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Investigating the Molecular Genetic Basis of Antioxidants in *Lactuca sativa* for the Enhancement of its Nutritional Qualities

by

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ABSTRACT

Consumer awareness of food nutritional properties has increased significantly. A healthy diet is now viewed as the first step towards the prevention of many age-related chronic diseases such as cancer and heart disease. In most cases oxidative damage is involved in the onset of these diseases, much of which can be prevented by a diet high in antioxidants. However, throughout hundreds of years of domestication, many crop species, such as lettuce, have been genetically manipulated with the purpose of increasing favourable traits. These selective processes have resulted in the unintentional decrease of the antioxidant nutritional content of species such as *Lactuca sativa*.

This project aimed to use natural resources for the enhancement of lettuce nutritional properties through the introgression of genes from a close relative of *L. sativa*. Initially, the total antioxidant content of baby lettuce leaves was investigated, with particular focus on carotenoids, chlorophyll and phenolic compounds. Wild lettuce (*L. serriola,* acc. UC96US23) was found to produce higher levels of total antioxidants, chlorophylls and carotenoids than the lettuce cultivated variety*,* Salinas cv. Subsequently, these traits were analysed in a Recombinant Inbred Line mapping population obtained by crossing these two lettuce species. Considerable transgressive segregation was observed and this data was then used to map Quantitative Trait Loci (QTL) on the population, which showed an additive effect of both parents contributing to these beneficial traits. Subsequent analyses of key genes involved in the synthesis of carotenoids and phenolic compounds revealed the presence of single nucleotide polymorphisms.

Understanding the processes involved in the synthesis of nutritional properties and identifying the genes underlying these traits is key to producing a better quality lettuce. This project demonstrates potential for the improvement of lettuce antioxidant nutritional qualities through breeding with a wild relative.

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The following accompanying material was produced for the human intervention trial study on blood plasma antioxidant levels following the consumption of fresh or stored green cos lettuce (Chapter 3):

1. Research Ethics Committee (REC) application form
2. Site Specific Information (SSI) form

DECLARATION OF AUTHORSHIP

I, GAIA FRANCESCA BIGGI, declare that the thesis entitled:

Investigating the Molecular Genetic Basis of Antioxidants in Leafy Salad Crops for the Enhancement of the Nutritional Qualities of *Lactuca sativa*

and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
- where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- where I have consulted the published work of others, this is always clearly attributed;
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- I have acknowledged all main sources of help;
- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- none of this work has been published before submission

Signed: ……..

Date:……14th March 2011…..…….

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LIST OF ACRONYMS AND ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
AFLP	Amplified Fragment Length Polymorphism
BCA	Bicinchloronic Acid
BSA	Bovine Serum Albumin
c.	*circa*
CCI	Chlorophyll Content Index
CVD	Cardiovascular Disease
CAD	Coronary Artery Disease
CHD	Coronary Heart Disease
CTAB	hexadecyltrimethylammonium bromide
DMF	Dimethyl Formamide
DW	Dry Weight
EDTA	Ethylenediaminetetraacetic Acid
EST	Expressed Sequence Tag
FRAP	Ferric Reducing Antioxidant Potential
FW	Fresh Weight
GER	Geophysical Environmental Research
G	Gravity
HDL	High-Density Lipoprotein
HPLC	High Performance Liquid Chromatography
ITCs	Isothiocyanates
LG	Linkage Group
LDL	Low-Density Lipoprotein
LOD	Log of Odds
4-MSB	4-methylsuphinylbutil
3-MSP	3-methylsuphinylpropyl
Nm	Nanometres
OD	Optical Density
PBS	Phosphate Buffered Saline
QTL	Quantitative Trait Locus

QR	Quinone Reductase
Rcf	Relative Centrifugal Force
RDA	Recommended Daily Allowance
RILs	Recombinant Inbred Lines
SBE	Single Base Extension
SDS	Sodium Dodecyl Sulfate
SSR	Simple Sequence Repeat
SEM	Standard Error Mean
SGH	Southampton General Hospital
SNP	Single Nucleotide Polymorphism
TAP	Total Antioxidant Potential
TEAC	Trolox Equivalent Antioxidant Capacity
UL	Tolerable Upper Intake Level
VAD	Vitamin A Deficiency
v:w	Volume per weight
WHO	World Health Organisation

CHAPTER 1 - INTRODUCTION

1.1 Overview

1.1.1 Industry demand

It is today common knowledge that a diet rich in fruit and vegetables is one of the key components of a healthy lifestyle, important in helping to prevent age-related degenerative diseases. However, the recommended daily allowance of five portions of fruit and vegetables (400g in total) are still rarely met in the UK. In fact, fresh vegetable consumption per person per day has decreased in the last four years, seeing a drop of 2.0% in purchases of these since 2007, and, in particular, a significant decline in fresh lettuce consumption of -16.3% since 2005-6 (from 44g to 37g in 2008) (http://statistics.defra.gov.uk/esg/publications/efs/default.asp). Baby salad leaves contribute significantly to the 'five-a-day' intake of fresh fruits and vegetables, recommended by professionals, as lettuce still remains one of the preferred vegetable crop species in Europe and the United States, perhaps due to the versatility and convenience of packaged salads.

Plant-based diets should ensure adequate nutrition, however, plants vary considerably in nutrient content and many staple food crops contain low concentrations of essential vitamins and/or minerals which may fail to meet the Recommended Daily Allowance (RDA) guidelines. As defined by Goldhaber (2003): the "RDA is the average daily intake level that is sufficient to meet the nutrient requirements of nearly all (97-98%) healthy individuals in a particular life stage and gender group". Furthermore, RDAs indicate the minimum levels of nutrient requirements, not those required for optimum health (DellaPenna 1999). Consequently, RDA guidelines may not reflect studies which show how elevated levels of many plant-derived nutrients help in the prevention of certain diseases such as cancer, cardiovascular disease or macular degenerative disease.

Therefore, in much of the industrialised world, where food is abundant as well as varied, and daily caloric intake is often excessive, nutrient deficiencies are surprisingly common, owing to poor eating habits and to diets generally poor in foods containing the highest levels of nutrients such as vitamins and minerals (Agudo et al. 2002;Casagrande et al. 2007;Krebs-Smith et al. 1997;Nebeling et al. 2007;Schulze et al. 2001;Slimani et al. 2002).

Finding ways to increase the intake of staple crops' nutritive substances in the population is an increasingly important field of research. The selective increase of certain plant-derived nutrient concentrations in key crop species, through modern breeding techniques and molecular genetic approaches, is important for creating food that is not only nutritionally significant but also, to a certain extent, therapeutic as in aiding in the prevention of certain degenerative diseases.

1.1.2 Phytonutrient properties of plants

1.1.2.1 Functional foods, nutraceuticals and nutrigenomics

A healthy diet is now viewed as the first step towards the prevention of many age-related chronic diseases such as cancer, cardiovascular disease, high cholesterol, arthritis and degenerative macular disease. The growing interest in the promotion of health and well-being through food has sparked the growth and development of new areas in the sciences of food and nutrition. One such area is known as 'functional foods', which can be defined as "those foods that when consumed regularly exert a specific long term health-beneficial effect beyond their nutritional properties (i.e., a healthier status or a lower risk of disease) which must be scientifically proven (International Life Science Institute; http://www.ilsi.org)" (Briskin 2000;Espin et al. 2011).

Functional food compounds provide benefits beyond basic nutrition, owing to their physiologically active components, such as certain phytochemicals in

plants (Hasler 2000). Functional foods include whole, fortified, enriched or enhanced products which have the potential to benefit the consumers' health, if consumed regularly and as part of a varied diet. In other words, the benefits to health from consumption of these foods may arise from long term use (e.g. for chemoprevention). These food-types have recently gained much attention owing, in part, to growing public awareness and to the overwhelming scientific evidence of the link between health and nutrition (Berger 2005;Grassi et al. 2008;Kaliora et al. 2006;Kris-Etherton & Keen 2002;Lau et al. 2005;Meydani 2002;Nicolle et al. 2004).

Another such growing field is that of nutraceuticals: "diet supplements that deliver a concentrated form of a presumed bioactive agent from a food, presented in a non-food matrix, and used with the purpose of enhancing health in dosages that exceed those that could be obtained from normal foods". The two terms are often wrongly interchanged as nutraceuticals are sold in presentations similar to drugs: pills, extracts, tablets, etc. The Food and Drug Administration (FDA; http://vm.cfsan.fda.gov) regulates dietary supplements under a different set of regulations than those covering conventional foods and drug products (however, no specific regulation exists in Europe to control nutraceuticals) (Espin et al. 2011).

Advances in plant genomics combined with modern breeding methodologies provide opportunities for the enhancement of key phytonutrients, vitamins and minerals in targeted plant species with lower nutrient levels. These methodologies have led to the development of the field of nutrigenomics**,** i.e. the application of high-throughput genomics tools in nutrition research applied to human health (Milner 2000;Van der Meer et al. 2001;Van Ommen & Stierum 2002).

1.1.2.2 Secondary metabolites, phytochemicals and phytonutrients

In order for them to protect themselves, many plants produce thorns, spikes and stinging hairs, or they develop thick barks as a means for protection from

predators. In addition to these mechanical or morphological forms of protection, plants have developed secondary metabolites as chemical defences against herbivores, viruses, bacteria, fungi and competing plants (Maffei et al. 2007;Taiz & Zeiger 1998). Secondary metabolites are phytochemicals which are very specific to the type of plant. More than 200,000 have presently been identified, such as nitrogen-free terpenes, polyphenolic compounds and nitrogen-containing compounds (Oksman-Caldentey et al. 2004;Wink M. 1999). It has been estimated that 25-30% of the genes in *Arabidopsis* encode metabolic enzymes. They are termed secondary metabolites as they do not appear to have any direct function in plant growth and development. Secondary metabolites are also important colour pigments which can serve as signal molecules for attracting animals as pollinators and seed-dispersers for fertilisation or as defence chemicals against damage by ultraviolet light. In addition to this, secondary metabolites function as signal molecules in plant-plant, plant-herbivore and plant-microbe interactions, and have roles in plant mechanical support and for reducing growth of nearby competing plants. Often the same compound will hold a second activity, for instance, carotenoids are essential in plant photoprotection, sheltering the photosynthetic membrane from excess energy by quenching the excited state of chlorophyll, through electron transfer and will also be important orange colour pigment molecules serving as attractants to pollinators and seed dispersers (Taiz & Zeiger 1998;Wink 1999).

Those secondary metabolites which are of nutritional interest to humans are referred to as phytonutrients. These are organic or inorganic compounds present in small amounts and are not used for energy but are nonetheless important in maintaining good health.

Secondary metabolites often serve as phytonutrients and antioxidants in commonly consumed plant foods. These compounds are distributed differently in plant tissues or organs, for instance, studies on hydrophilic and lipophilic antioxidants in lettuce varieties have shown that according to leaf

position, lipophilic antioxidant activity increased sharply from stem to outermost leaves, suggesting a protective role of these antioxidants in light exposed leaves (Cano & Arnao 2005). Exposure to light also influences the synthesis of a number of different plant compounds. For instance, Mou et al state that phytonutrient analysis of crisphead lettuce (an iceberg lettuce cultivar with a closed head structure) revealed that the content of β-carotene (provitamin A), Ca, and Fe was higher in the outer leaves than in the inner leaves due to differences in light exposure (2004). Generally, vulnerable tissues and those vital for survival and multiplication need greater protection than old, senescing tissues, thus they will accumulate or produce more defence compounds (Wink 1999). Seeds, seedlings, buds and young tissues will gather larger amounts of secondary metabolites either by actively synthesising them or by accumulating them from other parts of the organism. Concentration can often vary within the developmental periods of an individual and sometimes even diurnally. Changes can also often be seen between individuals of the same population as well as between populations. Many of these metabolites play specific roles in allowing adaptation to specific ecological niches.

Phytochemicals can be grouped into major or minor constituents depending on their average abundance in plants. Carbohydrates, lipids and proteins are present and necessary at gram levels per 100g of food portions, thus they are categorised as major constituents, whilst vitamins, health beneficial secondary metabolites and minerals can be found in the microgram to milligram range and are thus categorised as minor constituents (Grusak 2002). Although they only constitute on average 0.1 – 3% of a tissue's dry weight (Taiz & Zeiger 1998;Wink 1999), secondary metabolites are of great importance to the fitness of the plant. Minor constituents can be significantly increased, with minimal diversion of precursors and only limited modification in the plant's ability to store or sequester the target phytochemical. For instance: the iron content in rice was increased two-fold (Lucca et al. 2001); β-carotene was bioengineered in rice *ex-novo* and then again, further increasing the value up to 23-fold the first result (Ye, 2000; Beyer, 2002;

Paine, 2005); flavonol content in tomato has been increased seven-fold (Muir et al. 2001); and ascorbic acid has been enhanced seven-fold in tobacco and lettuce cultivars (Jain & Nessler 2000). Thus, although they are generally quantitatively small changes, they constitute large changes in the functionality of the food as they provide nutritional and health benefits at low doses.

Numerous polyphenol phytochemicals also fall under the category of phytonutrients (e.g. phenolic acids and flavonoids). These are not essential for human growth and development, as other types of nutrients such as vitamins, lipids and carbohydrates, but they are important in maintaining body functions and health throughout adulthood and the later phases in life, and can be defined as 'lifespan essentials' (Holst & Williamson 2008).

1.1.2.3 Historical uses of plant secondary metabolites

Secondary metabolites have been used by mankind for thousands of years as stimulants (e.g. caffeine and nicotine), dyes (e.g. indigo, shikonin), fragrances and flavours (e.g. essential oils, mustard oils, capsaicin, vanillin), hallucinogens (e.g. morphine, heroin, cocaine), insecticides (bio-pesticides – e.g. nicotine, pyrethrin), poisons (e.g. strychnine, aconitine) and for therapeutic reasons (e.g. quinine, atropine, codeine) (Wink 1999). A fourth of all prescribed drugs contain compounds derived directly or indirectly from plants. In fact, 11% of the 252 drugs the WHO considers basic and essential are derived from flowering plants.

1.1.2.4 Beneficial properties of phytonutrients

Many of these compounds, for instance glucosinolates and phenolic acids, have been shown or have been proposed to aid in the prevention of a number of diseases such as different types of cancer, by acting as antioxidants, thus protecting against mutagenesis and carcinogenesis (Appendix 1) (Chiao et al. 2004;Conaway et al. 2002;Gill et al. 2004;Hecht et

al. 2004;Muskiet 2005;Palaniswamy et al. 2003; van Ommen & Stierum 2002), helping to reduce cholesterol levels (Graf, 1992; Nicolle et al. 2004) or helping to prevent degenerative diseases such as age related macular degenerative disease (Johnson et al. 2000;Sommerburg et al. 1998). Several studies have also noted a correlation between age-related decline in cognitive function and lower status of dietary antioxidants. Dietary antioxidants may reduce the risk of dementia associated with vascular dysfunction and in the prevention of stroke and atheroschlerosis (Meydani 2002).

1.1.2.5 Oxidants and their damaging effects

A number of sources of oxidants exist, which have the potential to cause oxidative damage to DNA, proteins and other macromolecules, such as lipid membranes. These oxidants, also termed free radicals, are unstable atoms or molecules containing one or more unpaired electrons. They can be classified as being endogenous or exogenous, as they are mutagens produced by environmental agents such as sunlight, radiation, pollution and smoking but are also by-products of normal aerobic metabolism.

Endogenous free radicals are produced through normal human metabolism and are classified into two main categories: Reactive Oxygen Species (ROS), and Nitric Oxide Species (NOS). ROS are oxygen centred radicals normally produced as a by-product of the respiratory mitochondrial chain or by leukocytes. These are essential for cell signalling and for bacterial defence. ROS can attack any biochemical component of the cell and cause damage to vital proteins lipids and DNA (Gutteridge & Halliwell 1993). The other category of endogenous free radicals is a product of nitric oxide metabolism (NOS) and is the by-product of endothelial metabolism (Berger 2005). Endogenous oxidants comprise: phagocytic cells, which destroy bacteria, virus or parasite-infected cells with an oxidative burst of nitric oxide (NO), superoxide anions ($O_2^{-\bullet}$), hydrogen peroxide anions (H_2O_2) or

hypochlorite ions (OCl^-)* (Table 1.1); peroxisomes, organelles which degrade fatty acids and other molecules, produce peroxide anions (H_2O_2); cytochrome P450, a primary defence against natural toxic chemicals present in plants but resulting in oxidative by-products which can damage DNA; and by-products of aerobic respiration by mitochondria consuming O_2, reducing it sequentially to produce water whilst gradually leaking electrons (Fig 1.1).

Under normal conditions, about 1% of ROS escape the control of the endogenous antioxidant defence every day, and contribute to oxidative damage of surrounding tissues and therefore to the aging process. Thus, oxidative damage increases with age and has been described as the major type of endogenous damage leading to ageing (Ames et al. 1993). For instance, the free radicals: superoxide ($O_2^{-\bullet}$), peroxide anion ($O_2^{-2\bullet}$) and hydroxyl radical (•OH) and nitrogen oxides (NO_x) are highly reactive oxygen and nitrogen molecules which can increase the risk of disease by damaging cell membranes and contents (DNA, proteins and lipids).

Forms of exogenous sources of oxidants, for instance, include cigarette smoke** (which produce nitrogen oxides, NO_x), iron and copper salts, and even certain plant foods with large amounts of certain phenolic compounds such as chlorogenic or caffeic acid may generate oxides by redox cycling.

* Chronic infection can result in cancer, in fact one third of the cases of cancer in the world are due to endogenous oxidants (Ames, Shigenaga, & Hagen 1993).

** Cigarette smoke causes a third of the cancer cases and a quarter of the cases of heart disease in the US.

Table 1.1 Reactive Oxygen Species

Hydrogen peroxide	H_2O_2
Hypochlorite Oxide	OCl^-
Free radicals	
• Hydroxyl radical	$^{\bullet}OH$
• Hydroxyl ion	OH^-
• Superoxide anion and radical	$O_2^{-\bullet}$

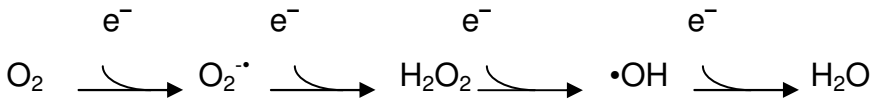


Fig 1.1 Oxidants generated from aerobic metabolism. The formation of superoxide ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical (•OH) result from the successive additions of electrons to O_2 (adapted from: Ames, Shigenaga, & Hagen 1993). Electron leakage is about 1-2% of partially reduced oxygen molecules which, instead of moving down the electron transport chain, jump directly to molecular oxygen forming the superoxide anion.

1.1.2.6 Antioxidants and their beneficial effects

Antioxidants are substances with the potential to inhibit or delay oxidation of a substrate while present in minute amounts. Antioxidant defences can be endogenous, which, in healthy subjects, can counteract the 1% daily leak of ROS, or exogenous, provided by nutrition (Halliwell & Gutteridge 1990). Dietary antioxidants seem to offer significant protection against the damage caused by oxidants and therefore help in the prevention of a number of age-related degenerative diseases due to oxidative damage including various

types of cancer, cardiovascular disease, decline of the immune system, cataracts, brain dysfunction as well as birth defects and childhood cancer, by counteracting the potentially damaging effects of oxidants. These antioxidants are mainly free radical scavengers and act through different mechanisms and in different compartments. They neutralise free radicals, reduce peroxide concentrations and repair oxidised membranes, quench iron to decrease ROS production, and, via lipid metabolism, short chain free fatty acids and cholesterol esters neutralise ROS (Parke 1999). The literature suggests that nutritional antioxidants also seem to help limit the damage caused by certain diseases and pathologies, i.e. they will not cure an installed disease such as gastrointestinal cancer but they may be able to prevent its promotion. For instance, antioxidants cannot cure ischaemia-reperfusion damage, but they may limit its ongoing extension, within the timings of a “therapeutic window” (Berger 2005).

Many phytonutrients, as well as vitamins and minerals, can act as antioxidants. Certain vitamins (A, C and E), and many phenolic compounds are potent antioxidants. They are free radical scavengers which can inhibit lipid peroxidation (Cook & Samman 1996) and the formation of DNA adducts which can lead to oncogenic mutations or cell death if not reversed by DNA repair mechanisms. Phytochemical antioxidant activities are due to their redox properties, which allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers (Drewnowski & Gomez-Carneros 2000;Kahkonen et al. 1999), reducing the adverse effects of free radicals. Antioxidant phytonutrients can be classified into those that protect lipid membranes from free radicals, such as vitamins A and E, and β-carotene (lipophilic). Whilst other antioxidant phytonutrients scavenge free radicals in the cytoplasm, such as vitamin C (hydrophilic).

1.1.3 Lettuce as a target species

The focus of the present study was on a selection of leafy crops such as the lettuce (*Lactuca sativa*) cultivars: salinas, green cos, red cos and lollo rosso,

their wild relative, *Lactuca serriola*, and two other salad crops, rocket (*Eruca sativa*, Shamrock Seeds Selection, standard variety) and spinach (*Spinacia oleracea,* Emilia cultivar, Pop Vriend, Holland), which are commonly consumed raw in many parts of the world (*L. sativa*, Salinas cv, and *L. serriola* and RIL population samples provided by the Genome Centre at UCDavis and other leaf samples provided by our industrial partners Vitacress Salads Ltd).

The focus of this project was solely on baby leaves, owing to the modern commercial trend of producing young leaves. This modern trend for baby leaves stems from the result of studies, such as those carried out on crucifers by J.W. Fahey et al. (1997) and Drewnowsky et al. (2000), which show that young plants, in this case sprouts, contain 10 to 100 times higher levels of secondary metabolites, including important phytonutrients, than their adult counterparts.

1.1.3.1 Classification

The genus Lactuca is classified in the Compositae family (Asteraceae), subfamily Cichorioidae, tribe Lactuceae Cass., subtribe Lactucinae Dumort. The family has a worldwide distribution divided into 17 tribes, with about 1,535 genera and 23,000 species. At least 98 wild Lactuca species have been described taxonomically.

The Compositae family include a number of economically important species including food and oil (lettuce and sunflower), medicinal (chamomile) and several horticultural crops (marigold, dahlia and chrysanthemum). Several important chemicals and insecticides (pyrethrum) and rubber (guayule) as well as detrimental weeds (thistle and dandelion) also originate from the Compositae family.

Despite current recognition as the largest family of flowering plants (23,000 species ± 1000) (Anderberg et al. 2007), the Compositae is home to

comparatively few important crop species. *L. sativa* and *Helianthus annuus* (sunflower) are the two most important species in the family, in economic terms, and have been extensively studied through molecular investigations of genetic structure and diversity. The scarcity of domesticated species in this family has been attributed to a variety of factors, chiefly the pervasiveness of secondary defence compounds, the lack of carbohydrates that can be digested by the human gut and the predominantly mechanical or wind-dependent seed dispersal syndrome (Dempewolf et al. 2008).

1.1.3.2 Domestication of lettuce

L. sativa is related to a number of wild Lactuca species, such as *L. saligna*, *L. virosa*, *L. perennis* and *L. indica*. However, analysis of 67 diverse cultivars and related species with RFLP markers suggested that domesticated lettuce may have evolved directly from the wild relative *L. serriola* or from a common ancestor (Kesseli & Michelmore 1996). The domestication process of lettuce started in the Eastern Mediterranean region as the centre of diversity for the genus when the climate of that part of North Eastern Africa was not as dry as it is today. Engravings on ancient Egyptian tombs suggest that domestication happened c. 4500BC, possibly as an oil seed crop, as depicted by rosettes of a tall large vegetable with subulate leaves painted on the walls of tombs at Thebes (Damania 1998).

Early human selection for non-shattering seed heads, late flowering, non-prickly leaves, decrease in latex content and hearting character (the tendency of leaves to congregate in layers in a heart-shaped head) is said to have led to its domestication as a leafy salad vegetable. Lettuce reached China around the 7th century AD where a special morphotype of *L. Sativa* was developed and reported by Fuchs in 1543 from Sinkiang (Damania 1998).

The domestication process of lettuce gradually generated about 100 different morphotypes and caused a number of morphological changes from its wild progenitor: the vegetative rosette has become exaggerated, branching has

been reduced, flowering and bolting were delayed, spininess, latex content and bitter compounds have been reduced, seed size and seedling vigour have been increased, germination times decreased, and shortening of internodes and non-shattering characteristics have been selected for (Lebeda et al. 2009) (Fig 1.2).

Lettuce, believed to be one of the first vegetables brought to the new world by explorer Christopher Columbus, has been grown in the United States since colonial times. In the early 1900s, the iced shipping industry was developed in the western states, expanding the range and popularity of lettuce. Today, in terms of production value, it is the leading vegetable crop in the United States and Europe. More than 90% of U.S. lettuce production is located in California and Arizona. The main types include head (iceberg, butterhead, Boston and Bibb), romaine and various leaf varieties (AGMRC: http://www.agmrc.org/commodities__products/vegetables/lettuce_profile.cfm)

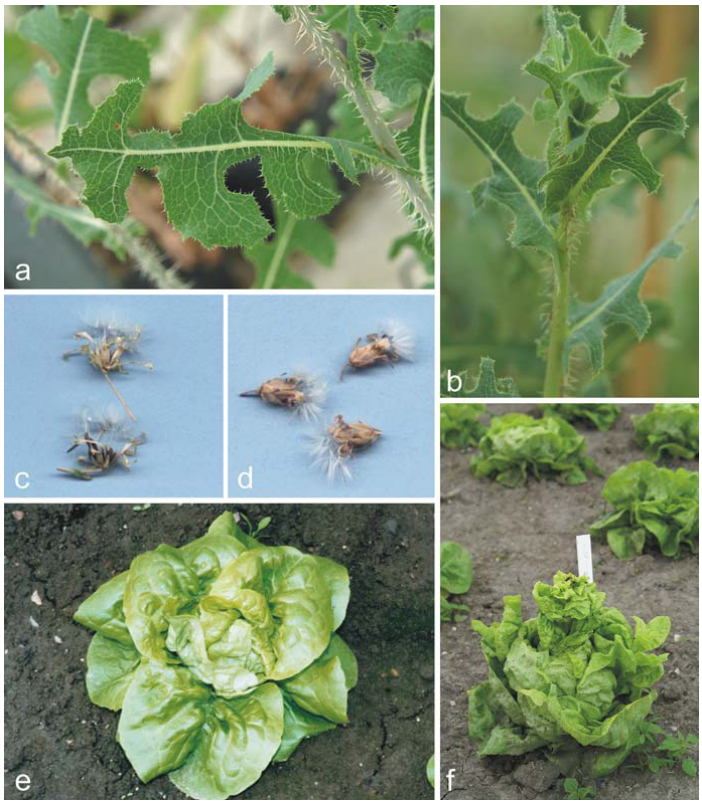


Fig 1.2 Lettuce phenotypic characteristics selected through domestication. Domestication of lettuce involved selection against latex content (a), leaf and stem spines (a, b), increase in seed size and non-shattering seeds (c, d), shortening of internodes, bunching of leaves (e), and selection for late bolting (f). (a),(b) and (c) *L. serriola*; (d),(e) and (f) *L. sativa* (Fig extracted from Lebeda et al. 2009).

1.1.3.3 Phylogeny and geographical distribution of wild Lactuca species

Lactuca sativa is part of the Compositae (Asteraceae) family, one of the largest and most diverse families of flowering plants (Anderberg et al. 2007). The Compositae family includes one tenth of all angiosperms (Funk et al. 2003) and is believed to have undergone rapid and extensive diversification, after originating in South America 30 to 100 million years ago. The family is divided into three subfamilies with 12 to 18 tribes, 1,100 to 2,000 genera and includes over 20,000 species (Kesseli & Michelmore 1996).

The worldwide distribution of the genus includes 17 species in Europe, 51 in Asia, 43 in Africa and 12 in the Americas (cultivated lettuce, *L. sativa*, is not considered in this section due to its worldwide commercial distribution). Some taxa (e.g. *L. serriola* and *L. saligna*) are naturalised in Australia and Tasmania. Lactuca is mostly distributed in the Northern hemisphere, in temperate and warm regions. Most of the species are well adapted to dry climatic conditions (xerophytes), except for some liana like endemic species found in the central African mountains and for eastern African tropical species (Lebeda et al. 2004). Lactuca species are very diverse and occur in many different habitats. Some more common European species are often ruderals (synanthropic) and therefore prefer disturbed habitats, such as *L. serriola*, *L. saligna* and *L. virosa*. However some species, e.g. *L. aurea, L. quercina, L. biennis* and *L. sibirica*, are common in woodland habitats. A number of typically Mediterranean varieties can be found on rocky slopes, e.g. *L. intricate, L. senerrima* and *L. vinimea* (calciphilous plants). In fact, the highest diversity of European Lactuca species occurs in the Mediterranean region, and some taxa are strictly confined to this region. *Lactuca serriola* in particular, is the most variable and most widely distributed species of the genus. It is a meridional-temperate, western Eurasian species which now has a synanthropic worldwide distribution. The species has recently spread throughout Europe as an invasive weed, occupying ruderal places, which supports the general characteristics of the species as an ‘r’ strategist, a pioneer plant which spreads easily with human activities (Lebeda et al. 2009).

1.1.3.4 Lettuce demand

Most families view lettuce and salads as essential components of their diets. Baby salad leaves contribute to the recommended intake of fresh fruits and vegetables and lettuce still remains one of the preferred vegetable crop species in Europe and the United States. While head (iceberg) lettuce, such as the Salinas cultivar used in this study, is still the dominant green salad, its yearly consumption decreased slightly from 9.57Kg in 2005 to 9.21Kg in

2007 per person (in the United States). Consumption of fresh market romaine and leaf lettuce increased from 4.81Kg in 2005 to 6.85Kg in 2008. The growing popularity of ready-to-eat packaged salad greens, introduced in the late 1980s, has contributed to the dramatic growth in the amount of romaine, leaf lettuce and spinach available for consumption in the United States (Amber Waves 2007 http://www.ers.usda.gov/AmberWaves/June07/PDF/Indicators.pdf). Interestingly, some of the increased popularity of romaine lettuce is due in part to the increased popularity of the Caesar salad. Leaf lettuce consumption has risen largely due to the popularity of salad bars, and has benefited from the introduction of packaged salads.

1.1.3.5 Lettuce production

Lettuce is a major horticultural crop with a worldwide production of over 21million tons in 2004 and accounts for $2.06billion in farm value in the USA. The United States is the second largest lettuce producing country behind China, which continues to dominate world production. U.S. production of lettuce in 2008 totalled 91 million pounds, while China produced more than twice as much (26 billion pounds). Chinese production accounted for 51 percent of global production compared to 22 percent for the United States. Lettuce production occurs year-round throughout the United States, through a sequence of production in Arizona and California which account for about 98% of US commercial domestic output, as well as commercial greenhouse hydroponic facilities. The total acreage of lettuce planted in 2008 was 282,400 acres (*Vegetables and Melons Outlook* /VGS-338/April 22, 2010; Economic Research Service, USDA http://www.ers.usda.gov/publications/vgs/tables/fresh.pdf).

The United States remains a leading lettuce exporter, second behind Spain, accounting for 20% of global exports (Amber Waves 2007 http://www.ers.usda.gov/AmberWaves/June07/PDF/Indicators.pdf). The main destinations of Spanish lettuce are Germany and the UK, which account for

55% of European imports of lettuce. Specifically, in 2008 the United Kingdom imported 172,414 tons of lettuce, while the German consumers bought 236,686 tons. In 2008 Spain exported 554,165 tons of lettuce of which 439,024 came from the Murcia region, which accounts for 80% of lettuce exported by Spain (Fresh Plaza: http://www.freshplaza.com/news_detail.asp?id=61014).

Home production of lettuce (whole head) in the UK was 117,3 thousand tonnes in the year 2009, with a value of 84,688 thousand pounds (http://www.defra.gov.uk/). During the same year, the UK imported 155,1 thousand tonnes of lettuce (with a value of 152,355 thousand pounds) and exported 5,7 thousand tonnes. The total supply in 2009 was 266,7 thousand tonnes, with Home Production Marketed (HPM) as a percentage of the total supply was 44.0%.

1.1.3.6 Lettuce breeding

L. sativa can easily be bred with closely related species in *Lactuca* such as *L. serriola* (also known as prickly lettuce), *L. saligna*, and *L. virosa*, and breeding programs for cultivated lettuce have included those species to broaden the available gene pool. These varieties are in fact important in current lettuce cultivar breeding programs as resistance genes donors (Kuang et al. 2008;Lebeda et al. 2004;McHale et al. 2009;Simko et al. 2009). Wild Lactuca species have been characterised into three gene pools on the basis of their relationship to *L. sativa*. On the basis of molecular studies, the primary gene pool which is also characterised by the absence of crossing barriers, includes species such as *L. serriola* (the probable progenitor of *L. sativa*), *L. altaica, L. aculeata and L. dregeana.* More distantly related species are included in the secondary (*L. saligna* and *L. virosa*) gene pool and the tertiary gene pool is represented by *L. quercina*, *L. tartarica, L. sibirica* and *L. viminea* (Koopman et al. 2001;Lebeda et al. 2004;Lebeda et al. 2001). The genetic resources of wild Lactuca species from the primary and secondary gene pools are considered important sources of many

characters in lettuce breeding, however only about 20% of species are available in germplasm collections worldwide and over 90% of accessions are represented by only three species: *L. serriola, L. saligna* and *L. virosa* mostly of European origin (Fig 1.3). The autochtonous species from other continents (Africa, Americas and Asia) which form c. 80% of Lactuca species richness are represented by only 3% of the accessions. Lebeda et al suggest that genebanks should focus on acquiring lettuce progenitors and wild relatives from the lettuce centre of origin and from areas with the highest genetic diversity of Lactuca species (2004). The highest levels of diversity have been found in the Mediterranean basin and South Western Asia, indicating these as hotspots for lettuce conservation (Lebeda et al. 2009).

