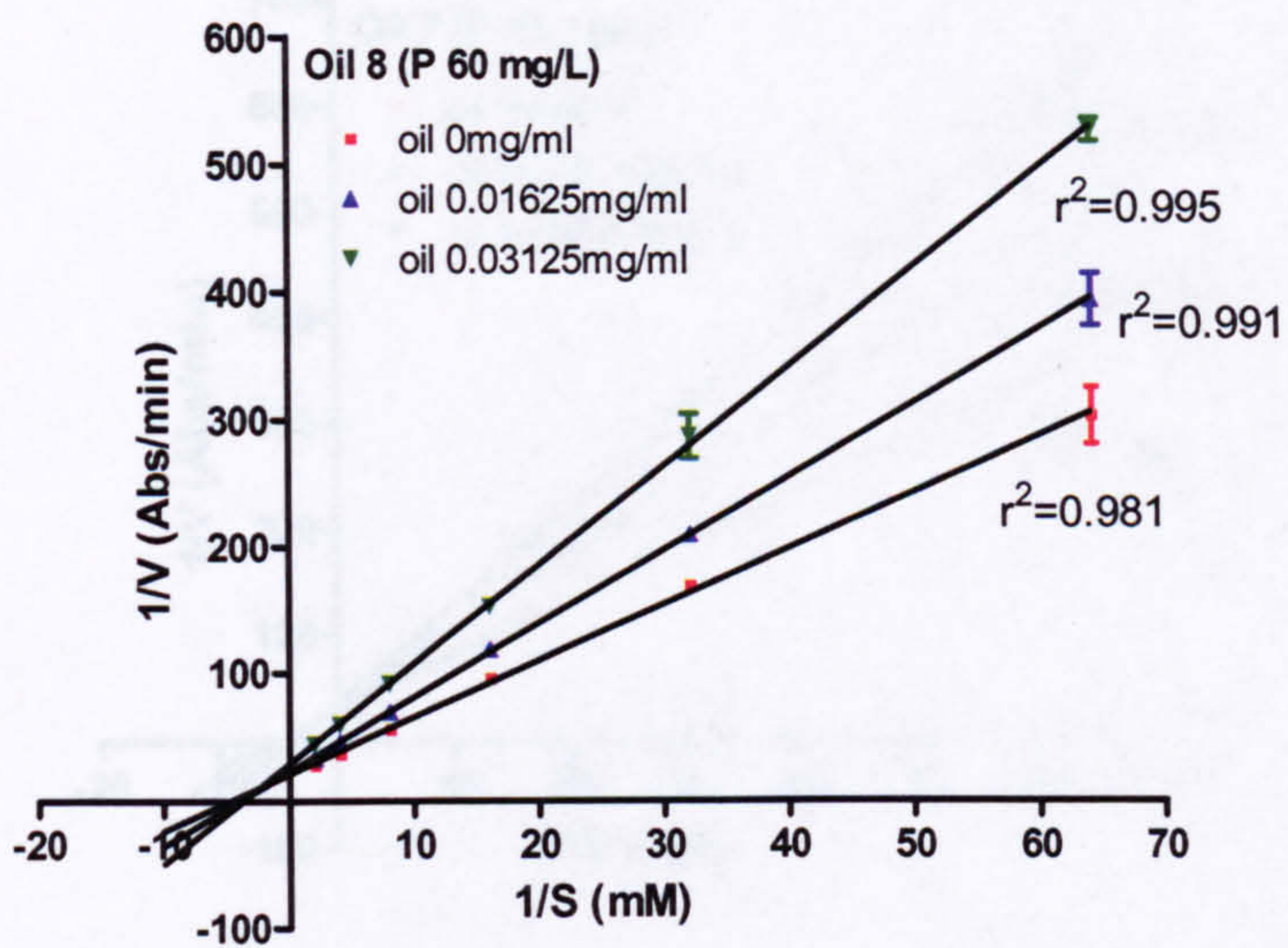
<i>P</i>	<i>V</i> _{max} (nmoles/min)			<i>K</i> _m (mM)		
	<i>control</i>	[I1] ^a	[I2]	<i>control</i>	[I1]	[I2]
60 mg/L	0.79±0.059	0.64±0.090	0.54±0.059	0.21±0.009	0.26±0.019	0.30±0.044
34 mg/L	0.83±0.134	0.69±0.116	0.61±0.102	0.22±0.019	0.26±0.022	0.29±0.024
12 mg/L	0.79±0.118	0.69±0.110	0.57±0.094	0.23±0.029	0.26±0.029	0.29±0.018
Average	0.80±0.013	0.67±0.017	0.57±0.020	0.22±0.006	0.26±0.00	0.29±0.003

^a [I1] and [I2] denote inhibitor concentration that ranged from 0.016mg/ml of oil to 0.0625mg/ml. [I2] was always greater than [I1].

^b Type of inhibition was characterised according to Engel (1981) and is non-competitive.

V_{max} and K_m values have been estimated from the reciprocals presented below

Oils 5, 8, 12 were used from P 60mg/L treatment; oils 1, 6 and 10 from P 34 mg/L treatment and oils 3, 7, 9 from treatment P 12 mg/L.