Fig 1.3 cultivated and wild Lactuca species included in the first and secondary gene pool. Wild Lactuca species involved in lettuce improvement: (a) cultivated *L. sativa*; wild species, (b) *L. serriola f. serriola*, (c) *L. serriola f. integrifolia*, (d) *L. saligna*, (e) *L. aculeata*, (f) *L. virosa* (extracted from Lebeda et al 2009).

1.1.4 Selected traits

Lettuce is often not renowned for producing large quantities of nutritional compounds compared with other varieties of leafy salad crops. However, a number of antioxidant phytonutrients do characterise lettuce leaves, including various vitamins and numerous polyphenols, such as quercetin and dicaffeoyl tartaric acid (DuPont et al. 2000;Ferreres et al. 1997;Llorach et al. 2008;Nicolle et al. 2004;Niizu & Rodriguez-Amaya 2005). Studies such as those carried out by Nicolle et al (2004) have shown that lettuce consumption exhibits a beneficial effect on lipid metabolism and on tissue oxidation. They conclude that regular consumption of lettuce should help protect against cardiovascular diseases (CVD) owing to their ability to decrease the total cholesterol LDL/HDL ratio and particularly liver cholesterol levels in rats, as well as improving antioxidant levels in the body. These antioxidant properties are due to the richness in the antioxidants: polyphenols, vitamins C and E, and carotenoids (Mou 2004;Mou 2005;Nicolle et al. 2004)

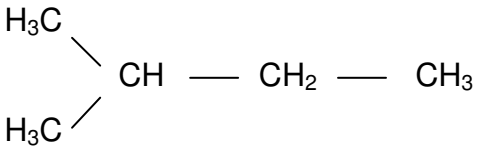
Thus, the following phytonutrients were chosen for the purposes of this study: total carotenoid (and correlated chlorophyll) content; polyphenolic compounds; vitamins C and E; and the total antioxidant content in general.

1.1.4.1 Carotenoids

Carotenoids in plants

Carotenoids are C_{40} isoprenoid polyene yellow, orange and red colour pigments (Farre et al. 2007). These compounds are part of a large category of secondary metabolites: the terpenes, or terpenoids (Taiz & Zeiger 1998).

The biosynthetic origin of terpenes derives from acetyl CoA or glycolytic intermediates and their basic structure is formed by the union of five-carbon elements whith the branched carbon skeleton of isopentane:



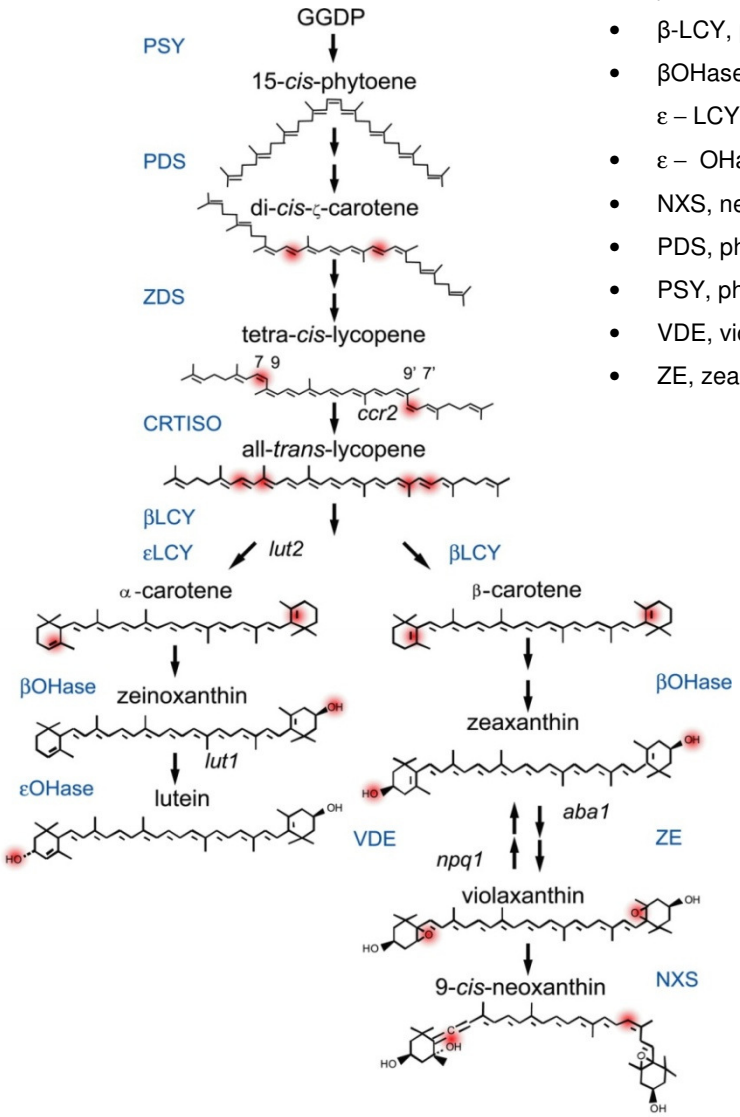
(the carotenoid biosynthetic pathway is shown in detail in Fig 1.4) Carotenoids are lipid soluble compounds and can be classified into two groups: the oxygenated xanthophylls (lutein, violaxanthin and zeaxanthin) and the hydrocarbon carotenes (β carotene, α carotene and lycopene (or ψ carotene) (Kopsell & Kopsell 2006). In higher plants, carotenoids are synthesised and localised in plastids where they are associated with light-harvesting complexes (LHCs) in the thylakoid membranes, or present as semicrystalline structures originating from the plastids (Faulks & Southon 2005;Fraser & Bramley 2004). Within the thylacoid membrane carotenoids are bound to specific protein complexes of photosystems I and II (Davuluri et al. 2005).

Some plants have a modified biosynthetic capacity and are able to synthesise compounds which are restricted to their genus or even species, for instance, Lactuca can produce lactucaxanthins and sesquiterpene lactones (which give most of the bitter taste in lettuce) as well as other carotenoids, which are found in the glandular hairs and serve as anti-herbivore agents (Taiz & Zeiger 1998).

In plants, carotenoids serve a number of purposes: they can function as pollinator attractants and seed dispersing animal attractants (owing to the orange colour they give to certain plant parts), as well as serving as accessory pigments in photosynthesis. Carotenoids play an important role in photo-protection of the photosynthetic membrane, which can be damaged by

the large amounts of energy absorbed by the pigments (Taiz & Zeiger 1998). They are termed ‘accessory’ as the light energy absorbed by carotenoids (mostly in the blue-green wavelength; 400-500nm region) is rapidly transferred to the photosynthetic core complex (CC) or reaction centres (RC). β–carotene in the PSII complex is concentrated in the CC whilst lutein is present in several light harvesting antennae components (Niyogi et al. 1997). Carotenoid pigments protect photosynthetic structures by quenching the triplet excited chlorophylls (^{3}Chl) and by binding to the reactive singlet oxygen ($^1O_2$), which are formed when the absorption of light radiation exceeds photosynthetic capacity (Tracewell et al. 2001;Young 2006). Carotenoid concentrations and chlorophyll* concentrations are in fact highly correlated (Mou 2005). The xanthophylls, such as lutein and zeaxanthin, are contained in the LHCI and LHCII and might also be involved in the reduction of lipid peroxidation and structural stabilisation of LHCs.

* Interestingly, chlorophyll also may have anti-carcinogenic function (Schreiner 2005)



Biosynthetic enzyme abbreviations:

- β-LCY, β-carotene cyclase;
- βOHase, β-carotene hydroxylase;
  ε – LCY, ε – cyclase;
- ε – OHase, ε – carotene hydroxylase;
- NXS, neoxanthin synthase;
- PDS, phytoene desaturase;
- PSY, phytoene synthase;
- VDE, violaxanthin deepoxidase;
- ZE, zeaxanthin epoxidase

Fig 1.4 Carotenoid biosynthetic pathway in land plants. Nearly all plant species share a common pathway (Arabidopsis mutations are shown in italics). The red highlights enzyme activity for generating the product at each step. Fig reproduced from Dellapenna and Pogson (2006).

Health benefits of carotenoids

In recent years, carotenoids have received a considerable amount of attention as potential anti-cancer and anti-aging compounds. A number of carotenoids, such as carotenes (α, β and γ), lutein, zeaxanthin and lycopene, have been shown to play important roles in the prevention of age related diseases (Zaripheh & Erdman 2002). Lutein, for instance, offers protection against macular degenerative disease, the leading cause of blindness and visual impairment among Americans 55 years or older (Seddon & Chen 2004). Lutein and zeaxanthin are the predominant carotenoids of the retina and are considered to act as photoprotectants preventing retinal degeneration (Stahl & Sies 2005). Epidemiological studies also suggest that the onset of chronic diseases such as certain cancers (e.g. prostate, colon and lung, albeit only in non-smokers), coronary heart disease and eye diseases such as cataracts may be reduced with consumption of carotenoid-rich foods owing to their antioxidant activities (Johnson et al. 2000;Mannisto et al. 2004;Slattery et al. 2000). Carotenoids are powerful antioxidants, protecting the cells of the body from damage caused by free radicals. β-carotene is also believed to enhance the function of the immune system (van den Berg et al. 2000). In addition to their antioxidant and immune-enhancing activity, carotenoids have shown the ability to stimulate cell to cell communication (Tapiero et al. 2004;van den Berg et al. 2000). Researchers now believe that poor communication between cells may be one of the causes of the overgrowth of cells, a condition which eventually leads to cancer. By promoting proper communication between cells, carotenoids may play a role in cancer prevention.

Carotenoids can be found in a number of fruit and vegetables, including lettuce varieties. There are more than 600 carotenoids found in nature, with 40 dietary carotenoids regularly consumed in the human diet (Kopsell & Kopsell 2006). The provitamin 'A' carotenoids, in particular α, β, γ and the xanthophyll β-cryptoxanthin, are used as substrates for retinal synthesis, the light-absorbing molecule, in many animals, making it a very important nutrient for healthy vision (DellaPenna 1999).

β-carotene is composed of two retinyl groups, and is broken down in the mucosa of the human small intestine by beta-carotene 15,15'-monooxygenase to retinal, a form of vitamin A (hence the term provitamin A). β-Carotene can be stored in the liver and body fat and converted to retinal as needed, thus making it a form of vitamin A for humans and other omnivores. The carotenes α-carotene and γ-carotene, due to their single retinyl group (beta-ionone ring), also have some vitamin A activity (though less than β-carotene), as does the xanthophyll carotenoid β-cryptoxanthin. All other carotenoids, including lycopene, have no beta-ring and thus no vitamin A activity, although they still have antioxidant activity and thus may help in the prevention of cancers.

Carotenoid toxicity and deficiency

Carotenemia or hypercarotenemia is excess carotene, but unlike excess vitamin A, carotene is non-toxic. Although hypercarotenemia is not particularly dangerous, it can lead to carotenodermia, an oranging of the skin.

β carotene is the most powerful provitamin A. Deficiency of this carotenoid can result in xerophthalmia, blindness and premature death (Mou 2005).

Carotenoid enhancement

Carotenoids in lettuce have been studied for comparisons between different lettuce cultivars and wild relatives (Mou 2004;Mou 2005;Mou & Ryder 2004); and for investigating the effects of supplemental ultraviolet radiation on carotenoid and chlorophyll content (Caldwell & Britz 2009). Carotenoid content enhancement has been employed in a number of crop cultivars. Strategies have been mainly focused on manipulating genes encoding carotenogenic enzymes, such as in cauliflower (Zhou et al. 2008). Successful carotenoid enhancement has also been applied to tomatoes (for carotenoid *and* flavonoid enhancement) (Davuluri et al. 2005); and in rice (Beyer, 2002; Paine, 2005).

1.1.4.2 Vitamin E

Vitamin E activity is produced by a large group of four tocopherols and four tocotrienols which together make up the tocochromanols (Schneider 2005). These tocochromanols are produced at various levels and in different combinations by all plant tissues and some cyanobacteria. Plant tissues vary considerably in their tocochromanol content and composition (Grusak & DellaPenna 1999). Photosynthetic tissues generally contain low levels of total tocochromanols but a high percentage of α-tocopherol, whilst seeds contain 10-20 times this level of total tocochromanols, with α tocopherol as a minor percentage of these.

Tocochromanols vary considerably in their vitamin E potential. The most potent vitamin E activity derives from α-tocopherol, followed by β-tocopherol and then by α-tocotrienol. These differences result from the preferential retention and distribution of α-tocopherols in animals, rather than differences in absorption of tocochromanol species during digestion (Traber & Arai 1999) (Fig 1.5).

Antioxidant activity of vitamin E

The antioxidant activity of tocochromanols helps to protect membrane lipids (especially polyunsaturated fatty acids - PUFAs) from oxidative damage by interacting with polyunsaturated acyl groups. Their antioxidant activity focuses on scavenging lipid peroxy radicals and quenching or chemically reacting with singlet oxygen ($^1O_2$*) and other ROS (Schneider 2005). A highly efficient charge-transfer mechanism achieves singlet oxygen quenching. The donation of a hydrogen atom from the tocochromanol hydroxyl ring terminates the free radical chain reaction of polyunsaturated fatty acids. This activity results in a ‘tocopherol radical’. A recycling of the tocopherol radical back to its corresponding tocopherol allows for the re-utilisation of the antioxidant, which then participates in a number of lipid peroxidation chain-breaking events before being broken down. This regeneration cycle activity

occurs in mammals, however it is unclear whether this process also occurs in plastids. Furthermore, tocochromanols are also able to scavenge a number of ROS and become converted to the corresponding quinone (Lass & Sohal 1998).

Tocopherols in plants

Tocopherols have been extensively studied mostly in animal systems owing to their health-promoting vitamin E and antioxidant activities (Schneider 2005;Zingg & Azzi 2004). Unlike in animal systems, tocopherol deficiency in plants is not lethal and, in Arabidopsis plants, their phenotypes are undistinguishable from their wild-type counterparts (Cheng et al. 2003;Maeda et al. 2005). Vitamin E biosynthesis occurs in the chloroplast envelope and knock-out mutations of tocopherol-cyclase in maize cause malfunctioning of a set of plasmodesmata, gap junctions which connect cell cytoplasms for the exchange of chemicals, proteins and RNAs, thus causing the mutants to accumulate anthocyanins, sugar and starch in leaves (Schneider 2005). However, the main function of these molecules in plants has been shown to be in the protection of seed storage lipids from oxidation during dormancy and germination (Sattler et al. 2004).

Health benefits of vitamin E

In humans, vitamin E has been shown to boost the immune system, delay the onset of Alzheimer's disease and increase resistance to oxidative injury associated with exercise. Studies have also shown that vitamin E may help in the prevention (but not the curing) of cardiovascular disease through a number of different mechanisms: by decreasing expression of proinflammatory cytokines, adhesion molecules and monocyte adhesion of the endothelial cells; by decreasing smooth muscle proliferation (α-tocopherol); by improving vessel relaxation; and by decreasing platelet aggregation. The antioxidant activity of vitamin E compounds may reduce lipid peroxidation due to ROS and RNS and the binding of their reactive

products to DNA, thereby preventing oxidative damage to DNA and, secondly, inhibiting the formation of nitric oxide species also known to react with DNA and other biological molecules. Vitamin E has also been shown to possess anti-cancer and cancer-suppression activities. These compounds may help in the prevention of tumour growth by inhibiting angiogenesis; by preventing its proliferation; by inducing apoptosis; and by inducing an immune response (Kalanithi 2007;Meydani 2002;Schneider 2005) (Fig 1.6).

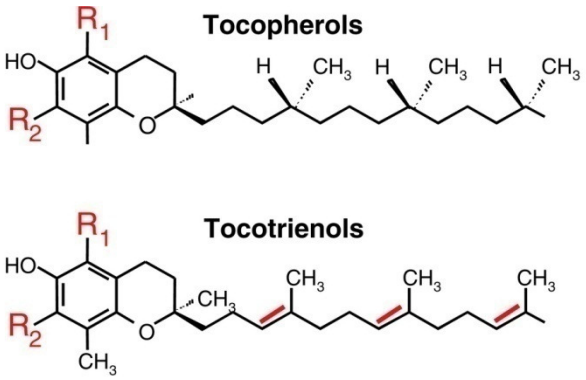
Vitamin E enhancement

Tocopherol enhancement has been achieved in a number of different crop species, such as *Brassica juncea,* an important oil seed crop, by overexpression of a cDNA encoding γ-tocopherol methyltransferase from *Arabidopsis thaliana* (Yusuf & Sarin 2007); and α-tocopherol in soybean seeds using cDNA encoding γ-tocopherol methyltransferase from green shiso (*Perilla frutescens)* (Tavva et al. 2007). In *Lactuca sativa,* Tocopherol enhancement has also been achieved, by overexpression of a cDNA encoding γ-tocopherol methyltransferase from *Arabidopsis thaliana* (Cho Eun et al. 2004) (the complete biosynthetic pathway is displayed in Fig 1.7).

Vitamin E deficiency and toxicity

Vitamin E deficiency in humans causes neurological problems due to poor nerve conduction. These include neuromuscular problems such as spinocerebellar ataxia and myopathies. Deficiency can also cause anemia, due to oxidative damage to red blood cells. However, vitamin E deficiency is very rare in humans and is almost never caused by a poor diet.

The RDA for vitamin E is 15mg. Because vitamin E can act as an anticoagulant and may increase the risk of bleeding problems, many agencies have set an upper tolerable intake level (UL) for vitamin E at 1,000 mg (1,500 IU) per day.



Activity versus α-tocopherol

Tocochromanol type	R_1	R_2	α-TPP binding	Vitamin E activity
α-tocopherol	CH_3	CH_3	100	100
α-tocotrienol	CH_3	CH_3	12.5	21-50
β-tocopherol	CH_3	H	38	25-50
β-tocotrienol	CH_3	H	nd	nm
γ-tocopherol	H	CH_3	9	8-19
γ-tocotrienol	H	CH_3	nd	nm
δ-tocopherol	H	H	1.5	<3
δ-tocotrienol	H	H	nd	nm

Fig 1.5 Basic structures of tocochromanols. The key differences between tocophenols and tocotrienols are indicated in red. The table indicates the number and position of ring methyls in each tocopherol and tocotrienol molecule (α–, β–, γ–, δ-). The binding of each tocochromanol to an α-tocopherol transfer protein (α-TTP) and the vitamin E activity in the rat resorption-gestation assay are expressed as a percentage relative to α-tocopherol, Fig extracted from Dellapenna et al. (2006).

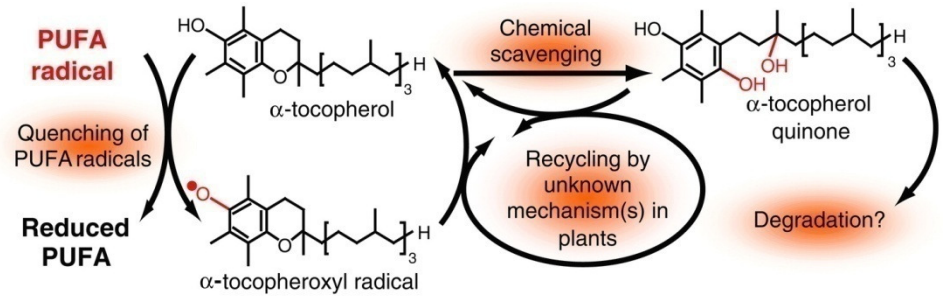
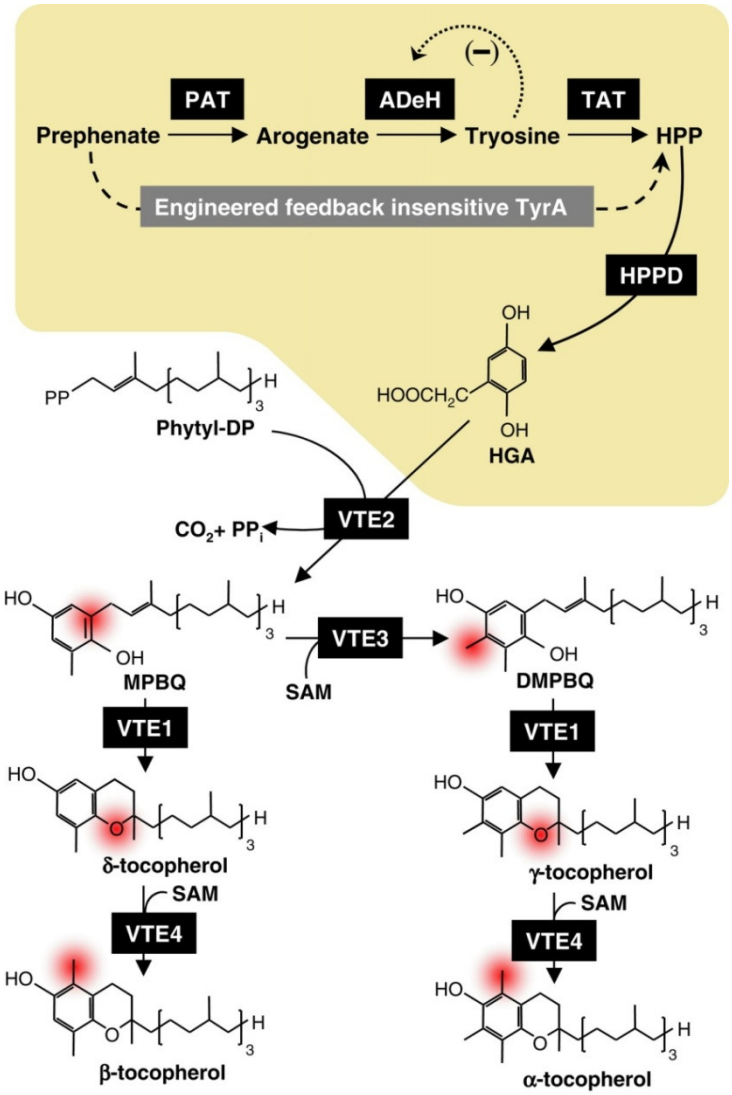


Fig 1.6 General diagram showing the quenching system of polyunsaturated fatty acids (PUFAs) and scavenging of Reactive Oxygen Species (ROS). The red indicates key differences between molecules, however not all reaction, intermediates and products are displayed, Fig extracted from Dellapenna et al. (2006).



Biosynthetic enzyme abbreviations:

- DMPBQ, 2,3-dimethyl-5-phytyl-1, 4-benzoquinone;
- HPP, p-hydroxyphenylpyruvate;
- HPPD, HPP dioxygenase;
- PAT, prephenate amino transferase;
- SAM, S-adenosyl methionine;
- TAT, tyrosine amino transferase;
- VTE1, tocopherol cyclase;
- VTE2, homogentisate phytyltransferase;
- VTE3, MPBQ methyltransferase;
- VTE4, γγ-tocopherol methyltransferase.

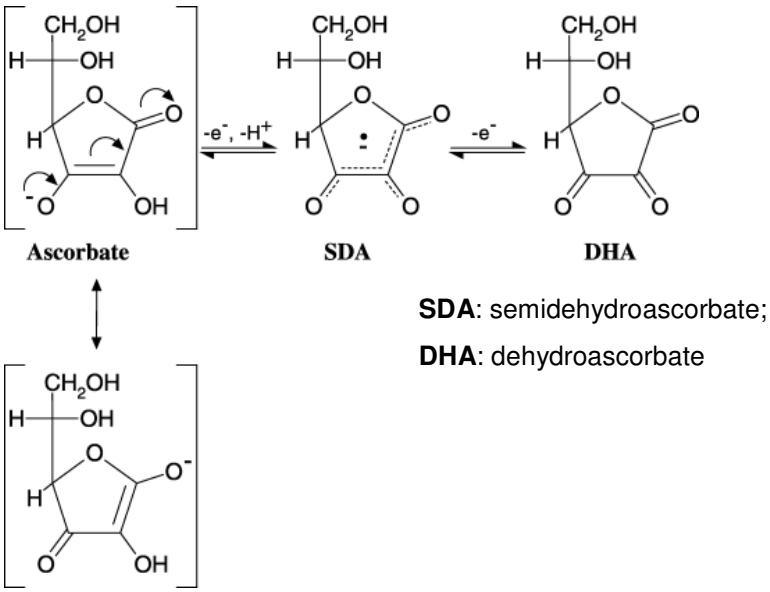
Fig 1.7 Tocopherol biosynthetic pathway in plants. The pathway and enzyme nomenclature, loci and genes are in reference to studies in Arabidopsis. Tocotrienols are biosynthesised using the same pathway, however, the phenyltransferase reaction (VTE2) in these organisms can also use geranylgeranyl diphsphate (GGDP) in addition to or in place of phytyl-diphosphate (phytyl-DP). α-tocopherol is the most abundant tocopherol produced in wild-type Arabidopsis leaves and in *Synechocystis* (sp. PCC6803). Whilst γ-tocopherol is the most abundant tocopherol in Arabidopsis seeds. The red highlights the acivity of the VTEs for generating the product for each step. Fig extracted from Dellapenna et al. (2006).

1.1.4.3 Vitamin C

Vitamin C in vertebrates and health benefits

There is an enormous amount of literature on vitamin C (L-ascorbate or L-ascorbic acid) intake and health in animals, cell cultures, and humans. Vitamin C is a potent reducing agent that efficiently quenches damaging free radicals (Fig 1.8); thus it is another antioxidant important in the prevention of age related diseases such as cardiovascular disease (CVD), in maintaining a healthy immune system and in aiding wound healing (Rowland 1999). Vitamin C is required for its function in collagen synthesis, L-carnitine and biosynthesis of certain hormones (Li & Schellhorn 2007), it is essential in the conversion of dopamine to norepinephrine. Furthermore, ascorbic acid is known to increase absorption of inorganic iron, to have essential roles in the metabolism of folic acid and of some amino acids and hormones, and to act as an antioxidant (Gershoff 2009).

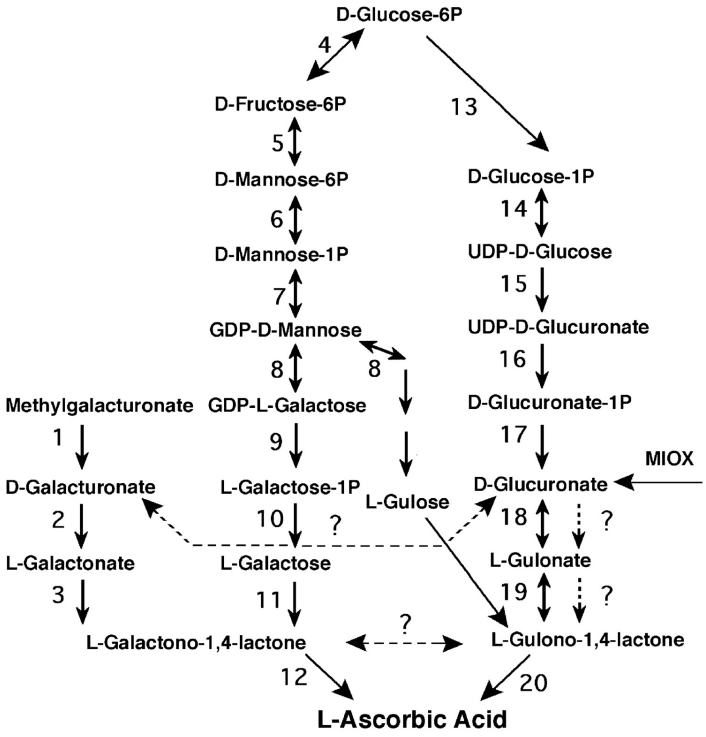
In vertebrates, the last step in vitamin C synthesis is the oxidation of L-gulonolactone to L- ascorbic acid by the enzyme L-gulonolactone dehydrogenase, which is associated with the endoplasmic reticulum membrane (Fig 1.9). Dietary intake of vitamin C is important in primates, guinea pig, and perhaps a few other vertebrates, as this last enzyme is absent due to mutations in its gene {Linster, 2007 469 /id}.



SDA: semidehydroascorbate;

DHA: dehydroascorbate

Fig 1.8 The three redox states of vitamin C. Ascorbate is the fully reduced form; SDA is the monooxidized form and DHA is the fully oxidized form. Stabilization of the ascorbate monoanion and SDA by electron delocalization are also shown. Figure extracted from Linster et al (2007).



Enzymes catalyzing the numbered reactions are:

1, methyl-esterase;

2, D-galacturonate reductase;

3, aldono-lactonase;

4, Glc-6-phosphate isomerase;

5, Man-6-phosphate isomerase;

6, phosphomannomutase;

7, GDP-Man pyrophosphorylase;

8, GDP-Man-3,5-epimerase;

9, phosphodiesterase;

10, sugar phosphatase;

11, L-Gal-1-dehydrogenase;

12, L-galactono-1,4-lactone (L-GalL) dehydrogenase;

13, phosphoglucomutase;

14, UDP-Glc pyrophosphorylase;

15, UDP-Glc dehydrogenase;

16, glucuronate-1-phosphate uridylyltransferase;

17, glucurono kinase;

18, glucuronate reductase;

19, aldono lactonase,

20, guluno-1,4-lactone dehydrogenase.

Fig 1.9 Vitamin C biosynthetic pathway in plants and animals. Reactions 1–12 are found in plant biosynthetic pathway and reactions 13–20 in animals. Four potential branch pathways operate in plants, the Man pathway, galacturonate pathway, the gulose pathway, and the MI pathway, are shown. Question marks indicate enzymatic reactions that have not been demonstrated in plants. Figure extracted from Lorence at al. (2004).

Ascorbate in plants

Ascorbate is abundant in plants and is found at concentrations of 25mM in chloroplasts (Wheeler, Jones, & Smirnoff 1998), with particularly high concentrations occurring in citrus fruits (oranges, lemons, limes, grapefruits), tomatoes, potatoes, cabbages, and green peppers. It helps plants deal with stresses from drought to ozone and UV radiation and it has been shown to be essential for plant growth. Ascorbate has been shown to be necessary for seedling growth, the absence of which causes immediate growth arrest upon germination and bleaching of the cotyledons (Dowdle 2007). Vitamin C provides protection against the harmful side-effects of light during photosynthesis thus plants are unable to grow without it (Colville & Smirnoff 2008). Interestingly, Arabidopsis low vitamin C mutants, *vtc1* and *vtc2*, which have between 10% and 25% of wild-type ascorbic acid, have been shown to exhibit microlesions, express pathogenesis-related (PR) proteins, and have enhanced basal resistance against infections caused by *Pseudomonas syringae*. The mutants also had a delayed senescence phenotype with smaller leaf cells than the wild type at maturity (Pavet et al. 2005).

Vitamin C is present in various foods, particularly of plant origin, but also in animal products, and in quantities that are generally quite high in comparison to other vitamins (typically 10-100mg/100g). This may be due to a simple biosynthetic process and to the fact that they are formed from sugars, which are abundant compounds (Fig 1.9) {Linster, 2007 469 /id}.

Vitamin C enhancement

A 4- to 7-fold increase in the Ascorbic Acid (AsA) content was obtained in lettuce (*Lactuca sativa*) and tobacco (*Nicotiana tabacum*) plants after constitutive expression of the rat (*Rattus norvegicus*) gene encoding L-GulL oxidase, the enzyme involved in the final step of the animal pathway (Jain & Nessler 2000). Ascorbic acid concentration was also increased in Arabidopsis, by constitutive expression of *miox4*, as *myo*-inositol (MI) is a

precursor of AsA biosynthesis found both in A*rabidopsis thaliana* (on chromosome 4) and in animal species. The levels of vitamin C were enhanced 2- to 3-fold in Arabidopsis leaves (Fig 1.9) (Lorence et al. 2004).

Vitamin C toxicity

Relatively large doses of vitamin C may cause indigestion, particularly when taken on an empty stomach. When taken in large doses, vitamin C causes diarrhoea in healthy subjects. The signs and symptoms in adults are nausea, vomiting, diarrhoea, flushing of the face, headache, fatigue and disturbed sleep, however, vitamin C exhibits extremely low toxicity.

Vitamin C deficiency

Perhaps the most notorious deficiency disease caused by inadequate intake of a vitamin form in humans is that of scurvy due to lack of vitamin C. Without vitamin C, the synthesized collagen is too unstable to perform its function. Scurvy leads to the formation of liver spots on the skin, spongy gums, and bleeding from all mucous membranes due to blood vessel fragility, connective tissue damage and is an ultimately fatal disease (Li & Schellhorn 2007). However, vitamin C deficiency occurs very rarely in most parts of the world today.

Recommended daily intake and tolerable upper intake levels

Recommendations for vitamin C intake have been set by various national agencies (Table 1.2).

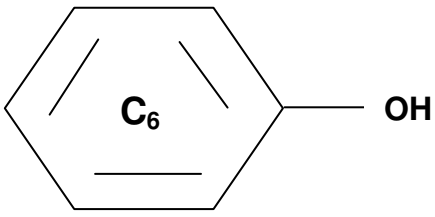
Table 1.2 Recommended daily intake and tolerable upper intake levels

75 milligrams per day: the United Kingdom's Food Standards Agency
45 milligrams per day: the World Health Organization
90 mg/day (males) and 75 mg/day (females): Health Canada 2007
60–95 milligrams per day: United States' National Academy of Sciences
The United States defined Tolerable Upper Intake Level for a 25-year-old male/female is 2,000 milligrams per day.

1.1.4.4 Polyphenolic compounds

Polyphenolic compounds are a large group of secondary metabolites responsible for carrying out a number of different functions such as defence against herbivores and pathogens, mechanical support, attracting pollinators and fruit dispersers, absorbing harmful UV radiation and reducing growth of nearby competing plants (Maffei et al. 2007;Taiz & Zeiger 1998). Overall, these compounds are abundant micronutrients in our diets as all plants synthesise a variety of different polyphenols and in different quantities and combinations. Several polyphenolic metabolites have been and are currently the subject of numerous investigations, as researchers and food manufacturers have become increasingly interested in these compounds. The main reasons for this increasing interest are the recognition of these as important antioxidant phytochemicals, the great abundance of these in our diets and their role in the prevention of age-related diseases such as cancer, cardiovascular and neurodegenerative diseases (Manach et al. 2004).

Several thousand polyphenol compounds have already been identified in higher plants, and several hundred in edible plants. These molecules are composed of a basic hydroxyl functional group attached to an aromatic ring (or benzene ring), which together form the phenol group:



The additions of hydroxyl groups, carbon groups, sugars, methyl ethers or modified isopentyl units to the basic carbon skeleton structure determine the type of phenol (Fig 1.10) (Taiz & Zeiger 1998). These compounds can be classified into several groups according to the number of phenol rings that they contain and to the structural elements that bind these to one another. These groups are the phenolic acids, flavonoids, stilbenes and lignans.

Furthermore, polyphenols may be associated with one another or with carbohydrates or organic acids.

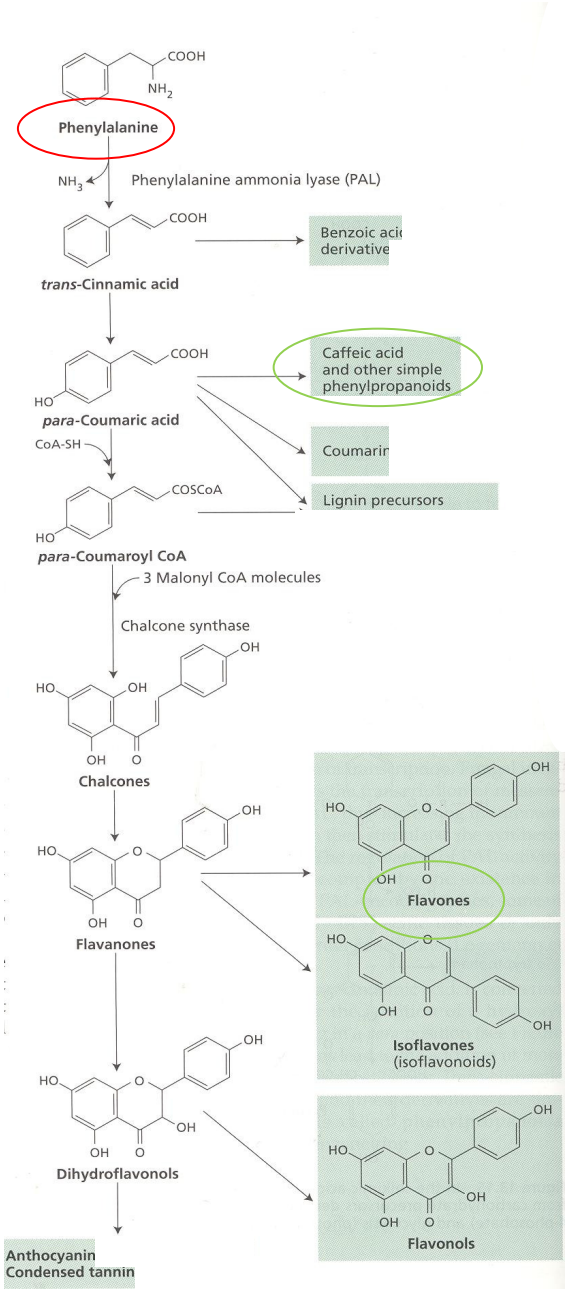
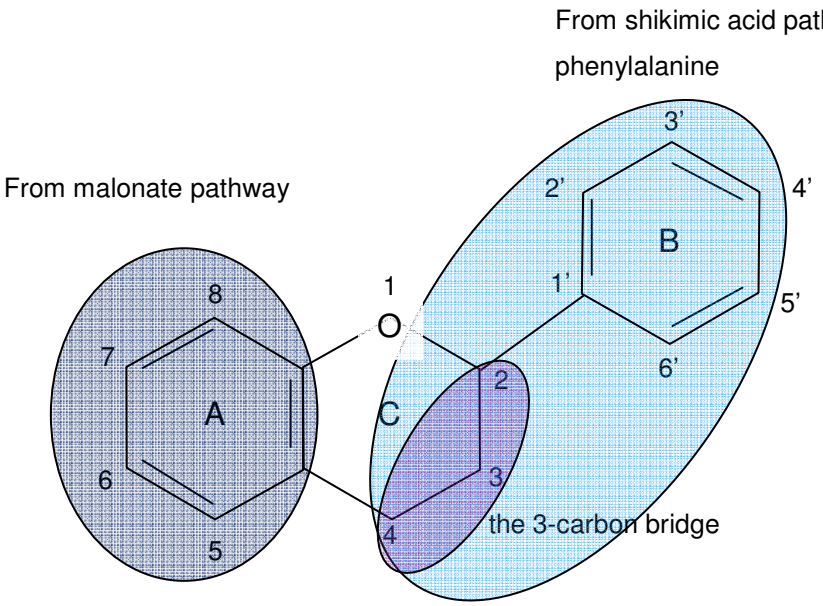


Fig 1.10 The polyphenol biosynthetic pathway. The most abundant phenols derive from phenylalanine via the elimination of an ammonia molecule to form the cinnamic acid. This reaction is catalysed by Phenylalanine Ammonia Lyase (PAL). Fig reproduced from Taiz and Zeiger (1998).

One of the largest classes of plant phenolics are the flavonoids, which are widely distributed in the leaves, seeds, barks and flowers of plants. Over 4,000 flavonoids have been identified to date (Heim et al. 2002). Flavonoids share a common structure of two aromatic rings (A and B) joined by a three-carbon bridge that form an oxygenated heterocycle (ring C):



The flavonoids may be subdivided further into eight sub-classes: flavonols, flavones, isoflavones, flavanones, anthocyanins, anthocyanidins, tannins and flavanols (catechins and proanthocyanidins) (Manach et al. 2004). Different flavonoids perform very different functions within the plant. They are important for attracting animals for pollination and seed dispersal (anthocyanins and anthocyanidins; responsible for the red, pink, blue and purple colours observed in plant parts); for protection against damage by ultraviolet light (flavones and flavonols); and for deterring feeding by herbivores (tannins).

Polyphenols in lettuce

Notable levels of certain polyphenolic compounds have been reported in lettuce, comparable to other important sources of these compounds such as onions, apples, tea and broccoli (Llorach et al. 2008;Nicolle et al. 2004). Significant levels of dicaffeoyl tartaric and chlorogenic acids (phenolic acids), together with isochlorogenic acid, several luteolins, caffeoyl malic acid, and kaempferols and several quercetin glucosides (flavonoids) in green lettuce leaves have been observed by Llorach et al. (2008).

The quantities of total phenolic content were shown to vary according to cultivar, and to leaf position, where, e.g. in romaine lettuce leaves, the content increased from stem to outermost leaves or between the greener outer leaves and the inner whiter leaves, where the flavonol concentration was more than 10 times lower. This was due to the fact that flavonol biosynthesis is stimulated by light, thus in fruit and vegetables there are marked differences in polyphenol concentrations, even within the same leaf or fruit sample, due to exposure to light (Cano & Arnao 2005;DuPont et al. 2000;Manach et al. 2004).

Interestingly, Liu et al (2007) observed that the cultivars with the highest antioxidant potential contained the highest total phenolic content, suggesting a strong correlation between the two. In agreement with Liu, an association between total antioxidant potential and total phenolic content was observed by Llorach et al (2004), and by Proteggente et al (2002) (who, in addition, noted an association between vitamin C and antioxidant activity in a number of different fruit and vegetables available in the UK). Nicolle et al (2004) also investigated the total antioxidant content of different lettuce varieties (three green varieties: butterhead, batavia and oak leaf, and one red cultivar: red pigmented oak leaf) and reported that 64% of the total antioxidant power of the leaves was due to their polyphenolic content. In particular the phenolic acid dicaffeoyl tartaric acid (or chicoric acid) accounted for over half of this power and the rest was due to caffeic and chlorogenic acids, and to the

flavonoid quercetin 3-*o*-glucuronide (in agreement with Proteggente et al., 2002). Thus polyphenolic compounds are a significant component of the total antioxidant potential of lettuce leaves.

Antioxidant potential of polyphenols and health benefits

Several studies have investigated the antioxidant potential and related health benefits of numerous polyphenolic compounds. For instance, a number of flavonoids have powerful antioxidant capacities and have been shown to aid in the prevention of heart diseases, cataracts, high cholesterol and allergies (Burda & Oleszek 2001;Kelly et al. 2002;Kris-Etherton & Keen 2002;Rowland 1999). Furthermore, different polyphenolic compounds have different degrees of antioxidant capacities. Interestingly, the antioxidant capacity of the flavonol quercetin, a significant polyphenolic compound found in lettuce, is 4.7mM expressed in Trolox Equivalent Antioxidant Capacity (TEAC), which is comparable to the antioxidant capacity of the flavanol epigallocatechin gallate found in tea (4.75 mM) and to the anthocyanidins found in red forest fruits (4.42 mM TEAC) (Kelly E Heim, Anthony, & Dennis 2002;Rice-Evans, Miller, & Paganga 1996).

The antioxidant potential of flavonoids has been attributed to their capacity to transfer electrons to free radicals, chelate metal catalysts, activate antioxidant enzymes, reduce α-tocopherol radicals and inhibit oxidases. These processes depend upon the arrangement of functional groups about the nuclear structure. The configuration and the total number of hydroxyl groups in the B ring substantially influence several mechanisms of antioxidant activity (Sekher Pannala et al. 2001). The ROS and RNS free radical scavenging capacity of flavonoids is primarily attributed to the high reactivity of the hydroxyl groups that participate in the following reaction:

$$F\text{-}OH + R^{\bullet} \longrightarrow F\text{-}O^{\bullet} + RH$$

Where F is the flavonoid; OH is the hydroxyl functional group and R is the free radical: the flavonoid hydroxyl group donates hydrogen and an electron to the hydroxyl, peroxyl and peroxynitrite radicals, stabilising them and creating a relatively stable flavonoid radical (Burda & Oleszek 2001). The antioxidant capacity increases linearly with the number of hydroxyl groups of the flavonoid species.

Conversely, the sugar moiety of the molecules has been shown to reduce the compound's antioxidant capacity due to its substitution to a functional hydroxyl group and to its lending hydrophylicity to the molecule thus limiting access to lipid peroxyl and alkoxyl radicals during propagation of lipid peroxidation (LPO) in membranes. Whether the sugar moiety is glucose, rhamnose or rutinose is also relevant. For instance, the scavenging efficiency of quercetin (Fig 1.11) is reduced if the glucose moiety is rhamnose instead of rutinose (Heim et al. 2002;Limasset et al. 1993).

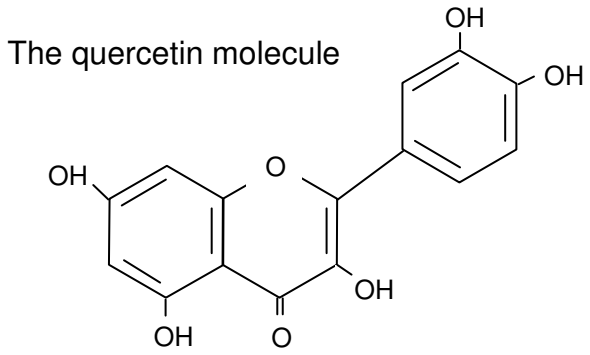


Fig 1.11 The basic structure of the quercetin molecule.

Simple phenols such as the phenolic acids have also been shown to aid in the prevention of a number of age-related degenerative diseases. For instance, the antioxidant activity of ferulic acids have been shown to help in the prevention of cancers and high cholesterol levels (Nicolle et al. 2004).

Interestingly, one study suggests that a number of polyphenolic compounds have even been found to be more effective antioxidants than vitamins C and E (Rice-Evans et al. 1997). In the Rice-Evans study vitamin C and E were reported to contain 1.0mM expressed in trolox equivalent antioxidant activity, whilst the flavonol quercetin contained almost five times as much (4.7mM Trolox equivalent antioxidant activity), thus these compounds might be major contributors to the protective effects of antioxidants in humans. Quercetin is in fact, one of the most abundant, potent and widely studied polyphenol (Heim et al. 2002).

Polyphenol deficiency

Unlike with vitamin C deficiencies or vitamin E and selenium (combined) deficiencies, a lack of dietary polyphenols does not cause any known clinical conditions. This is because polyphenols are not essential nutrients for growth and maintenance of a healthy metabolism but are compounds which aid in the prevention of diseases in the long run.

Phenolic contents: bitterness and toxicity

An important function of many secondary metabolites is protection against herbivore attacks and infection against microbial pathogen infection. Owing to their insecticidal, fungicidal and phytotoxic qualities, some secondary metabolites, e.g. nicotine, rotenone, quassin and pyrethrins, are and have been used in agriculture as natural plant protectants: ‘bio-pesticides’.

Several of these defence compounds also make the plant taste bitter and undesirable to animals, including humans (Acamovic & Brooker 2005). Bitterness, in fact, tends to be equated with dietary danger and can be detected in µmol amounts. Thus, during the history of agriculture, many important crops have been artificially selected for producing lower levels of these compounds, which had the adverse effect of making the plant also more susceptible to attacks by insects and disease (Drewnowski & Gomez-

Carneros 2000;Taiz & Zeiger 1998). Bitter phytonutrients, such as glucosinolates (other plant secondary metabolites), are particularly abundant in cruciferous vegetables, where they have traditionally been viewed as plant based toxins, however, recent studies showing that some of these also seem to lower the risk of cancer and cardiovascular disease (Drewnowski & Gomez-Carneros 2000), have driven studies aiming to investigate and enhance the levels of such compounds in plants through selective breeding or genetic improvement making them strong, natural options in the quest for the prevention of many diseases.

Polyphenol intakes

Polyphenol intake depends largely on dietary habits and preferences. There can be marked differences between populations, e.g. the Japanese have an average dietary intake of isoflavones of 30-40 mg/d due to their consumption of soy products, but also between individuals within populations, depending on the choice and frequencies of any plant derived foodstuffs. Major sources of polyphenols are beverages such as red wine, coffee, tea and certain fruit juices. For instance, heavy coffee drinkers will consume more phenolic acids than flavonoids, or chocolate eaters will have greater contribution of polyphenols, in particular, catechin and proanthocyanidin. The main sources of flavonols in Japan and the Netherlands are tea, wine in Italy and France and onions in the USA and Greece (Hollman 2000). In general, phenolic acids account for about one third of the total phenols and flavonoids account for the rest (Tapiero et al. 2002).

Bioavailability

The sugar moiety is an important determinant of the site of absorption of the polyphenol glycosides and thus of their bioavailability (Hollman 2000). Sugar conjugated flavonoids may be hydrolyzed by intestinal microflora or by hydrolases at the intestinal brush border membrane, after which the aglycone may diffuse across the membrane into the cell. Alternatively,

flavonoids may enter the cell as intact glycosides via the sodium-dependent glucose transporter (SGLT1), where they are then cleaved by cytosolic β-glycosidases (Day et al. 1998). Furthermore, different compounds can differ in absorption and excretion rates. For instance: Hollman states that the absorption rate of flavanols may be fast, reaching peak values between 0.5 and 4 h. Flavanols are also rapidly excreted, with elimination half-lives of 1 to 6 h. whilst, quercetin glycosides show rapid to slow absorption, peak values are reached between less than 0.5h and 9 h. Furthermore, the type of glycoside determines the rate of absorption. Whereas, excretion of quercetin glycosides is slow: elimination half-lives are 24 h, independent of the type of glycoside (2000). Thus, the chemical structure of polyphenols determines the rate and extent of their absorption in the intestine and nature of the metabolites circulating the blood plasma (Tapiero et al. 2002). Glucuronation, sulfation and methylation of polyphenols occur in humans. This is because the polyphenols must be hydrolysed by the intestinal enzymes or by the colonic microflora before they can be absorbed. This is a necessary metabolic detoxification process which restricts the potential toxic effects of the compounds and facilitates their biliary and urinary elimination by increasing their hydrophilicity.

Inter-individual variations have been observed, and absorption may also depend on a number of other variables, such as dosage, vehicle of administration, antecedent diet, sex differences and microbial population of the colon (Heim et al. 2002). Depending on the individual predisposition, including genetics and medication, a bioavailable dose may cause different magnitudes of effects in different people (Faye and German 2008). Age might affect the predisposition and thus the requirements phytonutrients (Holst & Williamson 2008). Furthermore, the polyphenols which are the most common in the human diet may not necessarily be the most active. This can be due to a lower intrinsic activity or because they may be poorly absorbed by the intestine, highly metabolised or rapidly eliminated. In addition, the metabolites found in the blood or in other target tissues, can have different biological activities from the original substance.

1.1.5 Safe upper levels of phytonutrient fortification

It is imperative before attempting the selective enhancement of plant vitamins and minerals, to research the safe upper limits of daily nutrient intake, or Tolerable Upper Intake Level (UL) of the nutrients considered, i.e. whether excessive intake of such nutrients might have any negative effects on health and what the highest level is that is likely to possess health benefits but pose no risk of adverse effects (Acamovic & Brooker 2005;Goldhaber 2003;Tang & Halliwell 2010).

For instance, nutrients such as the minerals iron, calcium, selenium and iodine, and a number of vitamins, such as folates, vitamins E, A and B_6, play significant roles in maintaining good health, yet they are nevertheless limited in diets worldwide (Acamovic & Brooker 2005;DellaPenna 1999;Grusak & DellaPenna 1999). Whereas the safe upper limits for these minerals range from 2 to 13 times the RDA, the safe upper limits for vitamin intake can be much higher, allowing for a greater manipulation range. An exception is vitamin A, or retinal, as only 5 times the RDA can already cause side effects. Fortunately, plants can only synthesise provitamin A carotenoids, which are then used by animals to synthesise retinol. Provitamin A, of which β-carotene is the major component in plants, has an upper limit which is 20 times that of retinol and 100 times that of vitamin A. Therefore, whilst the synthesis of vitamin A in a plant might bring some risks, the enhancement of provitamin A synthesis would prove to be a much safer alternative (DellaPenna 1999).

1.1.6 Influence of environmental factors and postharvest conditions

Harvesting time and methods, and postharvest treatments, such as storage conditions; processing; germination and degree of ripeness can also significantly influence the phytochemical status of a plant (Ferreres et al. 1997;Gil et al. 2006). Storage may affect phytochemicals which are easily oxidised, changes which may be beneficial, as in the case of black tea, or

harmful, as in the decline of vitamin C or the browning of fruit, to consumer acceptability (Manach et al. 2004).

Carotenoid accumulation in plant foods is strongly influenced by environmental growing conditions as well as by the genetic variation between species. The levels of these compounds are also variable at the subspecies level. Changes in the growing air temperature, irradiance level, irradiance photoperiod and nutritional fertility are all factors which can affect carotenoid accumulation. Changes in colouration are indicative of the carotenoid levels in vegetables and fruit. These concentrations can increase in leaf tissues with maturity but decline during senescence. Manipulation of growing conditions and time of harvest can therefore affect the carotenoid concentrations of vegetable and fruit crops (Kopsell & Kopsell 2006). Postharvest and processing activities can also alter carotenoid chemistry, and ultimately affect bioavailability. For instance, flavonoids and lycopene have been shown to be strongly affected by food processing in Mediterranean tomato varieties (Re et al. 2002). Vitamin C is also strongly influenced by postharvest factors, where it can decline rapidly during the first days of storage (Lee & Kader 2000). Interestingly, studies have shown that carotenoid levels do not decline during storage, O'Beirne has even noted an increase in their levels in a number of fruits and vegetables (O'Beirne & O'Kenny 2010).

The polyphenolic content, both quantitatively and qualitatively, is not only influenced by the genetic make-up of the plant, but can also be strongly affected by environmental factors (Drewnowski & Gomez-Carneros 2000;DuPont et al. 2000;Gil et al. 2006;Nicolle et al. 2004). Numerous factors can affect the concentrations and proportions of polyphenols. The type of cultivar; agronomic practices, such as irrigation, culture practice, (glasshouse, field, biological or hydroponic) soil composition and herbicide/pesticide treatments; and pedoclimatic conditions (sun exposure, rainfall and soil type) can all influence phytochemical levels in a plant, and therefore food quality. For instance, exposure to light has a considerable

effect on most flavonoids. Research carried out by Dussi and his colleagues (2006), on anthocyanins in red pears, have shown that sun irradiation, light intensity and quality seem to affect flavonoid biosynthesis (Manach et al. 2004;Nicolle et al. 2004). Seasonal variation was shown to affect quercetin levels significantly in vegetables such as lettuce endive and leek, where flavonol contents were up to five times higher in the summer than in other seasons (Hollman 2000). In general, phenolic acid concentrations decrease during ripening and anthocyanin concentration increases.

Many polyphenols, especially phenolic acids, are involved in the response to different types of stress (Manach et al. 2004). Thus their contents will increase following pathogen and herbivore attacks, and will initiate lignification as healing processes of damaged areas, they will synthesise antimicrobial properties and their concentrations will increase after infection (Taiz and Zeiger, 1999). These processes explain the higher levels of these compounds found in organically-grown crops and in crops grown in the open air rather than in enclosed environments such as glasshouses or hydroponic conditions. This was shown in studies involving strawberries, blackberries and corn (Asami et al. 2003) and in lettuce where all open-air samples had higher flavonol contents than the greenhouse ones (Romani et al. 2002). Another study involved lettuce plants, grown in growth chambers, which were exposed to mild stresses such as heat shock, chilling or high light intensity. The investigation found that in response to these stresses, there was a two to threefold increase in the total phenolic content and a significant increase in the total antioxidant capacity (Oh et al. 2009). However, these effects vary considerably between different plant families, for instance, genetic factors were more important than light exposure or climate in a study conducted on more than 500 qualities of red wine {Clifford, 1999 537 /id}.

1.1.7 Improvement approaches

Improving the nutritional content of lettuce crops can be achieved through a number of different methods. Conventional breeding; pre-harvest treatments

(such as hydration, light irradiation or temperature); lab-based marker assisted selection of crops with increased levels of the traits of interest; or through biotechnological methods employing transformation techniques have all been applied to different crops (Schreiner 2005).

To date, efforts have focused on the maintenance of nutritional quality, through pre-harvest methods, such as cultivar selection, and postharvest methods and for maintaining visual and sensory quality, such as cold-shock treatment, temperature control and modified atmosphere packaging (MAP). For instance, a recent study has focused on regulated water deficit to improve the phytonutrient and total antioxidant status of lettuce (Oh et al. 2010). Other types of pre-harvest stresses have also been applied to lettuce, such as heat shock (40°C for 10min), chilling (4°C for 1d) or high light intensity on five-week-old lettuce (*Lactuca sativa* L.) plants grown in growth chambers, for the increase of their phenolic content and thus of total antioxidant levels (Oh et al. 2009).

Transformation methods for the actual improvement of lettuce nutritional quality have focused on the increase of vitamin C levels by, for instance, expressing a rat cDNA encoding L-gulono-lactone oxidase. This led to the accumulation of up to seven times more ascorbic acid than untransformed lettuce plants (Jain & Nessler 2000).

1.2 Quantitative Trait Locus (QTL) mapping of the lettuce population, identification of QTLs for nutritional traits

1.2.1 QTL

Most quality traits show continuous variation which is not only influenced by their genotype, but also by environmental conditions. A large proportion of these phenotypic traits are controlled quantitatively, exhibiting continuous variation from extreme to extreme in a segregating population (Kearsey 1996). Such quantitative traits cannot be studied individually by the principles of Mendelian genetics in the same way as qualitative traits. The genetic

variation of such traits can be attributed to the joint action of many genes, which may also be affected by the environment and which can be mapped on the genome with genetic markers (Quantitative Trait Loci, or QTLs). QTL analysis is a statistical method that links phenotypic data (trait measurements) and genotypic data (usually molecular markers) in order to explain the genetic basis of variation of these complex traits (Falconer & Mackay 1996;Kearsey 1998). QTL analysis allows researchers in very diverse fields to link certain complex phenotypes to specific regions of chromosomes. The aim of this process is to identify the action, interaction, number, and precise location of these regions.

QTL have been studied for over a century as they are a common feature to all eukaryotes, and thus, typical of commercially important traits in crop plants, domestic animals as well as in vital traits in humans. Starting in 1889, with Galton's work on man, developments in molecular biology have since opened up a new era in QTL analysis. QTL analysis relies on accurate data and statistical software and focuses on searching for associations between the quantitative trait and the marker alleles segregating the population (Kearsey & Farquhar 1998).

1.2.2 The mapping population

Plant mapping populations can be developed in several different ways, depending on the mating system of the crop species. These can be F_2 generation progeny; backcross to the parental lines (BC); homozygous double haploid lines (DH); near-isogenic lines (NIL); and recombinant inbred lines (RIL) originating from a cross between two parent lines.

1.2.2.1 F_2 population

Genetic linkage mapping in the past relied heavily upon intercrossing two homozygous genotypes to produce a heterozygous F_1 generation. Selfing or intercrossing of heterozygous F_1 lines creates a population that shows segregation in the traditional Mendelian fashion, i.e. in the 1:2:1 ratio.

However, the phenotypic distributions between a dominant homozygous and a heterozygous genotype are often impossible to distinguish. The limitations of using F_2 generation samples are that plant material can only be used once as it cannot be replicated in the F_2 population, as heterozygosis prevents their maintenance by self-mating. It is therefore impossible to use the same population to repeat the experiment in several environmental conditions, different years and locations.

1.2.2.2 Backcross population (BC)

A backcross population can be obtained by crossing the heterozygous F_1 generation back to one of its inbred parents, thus creating 1:1 segregation for polymorphic alleles from the donor parent by operating some kind of selection that can be phenotypical or through a molecular marker. This population presents numerous advantages, in particular, through the introgression of exotic germplasm from a wild relative into a domestic type (Kearsey 1996). Obtaining a backcross population is relatively cheap, easy and quick to obtain. But the accuracy of using this population is often poor, as a large population size is needed, which is time-consuming from the point of view of fieldwork and genotype analysis. Furthermore, it has the same problem of unrepeatability as the F_2 population.

1.2.2.3 Recombinant inbred lines (RIL)

A Recombinant Inbred Line population is a population of fully homozygous individuals, in the case of this project in ninth generation, obtained by repeated selfing from an F_2 hybrid, which comprises about 50% of each parental genome in different combinations (Doerge 2002). The lines undergo several rounds of meiosis before the homozygous state is reached; therefore linked genes have a higher probability of being recombinant. With each passing generation the heterozygous individuals diminish and homozygosity is normally reached between the sixth and eighth generation, through selfing. A significant advantage of using RILs is that QTL can be measured much

more accurately using replicates of a true breeding population (i.e. near homozygous or completely homozygous lines). The greatest advantage of this type of population is that they are comprised of homozygous genotypes which can be replicated in different environments or different years. The main disadvantages are the time and cost of production of RIL populations, but once produced these populations are highly informative and more effective to use than previous, heterozygous generations.

1.2.2.4 Near-isogenic lines (NIL)

By selecting for recombination events in a particular region, it is possible to map QTL with more accuracy. Near-isogenic lines are obtained from a large-scale backcrossing strategy by crossing two genotypes that are identical apart from a relatively small defined region on a particular chromosome. The differences in phenotype between these two genotypes must be due to genes in the defined region. NILs can be used in plants to validate and fine-map QTLs, since all phenotypic variation can be associated with the lone introgressed allele (Alpert & Tanksley 1996).

Just like RILs, near-isogenic lines (also known as introgression lines (ILs) or backcross inbred lines (BILs)), are easily maintained by selfing as they are effectively homozygous. Therefore determination of phenotypic values can be based on multiple replicates, which reduces the environmental effects and increases the power to detect QTL. Furthermore, the same population can be analysed in multiple environments to test genotype x environment (GXE) interactions and thus the effects of each QTL in different environments can be estimated precisely (Vreugdenhil et al. 2005).

1.2.2.5 Homozygous double haploid population (DH)

Double haploids (DH), are produced by generation in Anther culture which generates chromosome doubling, thus effectively producing

homozygous/doubled haploid plants from a hybrid heterozygous starting plant in a single generation.

Genetically, DH individuals contain two identical gametes, and therefore each effectively contains only one uniquely informative gamete, equivalent to first-generation backcross individuals as recombinant in terms of information content. The advantage of the use of DH lines is that when several genes control a trait, recessive alleles at all loci can be seen in the DH population, while only a small proportion of homozygous recessive alleles can be seen in the F_2 population and cannot been seen in a backcross population if the recessive allele is from the donor genotype (Paterson 2002). The problem with using a DH population is that DH lines show segregation distortion in some crops due to selection during the DH population production. The main advantage of such populations is the combination of homozygosity with the speed at which a population can be made.

1.2.3 Phenotyping the mapping population and transgressive segregation

In QTL mapping, contrasting phenotypes in the parent lines of a mapping population must be firstly identified. These traits can then be investigated in each individual of the mapping population, which is called phenotyping the mapping population. A large sample of individuals must be collected to represent the total population, to provide an observable number of recombinants, and to allow a thorough assessment of the traits of interests.

A common occurrence in population phenotyping and thus QTL mapping is the incidence of transgressive segregation. Transgressive segregation is defined genetically as the appearance of individuals in segregating populations that fall beyond the parental phenotypes (usually with respect to quantitatively inherited characters) and are often observed in offspring of both intraspecific and interspecific matings. There are several potential causes of transgression including de novo mutation and unmasking of

recessive deleterious alleles due to inbreeding. However, the cause most often proposed for transgression is accumulation in certain progeny of complementary alleles at multiple loci inherited from the two parents." (Tanksley 1993). Most importantly, these effects suggest a potential for an efficient use of wild plant germplasm to improve agricultural performance of elite germplasm.

1.2.4 Molecular markers

The molecular basis of biological traits in plants can be investigated through detection and analysis of genetic variation. To sequence a species' genome is not always feasible, thus molecular markers and their correlations to phenotypes can provide the necessary landmarks for elucidation of genetic variation. A molecular marker is defined as a particular segment of DNA that is representative of the differences at the genome level (Agarwal et al. 2008). However, these markers may or may not correlate with phenotypic expression of a trait. Numerous molecular marker techniques have been developed, such as RFLP (restriction fragment length polymorphism), RAPD (random amplified polymorphic DNA), SSR (simple sequence repeats) and AFLP (amplified fragment length polymorphism) (Kearsey & Farquhar 1998). Furthermore, recent combinations of previous basic techniques have inspired the emergence of new advanced techniques, which also utilise a newer class of DNA elements, such as retrotransposons, mitochondrial and chloroplast DNA, which allow for an increased genome coverage (Agarwal et al. 2008).

Molecular markers offer a number of advantages compared to phenotypic markers as they are stable and detectable in all tissues at all times during the lifespan of an individual, and are not influenced by environmental changes or pleiotropic and epistatic effects.

A number of different markers have been developed owing to the advantages brought by the advent of the polymerase chain reaction (PCR). Basic marker techniques can be classified into two categories: non PCR-

based (RFLP) and PCR-based (or hybridisation based techniques) (RAPD, SSR, AFLP, microsatellite, SNP and CAP). Several molecular marker types are explained in more detail below and a schematic representation of the historical development of molecular marker techniques is displayed in Fig 1.12.

1.2.4.1 RFLP

Restriction fragment length polymorphism (RFLP) is a non PCR-based technique in which DNA polymorphisms are detected by hybridizing a chemically labelled DNA probe to a southern blot of DNA digested by restriction endonucleases, ending in differential DNA fragment profiles. Numerous samples can be screened simultaneously and repeatedly with different probes, however it is time consuming, often involves radioactive and toxic reagents (although more modern methods exist which do not require use of radioactive or toxic substances) and requires a large quantity of high quality genomic DNA (Agarwal et al. 2008).

1.2.4.2 RAPD

Random amplified polymorphic DNA (RAPD) is a type of PCR reaction, but the segments of DNA that are amplified are random. It is based on the use of short random oligonucleotide sequences of about 10 bases long and it detects polymorphisms due to rearrangements or deletions at or between primer binding sites (Williams et al. 1990). The major advantages are speed and efficiency, and that it requires no prior knowledge of the sequence (as the primers will bind somewhere in the sequence, although it is not certain exactly where). A major disadvantage is the requirement of large intact genomic DNA templates. Disease resistance genes in lettuce (Paran 1991), bean (Adam-Blondon et al. 1994), and tomato (Martin et al. 1991) have been investigated through the RAPD analysis of near isogenic lines.