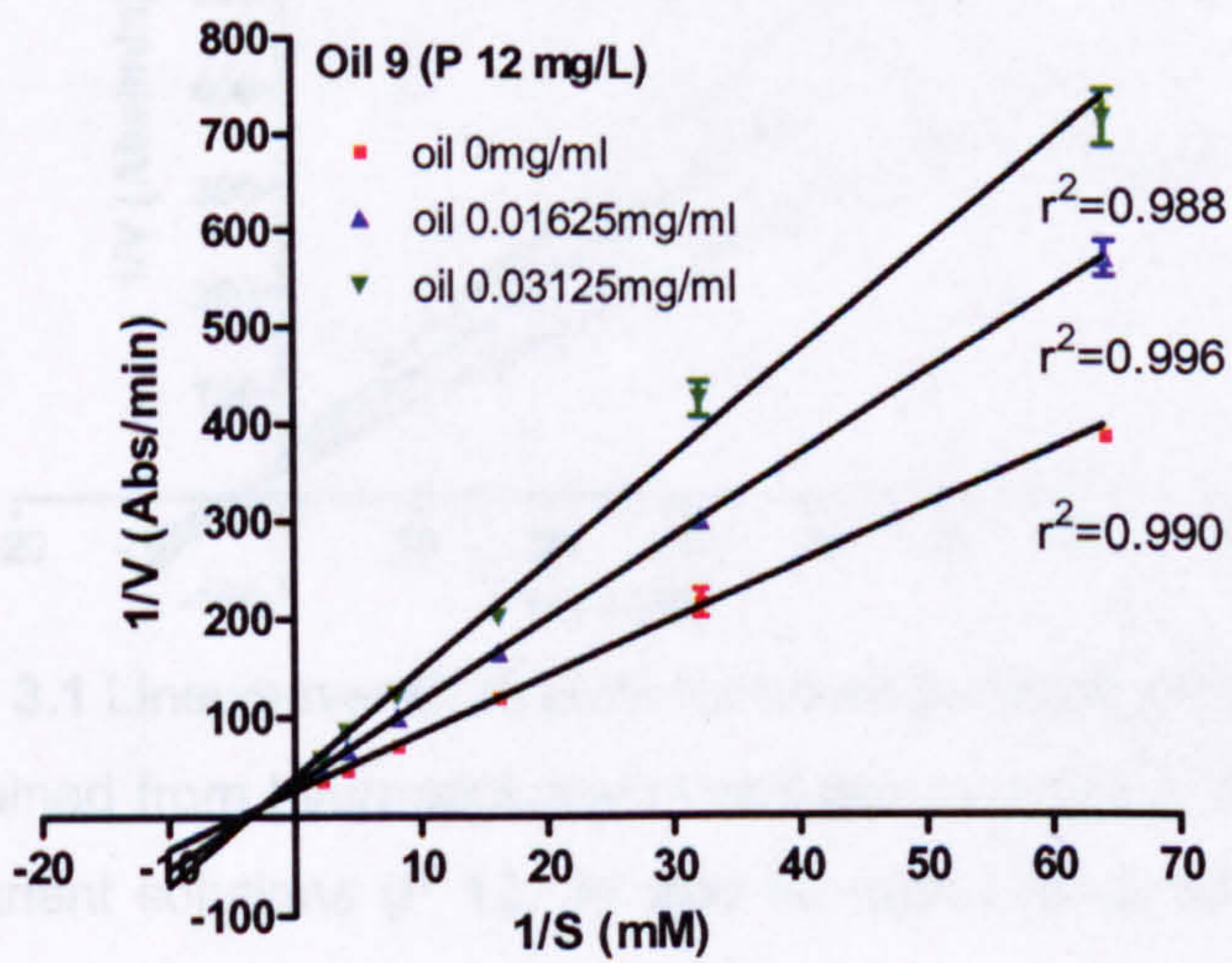
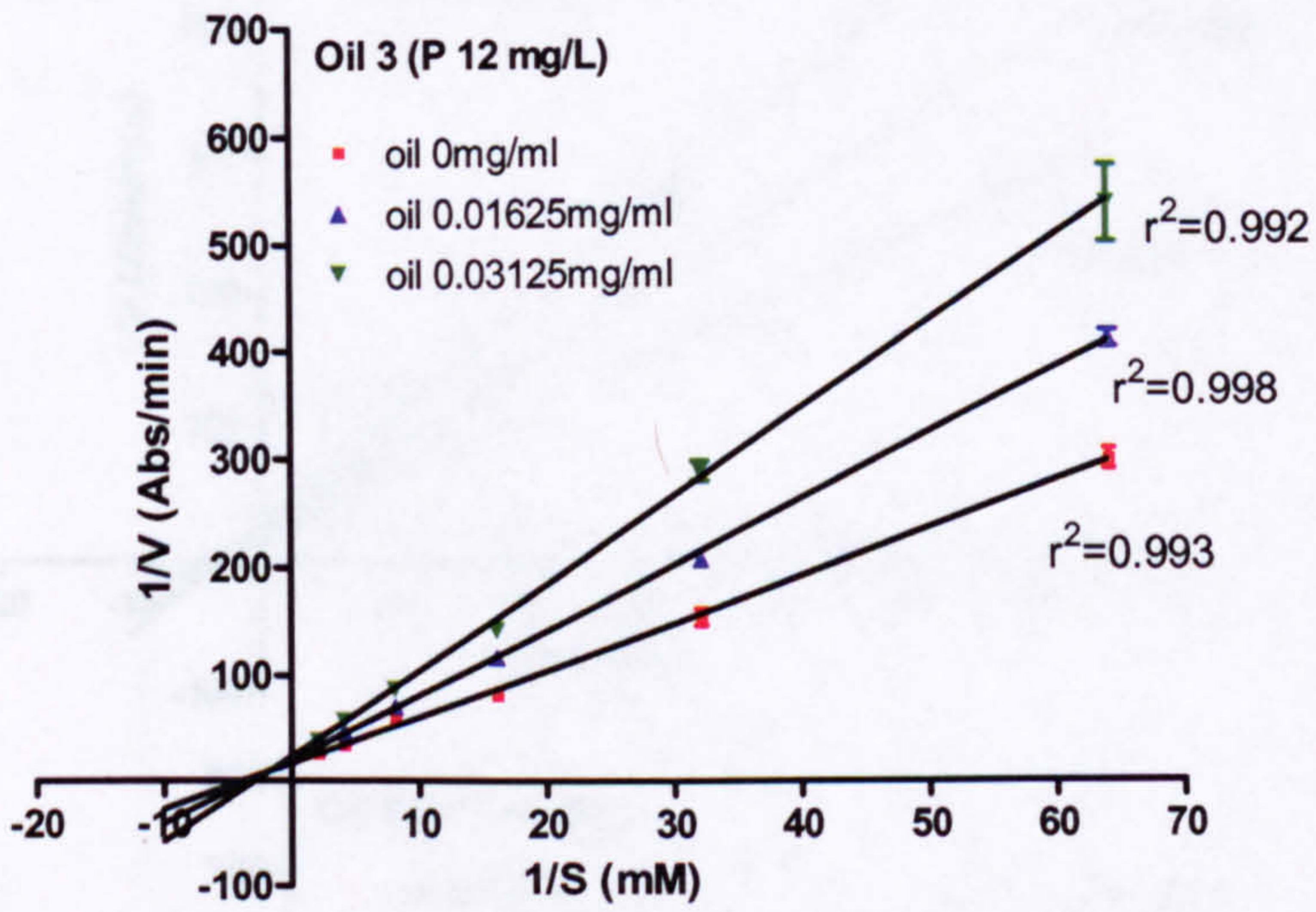
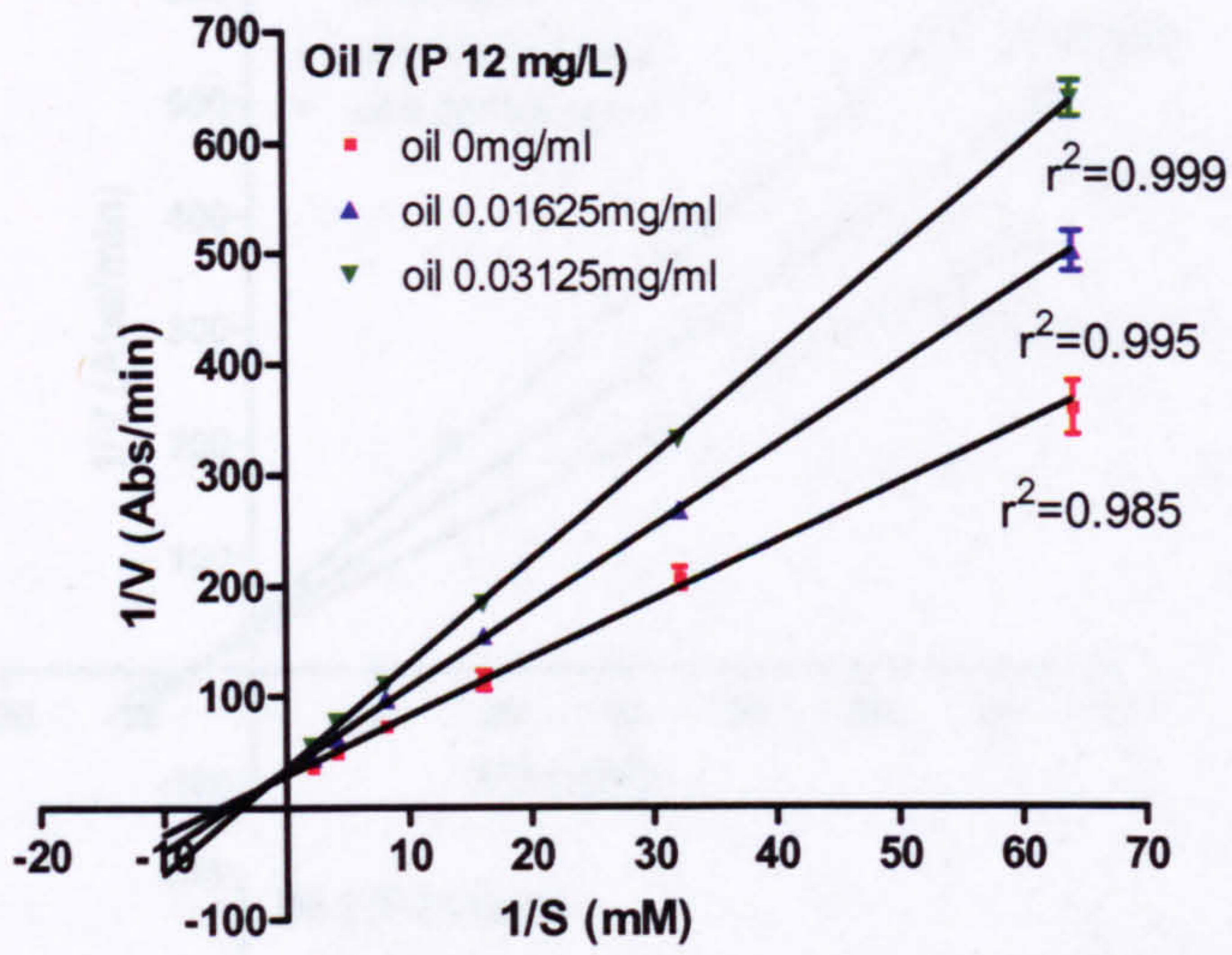