1.2.4.3 SSR

Simple sequence repeats (SSR), or microsatellite or short tandem repeats are monotonous repetitions of very short (one to five) nucleotide motifs, which occur as scattered repetitive constituents in all eukaryotic genomes (Tautz & Renz 1984).

Microsatellites are popular genetic markers because of their codominant inheritance, high abundance, enormous extent of allelic diversity, and the ease of assessing SSR size variation by PCR with pairs of flanking primers. Different research laboratories can efficiently use microsatellites and produce consistent data making the technique highly reproducibile (Agarwal et al. 2008). Locus-specific microsatellite markers have been reported in numerous plant species, including lettuce (Van de Wiel 1999).

1.2.4.4 AFLP

Amplified fragment length polymorphisms (AFLPs) were originally developed by Vos et al to overcome the limitation of reproducibility (1995). AFLPs are PCR-based markers for the rapid screening of genetic diversity. The assay uses restriction enzymes to digest genomic DNA, followed by ligation of adaptors to the sticky ends of the restriction fragments. A subset of the restriction fragments is then selected to be amplified and then visualized on denaturing polyacrylamide gels either through autoradiography or fluorescence methodologies.

AFLP has higher reproducibility, resolution, and sensitivity at the whole genome level compared to other techniques, as well as the capability to amplify between 50 and 100 fragments at one time (Mueller & Wolfenbarger 1999). In addition, no prior sequence information is needed for amplification (Meudt & Clarke 2007). The technique can be used to distinguish closely related individuals at the subspecies level (Althoff et al. 2007), to assess the degree of relatedness or variability among cultivars, to establish linkage groups in crosses or to map genes (Mian et al. 2002).

1.2.4.5 Newer techniques

A number of other, enhanced molecular marker techniques have also been described, such as: cleaved amplified polymorphic sequences (CAP); sequence characterised amplified regions (SCAR); and organelle microsatellites (chloroplast and mitochondrial microsatellites). These techniques are the result of recent technical advancements and genome based discoveries, and are a combination of advantageous characteristics of other techniques as well as increased sensitivity and resolution (Agarwal et al. 2008).

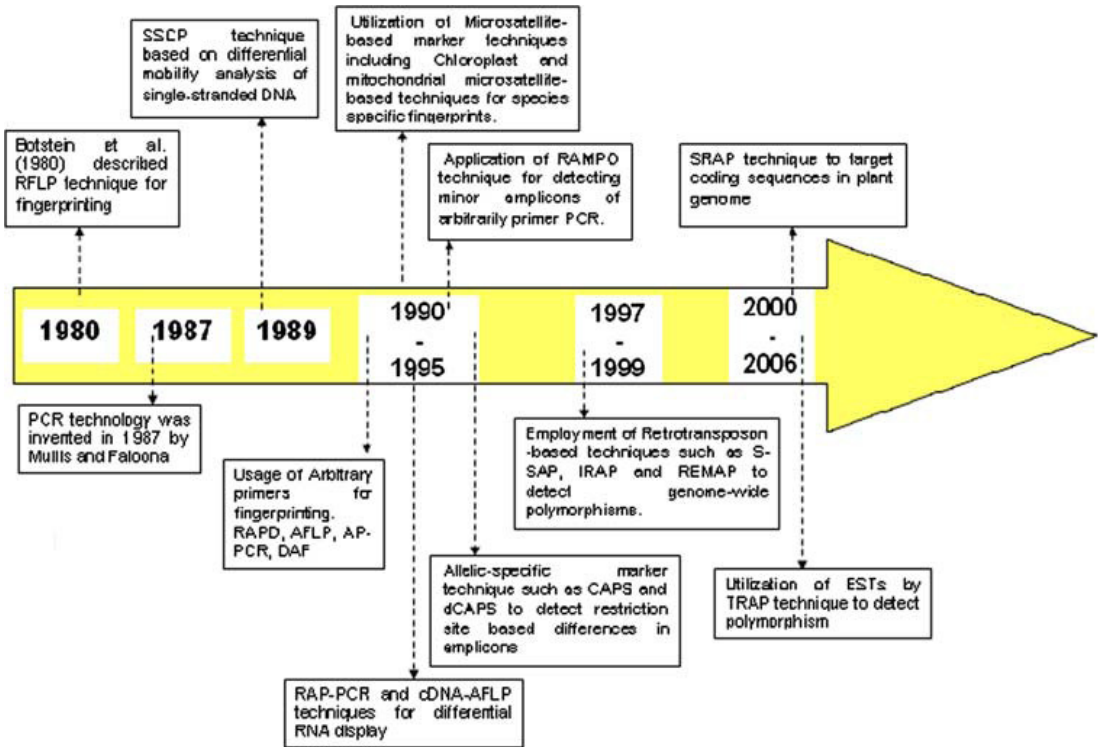


Fig 1.12 Schematic representation describing the development of molecular marker techniques and their advancements over last two decades. Fig extracted from Agarwaal et al. (2008).

1.2.5 Genetic linkage maps

A linkage map is a genetic map of a species or experimental population that shows the position of its known genes or genetic markers relative to each other in terms of recombination frequency, rather than as specific physical distance along each chromosome. Detailed linkage maps are of great importance in studies concerning selection, identification and organisation of plant genomes. Several genetic linkage maps have been constructed in lettuce species for the studies of disease resistance genes (Jeuken 2002;Syed et al. 2006), and for the identification of genes involved in morphological traits such as flowering, leaf and seed characteristics (Ryder 1996;Waycott & Taiz 1991).

The first lettuce linkage map was constructed by Landry et al. using RFLP, isozyme, disease resistance and morphological markers (1987). The markers were distributed into lettuce's nine linkage groups and covered 404cM, i.e. circa 25-30% of the lettuce genome. The flanking markers were used to study the source of variation in downy mildew resistance genes (*Dm*) and were also part of a strategy to clone resistance genes. Genetic linkage maps for lettuce have been subsequently published such as those created by Kesseli et al (1994) and Waycott et al (1999) assembled on populations derived from intraspecific crosses within cultivated lettuce, *L. sativa*. Two other maps were assembled from crosses between cultivated lettuce and its closest wild relatives *L. serriola* and *L. saligna*. Johnson et al. (2004) described a framework map of AFLP markers for QTL analysis of a population derived from an *L. sativa* and *L. serriola* cross.

Jeuken et al. (2001) used AFLP markers to generate an integrated consensus map of the nine chromosomes for two populations derived from crosses between *L. saligna a*nd *L. sativa*. They used the software program JoinMap 2.0 (Stam & Van Ooijen 1996) to construct the linkage map. This integrated map consisted of 476 AFLP markers and 12 SSRs on the nine linkage groups spanning 854 cM. It has been reported that AFLP markers

using the *Eco*RI/*Mse*I restriction enzyme combination tend to cluster around centromeric regions in other crops such as barley (Qi et al. 1998) and soybean (Young et al. 1999). Fortunately, the AFLP markers did not show severe clustering in Jeuken's map, therefore, this integrated genetic map provides good opportunities for use in QTL mapping and marker-assisted selection in lettuce cultivars. A further genetic map was constructed from a cross between *L. serriola* DHM21 and *L. sativa* cv. Dynamite (Syed et al. 2006). This map was developed using retrotransposon based markers as well as some AFLP markers in common with the map of Jeuken et al.

More recently a high-density genetic linkage map for lettuce has been constructed comprising of 2,744 markers from seven intra- and inter-specific mapping populations (Truco et al. 2007). 2,073 AFLP, 152 RFLP, 130 SSR, and 360 RAPD as well as 29 other markers were assigned to nine chromosomal linkage groups that spanned a total of 1,505 cM and ranged from 136 to 238 cM. The mean interval between markers was 0.7 cM and 43 cM was the maximum. The lettuce consensus map integrated four previously published maps of lettuce with data from three new populations: two maps generated from crosses between *L. sativa* and *L. serriola* and an additional map derived from *L. saligna* and *L. sativa*. By integrating data from all seven individual maps, the new map provides better coverage of all genomic regions. The integrated map was developed as a framework for mapping ESTs in one core mapping population relative to phenotypes that segregate in other populations. The study also aimed to provide large numbers of markers for marker assisted selection, candidate gene identification, and studies of genome evolution in the Compositae.

1.2.6 QTL mapping methods

1.2.6.1 Single marker tests

Single marker analysis uses statistical tests such as *t*-tests, ANOVA and simple linear regression statistics (Doerge 2002) and is based on the idea

that if there is an association between a marker genotype and a trait value it is likely that a QTL is close to that marker locus and therefore point to the existence of potential QTL. This method has the potential to identify numerous significant markers. However, the strength of the regression between markers and loci will decrease as the genetic distance between these increases. Thus this method loses accuracy if the QTL does not lie exactly at the marker position. Furthermore, the approach cannot distinguish between a QTL with a small effect tightly linked to the marker gene and a QTL with a large effect that is loosely linked. The single marker tests are used for quick scanning of all chromosomes as a means to identify individual markers that are segregating with a trait.

1.2.6.2 (Simple) interval mapping

Interval mapping uses an estimated genetic map as the framework for the location of QTL. It provides a systematic way to scan the whole genome for evidence of QTL by using two observable flanking markers to construct an interval within which to search for QTL. The analysis was made popular by Lander and Botstein and uses statistical methods to test whether a QTL is likely to be present within an interval defined by ordered pairs of markers. Whereas, in single marker analysis, only one marker is used in QTL mapping, thus effects can be underestimated and the precise QTL position cannot be determined. A map function (either Haldane or Kosambi) is used to translate from recombinational units to genetic distance and vice versa. The results of the tests are then expressed as LOD (logarithm of the odds) scores which is calculated at each increment in the interval and then for the whole genome. LOD is the probability of a QTL being located within a marker interval measured against the probability of the result occurring by chance. If the LOD score exceeds a predetermined threshold, the presence of a QTL is assumed. Interval mapping is certainly more powerful than single-marker approaches for detecting QTL. However, it is limited as a single QTL method and by being a one-dimensional search that does not allow interactions between multiple QTL to be detected.

1.2.6.3 Composite interval mapping

Composite interval mapping is an extension of simple interval mapping, the difference is that composite interval mapping attempts to statistically control for the genotype by including additional markers other than those immediately flanking the candidate QTL (Jansen 1993;Jansen & Stam 1994;Zeng 1994). Composite interval mapping adds background loci (markers are usually 20-40cM apart) to simple interval mapping. The purpose of this is to remove the variation that is associated with other (linked) QTL in the genome. It fits parameters for a target QTL in one interval whilst simultaneously fitting partial regression coefficients for background markers to account for variance caused by non-target QTL. Composite interval mapping has therefore, more power and precision than simple interval mapping as the effects of other QTL are not perceived as residual variance.

1.2.6.4 Multiple interval mapping

Like composite interval mapping, multiple interval mapping also includes additional markers as cofactors for the purpose of removing variation that is associated with other linked QTL in the genome. In addition to this it allows for the identification of multiple epistatic QTL (Kao et al. 1999) and to refine analyses during the process.

Composite and multiple interval mapping are more powerful and precise than simple interval mapping as they can potentially differentiate between linked and/or interacting QTL and reduce the effects of linked and ghost QTL. However, the limitations are that they are restricted to one-dimensional searches across the linkage groups and can be confounded by epistatic QTL effects. Another limitation is the risk of putting too many markers in the model as cofactors (Doerge 2002).

1.2.7 Marker assisted selection (MAS)

Marker-assisted selection (MAS) is a commonly used application of DNA markers. Once the traits of interest have been mapped and closely linked markers have been found, it is possible to screen large numbers of samples for rapid identification of progeny that carry desirable characteristics. the localisation of genes involved in the expression of these traits can be easily done for both simple heritable traits based on a single gene and more complex traits based on more genes (Kearsey 1996).

Marker assisted selection has been shown to have high potential in lettuce breeding (Asins et al. 2009). A number of studies have utilised MAS for the improvement of existing cultivars and the development of new 'ideal ideotype' by exploiting the potential value of wild germplasm (Kearsey & Farquhar 1998). For instance, Johnson et al identified QTL for root architectural traits and deep soil water exploitation in an F_2 generation derived from an interspecific cross between *Lactuca sativa* and the wild *L. serriola* (Johnson 2000). These results provided the opportunity for marker-assisted selection via the introgression of wild alleles into cultivated lettuce to improve soil water and nutrient acquisition from deeper soil zones, thereby minimizing water and fertilizer inputs and ultimately enhancing water quality. In a similar project, Jeuken and Lindhout have used wild germplasm from *L. saligna* to produce lettuce backcross inbred lines (2004). Other QTL and MAS studies in lettuce involved the investigation of seed traits (Argyris et al. 2005) and disease resistance genes (Jeuken 2002).

1.2.8 Genotype x Environment (G x E)

The successful employment of QTL mapping depends on the reliability and accuracy of the QTL analysis from which the information has been obtained. Several factors have an important influence on this accuracy: the capacity of the statistical method to detect the QTL, the experimental design (including the type of segregating population), its size, the heritability of the trait, the

number and contribution of each QTL to the total genotypic variance, their interactions, their distribution over the genome, the number and distance between markers, the percentage of codominant markers, the evaluation of the trait, and the reliability of the order of markers in the linkage map (Asíns 2002). Furthermore, two types of interactions may significantly affect a quantitative trait locus: genotype by genotype interactions (known as epistasis) and genotype by environment interactions (G x E) (Mackay 2001;Paran & Zamir 2003). Carrying out QTL investigations in different environments can be an important factor in testing the robustness of a QTL. The effects of a QTL can be influenced by the environment they inhabit and this sensitivity will result in phenotypic plasticity (Van Kleunen and Fischer 2005).

The number and contribution of each quantitative QTL that has significantly different effects across environments will be associated with substantial genotype by environment (G x E) interaction effects (Moreau et al. 2004). Such G x E interaction effects can be indicative of QTL that are specific to a particular environment; lack of a G x E interaction can suggest that a QTL is a more general growth regulator (Maloof 2003). G x E interaction is of critical importance to genetic breeding. In breeding practice, it is a risk to apply the superior genotype to different environments when it is achieved based on QTL information obtained in only one environment. If the superior genotypes predicted in different environments are very different, their superiority may be dramatically reduced in different environments. Thus, in order to develop broadly adaptable cultivars, it is necessary to partition the main QTL effects and G x E interaction effects, and to base genetic improvement only on the QTL with the main effects (Yang & Zhu, 2005). If a robust QTL shows G x E interaction then selection of genotypes adapted to specific environments may be achieved with more confidence.

1.3 Functional genomics to determine and map candidate genes

1.3.1 EST data mining for the identification of candidate genes for nutritional traits

Expressed Sequence Tags (EST) are short (200–800 nucleotide bases in length), unedited, randomly selected single-pass sequence reads derived from cDNA libraries. High-throughput ESTs can be generated at a reasonably low cost from either the 5' or 3' end of a cDNA clone to get an insight into transcriptionally active regions in any organism (Nagaraj et al. 2007). EST data mining is an efficient strategy used in the identification of candidate genes associated with phenotypic characteristics. EST databases are expanding at a high rate and constitute the largest proportion of the available DNA sequence databases. They are significant resources for the investigation of SNPs and have become an important tool for the understanding of plant genome structure and gene expression and structure.

For many plant species, extensive collections of ESTs exist in the public domain, which have made direct analysis of genetic variation at the DNA sequence levels possible (Soleimani et al. 2003). The CGP, has identified over 50,000, providing a unigene set of 19,523 in lettuce (http://www.cgpdb.ucdavis.edu). Lists of putative candidate ESTs linked to the traits of interest can be assembled from the database. *Arabidopsis* and lettuce blast searches are possible on the compositae database site and these enable lettuce ESTs to be identified for the genes of interest already well-studied in *Arabidopsis.* The lettuce ESTs identified by the Compositae Genomics Programme (CGP) may be used to investigate SNPs in Lactuca species. Since the ESTs are derived from a number of different genotypes, many polymorphisms are expected to exist between multiple EST representations of a gene, which can be analysed using computer software such as POLYBAYES (Rafalski 2002).

1.3.2 Single Nucleotide Polymorphism (SNP) detection

Single Nucleotide Polymorphisms (SNPs) are single nucleotide variations in the genome sequence of individuals of a population (Agarwal et al. 2008). Occurrence and distribution of SNPs vary between species, however, they are the most abundant molecular markers in the genome and are widely distributed throughout genomes. For instance, maize has 1 SNP per 60-120 bp (Ching et al. 2002) and tomatoes have 1 in 8,500 bp (Van Deynze et al. 2007), whilst humans have 1 SNP per c. 1,000 bp and over 1.4 million SNPs have been identified in the human genome (Sachidanandam 2001), and 56,000 have been identified in Arabidopsis by Cereon Corp. (Buckler & Thornsberry 2002).

SNPs are most common in non-coding regions of the genome. However, SNPs within a coding sequence will not necessarily change the amino acid sequence of the protein that is produced, due to degeneracy of the genetic code. A SNP in which both forms lead to the same polypeptide sequence is termed *synonymous* (sometimes called a silent mutation) — if a different polypeptide sequence is produced they are *nonsynonymous* (Agarwal et al. 2008). A nonsynonymous change may either be missense or nonsense, where a missense change results in a different amino acid, while a nonsense change results in a premature stop codon.

The majority of SNP genotyping assays are based on one or two of the following molecular mechanisms: allele specific hybridization, primer extension, oligonucleotide ligation and invasive cleavage (Sobrino et al. 2005). Perhaps the most direct and straightforward approach is sequencing of PCR products from a number of diverse individuals. PCR primers can be designed on the basis of known DNA sequences such as ESTs. DNA polymorphisms can then be easily detected in homozygous individuals, such as the Recombinant Inbred Lines. SNP analysis is a high-throughput genotyping method which makes these polymorphisms greatly attractive and convenient genetic markers. They are suitable for automation and are used

for a range of purposes, including rapid identification of crop cultivars and construction of ultra high-density genetic maps (Agarwal et al. 2008).

1.3.3 Co-location of QTLs and candidate genes for the traits of interest

The analysis of Quantitative Trait Loci (QTL) allows the location and estimation of the genetic components which control a selected trait by the joint study of segregation of marker genotypes and of phenotypic values of individuals or lines (Asins et al. 2009). Several studies have been carried out on co-located QTL and candidate gene mapping. Candidate genes for the traits of interest can be identified following the mapping of the putative candidate genes, derived from EST data-mining, at the same location as the QTL. A number of studies have effectively identified major co-location QTL and suggested candidate genes for future research (Causse et al. 2002;Zhang et al. 2007).

1.4 Aims

The aim of this study was to use natural lettuce variation for the enhancement of its nutritional properties. This would be achieved through the careful analysis of the phytonutrient status of a common lettuce cultivar (*Lactuca sativa*, cv salinas) and its wild relative, *L. serriola* (acc.UC92G489) to assess significant differences between these and possible variation during storage and other common postharvest procedures. Eventual inequalities would then be investigated in a segregating population of Recombinant Inbred Lines (RILs) obtained from a cross between the two lettuce lines. The project focused on quantifying a number of different phytonutrient levels in 60 RILs grown in different environments (field and glasshouse). These data were then used to map major Quantitative Trait Loci (QTL) which may subsequently be exploited commercially in a marker assisted breeding programme. Further in-depth analyses aimed to investigate SNPs in the genes involved in the biosynthesis of carotenoids and polyphenols.

The final aim of the project was then to carry out an intervention trial on human subjects to investigate the bioavailability of antioxidants in human blood after consumption and to study how the effects of storage on the antioxidant levels of lettuce leaves are transferred to the antioxidant concentrations in human blood.

CHAPTER 2 - MATERIALS AND METHODS

2.1 Plant material

2.1.1 Crop samples used for antioxidant evaluation

Plant samples chosen for antioxidant comparison studies were six lettuce varieties: *Lactuca sativa* cultivars (salinas, green cos, red cos, tango and lollo rosso cultivated varieties) and a wild relative, *Lactuca serriola* (acc. UC96US23). Other crop samples analysed were: rocket (*Eruca sativa,* Shamrock Seeds Selection, standard variety) and spinach (*Spinacia oleracea,* Emilia cultivar, Pop Vriend, Holland). Seed material for *L. sativa* Salinas cv. and *L. serriola* was supplied by Professor R.W. Michelmore at University of California, Davis. Crop samples were provided by Vitacress Salads Inc, UK (Fig 2.1). Samples were sown and harvested according to standard commercial practice.

Fig 2.1 Sample crops at Vitacress Salads Ltd., Mullens farm, Wiltshire, UK.
Clockwise: green cos; rocket; spinach; and red cos baby leaves.

2.1.2 Mapping population parent lines

Parent lines for the recombinant inbred line (RIL) mapping population were the cultivated lettuce *Lactuca sativa* (Salinas cv) and its wild relative, *L. serriola* (Fig 2.2).

(a) **(b)**

Fig 2.2 *Lactuca sativa* (salinas cv) (a) and *Lactuca serriola* (wild relative) (b) parent lines

2.1.3 Recombinant Inbred Lines (RIL) mapping population

Seeds were kindly provided by Professor R.W. Michelmore and colleagues at University of California Davis, and stored in the dark at 11ºC. The entire mapping population includes 113 RILs, in their ninth generation (F9). From these, 60 highly informative lines were previously selected by Michelmore et al based on their higher number of recombination events using MapPop (Vision et al. 2000) and GenoPlayer (http://compgenomics.ucdavis.edu/genoplayer/). This provided a population that had nearly as many recombination breakpoints and was therefore as informative as a population of about 90 RILs (Fig 2.3).

Parent lines:

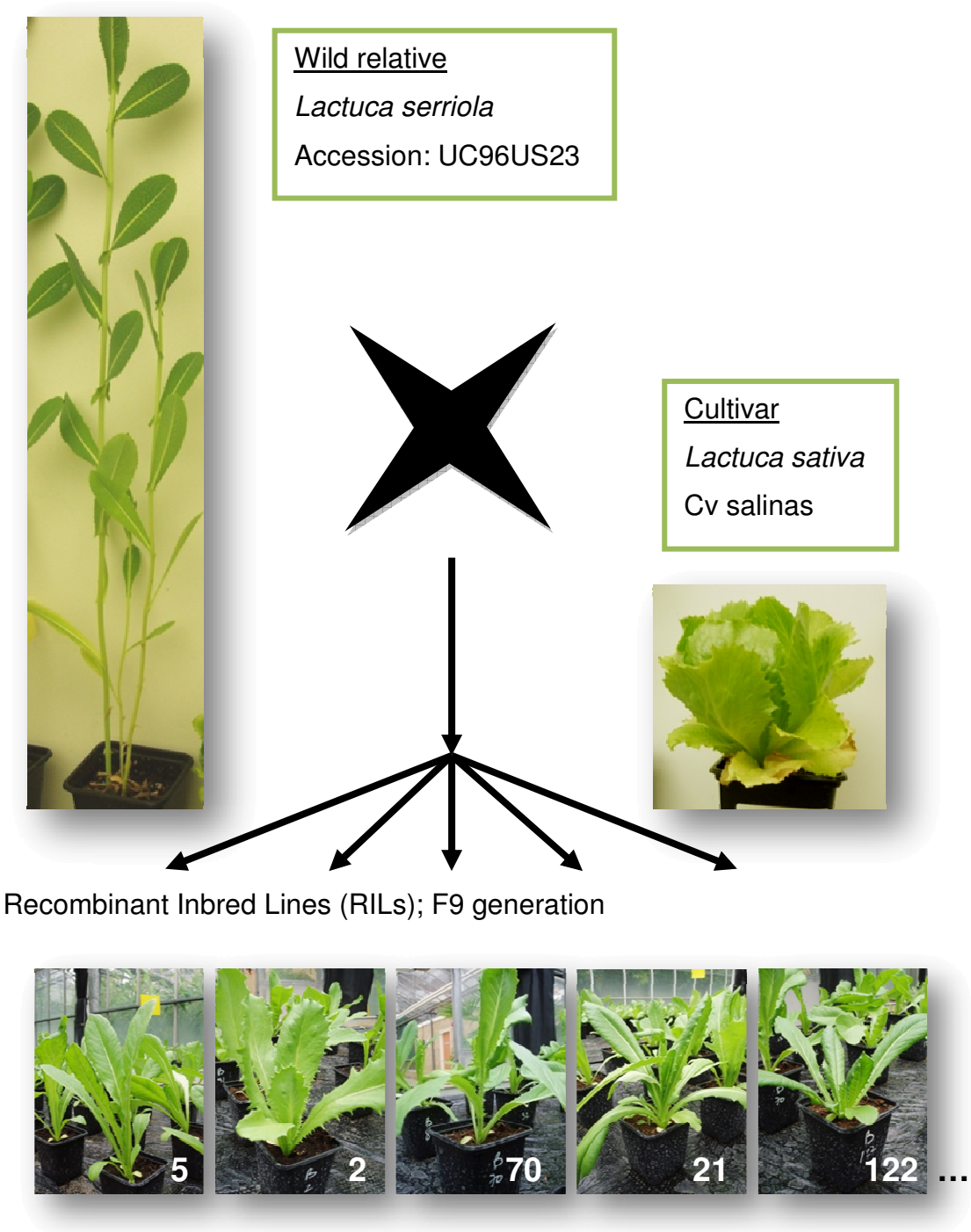


Fig 2.3 Generating a population of Recombinant Inbred Lines (RILs). A cross between two parent lines (*L. serriola* and *L. sativa*) gave rise to a breeding population of Recombinant Inbred Lines (RILs), now in the ninth generation. 1334 genetic markers (RAPD and AFLPs) were used to enable to track the origins of sections of the genome in each RIL. 115 lines were generated, the 60 best lines, i.e. those with the highest number of recombination events were chosen for this study.

2.2 Field and glasshouse trials

2.2.1 Field trials

The 60 F_9 RILs and their parent lines were planted according to a computer-generated fully randomised experimental design using the statistical software package Minitab 14.0 for Windows (Minitab Inc., Philadelphia, PA, USA). The pattern included nine replicates of each of the 62 lines, arranged into 3 blocks, A, B and C (Fig2.4). Batches of four seeds were planted for each line per biological replicate, with 10cm between each plant. Field trial measurements were 1.2 X 35m with two rows of cos lettuce planted around each block to avoid 'edge effects' (Zhang et al. 2007). The field trials were carried out on commercial farms with standard industry maintenance.

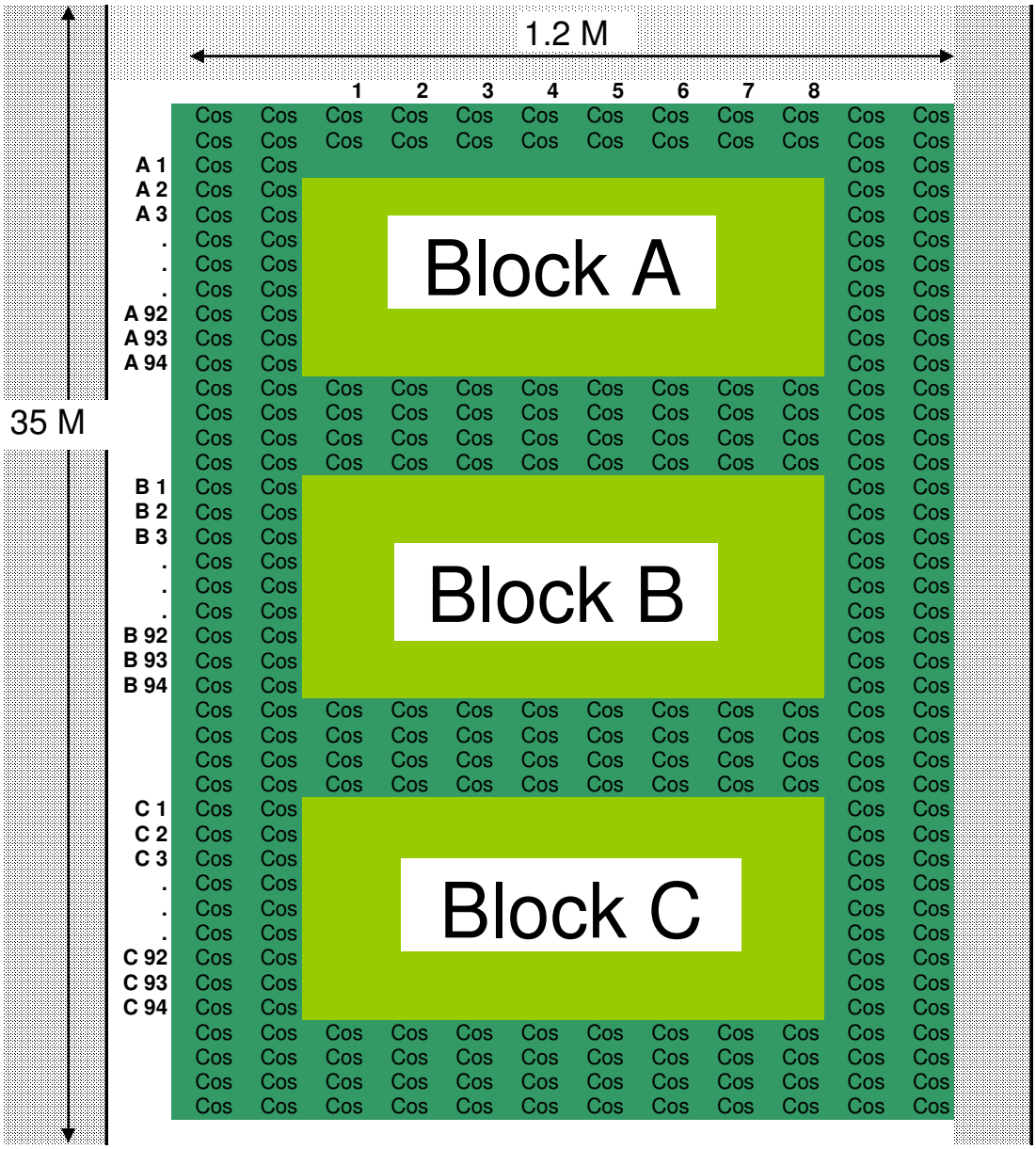


Fig 2.4 Randomised distribution of the 60 RILs and parent lines for field trials in Spain (January-March 2006) and in the UK (June-August 2006). Each block consisted of three replicates of each line sown in batches of four each, in a randomised pattern and surrounded by green cos to prevent edge effect.

Two contrasting field sites were chosen for this study: the first was at the Vitacress España farm near Àguilas (Murcia), Spain (Latitude 37°24'N, longitude 1°34'W) (Fig 2.5). The second site was at the Pinglestone Vitacress Farm near Winchester, UK (Latitude 51°5'N, longitude 1°11'W). Samples were sown in Spain and in the UK according to standard

commercial practice. The choice of location was due to the generally better climatic conditions and longer daylight hours than in the UK during the same period.

Seeds were planted at the Spanish site on the 20th January 2006 and harvested after 10 weeks, on the 30th of March (69 days) (Fig 2.6). In the UK, seeds were planted on the 30th of June and harvested after 7 weeks, on the 10th August 2006 (41 days) (Fig 2.7). Average temperatures in Spain from January to March ranged between 10.5°C and 15.5°C (January and February) and reaching 28.4°C by the end of March; average temperatures for the UK in July ranged between 25°C and 34°C with initial temperatures during the first week of early July around 22°C during some spells of bad weather with thunderstorms. The sites in Spain and the UK span the range of environmental conditions currently used for much of the baby leaf grown for the UK market.

The leaf samples were collected from Spain and transported back to the UK in a commercial refrigerated lorry (Vitacress Salads Ltd), the journey took three days. Collection from the factory at Saint Mary Bourne on the day of arrival, samples were kept in the dark, at 10°C overnight and were snap-frozen in liquid nitrogen on the following day.

Length of day in Spain ranged between 9h56m (20/01/06) and 12h32m (30/04/06). Length of day in the UK ranged between 16h28m (30/06/06) and 14h51m (10/08/06) (http://www.timeanddate.com/). After harvesting, samples were brought back to the lab were they were snap-frozen immediately in liquid nitrogen.