Figure 3.1 Lineweaver-Burk plots for the reaction of the enzyme with the substrate in the presence of different concentrations of oil obtained from the nutmeg. The y-axis represents the reciprocal of the initial reaction velocity (1/V) and the x-axis represents the reciprocal of the substrate concentration (1/S). The r^2 indicates goodness of fit of the linear regression. Values represent the mean and error bars for three independent experiments.

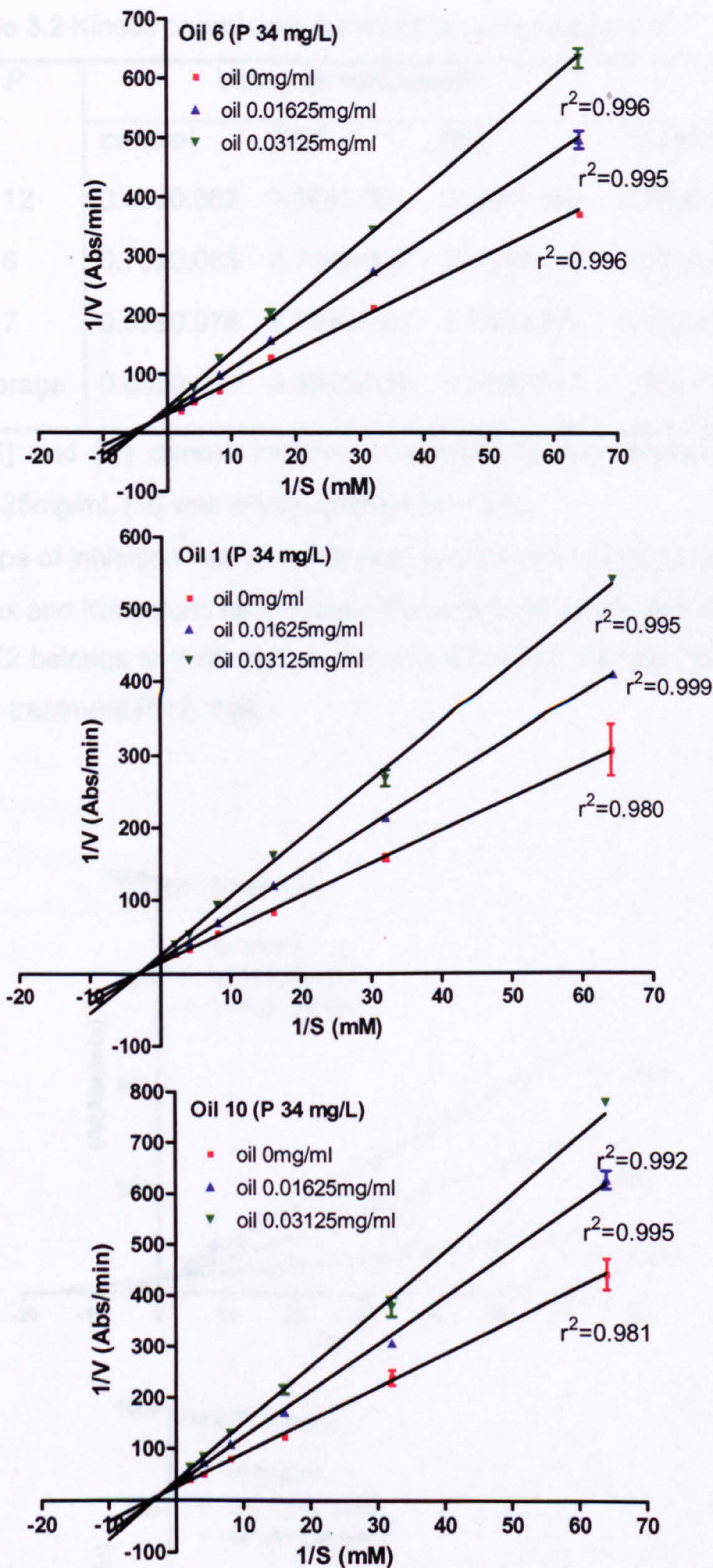


Figure 3.1 Lineweaver-Burk plots for horse serum BuChE by *Salvia officinalis* var *purpurea* oil obtained from hydroponically raised plants under three concentration of phosphorus in the nutrient solutions (P 12, 34 and 60 mg/L). S denotes substrate concentration and V enzyme velocity

r² indicates goodness of fit of the linear regressions

Values represent the means and vertical bars the standard error of the mean (n=4)

Table 3.2 Kinetic parameters for inhibition of human AChE

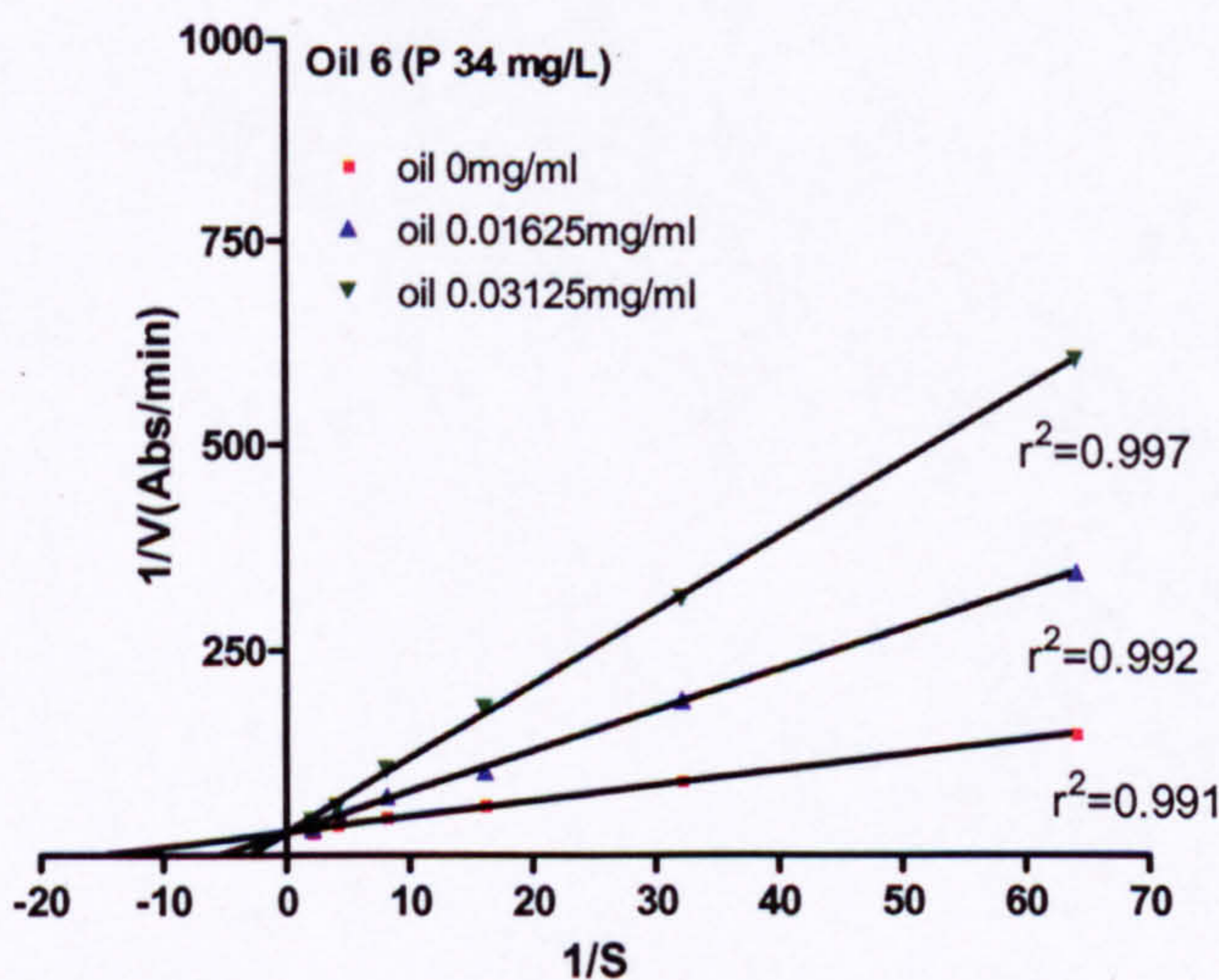
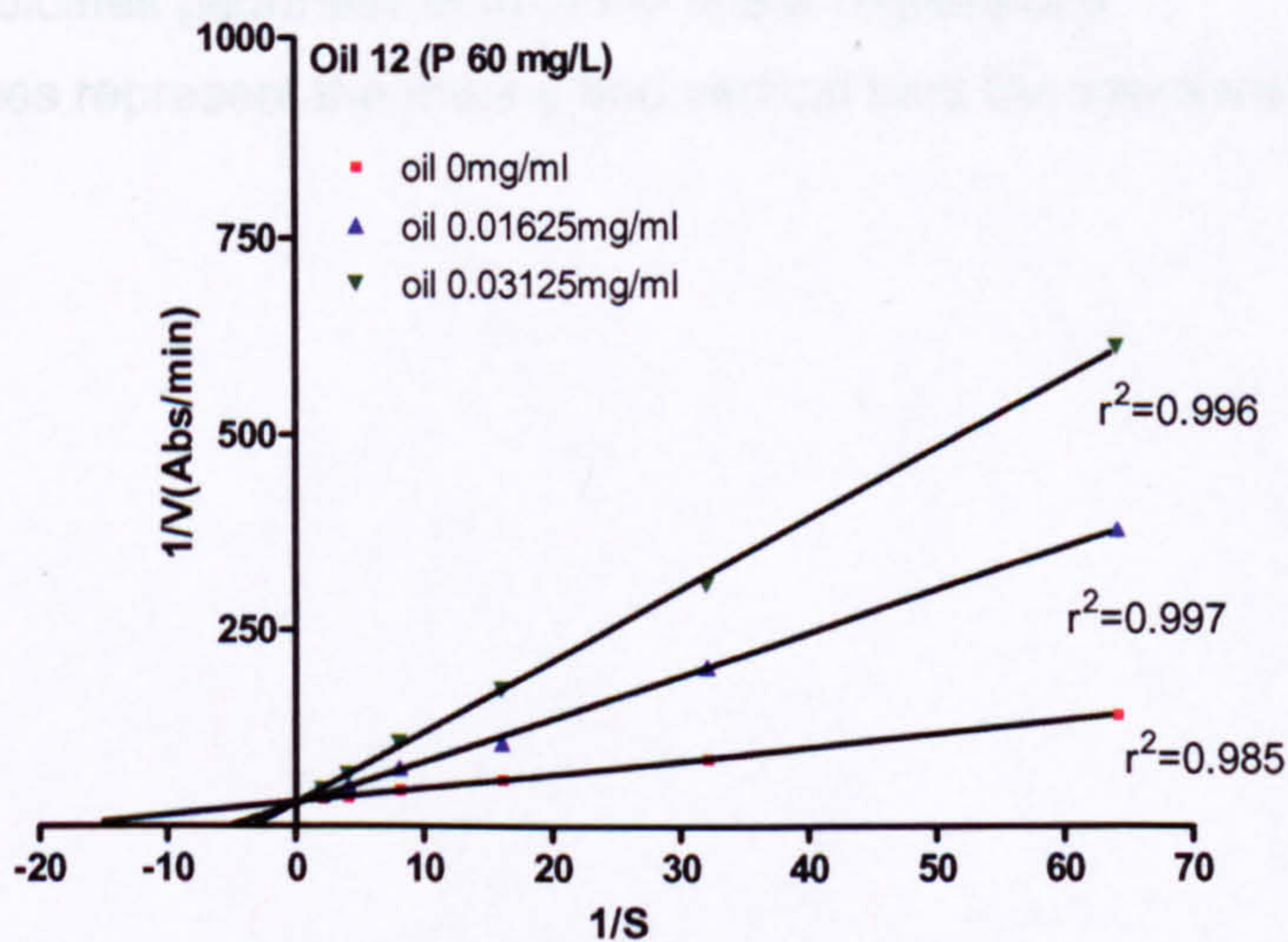
<i>P</i>	<i>V</i> _{max} (nmoles/min)			<i>K</i> _m (mM)		
	control	[I1] ^a	[I2]	control	[I1]	[I2]
Oil 12	0.68±0.082	0.68±0.051	0.68±0.062	0.06±0.005	0.19±0.020	0.32±0.026
Oil 6	0.70±0.063	0.70±0.069	0.71±0.058	0.07±0.006	0.18±0.011	0.34±0.023
Oil 7	0.59±0.076	0.59±0.060	0.59±0.069	0.05±0.006	0.14±0.009	0.30±0.024
Average	0.66±0.033	0.66±0.034	0.66±0.037	0.06±0.004	0.17±0.015	0.32±0.012