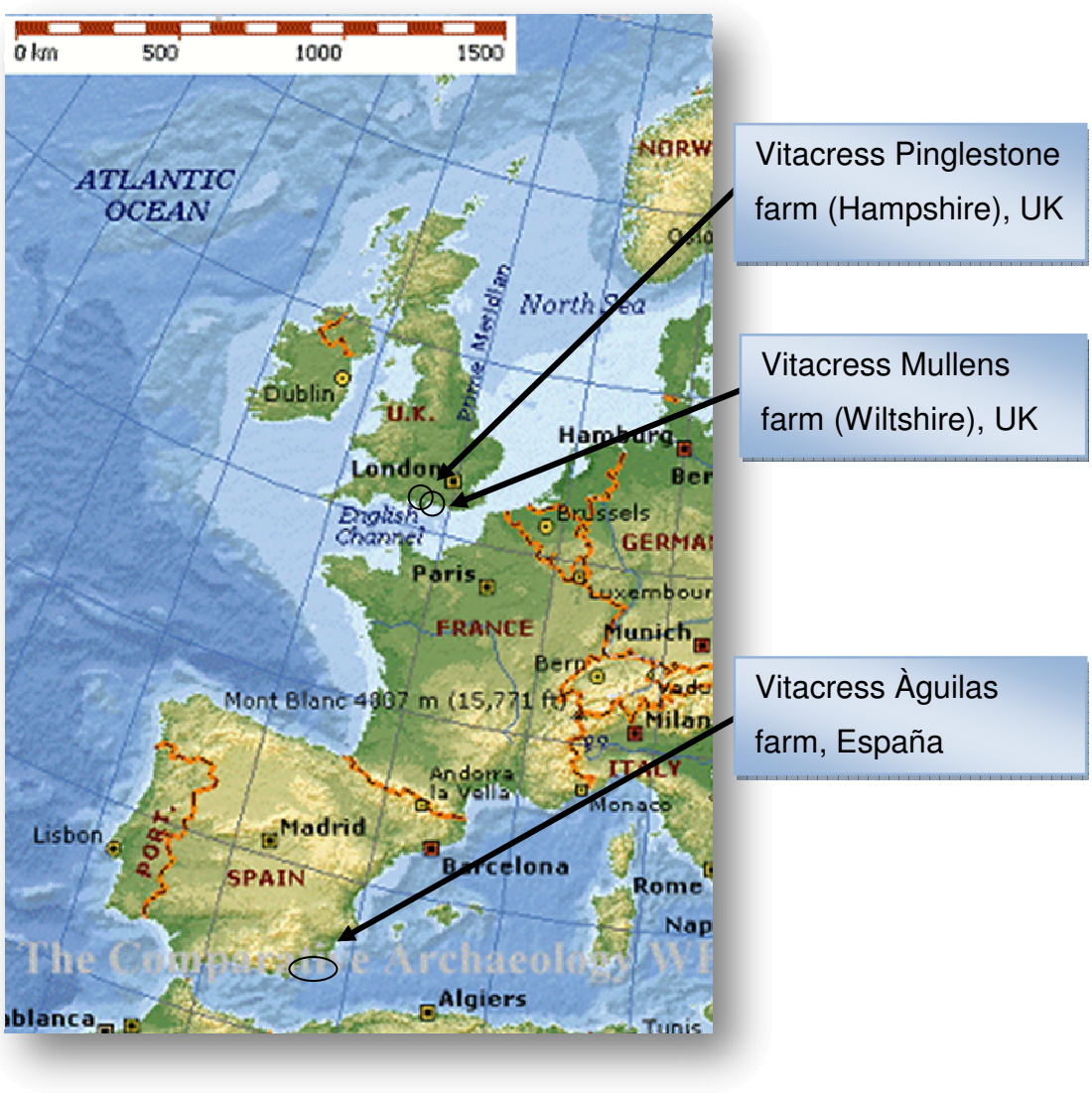


Fig 2.5 Field site locations. Partial map of Europe showing the three field sites: one in Spain and two in Southern England (http://www.comp-archaeology.org/EuropeMapCAWEB.gif).

Fig 2.6 Field site at the Vitacress farm in Spain. Field site near Àguilas at the stages of sowing in January (a) and of harvesting, after 10 weeks (b).

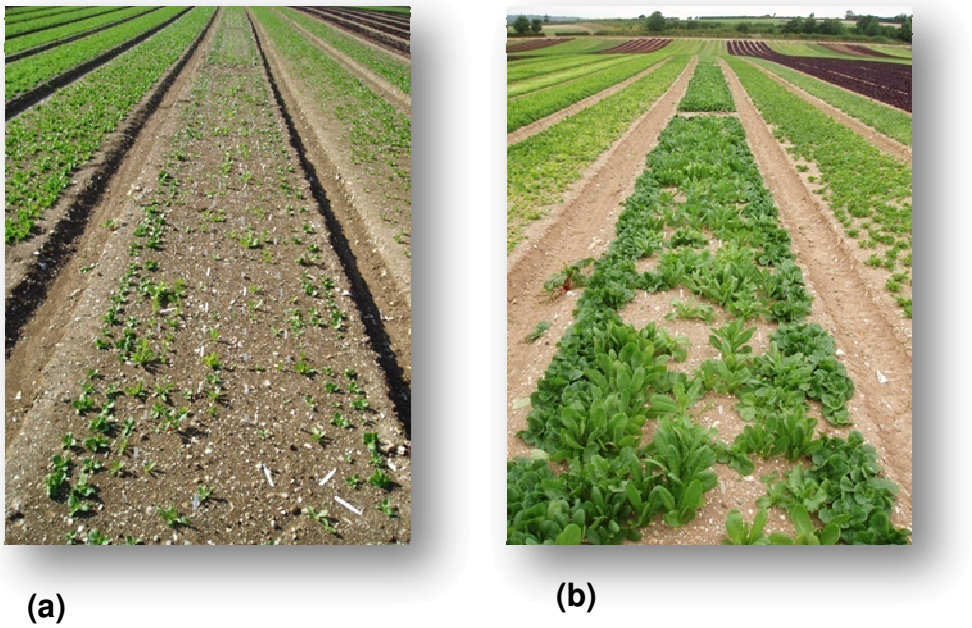


Fig 2.7 Field site at the Vitacress farm in the UK. Field site in Pinglestone, UK, four weeks after planting (a) and seven weeks after planting (b).

2.2.2 Glasshouse trial

The selected 60 RILs were planted in the glasshouse as a controlled environment trial (Fig 2.8). Seeds were planted on the 23/05/2006 of May and harvested 28 days later on the 20/06/2006 June.

Single pots were used for each set of four seeds and subsequently thinned out so that only one plant remained per pot. Seeds were planted in 7 X 7 X 8cm square pots in 100% blended peat, seed and modular growing media, pH 5.5 (Vapogro, Kekkilä and Avoncrop Ltd). Pots were kept in the glasshouse, day temperature in the range of 18ºC to 27ºC, night temperature at around 18ºC and approximately 16h day length, for 28 days before trials. Pots were placed in the randomised pattern described in section 2.2.1 and watered with a lance when needed.

(a) **(b)**

Fig 2.8 Glasshouse trial, June-July 2006. Day of sowing (a), and at time of harvest four weeks later (b).

2.3 Sample treatments for determination of total antioxidant potential

2.3.1 Comparison between Lactuca sativa *and* L. serriola

L. sativa and *L. serriola* samples were grown in the growth room with day and night temperature set at 25ºC and at 23ºC respectively with a 12h day length, for five weeks before sampling. Leaves of the same age (the fourth leaf of each plant) were used for the comparison. Eight biological replicates (individual leaves) and three technical replicates were sampled for total antioxidant potential, using the Ferric Reducing Antioxidant Potential assay or FRAP assay (section 2.4), after measurement of fresh weight (FW) and extracted sap. (The TEAC III assay for antioxidant potential was also developed and used to compare the parent lines, see Appendix 3).

2.3.2 Comparison between young and old leaves

For this comparison, the youngest and the oldest leaf of each plant were sampled. Plants were grown in the growth room, conditions as described in section 2.3.1. Eight biological replicates and three technical replicates for each biological replicate were sampled for total antioxidant potential, using the FRAP assay after FW had been measured.

2.3.3 Leafy salad crop evaluations

Commercial samples of green cos, red cos, lollo rosso, rocket (Shamrock Seeds Selection, standard variety) and spinach (Emilia cultivar, Pop Vriend, Holland) leaves from lettuce harvested at the Vitacress farm in Wiltshire (Mullens Farm) were sampled for total antioxidant potential, using the FRAP assay. The crops were sown and grown according to standard commercial practice.

The crop samples were harvested at four time points during the day, once every six hours: evening (19:00), night (01:00), morning (07:00) and

afternoon (13:00). The sampling started on the 4th of September 2007. Five samples of each cultivar, at each time point were immediately snap-frozen on site in liquid nitrogen, then transported in dry ice back to the lab (and stored at -80℃).

To assess for potential knock-on effects of different harvesting times, another five samples of each cultivar harvested at the four different time points were bagged on location and stored in the dark at 4℃ for seven days before being snap-frozen in liquid nitrogen and stored in the -80°C freezer. All samples were then analysed simultaneously using the FRAP assay.

2.3.4 Green cos and lollo rosso shelf-life

Commercial packaged mixed lettuce containing green cos and lollo rosso lettuce was collected from the Vitacress processing factory at Saint Mary Bourne. Samples were kept for two, five, eight and eleven days in a standard commercial fridge at 4°C, before being tested. Lollo rosso and green cos leaves were selected for the test. Twelve biological replicates (individual leaves) taken from six bags and three technical replicates for each biological replicate were sampled for total antioxidant potential on each sampling day, using the FRAP assay.

2.3.5 Tango shelf-life

The shelf-life trial was repeated for commercial Tango lettuce. Samples were washed in distilled water and chlorine (1:40 dilution according to standard commercial practice) for one minute on minimum speed using a twin-tub washing machine to mimic the industrial washing process. The samples were spun dry for 20 seconds then bagged and sealed, and kept for one, four, seven and ten days in the fridge at 4°C, before being opened and tested for antioxidant potential. Three biological replicates, consisting of five leaves each, were carried out for each treatment and three technical replicates were carried out for each biological replicate.

2.3.6 Light intensity trial

L. sativa and *L. serriola* parent lines were grown in the glasshouse for 36 days, between the 11th of June and the 17th of July 2008. Seeds were planted in 7 X 7 X 8cm square pots in 100% blended peat, seed and modular growing media, pH 5.5 (Vapogro, Kekkilä and Avoncrop Ltd). Eight replicates for each parent line were grown on top of the bench and eight of each under the bench, covered on the sides with a light muslin cloth, so as to recreate "shady" conditions (Fig 2.9). Whole head plants were used for the comparison. Four biological replicates and three technical replicates for each treatment were sampled for total antioxidant potential, using the FRAP assay.

All seeds were initially sown and kept above the bench, under sunny conditions. Seedlings were then thinned out on the 26th of June and on the 30th of June half of the samples were moved under the shaded area. Measurements were taken at 1:00pm every day: sunlight levels averaged 494.19µmol/s/m^2/µA for the sunlight treatment and 27.56µmol/s/m^2/µA for the shade treatment and at the time of harvest sunlight levels were 528.0 µmol/s/m^2/µA and 5.4 µmol/s/m^2/µA respectively. Sunlight measurements were taken with a LI-COR Quantum/ Radiometer/Photometer model LI-250 light meter (measurements were taken with 15sec averages).

Day temperatures averaged 25.5℃ for samples kept under sunny conditions and 22.9℃ for samples kept under shady conditions. At the time of harvest temperatures were 23.0℃ for samples in sunlight and 20.4℃ for those in the shade. Temperature measurements were taken using a datalogger Testo model 174.

(a)

(b)

Fig 2.9 Sunlight irradiation and temperature measurements. Measurements were taken with a LI-COR Quantum/ Radiometer/Photometer model LI-250 light meter, with 15sec averages. Samples exposed to normal glasshouse sunlight levels were kept on top of the bench (a) and samples kept in shady conditions were positioned underneath the bench, a light muslin cloth hanging from the bench was also attached to keep the plants in the shade (b). Temperature measurements were taken with a Datalogger Testo model 174.

2.4 The Ferric Reducing Antioxidant Potential (FRAP)

2.4.1 Introduction

The FRAP assay, first developed by Benzie and Strain for the analysis of antioxidants in blood samples (Ferric Reducing Ability of Plasma) (Benzie, 1996), is now also commonly used for measurement of the antioxidant potential of fruit and vegetables (Ferric Reducing Antioxidant Potential) (Stratil, 2006;Llorach, 2008;Proteggente, 2002;Llorach, 2004). The technique was modified in this study from the original Benzie and Strain protocol (1996), for use on plant tissue material. The reaction is based on the ability of the antioxidant to reduce the ferric 2,4,6,-tripyridyl-*s*-triazine substrate in the reagent to ferrous ions (Fe^{3+} to Fe^{2+}). The ferrous form has a maximum absorption at 570nm wavelength which is measured in a spectrophotometer. The reaction is carried out in a 96-well microtitre plate such as the one shown in Fig 2.10.

2.4.2 Method

Whole leaf samples were snap frozen in liquid nitrogen, ground with dry ice to keep the samples frozen, then centrifuged at rcf=15,550x*g* for 5min. Supernatant was harvested, immediately snap frozen again and kept at -80℃ until required.

FRAP reagent was prepared fresh each time using 300 mM acetate buffer, pH 3.6 = 3.1g $C_2H_3NaO_2 \cdot 3H_2O$ (Sodium Acetate Trihydrate) and 16ml $C_2H_4O_2$ (Acetic Acid) /L of buffer solution; 10mmol TPTZ (2,4,6-tripyridyl-*s*-triazine) in 40mmol HCL; and 20mmol $FeCl_3 \cdot 6H_2O$ (Ferric Chloride Hexahydrate).

Serial dilutions, in the range of 0.25 – 6mmol, of aqueous solutions of known Iron Sulphate Heptahydrate (Fe^{2+}) concentration were prepared for use as a standard.

Using a flat-bottom microtitre plate, 300µl of FRAP reagent were added to each well containing either 10µl of sample, or 10µl of Fe^{2+} standard or 10µl H_2O (negative control). Three technical replicates were carried out for each sample. For blood serum samples 30µl of H_2O was added to each sample and standard.

Samples were added to the wells before the FRAP reagent, to ensure even distribution. Optical density (OD) was measured after 2 minutes, at absorbance 570nm, using a plate-reading spectrophotometer (Anthos Labtec Instruments).

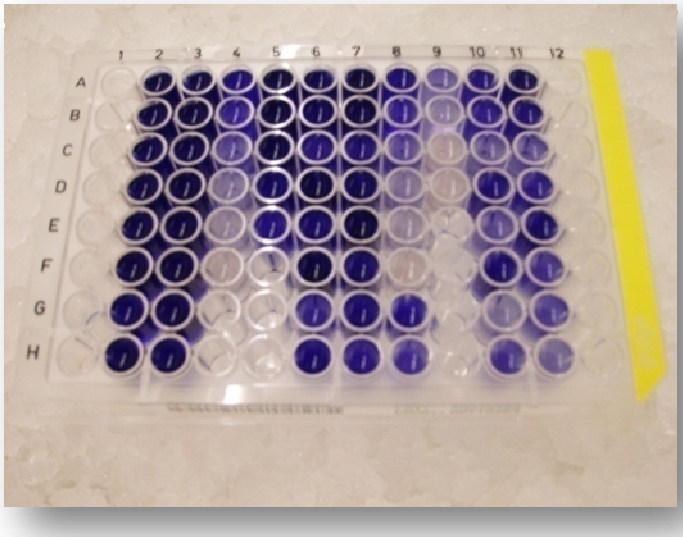


Fig 2.10 The Ferric Reducing Antioxidant Potential (FRAP) assay. The assay is based on the reduction of ferric ions with the addition of the antioxidant sample. The darker the colour of the assay the greater the absorbance (at 570nm) of the formed ferrous ions indicating the level of antioxidant potential of the sample.

2.4.3 Analysis of FRAP measurements

FRAP values were obtained by comparing the absorbance change at 570nm in test mixtures with those containing ferrous ions in known concentrations and were expressed as mmol Fe^{2+} equivalents /g fresh weight (FW) using Excel 2003 for Windows.

2.5 Determination of vitamin C content in the parent lines, in tango lettuce and in spinach samples

Commercial tango lettuce samples were sent to Leatherhead Food International to be analysed for vitamin C content. Frozen leaf samples were taken from the tango shelf-life trial. Vitamin C content was also determined for the two parent lines grown in the glasshouse, three replicates for each parent were analysed.

2.6 Chlorophyll and carotenoid extraction and quantification

The standard solvent dimethylformamide (DMF) was used to extract chlorophylls a and b and total carotenoids from the RILs and from the parent lines (Minocha et al. 2009;Wellburn 1994).

Three 1cm discs were taken from leaf number four of each plant grown in the glasshouse, from the tip and from the left and right of the mid-rib vein, avoiding other major veins (Fig 2.11). Leaf samples were placed in Eppendorf microfuge tubes and stored in the dark at 4°C for over 24 hours. Chlorophyll and carotenoid pigment optical density (OD) were measured at 664, 647 and 480 nm wavelengths in a spectrophotometer (U-2000, Hitachi, Wokingham, UK). The chlorophyll a concentration was calculated according to the following formula (Wellburn 1994): C_a=11.65*A_{664}-2.69*A_{647}, where C_a is the chlorophyll a concentration in the DMF solution in µg/ml. Chlorophyll b concentration was calculated using the formula: C_b=20.81* A_{647}- 4.53*A_{664}, where C_b is the chlorophyll b concentration in the DMF solution in µg/ml and total chlorophyll (C_t) content was calculated by simply adding the two chlorophylls together. Carotenoid concentration was calculated according to the following formula: *Carot*=(1000*A480-0.89*C_a-52.02*C_b)/245, where *Carot* is the carotenoid concentration in µg/ml. From the measured chlorophylls and carotenoid concentrations and the sample disc areas, chlorophylls a and b and carotenoid contents were calculated using the

following formula: $C=C_{(a,\ b\ or\ t)}/96.78*3$, where C is the pigment content in $\mu g/mm^2$ (Cunningham & Gantt 1998;Wellburn 1994).

Fig 2.11 Chlorophyll and carotenoid pigment measurements. Cutting out 1cm discs for pigment extraction in DMF.

2.7 Analysis of antioxidant phytonutrient levels in human blood plasma after lettuce ingestion.

Postprandial antioxidant levels were investigated in human volunteers after the ingestion of lettuce leaves. The study aimed at comparing the levels found in blood serum after consumption of fresh leaves and after consumption of leaves stored for one week.

2.7.1 Subject selection and recruitment

The following approaches were used to attract 20 volunteers:

1. Personal contact with colleagues.

2. Advertisements in University of Southampton publications and in the Southampton general press (Appendix 5, section b.).
3. E-mail messages to staff in the School of Medicine and School of Biological Sciences.
4. Posters within the University of Southampton and Southampton General Hospital (SGH) (Appendix 5, section a.).

The advertisements invited a personal, telephone or e-mail enquiry.

Suitable volunteers were invited to an interview (of about 20 minutes) in order to discuss the project, to assess their general health and suitability, and to record their height and weight. After a brief discussion, volunteers who appeared to be suitable were sent the participant information sheet (Appendix 5 section f.).

In order to reduce bias and variability and therefore to increase the power of the study, the criteria for the selection of suitable participants were: healthy Caucasian (or similar ethnic background) men 18 to 45 years of age, non-smokers, with body-mass-index between 20kg/m^2 and 25kg/m^2, with fasting plasma triglyceride concentration less than 2.5mM and cholesterol less than 8mM (lettuce antioxidants have been shown to have an effect on rat cholesterol levels) (Nicolle et al. 2004) (Herman et al. 2009;Wang & Bakhai 2006;Young 2003)

Those not fulfilling the inclusion criteria were those who have diagnosed type 1 or type 2 diabetes mellitus or are receiving anti-coagulant therapy; those who were taking vitamin supplements; vegetarians or vegans; and subjects who exercised daily and were unwilling to suspend these activities for the duration of the study.

The subjects were then asked to attend the Wellcome Trust Clinical Research Facility (WTCRF), at the Southampton General Hospital (SGH) where sampling took place. The participants were asked to sign a consent

form (Appendix 5, section k.) and to fill out a brief questionnaire on their activities and food and drink consumption (Appendix 5, section l.) during the previous 24 hours. The volunteers were paid £40 each.

2.7.2 Study design

Lettuce samples (bags of 120g of green cos; Appendix 5, section q.) were provided by Vitacress Salads Ltd. Samples for the trial run (an 80g bag) were harvested, washed and bagged on the 10th of July, and samples for the main study were harvested, washed and bagged on the 15th of July.

The trial run was carried out on the 11th of July 2008. The participant was given an 80g bag of lettuce and a blood sample was collected before lettuce consumption (time 0min), then after 15; 30; 60; 90; and 120min.

Based on the results of the trial run, the protocol for the main trial was slightly modified and conducted as follows.

Each participant took part in a trial in which they were given 120g of either fresh or stored (for one week) baby leaves of *Lactuca sativa* (green cos cultivar). The sampling took place on four separate days: the 16th, 17th, 23rd and 24th of July 2008. Five volunteers were present on each sampling day, except on the 23rd when there were only four due to a volunteer developing a cold and who was therefore unable to participate. The first two sets of five participants (on the 16th and 17th of July) were given fresh lettuce to consume, whereas on the 23rd and 24th of July the volunteers were given lettuce which had been stored at 4°C in the cold room at Southampton University for the previous week (Fig 2.12).

Subjects were asked to fast for 12 hours until the start of the trial on the following morning (about 12 hours). On arrival at the WTCRF, participants were requested to remain resting on a bed as much as possible. A venous cannula was placed in a forearm vein and a baseline blood sample collected

(15ml) (Fig 2.13). The participants then consumed their breakfast composed of a packet containing 120g of either fresh or stored lettuce leaves. They were allowed free access to water but were not allowed to eat anything else throughout the study. Venous blood samples (15ml each) were drawn at 0; 15; 30; 60; 90; 120; 150min and stored in microfuge tubes containing heparin to prevent blood coagulation. Participants were offered lunch and drinks at the end of the trial.

Plasma was isolated from the blood by centrifugation at 140 *x g* for 10min and stored at -80°C on the WTCRF site until all samples had been collected and centrifuged, samples were then transported in dry ice to the University lab for analysis. Total antioxidant content was measured using the FRAP assay.

Each participant's identity was kept anonymous to anyone except for the chief investigator, by labelling the results with the participants' initials on the centrifuge tubes and in the results and analysis files.

Diagram of the intervention trial protocol

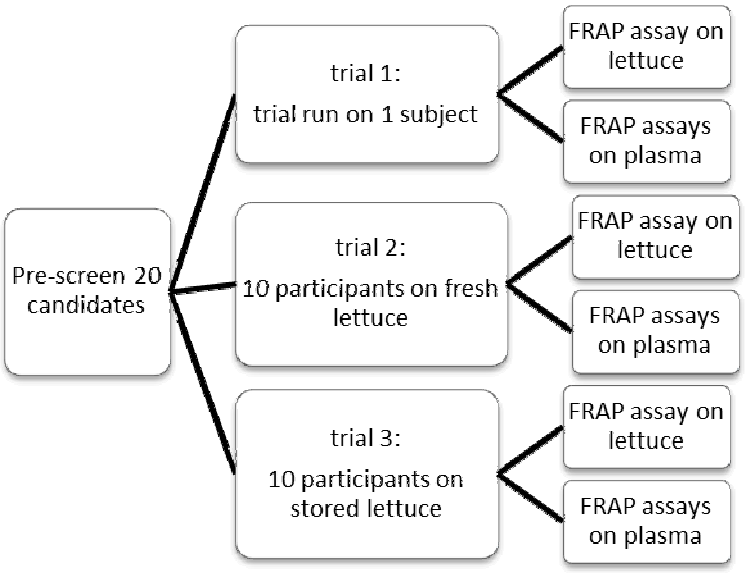


Fig 2.12 Diagram describing the intervention trial procedure. Following an initial trial run on one participant, 10 participants were each fed a 120g bag of fresh green cos lettuce (harvested on the previous day), blood samples were collected, centrifuged and stored at -80℃ on the same day. One week later, 10 different participants were fed each a 120g bag of one week old green cos lettuce. Blood samples were collected, centrifuged and stored at -80℃ on the same day. All blood and lettuce samples were then analysed simultaneously using the FRAP method.

Fig 2.13 Representation of a participant eating lettuce whilst being cannulated. Photograph taken by the Fresh Produce Journal for publication of an article regarding the present study (Appendix 5, section m.).

2.7.3 Study progress and conclusion reports for the ethics committee

Reports notifying the Ethics Committee of the study's progress (the Annual Progress Report. Appendix 5, section n.) and conclusion (Declaration of the end of a study form Appendix 5, section o.) were compiled and returned on the 07/08/2008.

All relevant paperwork are displayed in Appendix 5, sections a. – q. Copies of the completed Research Ethics Committee (REC) application form and Site Specific Information (SSI) form are attached as accompanying material.

2.8 Statistical methods

Statistical analyses of all phytonutrient results and of the effect of lettuce consumption on postprandial changes in blood antioxidant levels were carried out with one-way and two-way repeated measures ANOVA (Minitab 14). Tukey and Bonferroni's post hoc corrections were employed for multiple comparisons between groups. Graphs were plotted using SigmaPlot 10.0.

A power analysis was carried out before commencement of the intervention trial study to establish the ideal number of participants to the study (Lenth, R. V., http://www.stat.uiowa.edu/~rlenth/Power).

2.9 Genotypic analysis of the mapping population

2.9.1 Quantitative Trait Locus (QTL)

Previous researchers working on the RILs have selected a set of framework markers for Quantitative Trait Locus (QTL) studies by using a dense molecular marker linkage map developed for the RIL population (Zhang et al. 2007). A series of 1334 markers was used as a framework map for QTL analysis, these had been selected to maximise genome coverage and

marker information content. QTLs were detected using the software WindowsQTL Cartographer version 2.5 (Wang 2007) by composite interval mapping (CIM). The threshold value for the logarithm of odds (LOD) for declaring QTL significance at P≤ 0.05 had been set at 3.7 following previous estimations by permutation analysis for each trait using 1000 iterations (Churchill & Doerge 1994). The graphical representation of the linkage groups and the associated QTLs was prepared using MapChart (Voorrips 2002).

2.9.2 Carotenoid and polyphenol gene investigations and primer design

Primer sets were designed by searching for 21-24bp in the most homologous regions, with a reasonable G-C content (40-50%) and for a T_m ≥60°. The primers were ordered from Invitrogen Ltd (Tables 2.1 and 2.2) from the web based program: http://frodo.wi.mit.edu/.

Table 2.1 Carotenoid primer sequences. *LC primers for the amplification of lycopene ε- cyclase; and CH primers for the amplification of β-carotene hydroxylase. Tms were calculated manually using the following formula: 4(G+C) + 2(A+T).*

Primer	Sequence	T_m ℃
LCF1 forward	CCAGATCTAATGGTGTTCATGG	64
LCF2 reverse	GGTCGTATATCCCCGTAGGTGG	70
LCR1 forward	CTGACAAAGATCGGACAACTG	62
LCR2 reverse	GGCTCGCTGTCTTTTCCTTTC	64
CHF1 forward	GAGATGTTTGGGACATTTGCTCT	66
CHF2 reverse	AAACGCCGTTCCGGCGATTGCG	72
CHR1 forward	CATGTACGCCATTCCAAACACCG	70
CHR2 reverse	GGGTCATTTGCAATGGGACCCAC	72

Table 2.2 Polyphenol primer sequences. *PAL primers for the amplification of phenylalanine ammonia-lyase; HC/BT primers for the amplification of hydroxycynnamoyl transferase; HCT primers for the amplification of p-hydroxycinnamoyl-CoA quinate and shikimate p-hydroxycinnamoyl transferase.*

Primer	Sequence	T_m ℃
PAL 1 forward	AGAGGAGTTCGACAGGGTGTT	64
PAL 1 reverse	CAGAATATTACAAATACAATTCAAACG	70
PAL 2 forward	GCGGTGCTTTACAGAAGGAG	62
PAL 2 reverse	ACAGAACCATGGAAGCCATC	60
HC/BT 1 forward	GCATTGGCTATTTGCATTGAT	58
HC/BT 1 reverse	CTAAAACCGAGTGCACGTACC	64
HC/BT 2 forward	CCTGGCGTCAAGTTCATCTAC	64
HC/BT 2 reverse	TTGAATGGATAACAATGGCAAC	60
HCT forward	GGTGTGGAGTGCACCATACTT	64
HCT reverse	TGGTGGTGGGTGGTACTCTAC	66
40S S16 forward	CGGAGATCCTCCGTTACAAG	62
40S S16 reverse	TCTGAGAGGTGTGACCACCA	62
PAL 3 forward	ACAACATCACCCCTTGTTTACC	64
PAL 3 reverse	AACGTCTTGATTGTGTTGTTCG	62
PAL 4 forward	TGATTGGGTGATGGAGAGTATG	64
PAL 4 reverse	GGTGATGCTTCAATTTGTGTGT	62
HCT 2 forward	CGGTTGTGGTGTGTTTCATACT	64
HCT 2 reverse	CCACGTATCAAAGCCGATAAAT	62
HCT 3 forward	GTGTTTTGTTTGTGGAAGCTGA	62
HCT 3 reverse	AGGGAGTGGAGGGTTCAATCT	54
4CL forward	GGCTCCAAGTTGGTGATGAT	60
4CL reverse	GTCAGGCGACGACACTACAA	62
C4H forward	TTTAGGCCCGAGAGGTTTTT	58
C4H reverse	TCTTAGTCTGTCCGGGTGGT	62
C3H forward	TAATGGGTTGAGCCGAGAAG	60
C3H reverse	AATGTAACCGACGCGATACC	60

2.9.3 Genomic DNA Extraction

Genomic DNA was extracted using the following two-day method.
On the first day:

1. Leaf material was ground in liquid nitrogen using pestle and mortar. A small amount was placed into a labelled 1.5ml Eppendorf tube. Samples were kept cold until step 2.
2. 900µl of pre-warmed CTAB (hexadecyltrimethylammonium bromide) (65^{o}C) was added to the samples, mixed thoroughly and incubated in a water bath at 65^{o}c for 45 minutes.
3. 900µl Chloroform/Isoamyl (24:1; CHISAM) alcohol was added into each tube. The mixture was centrifuged for ten minutes at maximum speed.
4. 500µl of the supernatant was placed into a fresh Eppendorf with the addition of 50µl 3M sodium acetate and 333µl cold isopropanol.
5. The mixture was incubated at -20 ^{o}C for 15 minutes prior to centrifugation for 10 min at rcf=15,550x*g*.
6. The liquid phase was removed and the DNA pellet (DNA) was dried by inverting the tube and blotting it gently on tissue paper. The tubes were then centrifuged for 15 seconds and any remaining liquid was discarded.
7. The pellets were washed with 500µl cold 70% ethanol and centrifuged twice for 10 minutes at maximum speed before discarding the liquid. The pellets were air dried for 20 minutes.
8. DNA pellets were gently dissolved in 50µl of TE with 1µl of RNase (10mg/ml) and left at room temperature overnight.

On the second day:

1. The volume was increased with 200µl TE (10mM TRIS-HCl and 1 mM EDTA·Na_2) followed by 100µl of 3M sodium acetate and 1ml cold absolute ethanol. The solution was mixed well and centrifuged for 10 minutes at rcf=15,550x*g*.
2. The liquid phase was discarded, the tube was briefly centrifuged for 15 seconds prior to removal of any remaining liquid. The pellets were

air dried for 20 minutes before being dissolved in 50µl water or TE solution.

2.9.4 RNA extraction and DNAase-free treatment

RNA from the parent lines was extracted from whole head plants using the QIAGEN RNeasy Mini kit according to manufacturer's instructions. RNA was quantified using a NanoDrop ND 1000 spectrophotometer.

RNA samples were cleaned of any residual DNA contamination using the (QIAGEN RNase-free DNase set kit) with the following procedure: 1µl 10X DNase1 buffer (0.1 volume), 0.5µl γDNase1 and 3µl RNAase-free H_2O were added to 5µl of the RNA and mixed gently. The master mix was incubated at 37℃ for 30min (in a warm room). 0.5 γDNase1 was added to the mixture which was incubated for a further 30min. 1µl DNase inactivation reagent was added before being incubated at room temperature for two minutes, mixing occasionally. The mixture was centrifuged at 9,201 x *g* for 1.5 minutes. The supernatant containing cleaned RNA was transferred to a fresh tube and stored at -20℃.

2.9.5 Reverse Transcriptase PCR for cDNA synthesis

cDNA was synthesised from the parent line RNAs using 200ng of sample total RNA. The Reverse Transcriptase – PCR protocol was carried out as follows. 0.5µl of RNA was mixed with 1µl of oligo dT and 3.5µl nuclease-free H_2O. The mix was heated to 70℃ for five minutes and chilled immediately in ice water for five minutes. The tubes were subsequently centrifuged for 10 seconds at maximum speed.

The reverse transcription reaction was set up on ice water as follows. 5.4µl of nuclease-free H_2O was added to 4.0µl of ImProm-II 5X reaction buffer; 3.6µl $MgCl_2$; 1.0µl dNTP; and 5µl of the prepared RNA. Lastly, 1.0µl ImProm-II

Reverse Transcriptase was added to the master mix which was incubated in a Biometra Uno II Thermoblock as follows:

1. Annealing at 25℃ for five minutes
2. Extension at 42℃ for one hour
3. Enzyme inactivation at 70℃ for 15 minutes.

2.9.6 PCR amplification and cleaning of end product

PCR amplification was carried out using 2x BioMix™, from Bioline: 5µl BioMix; 0.5µl of 20mM forward and of reverse primers; 3µl H_2O; and 1µl sample. Thermal cycles were carried out in a Uno II Thermoblock, Biometra as follows:

1. Initialisation at 94℃ for 15 sec
2. Denaturation at 94℃ for 20 sec
3. Annealing at 55℃ for 15 sec
4. Extension at 72℃ for 2min

} 35 cycles

5. Final extension at 72℃ for 5min
6. Final hold at 16°

Amplified products were checked by electrophoresis on a 1.2% (w/v) agarose gel.

Following PCR amplification, the products were treated with Exonuclease 1 (20u/µl; New England Biolabs) and Shrimp Alkaline Phosphatase (1u/µl; Promega) to remove leftover primers and dNTPs (incubation at 37°C for 45min followed by heat inactivation at 80°C for 15min) for subsequent sequencing.

2.9.7 Real time PCR amplification

Real time PCR (rtPCR) amplification was carried out using 10µl 2q PCR master mix; 1µl of 20mM forward and of reverse primers; 7µl H_2O; and 1µl cDNA. Q-RT-PCR analysis was performed in a Chromo 4 Real Time 4-color 96-well plate PCR system with Optical 3 Analysis Software (MJ Research Masschusetts, USA) using SYBR Green monitor double-stranded (ds) DNA Synthesis. Thermal cycles were carried out as follows:

1. incubation at 95°C for 10 min
2. incubation at 94°C for 30 sec
3. incubation at 58°C for 1 min
4. incubation at 72°C for 1 min
5. plate read

(2–5: 35 cycles)

6. incubation at 72°C for 10 min
7. melting curve from 65 – 90°C, read every 0.2°C
8. Final hold for 1 sec
9. Result calculation by REST software

rtPCR analysis was conducted according to Pfaffl's method (Pfaffl 2001). The threshold value for the Comparative threshold level (C(t)) measurement was set to 0.025 relative fluorescence unit (rfu) and the baseline average over the first ten cycles was subtracted to explain any well intensity anomalies. C(t) values were the mean of three technical replicates which were amplified in separate wells. The rtPCR amplification efficiency (E) of the target and reference genes was generated using the Opticon Monitor software and based on the gradient of the linear phase of amplification. The difference between the C(t) values of a target gene versus the reference gene is presented to determine the relative expression ratio (R) or fold change.

The equation for calculation of R of a target in comparison to a reference gene is:

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta C(t)\ \text{target (control-treated)}}}{(E_{\text{reference}})^{\Delta C(t)\ \text{reference (control-treated)}}}$$

Where:

E_{target} = the rtPCR efficiency of target gene transcript

$E_{\text{reference}}$= the rtPCR efficiency of reference gene transcript

ΔC(t) target = the C(t) variation of control – treated sample of the target gene transcript

ΔC(t) reference = the C(t) variation of control – treated sample of the reference gene transcript

(Pfaffl 2001)

2.9.8 Sequencing and Single Nucleotide Polymorphism (SNP) analysis

The amplified products were submitted to the Dundee Sequencing Service (http://www.dnaseq.co.uk/) for sequencing.

Sequences were visualised using the Chromas Pro (version 1.41) software and sequence alignment between the two parent lines was carried out manually.

SNPs found in the parent lines were investigated in the RILs. Previously extracted cDNA from the RILs was used to investigate the polymorphisms by sequencing each line and aligning using VectorNTI Advance software (sequencing and alignment were carried out at the John Innes Genome Centre by David Baker).

CHAPTER 3 – ANALYSIS OF ANTIOXIDANT PHYTONUTRIENTS AND THEIR BIOAVAILABILITY IN COMMERCIAL BABY SALAD LEAVES

3.1 Introduction

Different qualities of salad leaves, grown according to standard commercial practice at the Vitacress Mullens farm, were analysed using the Ferric Reducing Antioxidant Potential (FRAP) to assess differences in antioxidant potential between cultivars. The FRAP assay is a rapid colorimetric assay which measures the reduction of metal ions upon contact with an antioxidant compound.

To investigate how the antioxidant potential declines in time, leaves were also stored for a number of days at 4℃ in a conventional household fridge and tested at different time points, thus simulating consumer storage methods before consumption.

Furthermore, to investigate antioxidant values at different times of harvest, a trial was carried out where baby leaves of different species and subspecies were harvested at four different time points during the span of 24 hours.

An intervention trial was then carried out on human subjects to investigate the bioavailability of antioxidants in human blood after consumption and to study how the effects of storage on the antioxidant levels of lettuce leaves are transferred to the antioxidant concentrations in human blood. The volunteers were fed either fresh lettuce or lettuce stored for one week and blood samples were harvested at different time points then analysed using the FRAP method.

Aims

1. To evaluate differences in antioxidant potential between different salad cultivars;

2. To evaluate pre-harvest differences in antioxidant potential of different cultivars harvested at different time points and the potential knock-on effects of harvesting at different time points;

3. To investigate the effects of storage on lettuce cultivars' antioxidant potential;

4. To determine how the ingestion of fresh and stored baby leaf salad leaves influence antioxidant bioavailability in human blood plasma.

3.2 Results

3.2.1 Spinach and red cos baby leaves have a significantly higher antioxidant potential than rocket, followed by green cos leaves

FRAP analysis of four different commercially available leafy salad crops, was carried out to investigate inter-specific differences in total antioxidant potential. Spinach (Emilia cultivar, Pop Vriend, Holland), red cos, rocket (Shamrock Seeds Selection, standard variety) and green cos baby leaves were harvested three weeks after sowing at the Vitacress Mullens farm in Wiltshire, UK.

The FRAP results showed that the antioxidant potential of green cos was significantly lower than that of the other cultivars tested. The highest levels were held by spinach (Emilia cultivar) and red cos (Fig 3.1).

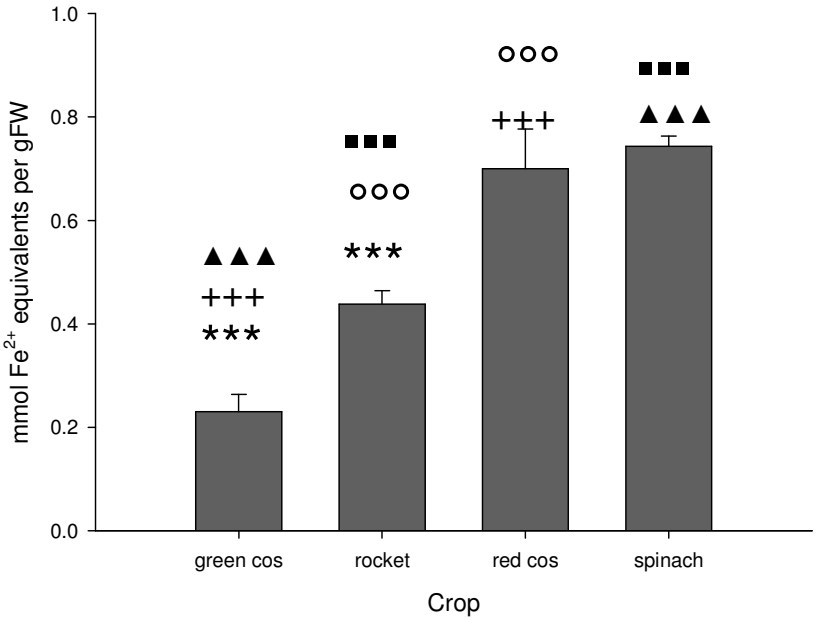


Fig 3.1 Total antioxidant levels of spinach, red cos, rocket and green cos. Samples were harvested at the Vitacress Mullens farm in the UK, on 05/09/2007. Five replicates per crop were sampled. One-way ANOVA statistical analysis and Tukey 95% Simultaneous Confidence Intervals showed a significant difference in antioxidant potential between green cos and rocket ($F_{3,16}$=28.40; ***P <0.001); green cos and red cos ($F_{3,16}$=28.40; $^{+++}$P <0.001); green cos and spinach ($F_{3,16}$=28.40; $^{▲▲▲}$P <0.001); red cos and rocket ($F_{3,16}$=28.40; ^{ooo}P <0.001); and rocket and spinach ($F_{3,16}$=28.40; $^{▪▪▪}$P <0.001).

3.2.2 Total antioxidants decline with storage

Shelf life trials were carried out to establish how total antioxidant levels drop with time and to assess this effect in different varieties of lettuce. Commercial lollo rosso, green cos and tango baby leaves were chosen for the study as green and red leaves were shown to contain significantly different amounts of antioxidants. Tango lettuce leaves were chosen owing to their similarity to the Salinas cultivar (cv) leaves studied as a parent line in the mapping population studied in the following chapters.

Leaves were kept in the dark at 4℃ for ten days. The FRAP analysis on lollo rosso and green cos samples showed a statistically significant decline in antioxidant potential with time (Fig 3.2 and 3.3). Lollo rosso samples also seem to decline more steadily than green cos samples. However, tango cv samples' (Fig3.4) antioxidant potential did not decline during storage.

The antioxidant potential of lollo rosso declined starting from the first day of storage. A significant decline in antioxidant potential was observed between days one and days four, seven and ten, losing about half of its potential by day seven. Whereas, green cos only showed a significant decline in antioxidant potential after the fourth day, reaching less than a third of its potential by the seventh day of storage. Interestingly, tango leaves, which started off with the lowest antioxidant potential of all, remained at a constant level for all of the ten days.

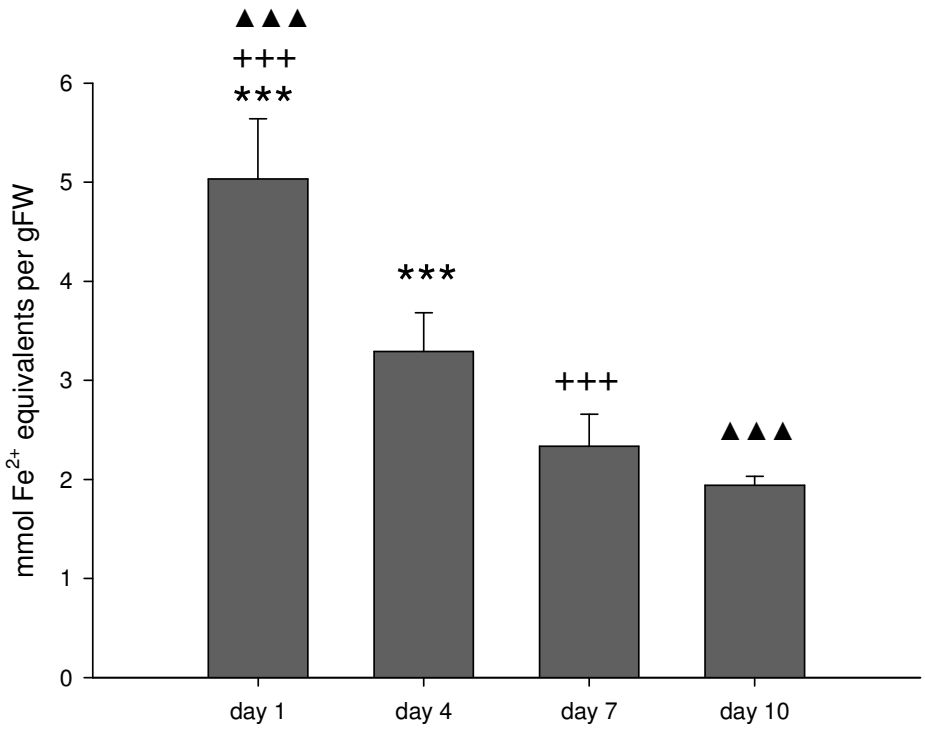


Fig 3.2 Effects of storage on total antioxidant potential of commercial lollo rosso during storage. Leaves were kept in closed bags at 4ºC for up to 10 days. The ANOVA General Linear Model statistical analysis showed a general decline in antioxidants ($F_{3,70}$=8.15, ***P<0.001). Tukey's 95% Simultaneous Confidence Intervals post hoc test, revealed a decline in antioxidant potential between days 1 and 4 ($F_{3,36}$=7.21; ***P=0.001); days 1 and 7 ($F_{3,36}$=7.21; $^{+++}$P=0.001); and days 1 and 10 ($F_{3,36}$=7.21; $^{▲▲▲}$P=0.001).

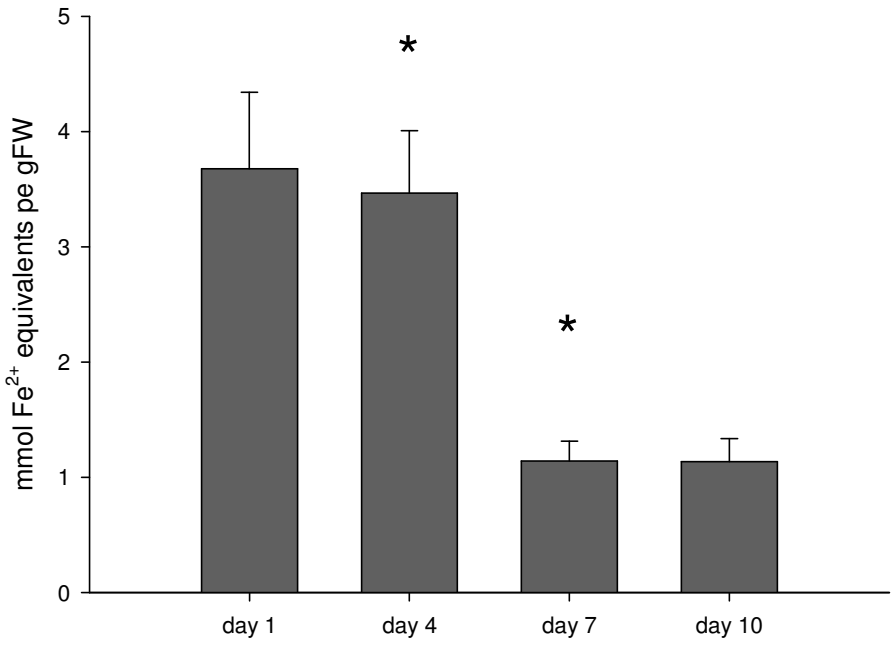


Fig 3.3 Effects of storage on total antioxidant potential of commercial green cos during storage. One way ANOVA analysis and Tukey 95% Simultaneous Confidence Interval tests of Green cos samples showed a significant decline in antioxidant potential between days 4 and 7 ($F_{3,20}$=4.82; *P<0.05).

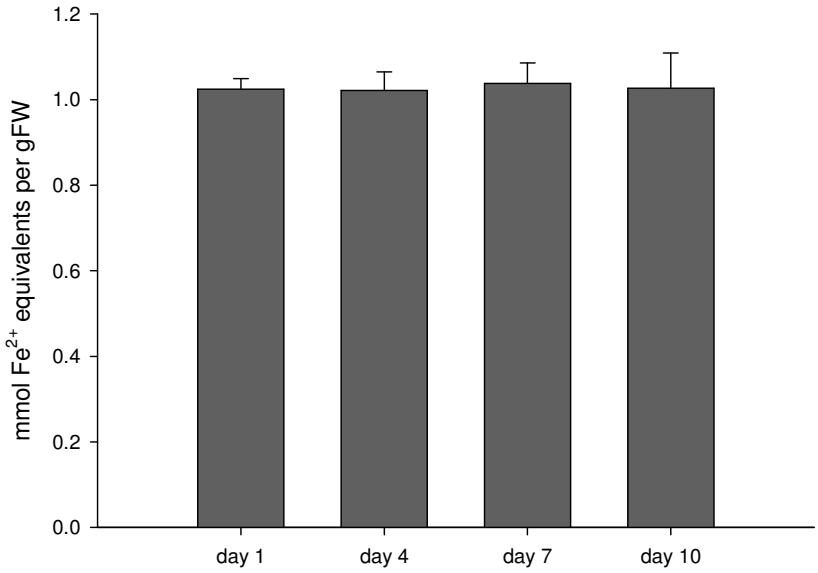


Fig 3.4 Effects of storage on total antioxidant potential of commercial tango lettuce. A one-way ANOVA showed no significant effects of storage on the tango cultivar antioxidant potential.

3.2.3 How vitamin C levels in lettuce decline during storage

Vitamin C analysis of stored tango lettuce was carried out at Leatherhead Food International by using High Performance Liquid Chromatography (HPLC). The aim was to relate variation in vitamin C levels to the total antioxidant potential during shelf life.

Vitamin C levels in tango lettuce remained constant for the first four days of shelf life after which levels started to decline significantly. There was a significant drop in vitamin C levels between days one and ten, four and ten, and seven and ten (Fig 3.5).

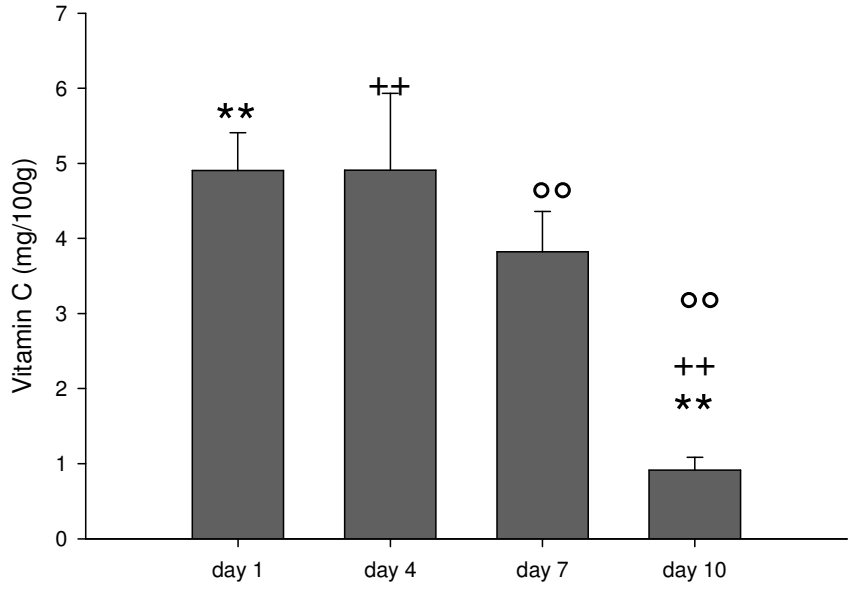


Fig 3.5 HPLC analysis of vitamin C in commercial tango leaves during storage. The leaves were washed in distilled water and chlorine (1:40 dilution) and stored in closed bags at 4ºC for up to ten days. One-way ANOVA and Tukey 95% Simultaneous Confidence Intervals post test showed that there was a significant decrease in vitamin C levels between days 1 and 10 ($F_{3, 16}$ = 8.79,**P< 0.01), days 4 and 10 ($F_{3, 16}$ = 8.79,$^{++}$P< 0.01) and between days 7 and 10 ($F_{3, 16}$ = 8.79,^{oo}P< 0.01).

3.2.4 Harvesting at different times of the day has an effect on the antioxidant content of red cultivars but not on green cultivars

Leaf samples were harvested at four different time points over 24 hours to assess the effects of harvesting at different times of the day on the antioxidant potential of different varieties of salad leaves.

Spinach, red cos, green cos and rocket were harvested at 01:00, 07:00, 13:00 and 19:00 hrs. A significant difference was observed in the antioxidant potential of red cos at times 01:00 and 13:00; and between times 01:00 and 19:00, where samples harvested early in the morning had significantly lower levels of antioxidants than those harvested later on in the day (Fig 3.6). However, no significant difference was found for green cos, spinach or rocket samples harvested at different times of the day. Although there is a trend for samples harvested at 13:00 in the afternoon to have higher antioxidant levels.

The trial also aimed to investigate the potential knock-on effects of different harvesting times on the antioxidant potential of each of the crops after storage for one week at 4℃ in the dark. The aim was to investigate what happens in reality, with salads being commercially available a few days after harvest. There was a definite trend in antioxidant decline for all harvest hours with a significant decline in green cos antioxidant potential between fresh and stored samples harvested at 07:00 (Fig 3.7). There was also a significant decline in antioxidant potential for all stored rocket samples except for those harvested at 19:00 (Fig 3.8).

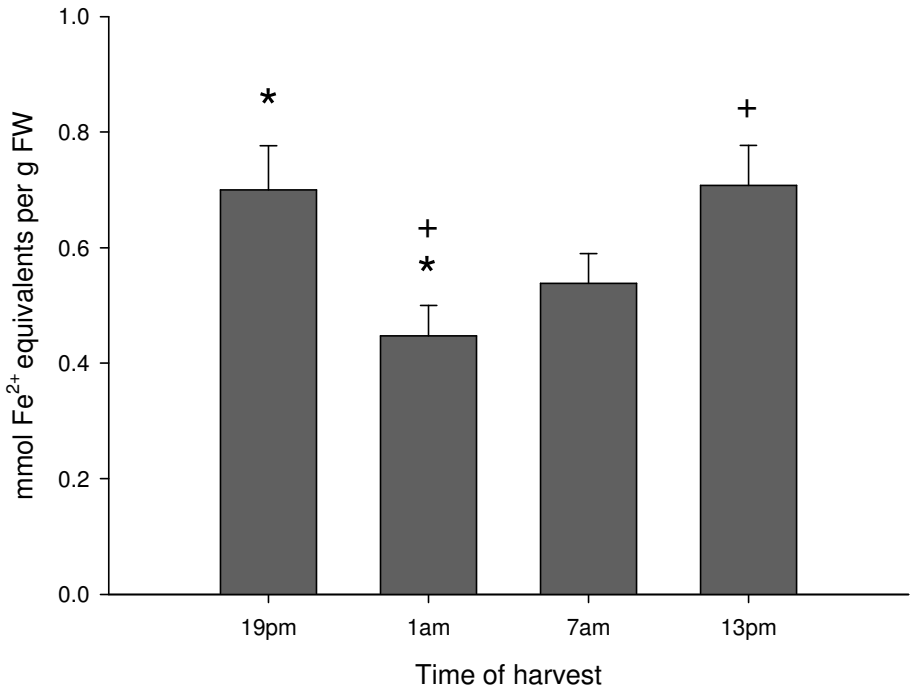


Fig 3.6 Effects of time of harvest on total antioxidant potential of commercial red cos. Commercial red cos samples were harvested at 19:00; 01:00; 07:00; and 13:00 hours. One way ANOVA analysis showed significantly lower antioxidant potential for samples harvested at 01:00 than at 19:00 ($F_{3,17}$=4.02;*P<0.05); and between samples harvested at 01:00 and 13:00 ($F_{3,17}$=4.02;$^{+}$P<0.05).

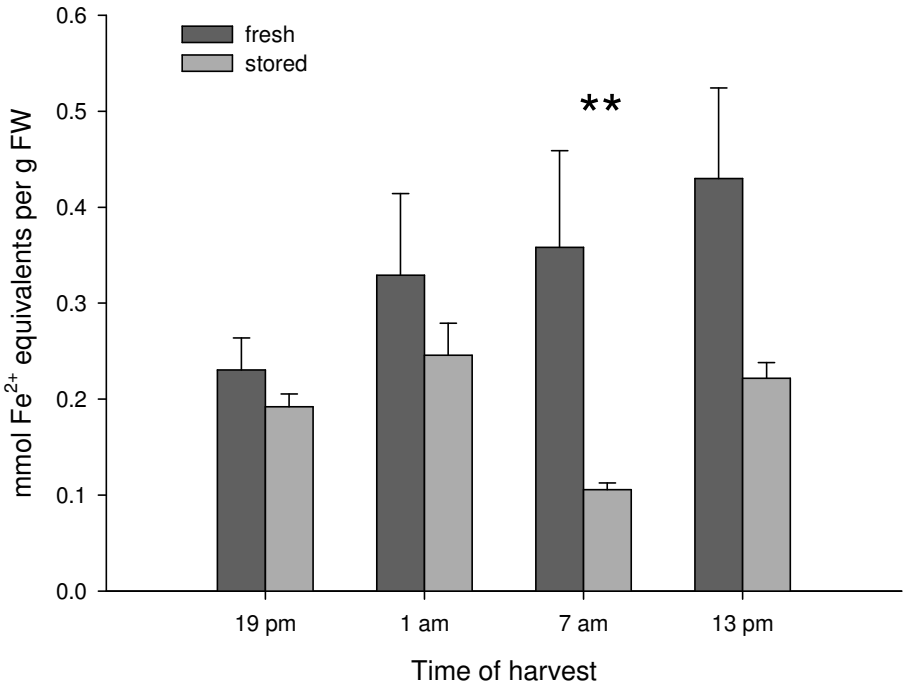


Fig 3.7 Effects of storage on total antioxidant potential of commercial green cos after 1 week. Commercial green cos samples were harvested at 19:00; 01:00; 07:00; and 13:00 hours. ANOVA General Linear Model analysis showed a consistent decline in antioxidant potential for each harvest hour after seven days of storage in the dark at 4℃ and a significant difference between fresh and stored samples harvested at 7:00am ($F_{3,36}$=11.69; **P<0.01).

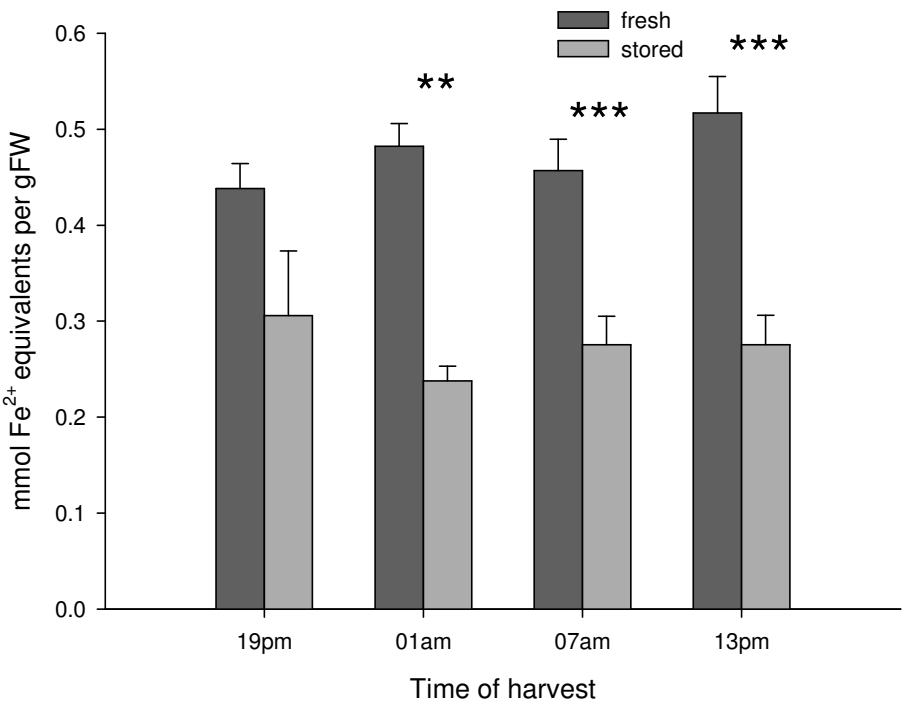


Fig 3.8 Effects of storage on total antioxidant potential of commercial rocket after 1 week. Commercial rocket samples were harvested at 19:00; 01:00; 07:00; and 13:00 hours. ANOVA General Linear Model analysis showed a general decline in antioxidant potential after seven days of storage at 4℃ for each harvest time. Analysis of storage effects on time of harvest using One Way ANOVA determined a significant decline in antioxidant potential for samples harvested at 01:00hrs ($F_{1,8}$; ***P<0.001); 07:00hrs ($F_{1,8}$; **P<0.01); and at 13:00 ($F_{1,8}$;***P=0.001).

3.3 Antioxidant levels in blood plasma are greater after consumption of fresh lettuce than after consumption of stored lettuce

An intervention trial was carried out on postprandial antioxidant bioavailability after consumption of fresh or stored lettuce. The aim was to relate these results to those obtained from lettuce shelf life analyses and thus to investigate whether eating a portion (equal or above the recommended daily allowance) of fresh or stored lettuce significantly increases the levels of antioxidants in human blood plasma.

A pilot study was carried out on a single subject two weeks previous to the main trial for a number of reasons: to establish an optimal amount of lettuce leaves to serve the volunteers; to ascertain a sufficient quantity of blood plasma to be measured; and to determine the time points for harvesting the plasma samples. The results indicated that the initial 80g of sample would need to be increased to 120g and that the collection times could be extended to 150min (data not shown).

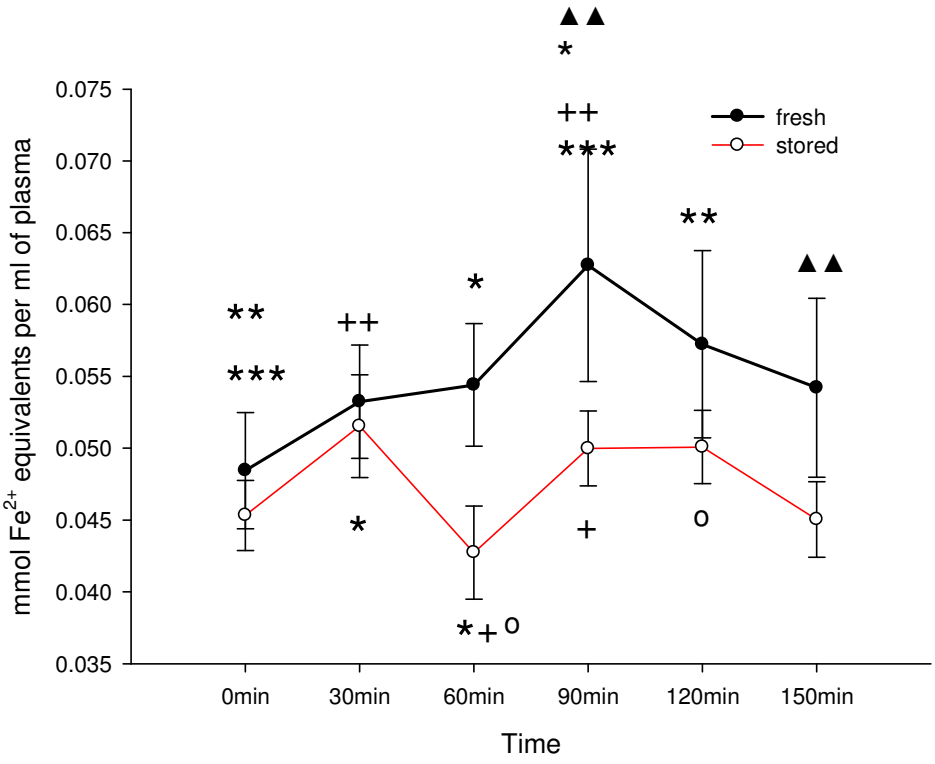
16 volunteers, Caucasian, non-smokers and between the ages of 18 and 45, were recruited for the trial. Only male volunteers were selected due to the monthly hormonal fluctuations of female subjects which may influence trial results. The volunteers were given fresh or stored lettuce samples to eat on an empty stomach. Green cos cv lettuce samples were grown and harvested locally according to standard commercial practice at the Vitacress Salads Ltd, Hampshire farm. 120g of fresh or stored lettuce were given to each of 16 (eight volunteers had fresh and eight had stored lettuce) volunteers who had fasted for the previous 12 hours and who had eaten meals containing low levels of antioxidants for the previous 24 hours. 15ml of blood were harvested before consumption (0min), then at five different time points for each volunteer: 30min; 60min; 90min; 120min; and 150min. The antioxidant status of the plasma samples were then measured using the FRAP method.

Results show a significant increase in antioxidant levels after consumption of fresh lettuce, but not after consumption of stored lettuce (Fig 3.9 (a)). Levels of antioxidants in plasma after eating fresh lettuce significantly increased at times 90min and 120min from the starting levels at time 0min, before lettuce consumption. Antioxidant levels are also significantly lower at time 30min and 60min than at time 90min, indicating a rise in antioxidant absorption during the first 90min after ingestion. A distinct peak in antioxidant absorption occurs at time 90min postprandial, after which the levels drop off again, with a significant decline in levels between 90min and 150min.

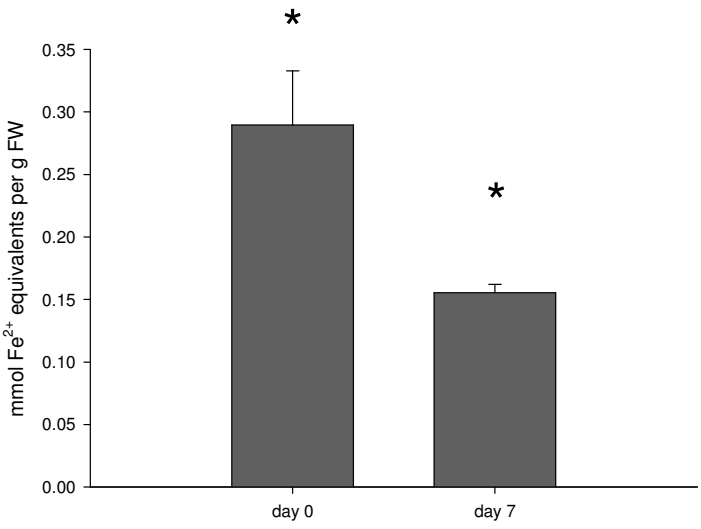
Results also show that after eating stored lettuce there is no significant increase of total antioxidant levels in plasma (Fig 3.9 (a)). However, they do show an interesting decline in antioxidant potential at time 60min compared to values below those at time 0min.

The antioxidant levels of the green cos samples used for this trial are shown in Fig 3.9 (b). A significant decline to about half the antioxidant potential is observed after one week of storage. This correlates well with plasma results in Fig 3.9 (a).

Two-way ANOVA indicated a significant interaction effect between the variables time and fresh/stored treatments, i.e. the type of lettuce treatment responded differently to the different time points, showing an impact of the lettuce age over antioxidant absorption over time (Fig 3.10).



(a)



(b)

Fig 3.9 Antioxidant levels of human blood plasma before and after lettuce consumption. Volunteers were given 120g of fresh or stored (for one week) green cos lettuce samples to consume on an empty stomach. Blood samples were harvested before ingestion (0min), then at time intervals 30min; 60min; 90min;

120min; 150min post lettuce consumption. Two-way ANOVA and Bonferroni post hoc tests were used to assess significance of the differences between treatments and between the different time points (a): time 0min and 90min ($F_{5,20}$=6.001, ***P<0.001); time 0 and 120min ($F_{5,20}$=6.001, **P<0.01); time 30min and 90min ($F_{5,20}$=6.001, $^{++}$P<0.01); time 60min and 90min ($F_{5,20}$=6.001, *P<0.05); and time 90min and 150min ($F_{5,20}$=6.001, $^{\blacktriangle\blacktriangle}$P<0.01). Differences in antioxidant levels of fresh and stored green cos samples used in the trial were analysed using one-way ANOVA ($F_{1,4}$=9.39; *P<0.05) (b).