^a [I1] and [I2] denote inhibitor concentration that ranged from 0.016 mg/ml of oil to 0.0625mg/ml. [I2] was always greater than [I1].

^b Type of inhibition was characterised according to Engel (1981) and is competitive.

*V*_{max} and *K*_m values have been estimated from the reciprocals presented below

Oil 12 belongs to P 60 mg/L treatment; oils 6 to P 34 mg/L treatment; whereas oil 7 comes from treatment P 12 mg/L.



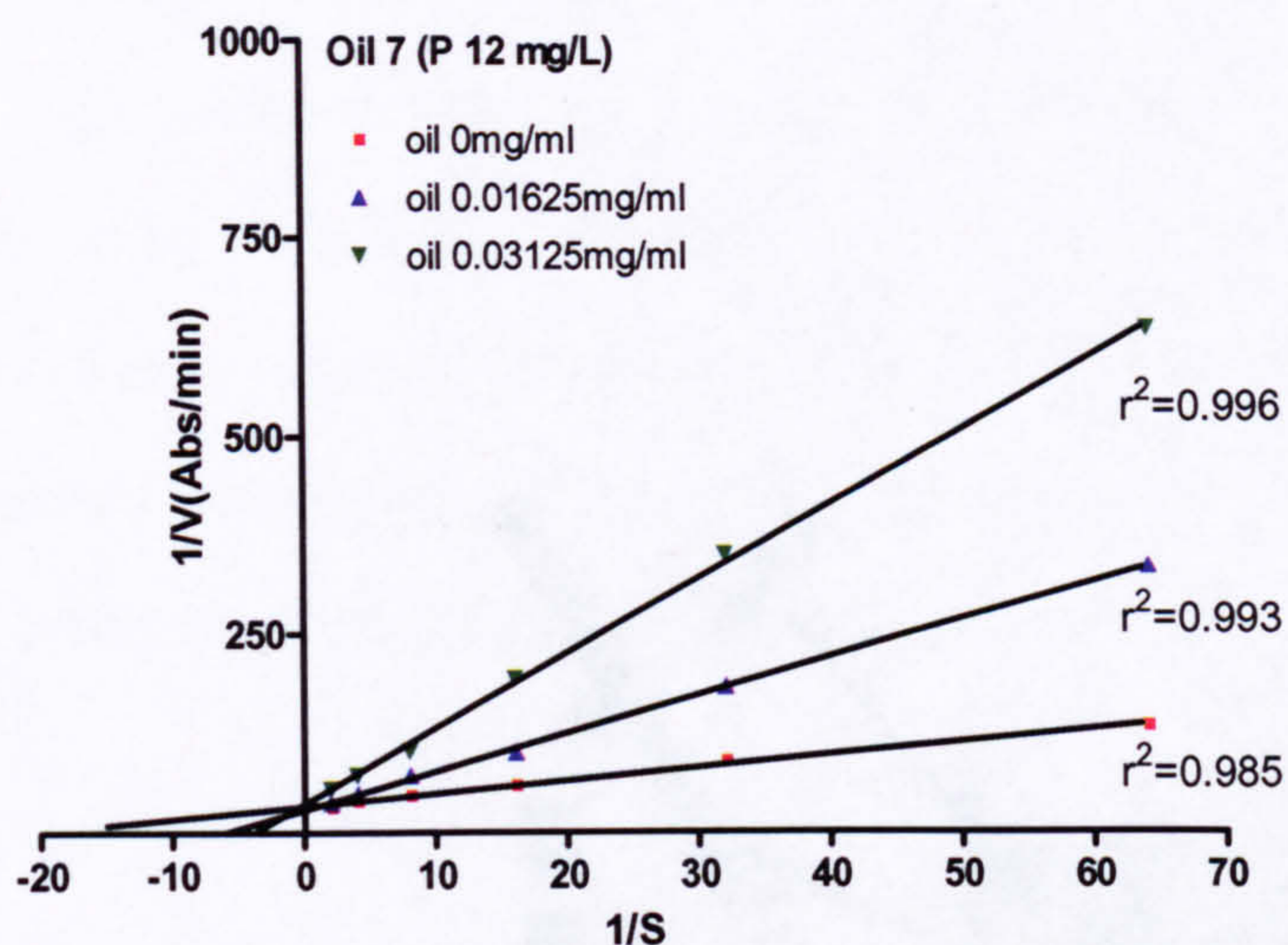


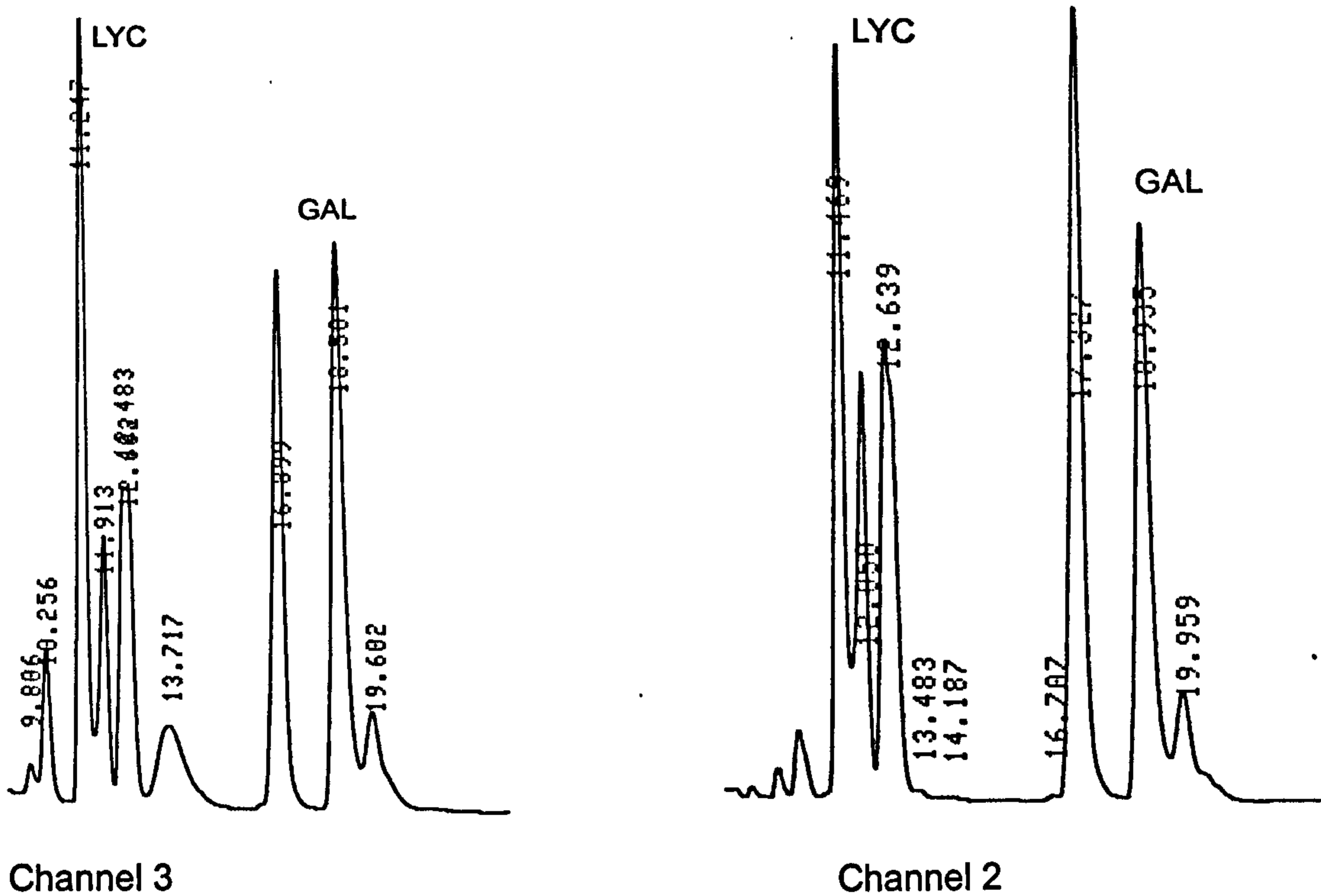
Figure 3.2 Lineweaver-Burk plots for AChE human erythrocytes by *Salvia officinalis* var *purpurea* oil deriving from plants hydroponically raised under three concentration of phosphorous in the nutrient solutions (P. 12, 34 and 60 mg/L). S denotes substrate concentration and V enzyme velocity

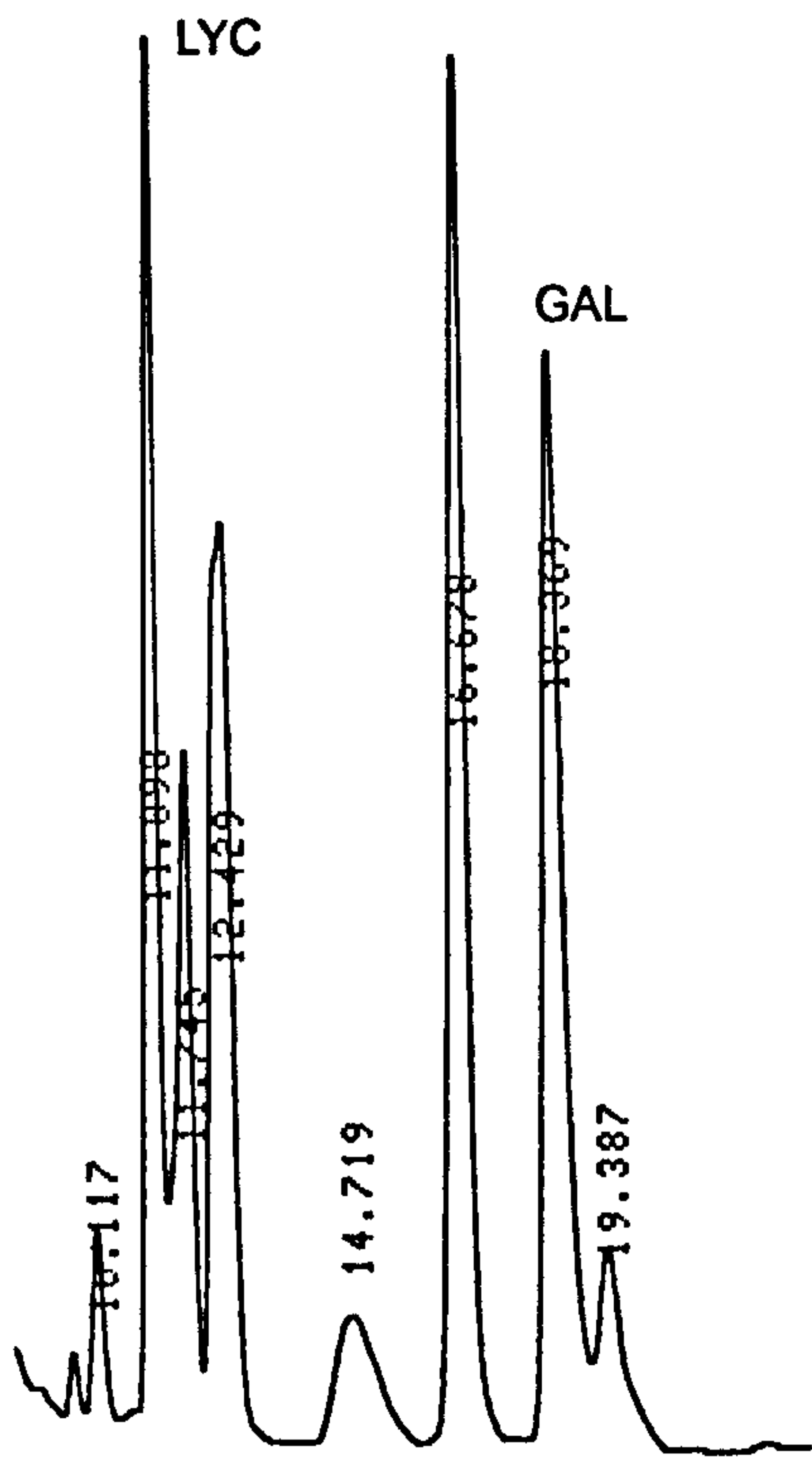
r^2 indicates goodness of fit of the linear regressions