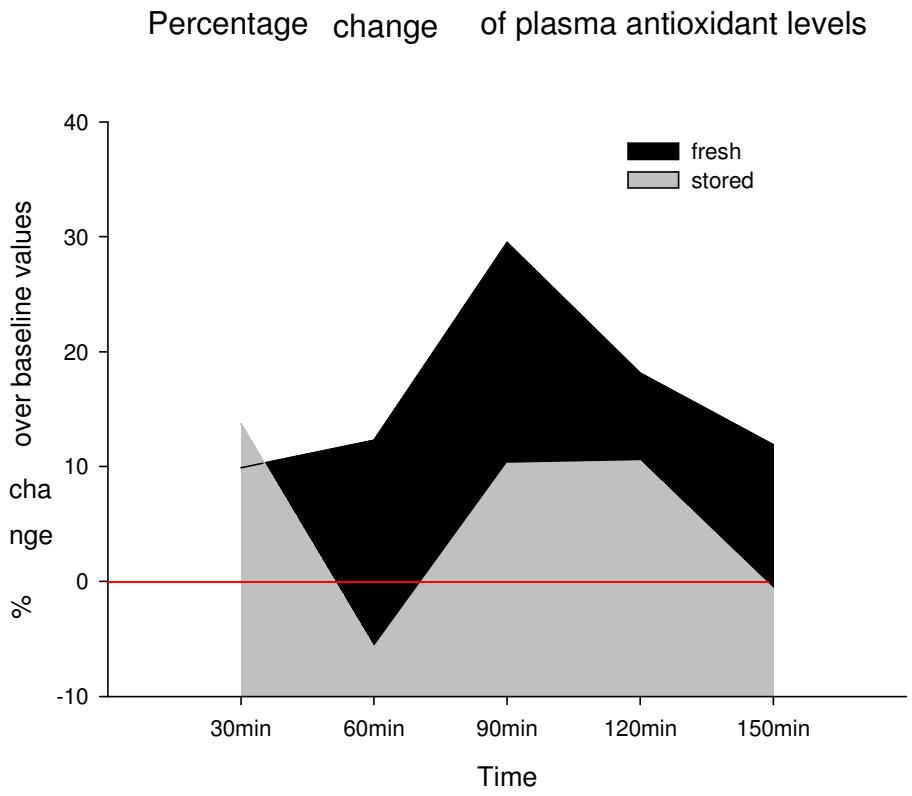


Fig 3.10 Percentage change of plasma antioxidant levels over baseline value. ANOVA testing found a significant interaction between treatments (fresh vs stored) and time, suggesting an impact of the lettuce treatment (fresh or stored) over the antioxidant absorption over time ($F_{5,20}$=2.434, *P<0.05).

All relevant paperwork necessary for the intervention trial are displayed in Appendix 5, sections a. to q. Copies of the completed Research Ethics Committee (REC) application form and Site Specific Information (SSI) form are attached as accompanying material.

3.3 Discussion

Numerous epidemiological studies have shown an inverse association between fruit and vegetable consumption and many chronic diseases, owing to the protective effects of various phytochemicals (Ames et al. 1993;Manach et al. 2004;Valko et al. 2007;Verhoeven et al. 1996). Thus, consumer awareness of food nutritional properties has increased significantly over the last couple of decades. Many such properties, such as antioxidants, have been shown to be an important part of healthy diets, aiding in the prevention of a number of degenerative diseases, by protecting against mutagenesis caused by oxidative damage and thus decreasing the chances of tumour development or carcinogenesis.

Understanding how these antioxidant values vary in different leafy salad crops, at harvest time, or with time since harvest, is important for the plant breeder as well as for the consumer. Thus the study focused on giving a general picture of total antioxidant potential in various salad leaves, on tracking changes in antioxidant potential following differences in harvest procedures and establishing their decline with storage. Finally, the study also aimed to establish the bioavailability of lettuce antioxidants in human blood plasma and the difference in bioavailability after consumption of fresh versus stored lettuce.

The key method used in this study was aimed at measuring the antioxidant content of the leaves investigated. A number of different methods can be employed for this purpose, such as the Trolox Equivalent Antioxidant Capacity (TEAC), the Ferric Reducing Antioxidant Potential (FRAP), or the Oxygen Radical Absorbance Capacity (ORAC). These three methods have been compared in previous studies and seem to consistently present similar values (Etminan et al. 2005;Kalt et al. 1999;Proteggente et al. 2002;Stratil et al. 2006). For the purpose of the present study, the FRAP assay was selected because it gave the following advantages: efficiency, as many samples can be analysed simultaneously, and therefore within a short time;

and versatility, owing to the fact that both plant material and blood plasma can be measured using the same assay, thus providing a certain degree of consistency.

3.3.1 Inter-specific and intra-specific differences in phytonutrient content

As a preliminary test for the FRAP assay, a comparison between different leafy salad crops, spinach (*Spinacia oleracea,* Emilia cultivar, Pop Vriend, Holland), rocket (*Eruca sativa* Shamrock Seeds Selection, standard variety), red cos and green cos (both *Lactuca sativa*), was carried out with the intention of placing lettuce on a nutritional scale in terms of its antioxidant potential. The assay was modified from the original protocol (Benzie & Strain 1996), which had been designed for blood samples, therefore these preliminary comparisons were also carried out to test the sensitivity of the assay and to optimise the technique on plant samples.

The results of this study are in accordance with previous publications on intra-specific differences in total antioxidant potential between *Lactuca sativa* cultivars (DuPont, Mondin, Williamson, & Price 2000;Liu et al. 2007;Llorach, Martinez-Sanchez, Tomas-Barberan, Gil, & Ferreres 2008), as well as inter-specific differences (Proteggente, Pannala, Paganga, Buren, Wagner, Wiseman, Put, Dacombe, & Rice-Evans 2002). The results showed that spinach (0.71mmol Fe^{2+} equivalents/g FW ±0.03) and red cos (0.70mmol Fe^{2+} eq/g FW ±0.08) had a significantly greater antioxidant potential than rocket (0.44mmol Fe^{2+} eq/g FW ±0.03), and that green cos had significantly less antioxidant potential than all of the other types of leaves (0.23mmol Fe^{2+} eq/g FW; ±0.03) (Fig 3.1). From these results it emerged that green leaf lettuce varieties (green cos and tango) consistently showed a significantly lower antioxidant potential than other types of salad leaves, in accordance with Proteggente et al (2002), who reported that green leaf lettuce showed little antioxidant activity in comparison to all the vegetable and fruit cultivars analysed.

In general results indicate that red or darker pigmented leaf cultivars have higher antioxidant potential than the light green cultivars, grown under the same conditions. These findings are in agreement with previous studies that found that red varieties contained the highest levels of antioxidants and polyphenols (Liu et al. 2007;Llorach et al. 2008). Liu et al compared 25 different lettuce cultivars including leaf, romaine, crisphead and butterhead types grown in Colorado, whilst Llorach et al compared five lettuce cultivars (iceberg, romain, continental, red oak leaf and lollo rosso) grown in Spain. Thus the pigment compounds such as flavonoids and carotenoids present in greater quantities in red leaf varieties of lettuce seem to confer their greater antioxidant potential.

In conclusion, the results of these experiments proved that different varieties of *Lactuca sativa* provide important antioxidant compounds in different quantities. They suggest that on a scale of antioxidant potential in salad leaves, green cos lettuce can be placed on a relatively low level in comparison to other, darker leafed cultivars.

3.3.2 Pre-harvest antioxidant evaluations and storage effects on phytonutrient content of salad leaves

The effects of storage on the quality of lettuce crops is an important issue for the producer who wants to maintain their products' nutritional qualities pre and post-harvest and for the consumer who is increasingly concerned with improving their health with good diet. Post-harvest losses in nutritional quality, particularly antioxidants and vitamin C, can be significant and may be enhanced during storage times (DuPont et al. 2000;Ferreres et al. 1997;Gil et al. 2006).

To mimic the storage effects and the potential losses of nutritional quality in three common lettuce varieties, tango, green cos and lollo rosso baby leaves

were kept for up to 10 days in the dark at 4ºC. FRAP analyses were then carried out to quantify antioxidant decay during storage.

Lollo rosso leaves had significantly lost 36% of their antioxidant potential by the fourth day of storage and over half (52%) by the seventh day with very little potential left by day 10 (Fig 3.2). Whereas green cos leaves seemed to maintain their antioxidant potential for the first four days, after which they lost over 70% by the seventh day (Fig 3.3).

Interestingly, tango leaves showed no significant decrease in antioxidant potential for the whole trial (Fig 3.4), indicating that decay is not appreciable within the first week to ten days of shelf life, after which browning of the leaves occurs. In fact, this type of lettuce seems to visually spoil before any significant nutrient loss occurs.

An important aspect is that salad types with the higher antioxidant content such as lollo rosso (5mmol Fe^{2+} eq/gFW) and green cos (3.6mmol Fe^{2+} eq/gFW) lost a large portion of their potential during storage, whereas salads with lower quantities of antioxidants, such as tango lettuce (1mmol Fe^{2+} eq/g FW) maintained their levels constant for a longer period. However, it is important to note that despite losing about 50-70% of their antioxidant capacity by the seventh day, the amounts present in lollo rosso samples on day 10 were still just as high as the tango leaves' were for the extent of the trial. This may indicate the presence of a large portion of unstable antioxidants in lollo rosso and green cos but not present in tango leaves.

Interestingly, vitamin C levels do not seem to correlate with the total antioxidant content in tango lettuce leaves: tango leaves' vitamin C levels decreased by 22% from day one to day seven (Fig 3.5). A further, significant, decline in vitamin C levels occurred after day seven, reaching almost a fifth of its original amount by day 10 (81% decrease). In fact, studies on antioxidant phytonutrients in lettuce leaves have shown that vitamin C is not one of the major constituents of the total antioxidant capacity of lettuce

leaves (Nicolle et al. 2004). Vitamin C loss during storage has been investigated in numerous studies and seems to vary considerably according to the type of vegetable or fruit investigated (Giannakourou & Taoukis 2003;Gil et al. 2006;Kalt et al. 1999); for instance, Gil et al (2006) report that the losses in vitamin C after 6 days at 5 ℃ were ≤5% in mango, strawberry, and watermelon pieces, 10% in pineapple pieces, 12% in kiwifruit slices, and 25% in cantaloupe cubes.

The total antioxidant capacity of a number of fruit and vegetable species has, conversely, been highly correlated with their polyphenolic content (Kalt et al. 1999;Llorach et al. 2004;Nicolle et al. 2004;Proteggente et al. 2002). Dupont et al reported that storage of lettuce resulted in significant losses of its flavonoid content. Lettuce whole heads were kept at 1℃ in the dark for one week resulting in losses of total flavonol glycosides (such as quercetin glycosides, the main flavonoid compounds found in lettuce leaves), in the range of 7- 46%.

These findings on the rate of antioxidant decrease within different leafy salad varieties during storage are important for consumers, who want to know whether their product's nutritional content is spoiling at a high rate within the first few days of storage.

Harvest time is another important aspect of leafy salad crop production. As standard commercial practice, harvesting is generally carried out in the early hours of the morning so that the leaves are then processed at the factory and ready to be despatched before the end of the day. Previous studies have found that harvesting at the end of the day rather than during early morning hours, has a positive impact on the shelf life of the salad leaves (Clarkson et al. 2007). Thus, an analogous trial was carried out to investigate whether harvesting in the evening had an effect on the antioxidant potential of different qualities of salad leaves.

Red cos, green cos, rocket (Shamrock standard variety) and spinach (Emilia cultivar) baby leaves were harvested at different time points and analysed using the FRAP method (Fig 3.6). Results showed that red cos had significantly higher levels of antioxidants when harvested in the afternoon or in the evening (i.e. at times 13:00 or 19:00). These higher levels of antioxidants could be due to the fact that the flavonoid and carotenoid pigment levels of these red leaves increase owing to the light irradiation they receive during the day. These data suggest that harvesting red lettuce leaf types later on in the day rather than in the early hours of the morning, would lead to an increase in their antioxidant levels. These data are supported by a number of studies (Li & Kubota 2009;Oh et al. 2009), which state that an increase in light irradiation led to a two to threefold increase in the total phenolic content and a significant increase in the antioxidant capacity of lettuce. Studies on seasonal effects on the increment of total polyphenolic and total antioxidant content also support this view (Howard et al. 2002). Howard et al (2002) found that spinach leaves planted in late autumn and harvested in the spring, had much higher levels of total phenolics and antioxidant capacity than spinach planted in early autumn and harvested in late autumn.

Surprisingly, a change in harvest time had no significant effect on fresh green cos, rocket (Shamrock Seeds Selection, standard variety) or spinach (Emilia cultivar) cultivars. However, there was a knock-on effect of harvest times in rocket and green cos after seven days storage. The decline in antioxidant potential in stored green cos leaves harvested at 07:00 in the morning was significantly greater than for leaves harvested at other times of the day. Furthermore, the decrease in antioxidant levels of fresh or stored rocket leaves seemed to be significant for all harvest times except for those harvested at 13:00 in the afternoon. Taken together, these findings suggest that harvesting rocket (of the variety tested here) and green cos leaves in the afternoon may increase their shelf life, in terms of antioxidant potential.

The results emerging from these experiments have important consequences with regards to the salad crop industries, as they would suggest importance in the selection of harvest times for different crops as well as importance in the choice of lettuce quality at the species and subspecies level. Thus these data suggest that it is important for the lettuce producer to consider environmental factors when selecting lettuce cultivars for enhanced antioxidant capacity.

3.3.3 Bioavailability of lettuce antioxidants

Numerous epidemiological studies have shown that a predominantly vegetarian diet is associated with better antioxidant status, cancer and coronary heart disease risk profiles than diets with low consumption levels of fresh fruit and vegetables (Chiao et al. 2004;Conaway et al. 2002;Etminan et al. 2005;Manach et al. 2004c;Reddy & Katan 2004;Trichopoulou & Vasilopoulou 2000;Valko et al. 2007). However, often these fruit and vegetables are consumed days after being harvested, both because of processing and shipping times and because the consumer may not eat them on the day of purchase. It is therefore important to establish the effects of storage on their beneficial properties, such as their antioxidant potential, and how this reflects on their phytonutrient bioavailability in humans.

The study's preliminary analyses on the effects of storage on green cos lettuce leaves have shown a reduction in antioxidant potential starting from the fourth day of storage and resulting in less than a third of the initial potential by the seventh day (Fig 3.3). An intervention trial in human volunteers was therefore set up and carried out to relate these shelf life results to the bioavailability of antioxidants from fresh and stored lettuce in human blood plasma.

The bioavailability of antioxidants in plasma samples of male volunteers was examined after ingestion of intact fresh or stored (in the dark, for one week at 4℃) baby leaf lettuce samples. Green cos leaves were used as they

represent a leaf cultivar available in most shops and commonly used as basis for salads or in sandwiches. Consumer sized bags (120g) of baby leaf lettuce such as the commercial varieties commonly found in Europe and the UK were fed to fasting volunteers. Whole baby leaves were used in the present study as plant metabolites start to decline soon after harvest, therefore, further chopping of leaf material would result in further declines in a number of metabolites, as well as increases in certain polyphenols, thus introducing other elements of variation to the samples (Kang & Saltveit 2002). The choice of using whole baby leaves also followed from the modern trend for bagged baby leaf salads.

The intervention trial results demonstrate that consumption of fresh lettuce significantly increases the total antioxidant potential found in blood plasma and illustrates how it increases with time after consumption before declining again (Fig 3.2). A significant rise in antioxidant status in blood plasma occurred soon after lettuce consumption, between 30min postprandial and 90min there was a dramatic and significant rise. A peak in antioxidant potential was found 90 minutes after ingestion after which the levels started to diminish again. The results showed that there was a gradual but significant decline in antioxidant status of blood plasma after 90min and that it was still significantly higher at 120min than at time 0min. Furthermore, whilst the decline from the antioxidant peak at 90min to 150min was significant, the levels at 150min are still greater than at time 0min, thus indicating a gradual decline in antioxidant phytonutrients in the blood.

A three-fold significant increase was noted within 1.5hrs ingestion of fresh lettuce, whereas after ingestion of stored lettuce antioxidants did not rise significantly in blood. Interestingly, in the latter case, the antioxidant potential showed a declining trend at times 60min and 150min, towards levels lower than the initial levels at time 0. This unexpected decline in antioxidants could be a consequence of changes in metabolism due to the volunteers' fasting period before the trial and to their low antioxidant intake during the previous 24 hours. This study is in partial accordance with Serafini et al (2002) who

found that eating chopped lettuce stored for three days under modified atmosphere packaging (MAP) had no benefit on plasma antioxidant levels (Serafini et al. 2002).

Previous studies have been carried out examining the effects of postprandial blood antioxidant status after consumption of other types of food or beverages, such as vegetable-based meals (Van het Hof et al. 1999), fruit and berry mixtures (Jensen et al. 2008), coffee (Nardini et al. 2002), orange juice (Riso et al. 2005), and numerous other foodstuffs (John et al. 2002). However, no other study had focused on the postprandial effects of packaged fresh and stored intact baby leaf lettuce on human blood antioxidant status. This study has shown that eating fresh lettuce increases the antioxidant status of blood plasma significantly whereas eating stored lettuce does not induce a significant change in antioxidant levels (Fig 3.9). Therefore, the study expands our understanding of the true nutritional value of modern convenience packed salad as it underlines the importance of eating sufficient quantities of fresh leaves rather than stored ones.

Further studies would focus on a breakdown of the individual metabolites which compose the total antioxidant potential present in blood serum after lettuce consumption: such as polyphenols and vitamins. It would be interesting to analyse the bioavailability of postprandial lettuce phenolic acids and flavonoids, such as quercetin, dicaffeoyl tartaric acid and chlorogenic acid and the differences after consumption of fresh or stored lettuce.

3.4 Conclusions

1. There can be considerable variation in the antioxidant content of different leafy salad crop cultivars;

2. The red *Lactuca sativa* cultivar studied here tends to have higher antioxidant potential than the green leaf varieties;

3. The nutritional value of lettuce leaves may be increased by pre-harvest factors such as time of harvest;

4. There is considerable variation in antioxidant potential in response to storage of different cultivars: green leaf varieties start off with lower values but tend to preserve their antioxidant status longer; red leaves start off with higher values which decline more rapidly;

5. Consumption of fresh green cos leaves contributes a significant amount of antioxidant potential to human blood plasma, peaking at 90min post-consumption;

6. Significant levels of antioxidants in lettuce can be absorbed by the body if fairly fresh lettuce is consumed.

CHAPTER 4 – ANALYSIS OF ANTIOXIDANT PHYTONUTRIENTS IN THE PARENT LINES AND IN THE RECOMBINANT INBRED LINES

4.1 Introduction

The phytonutrient qualities of the Recombinant Inbred Lines (RILs) and their parent lines, *Lactuca sativa* (cv Salinas) and its wild relative *Lactuca serriola* (acc UC96US23), were examined. Firstly, the total antioxidant potential and specific antioxidants such as carotenoid, chlorophyll, vitamin C and vitamin E contents were investigated in the parent lines. The objectives were to establish significant differences in the nutritional qualities between these two lines, and to give a general picture of their allocation in different leaves of the same plant and of their resistance to the effects of storage and reduced light irradiation intensity. The results would then influence the choice of analyses to be carried out on the RIL mapping population with the purpose of finding an ideal line which would have a good combination of high nutritional qualities and pleasant taste from either parent.

Aims

1. To establish significant phytonutrient differences between the parent lines;

2. To study these differences in nutritional qualities in the Recombinant Inbred Lines;

3. To investigate the environmental effects on the antioxidant potential of the RILs;

4. The selective increase of these traits in the cultivated species through breeding techniques.

4.2 Young leaves have a higher antioxidant level than old leaves

Following from the modern trend for fresh baby leaf packaged salads, a study was set up to investigate differences in antioxidant potential between leaves of different ages. Young and old leaves harvested from the same individuals were compared to investigate variation in allocation of antioxidant phytochemical quantities in different leaves of the same plant. The leaves were designated numbers according to age, where leaf one was the oldest leaf and leaf eight was taken as representing the young leaves.

For the analysis of the parent lines and subsequently of the RILs, the Ferric Reducing Antioxidant Potential (FRAP) assay was selected owing to its efficiency, as many samples can be analysed within a short time. It was therefore a practical assay for the analysis of the large numbers of lines in the complete mapping population.

FRAP analysis in *L. sativa* showed a significantly higher level of antioxidants for young leaves compared to old leaves. Young leaves (leaves #8) produced twice as much antioxidant potential than old leaves (leaves #1) taken from the same plant (Fig 4.1 2). Thus all subsequent analyses were carried out on young plants.

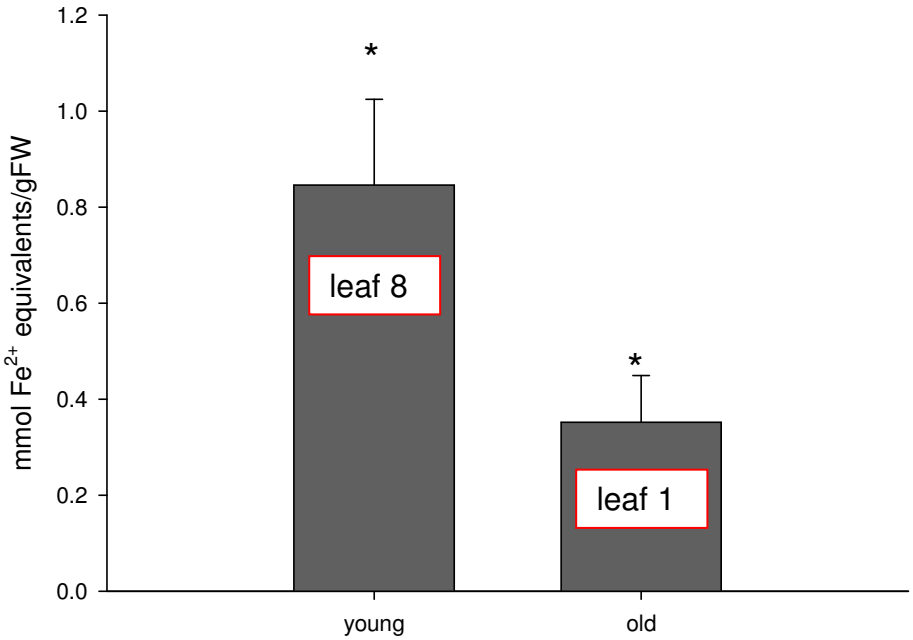


Fig 4.1 Total antioxidant levels in differently aged *L. sativa* leaves. Sets of one young (leaf 8) and one old leaf (leaf 1) were taken from seven one-month old *Lactuca sativa* plants. One way ANOVA analysis showed a significantly higher level of antioxidant potential in young leaves than in older leaves ($F_{1,26}$= 5.18;*P<0.05).

4.3 The wild parent line L. serriola *contains higher levels of antioxidants than the parent line* L. sativa *(cv)*

L. sativa and *L. serriola* were analysed and compared for total antioxidant potential. Differences between the two parent lines gave an indication of what could be present in the RILs and encourage investigations of these traits.

Interestingly, FRAP data on the parent lines five weeks post-germination showed that the wild subspecies, *L. serriola,* contained three times as much antioxidant potential than the cultivar, *L. sativa* (Fig 4.2). These results

suggested that the cultivar could be improved in terms of antioxidant capacity by breeding with the wild lettuce.

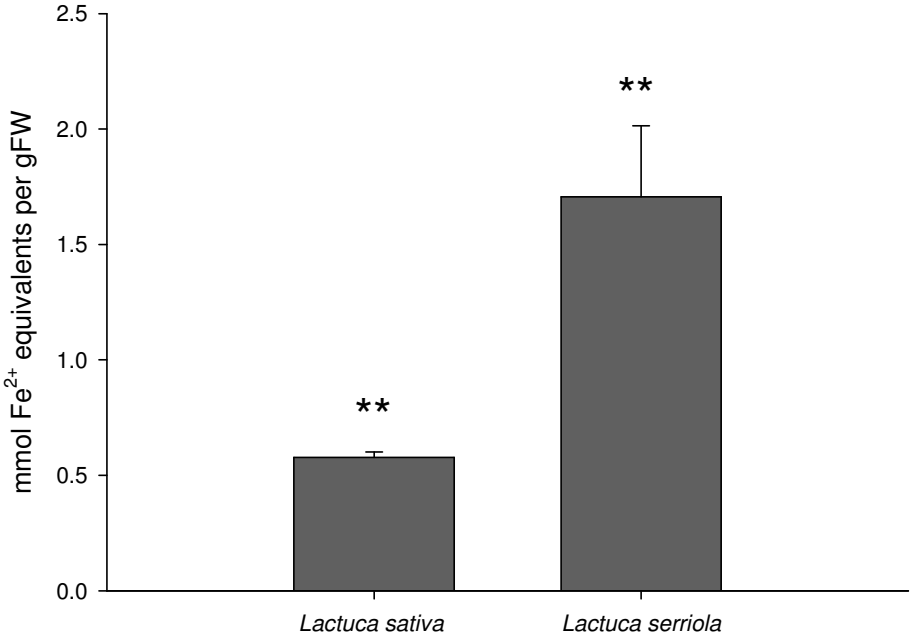


Fig 4.2 Total antioxidant levels of *L. sativa* and *L. serriola* as determined by the FRAP colorimetric assay. *L. sativa* and *L. serriola* leaves of the same age (the fourth from the youngest) were tested for total antioxidant potential. Nine biological replicates and three technical replicates for each biological replicate were sampled. One way ANOVA analysis showed significant difference between the wild parent, *L. serriola*, and the cultivar, *L. sativa* ($F_{1,16}$=13.29; **P<0.01).

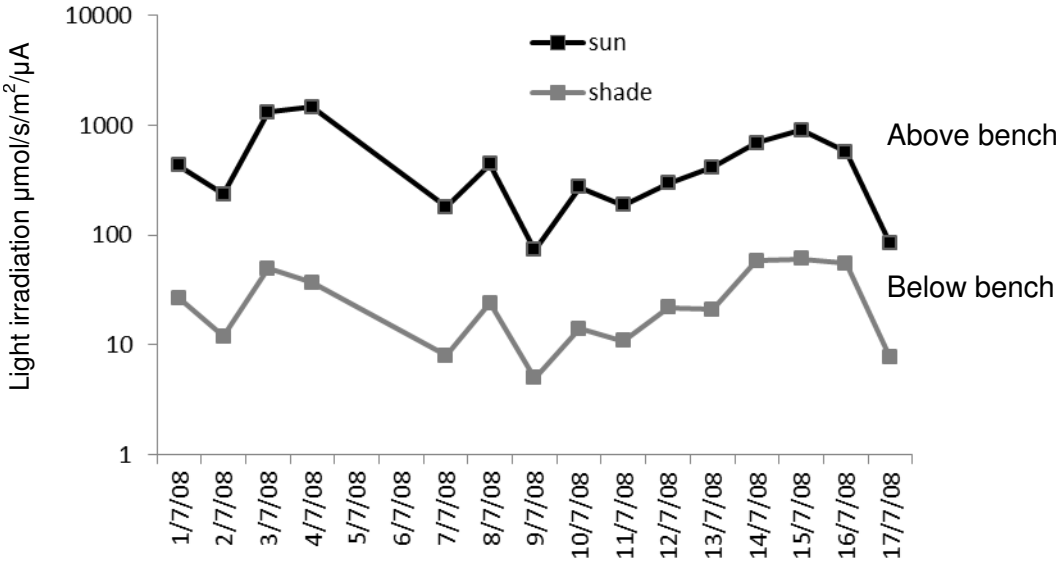
4.4 Light irradiation intensity affects total antioxidant levels in the wild parent more than in the cultivar

Lettuce varieties for salads are grown in very different geographic locations, such as Spain, Kenya or the UK, with different light levels. To ascertain whether different light levels had an effect on antioxidant potential the parent lines, *Lactuca sativa* and *L. serriola* were grown under two different light irradiation intensities.

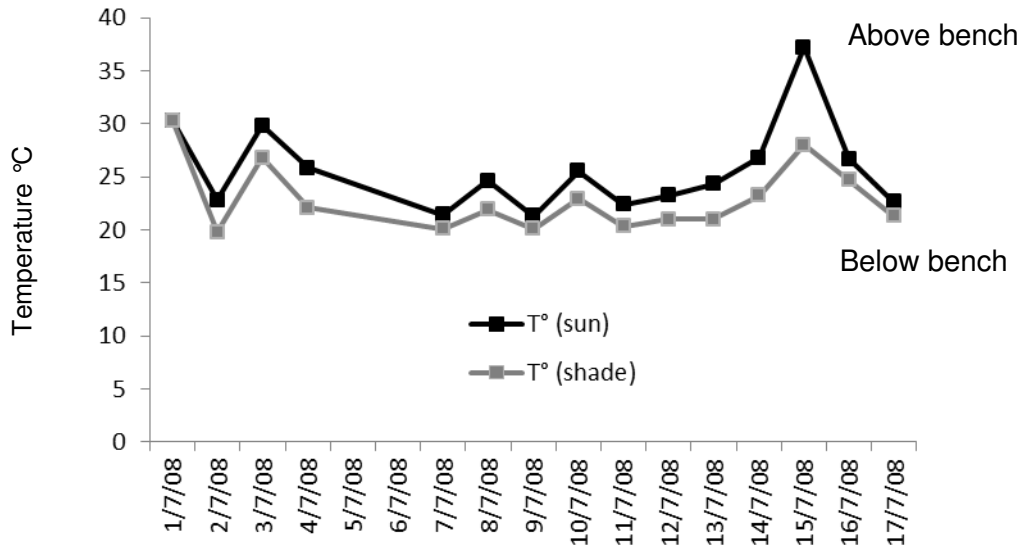
FRAP measurements on *L. sativa* and *L. serriola* leaves kept in the glasshouse (above bench) were compared to leaves kept in shady conditions (below bench). Averages of temperatures (°C) and light irradiation intensities (µmol/s/m^2/µA) for the period running from 01/07/08 to 17/07/08 are shown in Table 4.1 and Fig 4.3.

Table 4.1 Glasshouse temperature and light irradiation conditions during early summer 2008. *Averages of temperatures (°C) and light irradiation (µmol/s/m^2/µA) for the period 25/06/08 to 17/07/08 and at time of harvest are shown.*

	Light (25/06/08 - 17/07/08)		***Shade (01/07/08 – 17/07/08)***	
	Temperature (°C)	**Irradiation (µmol/s/m^2/µA)**	**Temperature (°C)**	**Irradiation (µmol/s/m^2/µA)**
Averages	25.52	494.19	22.90	27.56
At harvest time	23.00	528.00	20.40	5.40



(a)



(b)

Fig 4.3 Glasshouse sunlight irradiation measurements from 01/07/08 to 17/07/08. Sunlight measurements (a) and temperature measurements (b) were taken between 1:30 and 2:30 pm on each day.

The FRAP assay results showed that antioxidant levels were significantly higher when plants were grown in higher sunlight irradiation and temperature levels (Fig 4.4). There was a significant increase in antioxidant potential in both parent lines, however, the effect was greater in the wild parent, *L. serriola* than in the cultivar, *L. sativa*. The wild relative increased about seven-fold when grown under higher light and temperature conditions whereas the cultivar increased about two-fold. Interestingly, under shade conditions the antioxidant potential of both salad lines was comparable, thus wild lettuce responds better to light and temperature stimuli in terms of antioxidant production.