Values represent the means and vertical bars the standard error of the mean (n=4)

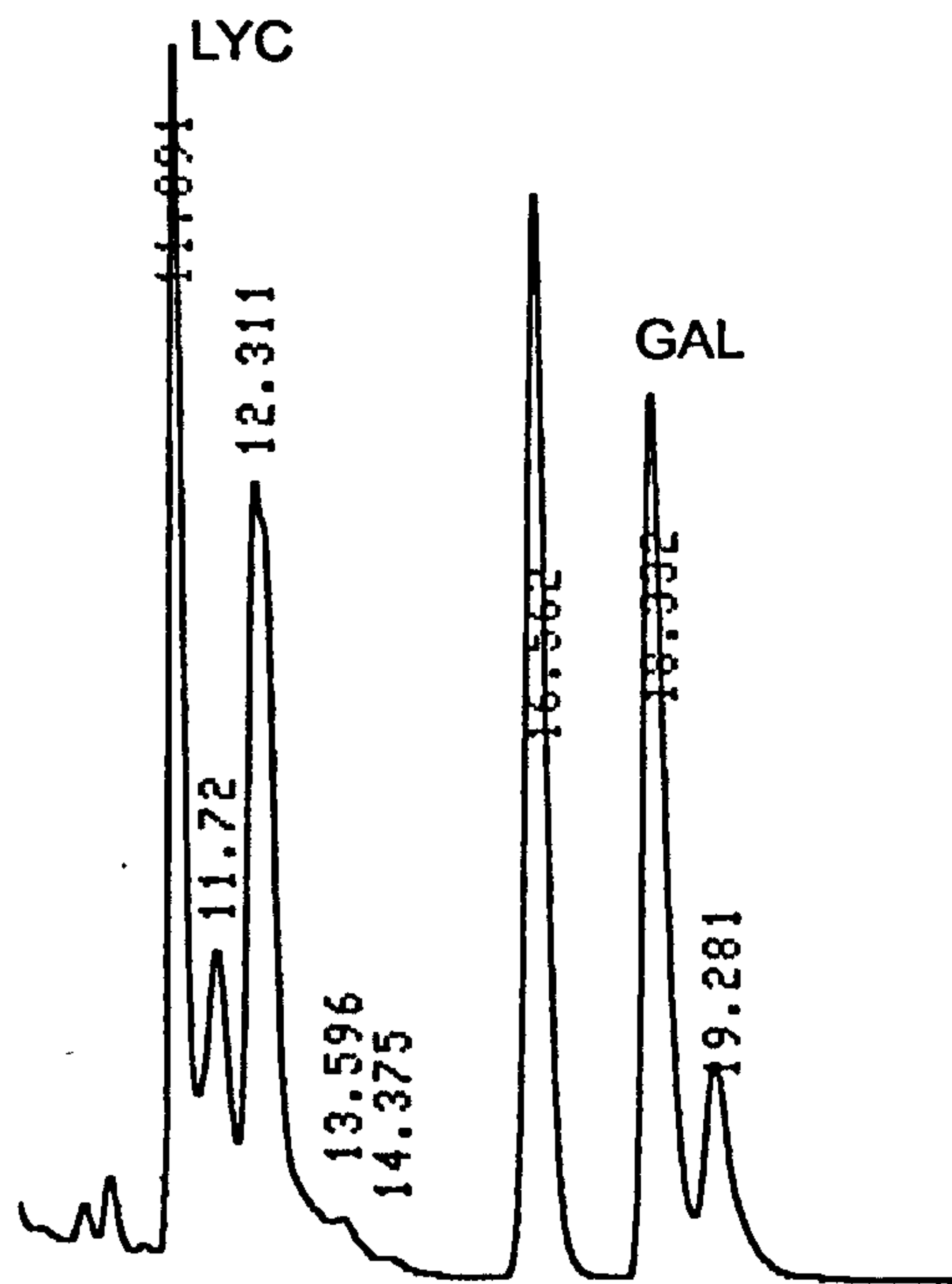
Appendix 5 HPLC chromatograms

Crude bulb extracts in acidic water from *Narcissus* "Ice Follies" grown under 100 and 180 mg/L nitrate supply and harvested during vegetative and anthesis stage were analysed by High Performance Liquid Chromatography (HPLC) in order to determine their alkaloid content. Examples of alkaloid chromatograms are presented here. Extraction method and HPLC conditions are described in detail at the general material and methods (section 2.8 and 2.9 respectively).

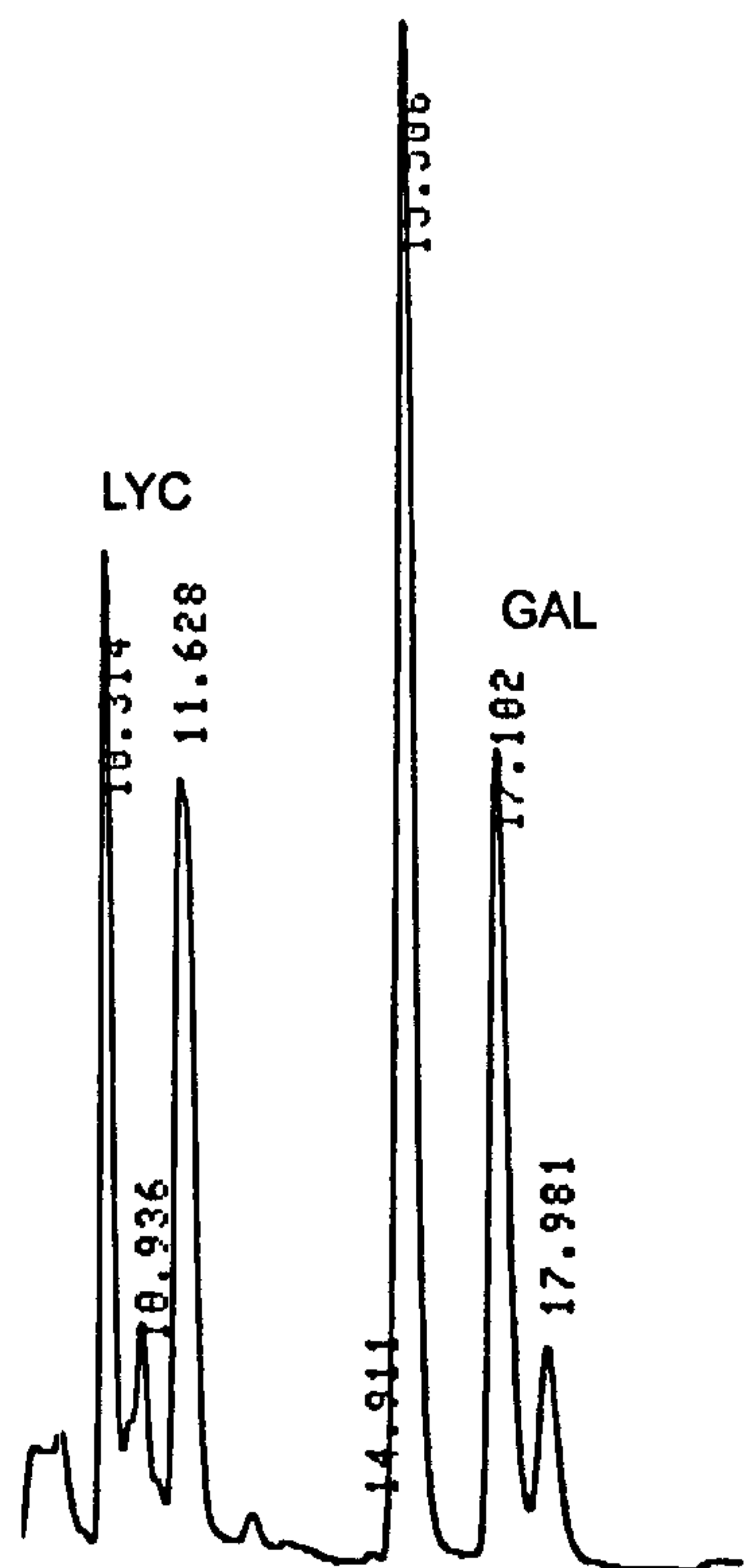




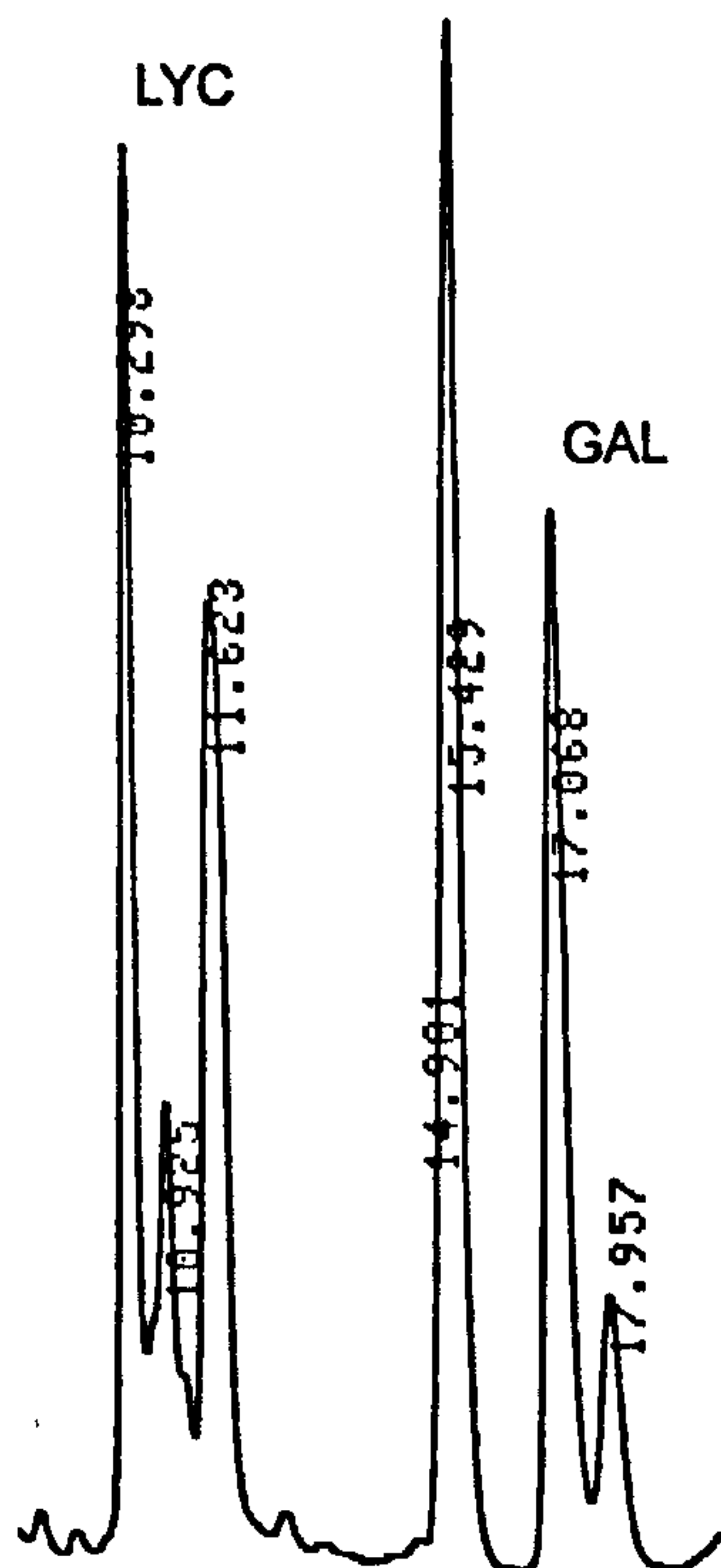
Channel 4



Channel 7



Channel 3 (2nd harvest)



Channel 8 (2nd harvest)

Figure 5.1 Crude alkaloid extract chromatograms from *Narcissus* "Ice Follies" bulbs hydroponically cultivated under two different nitrate levels (100 and 180 mg/L N-NO₃) in the nutrient solution. Bulbs were harvested first at the vegetative stages and then during anthesis. Channels 2, 3 and 6 belong to 100 mg/L treatment and channels 4, 7 and 8 to 180 mg/L N-NO₃.