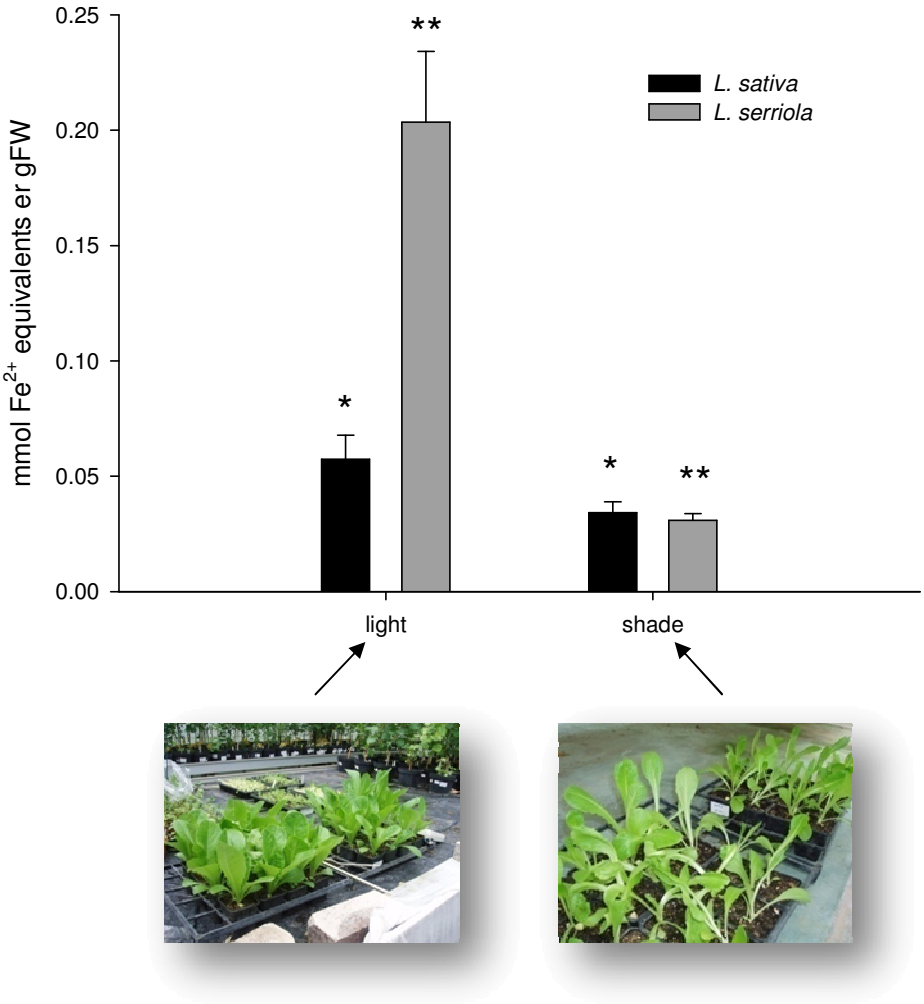


Fig 4.4 Light irradiation effects on the total antioxidant potential of the parent lines, *L. sativa* and *L. serriola*. Samples were grown under two different light intensities in the glasshouse. Unpaired, one tail t-test showed a significant increase in antioxidant potential for *L. sativa* plants grown in higher levels of light irradiation ($F_{3, 12}$=4.83; *P<0.05). Unpaired one tail t-test with Welch correction of F ratio for *L. serriola* plants also showed that plants grown in higher irradiation levels had a significantly higher antioxidant potential ($F_{3, 12}$=116; **P<0.01).

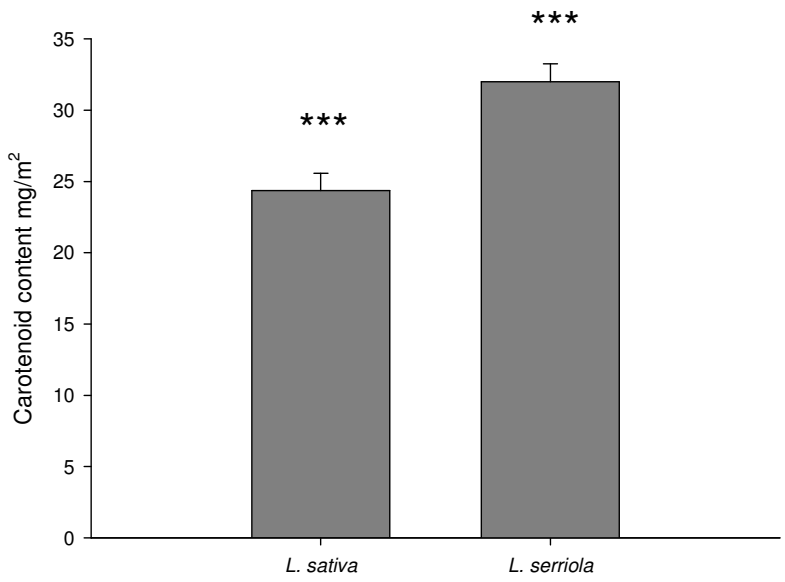
4.5 L. serriola *produces higher levels of carotenoids and chlorophylls a and b than* L. sativa

4.5.1 Comparison of different spectrophotometric methods

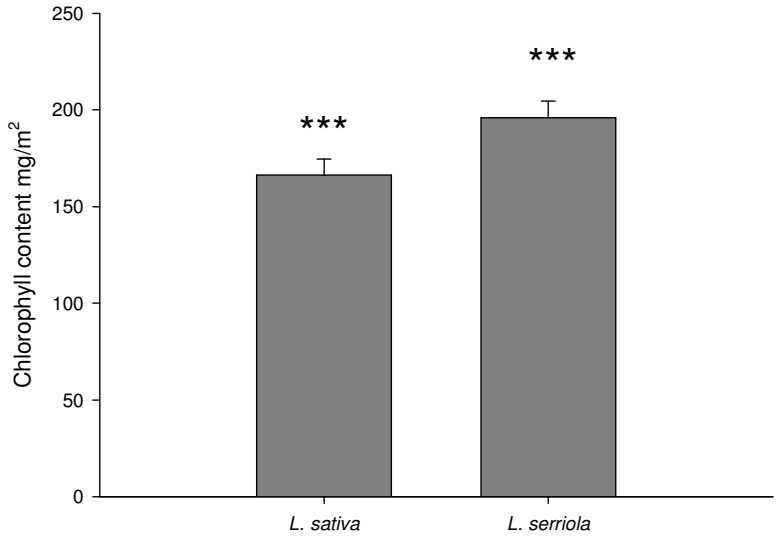
Three different methods were compared for the measurement of total carotenoid and chlorophyll contents in the parent lines. The standard chemical extraction method: DMF (Dimethyl Formamide), and two non-destructive methods: GER 1500 (Geophysical Environmental Research) and CCM – 200. Besides the evident advantage of being non-destructive methods, the GER assay does not quantify total carotenoid or chlorophyll contents of the leaves, but only gives you an exploring spectrum. Furthermore, the CCM was only designed to determine chlorophyll concentration, but not for the carotenoid content. Therefore, the chemical extraction method, DMF, proved to be the more accurate method for quantifying total carotenoid and chlorophylls a and b contents.

Carotenoids were sampled in the parent lines by the standard DMF pigment extraction and spectrophotometric assay, to compare levels in the parent lines and to help establish the relative importance of these nutrients as part of the total antioxidant contents of the parent lines. Samples were also taken from leaves of different ages, young (leaf 8), old (leaf 2) and senescing (leaf 1), to ascertain differences in carotenoid content in differently aged leaves.

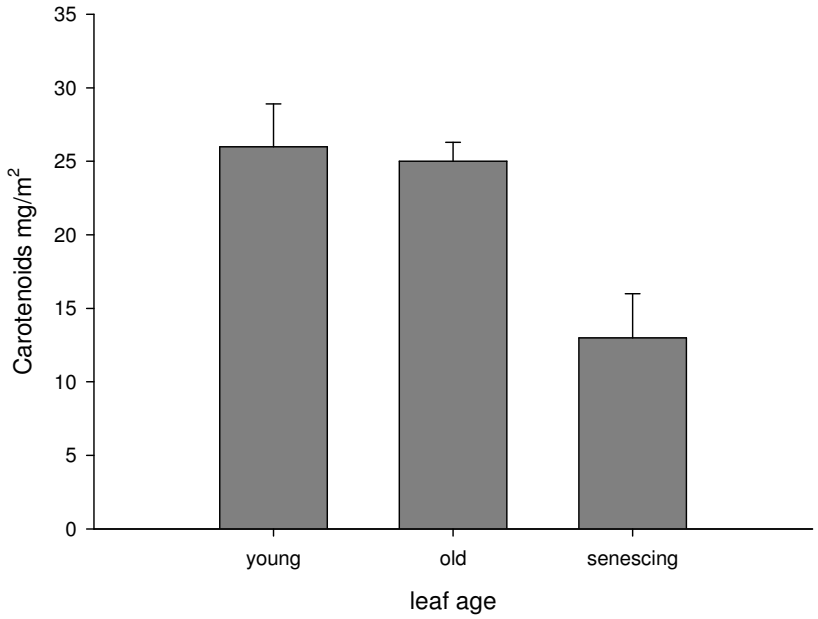
The wild relative, *L. serriola,* contained significantly greater amounts of chlorophylls a and b and of total carotenoids than the cultivar, *L. sativa* (Fig 4.5a and b). Furthermore, pigment levels in both lines did not seem to decline with age, until the leaves reached a senescing state (Fig 4.5c).


***
***
35
30
25
20
15
10
5
0
Carotenoid content mg/m2
L. sativa
L. serriola

(a)


***
***
250
200
150
100
50
0
Chlorophyll content mg/m2
L. sativa
L. serriola

(b)



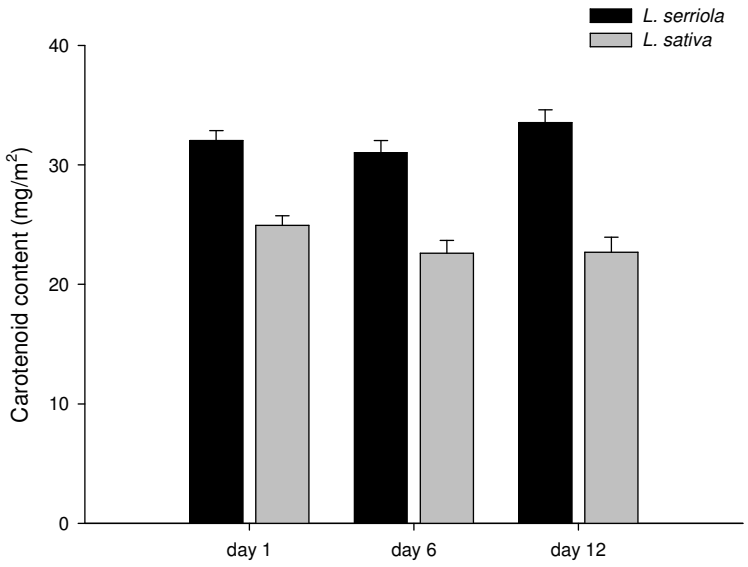
(c)

Fig 4.5 Chlorophyll and carotenoid pigment contents in *L. sativa* and *L. serriola*. DMF sampling for carotenoid and chlorophyll contents analysed as an average of three 1cm discs taken from each of 10 leaves for each parent line Averages of carotenoid (a) and chlorophyll (b) contents and for different leaf ages in *L. sativa* are shown (c). One-way ANOVA showed a significant difference between subspecies for both carotenoids ($F_{1, 64}$ = 17.90; ***P<0.001) (a) and chlorophyll ($F_{1, 64}$ = 19.59; ***P<0.001) (b), however, there was no significant difference in *L. sativa* carotenoid levels between young and old leaves (c).

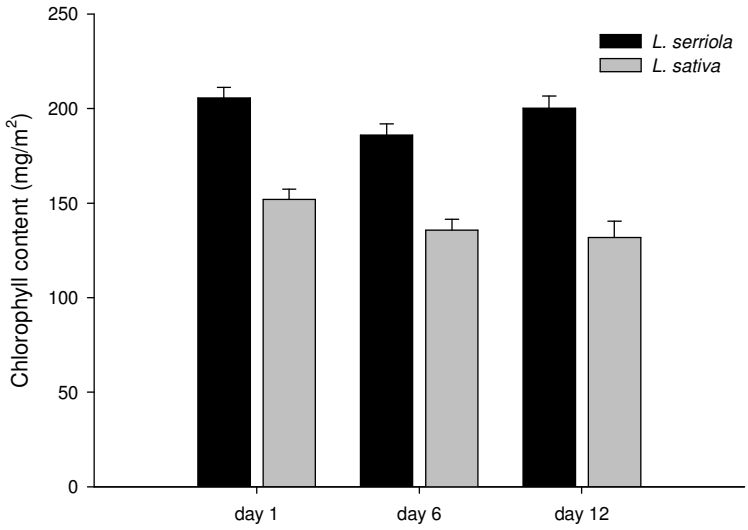
4.6 Chlorophyll and carotenoid contents in the parent lines do not decline during storage

Chlorophyll and carotenoid contents were analysed in parent lines harvested and stored for up to 12 days in the dark at 5℃ to assess variation of these particular antioxidant compounds during storage. The analysis was carried out by DMF chemical extraction and quantification.

Chlorophyll and carotenoid levels did not vary significantly with storage in neither *Lactuca sativa* nor *L. serriola* spp (Fig 4.6 (a) and (b)).



(a)



(b)

Fig 4.6 Analysis of carotenoid and chlorophyll pigment contents in *L. sativa* and *L. serriola* samples during storage. DMF sampling was used to quantify chlorophyll and carotenoid contents. One-way ANOVA showed that there was no significant change in carotenoid content or chlorophyll content with storage time.

4.7 Vitamin A and related compounds are the major vitamin phytonutrients present in the parent lines

A breakdown of the antioxidant phytonutrients found in the parent lines was carried out at Campden Chorleywood Food Research Association. The aim was to establish the relative importance of vitamins C and E and carotenoid compounds as part of the total antioxidant potential of the parent lines.

High Performance Liquid Chromatography (HPLC) showed that the predominant carotenoids present in the parent lines were zeaxanthin, lutein and β-carotene (Fig 4.7). Moreover, there was a definite trend suggesting that the carotenoid contents and metabolites were greater in *L. serriola* (Table 4.2).

Vitamin C compounds seemed to be comparable between the two subspecies.

Vitamin E content was considerably higher in the wild parent line. However, no statistical analyses were possible due to necessary pooling of the *L. serriola* samples. Thus further investigations of this vitamin would be interesting.

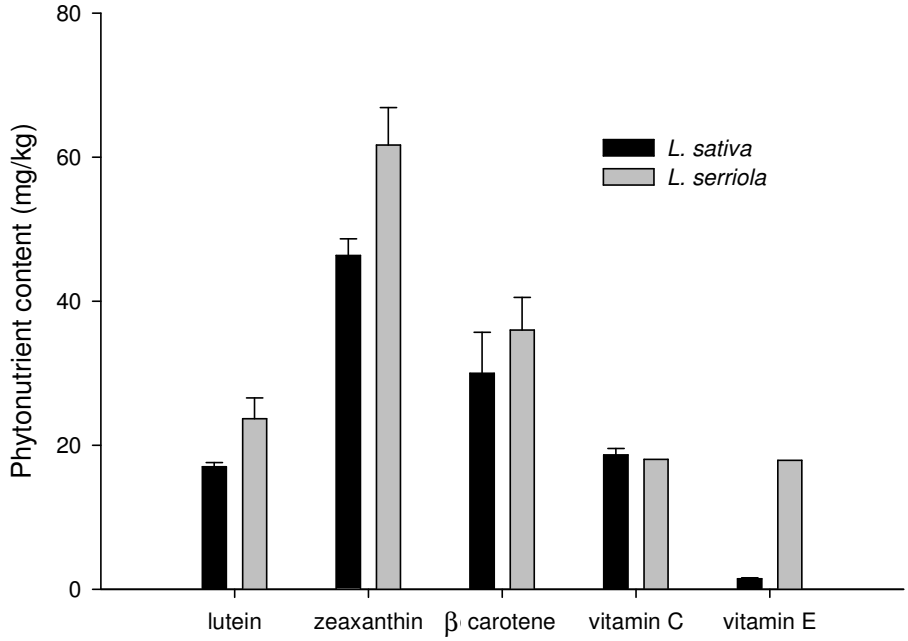


Fig 4.7 Breakdown of vitamin phytonutrient metabolites in the parent lines. HPLC analysis of carotenoids, vitamin C and vitamin E in *Lactuca sativa* and *L. serriola* parent lines. Analysis showed that the predominant carotenoids were lutein, β-carotene and zeaxanthin. Three biological replicates for each sample were investigated. One-way ANOVA found no significant difference between the parent lines was revealed for the carotenoid compounds. No statistical analyses were possible for the vitamin E as *L. serriola* samples had to be pooled.

4.8 Levels of vitamin C in L. sativa *are comparable to those in* L. serriola

Vitamin C analysis of the parent lines was also carried out at Leatherhead Food International using HPLC analysis. The aim was to extend the previous analyses carried out at Campden.

Statistical analysis of the comparison between the two parent lines did not show a significant difference (Fig 4.8).

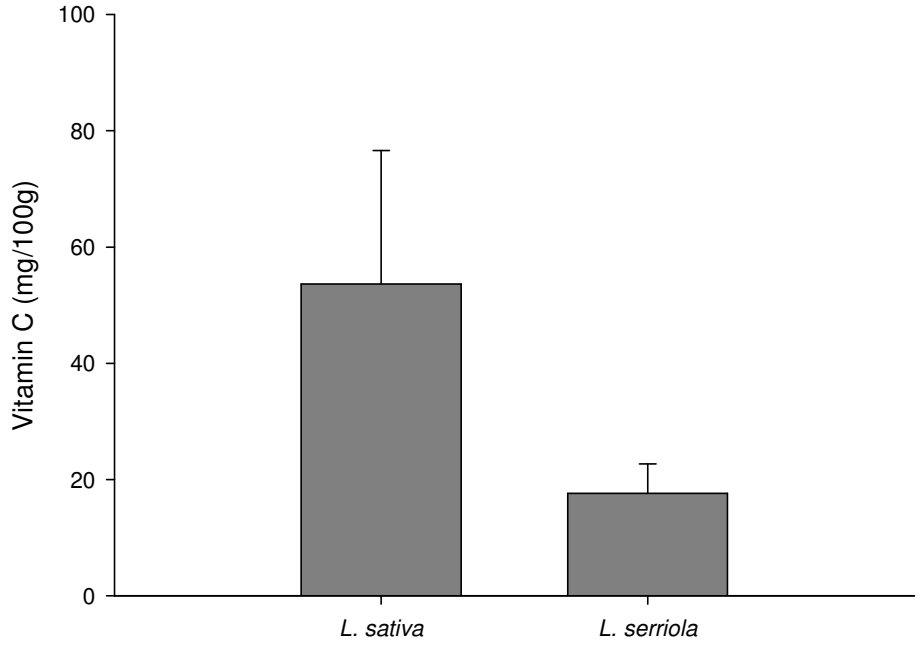


Fig 4.8 HPLC analysis for vitamin C of the cultivar *L. sativa* and its wild relative *L. serriola*. Samples were grown in the glasshouse and analysed at Leatherhead Food International. Three replicates were sampled for each *Lactuca* subspecies. One-way ANOVA found no significant difference between *Lactuca sativa* and *L. serriola*.

***Table 4.2 Nutritional evaluation of* L. sativa *and* L. serriola.** *Samples were grown in the glasshouse. Total antioxidant content, total carotenoid content and, total chlorophyll content were analysed in-house; lutein, zeaxanthin, β-carotene, vitamin C and vitamin E evaluations were carried out at Campden BRI.*

	L. sativa	***L. serriola***
TAP[a]	0.58±0.02	1.71±0.31
Total Carotenoids[b]	22.76±1.87	28.11±2.06
Total Chlorophylls[b]	147.76±11.16	180.74±11.55
Lutein[c]	17.00±0.58	23.67±2.91
Zeaxanthin[c]	46.33±2.33	61.67±5.21
β-carotene[c]	30.00±5.69	36.00±4.51
Vitamin C[cd]	18.67±0.88	18.00±(n/a)
Vitamin E[c]	1.47±0.13	17.90±(n/a)

[a] Total Antioxidant Potential expressed in mmol Fe^{2+} equivalents/gFW

[b] Values expressed in mg/m^2

[c] mg/kg

[d] Campden result

4.9 Determining the antioxidant potential of the Recombinant Inbred Lines (RILs)

Previous investigations on the antioxidant potential of the two parent lines showed that the wild relative*, L. serriola*, produced three times as much antioxidant potential than the cultivar, *L. sativa*. These analyses gave an indication of what may be present in the Recombinant Inbred Lines (RILs), i.e. that the values of the progeny could fall between the parent line values, but also above and/or below. and thus potential for improving the antioxidant content of *L. sativa*.

Thus, the 60 RILs and their parent lines were grown in the glasshouse in three blocks each containing three replicates per line. The plants were harvested at four weeks post germination and analysed for total antioxidant potential using the FRAP assay.

There was a wide range in the levels of antioxidant potential between the different RIL lines. This allowed for the identification of the five lines producing the highest levels of antioxidants: lines 112, 99, 20, 11 and 63, and the five lines producing the lowest levels of antioxidants: 89, 90, 106, 80 and 107 (Fig 4.9; Tables 4.3 and 4.4). All of the lines in the high end of the range, i.e. above *L. serriola*, and even a number of lines below *L. serriola* (down to line 19) contained antioxidant levels which were significantly higher than those of the cultivar, salinas, and were comparable to the antioxidant level of *L. serriola*. Lines 78, 22, 53 and 96 were significantly different from both the highest (line 112) and the lowest (*L. sativa*) lines in the range. Line 96 was also significantly lower than line 99. Lines 121, 83 and 21 were significantly lower than lines 99 and 112 but there was no significant difference between these and the cultivar. Thus the cut-off line between the highest antioxidant levels in the range and the low end of the scale is between the lines 19 and 121. The distribution follows a Gaussian shape, showing how the majority of the RIL lines fall between the two parent lines, indicated with black arrows. The antioxidants present in the RILs indicate

that the levels of antioxidants in *L. sativa* (Salinas cv) could be significantly increased by breeding with *L. serriola*.

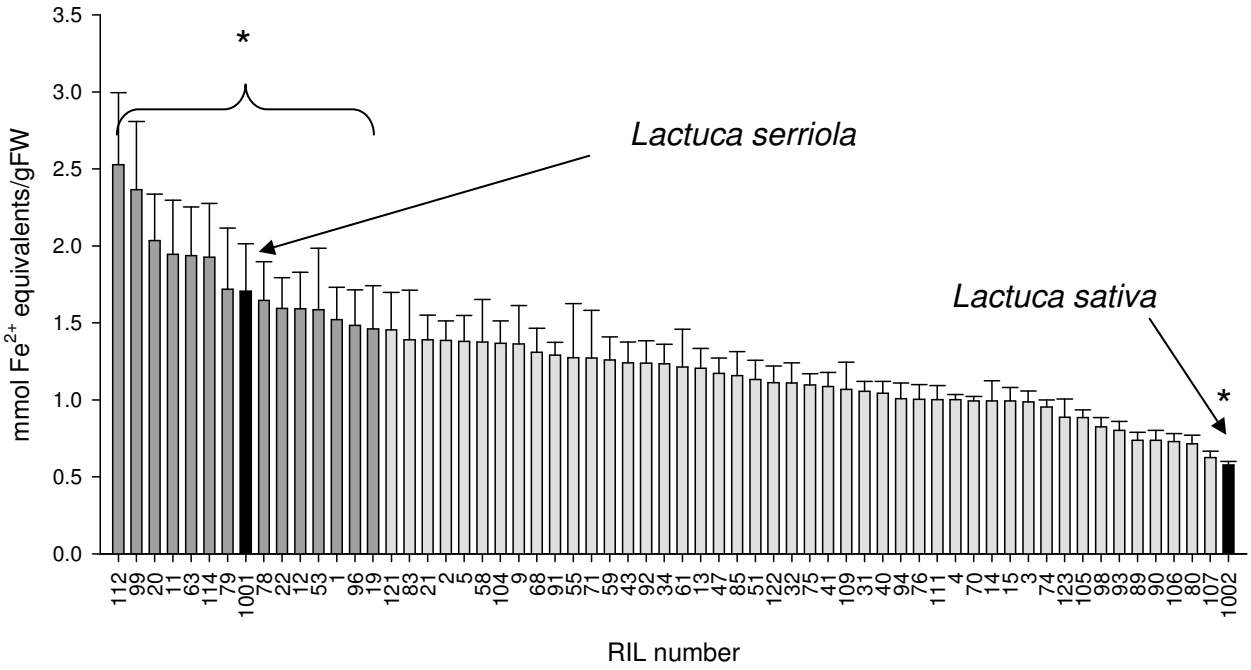


Fig 4.9 Antioxidant potential of the complete mapping population grown in the glasshouse. The lines were grown in the glasshouse during summer 2006 and organised into three blocks of three randomised replicates of each line per block. Nine replicates for each RIL line were analysed. An average of the antioxidant potential for each line is shown. The black bars show the antioxidant potential of the parent lines. The dark grey bars indicate the lines with significantly higher levels of antioxidants than the cultivar. One-way ANOVA and Fisher 95% Individual Confidence Intervals show the lines in the high end of the range, including the wild relative (line 1001), which contain significantly higher levels of antioxidant potential than the cultivar (line 1002) ($F_{18,162}$ =1.81;*P=0.05).

Table 4.3 Details of the significant differences between the individual extreme lines *(One-way ANOVA and Fisher 95% Individual Confidence Intervals; Simultaneous Confidence Level = 30.73%).*

	11	*20*	*63*	*99*[a]	*112*[b]	*1001*
80	***	***	***		***	***
89	***	***	***		***	***
90	***	***	***		***	***
106	***	***	***	***	***	***
107	***	***	***	***	***	***
1002	***	***	***	***	***	***

[a] 99 was also significantly different to: 11, 63 and 1001

[b] 112 was also significantly different to: 11, 20, 63 and 1001

Table 4.4 Antioxidant values of the extreme RIL and parent lines. *Lines were grown in the glasshouse during summer 2006.*

	RIL	**Antioxidant potential (mmol Fe^{2+} equivalents per g FW)**
Highest	112	2.53±0.47
	99	2.36±0.44
	20	2.04±0.30
	11	1.95±0.35
	63	1.94±0.32
	L. serriola (1001)	1.71±0.31
Lowest	89	0.74±0.05
	90	0.74±0.06
	106	0.73±0.05
	80	0.72±0.06
	107	0.62±0.04
	L. sativa (1002)	0.58±0.02

4.10 Changes in environmental conditions affect the total antioxidant potential of the RILs

To establish how environmental conditions affected the antioxidant potential, the complete mapping population (using the same randomised pattern of nine replicates per line organised into three blocks; Fig. 2.4) was grown in the field in the UK during July/August and in Spain during February/March.

Unfortunately, due to extreme weather conditions both in the UK, and in Spain, a number of lines did not germinate or only had one replicate. A FRAP analysis was nevertheless carried out on the complete mapping population grown at Pinglestone (Fig 4.10).

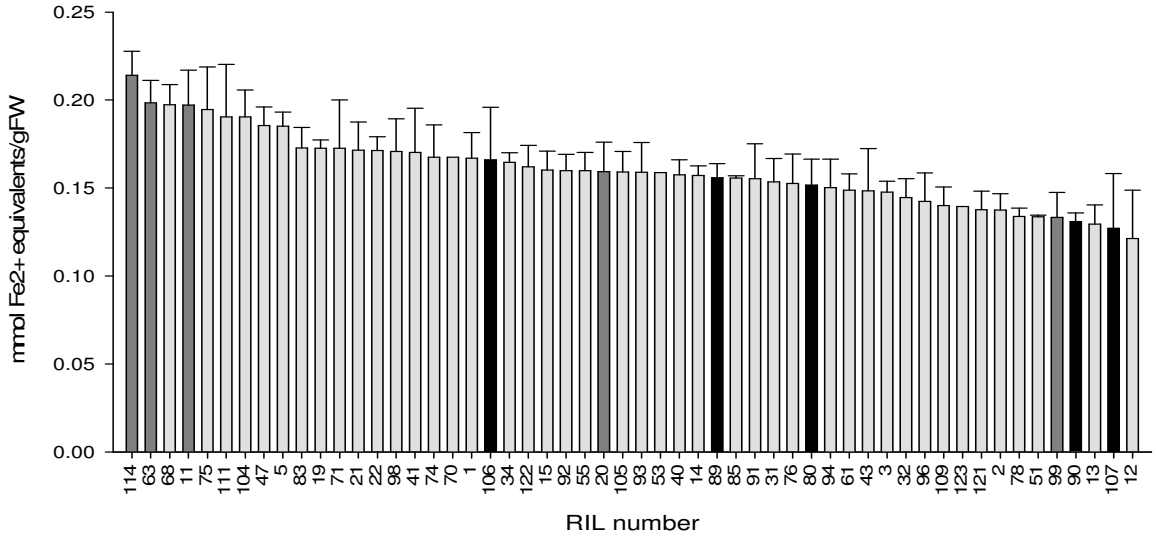
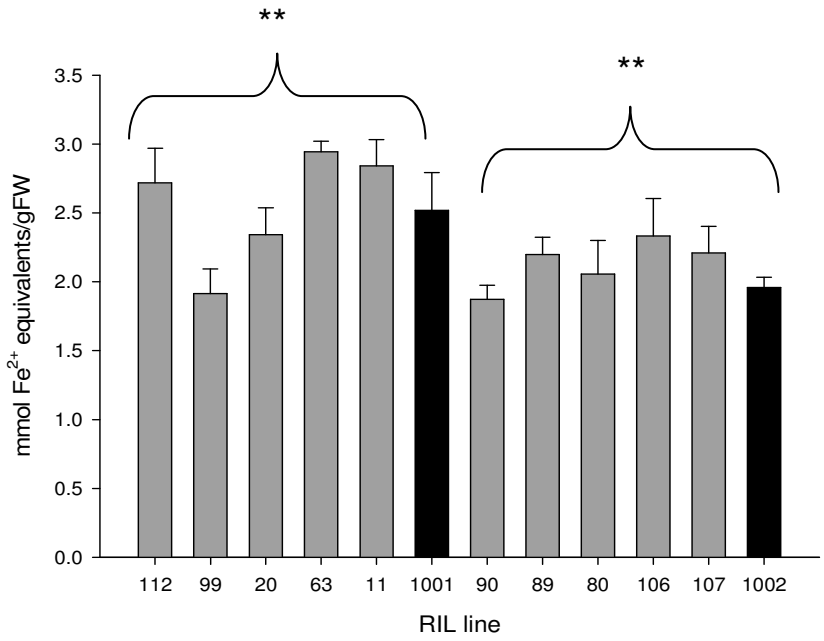
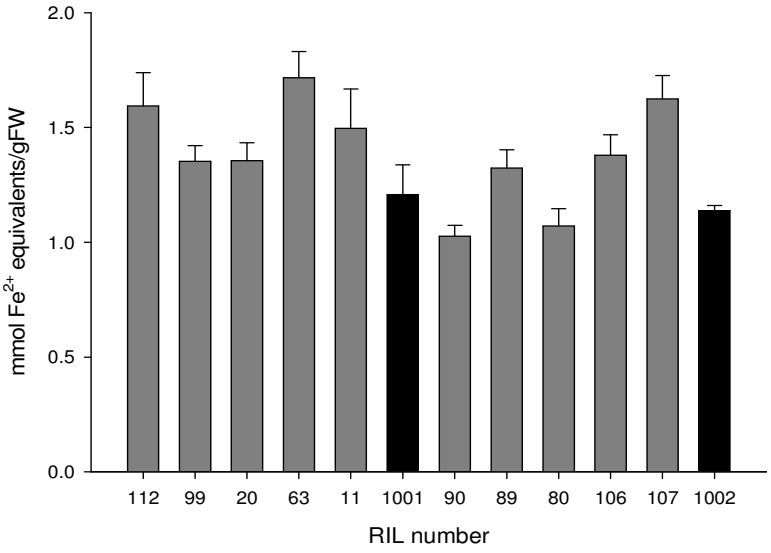


Fig 4.10 Antioxidant evaluation of the complete mapping population grown in the field, in the UK. The lines were grown at Pinglestone farm (Hampshire, UK) and organised into three blocks of three randomised replicates of each line per block. An average of the antioxidant potential for each line is shown. The dark grey bars show the antioxidant potential of the glasshouse RIL lines which had the highest potential (lines 99, 20, 11, 63 and 114) and the black bars indicate the six lines with the lowest levels (lines 106, 89, 80, 90, and 107). Unfortunately, lines 4, 9, 58, 59, 79, 112, 1001, 1002 did not grow; and only one replicate grew for lines 53, 70, 123.

The extreme lines and the parent lines of the trial carried out in Spain were FRAP analysed, and a set of extreme lines grown again at the Pinglestone site. One-way ANOVA detected a significant difference in antioxidant potential between the five RILs with the highest antioxidant potential (together with the wild parent) and their commercial parent line, cv salinas, grown in the field in the UK (Fig 4.11a; Table 4.5). However, there was no significant difference between the highest and lowest lines for the samples grown in Spain (Fig 4.11b).



(a)



(b)

Fig 4.11 Extreme lines grown in the field. Lines grown during summer months at Pinglestone, UK (a). Lines grown during winter months in Spain (b). The parent lines, *L. serriola* (1001) and *L. sativa* (1002) are shown in black. Highest level lines (lines 112, 99, 20, 63 and 11) are shown to the left of *L serriola*; lowest level lines (lines 90, 89, 80, 106 and 107) are shown to the right of *L serriola.* One-way ANOVA detected a significant difference between the averages of the highest and averages of the lowest lines grown at Pinglestone farm in the UK ($F_{1,62}$=9.71; **P<0.01) (a); no significant difference was found for the averages of the extreme lines grown in Spain (b).

Table 4.5 Details of the significant differences between the individual extreme lines grown in the field at Pinglestone (UK) *(One-way ANOVA and Fisher 95% Individual Confidence Intervals; Simultaneous Confidence Level = 30.61%).*

	11	20	63	99[a]	112	1001
80	**	**			**	
89	**	**			**	
90	**	**			**	**
106						
107	**	**			**	
1002	**	**			**	**

[a] 99 was also significantly different to: 11, 63, 112 and 1001

Interestingly, statistical analyses of the averages of all the highest lines and of all the lowest lines for each trial showed that the difference between these means decreased due to environmental changes. Whilst there was a significant difference between the means of the highest and lowest lines grown in the glasshouse, the significance value decreased when these lines were grown in the UK and there was no significant difference for the means of those grown in Spain (Fig 4.12). Thus, differences between extreme lines were reduced as a consequence of environmental change. A comparison of all the highest lines grouped by environment showed a significant difference between the groups as did a statistical analysis of the groups of lowest lines (Fig 4.12).

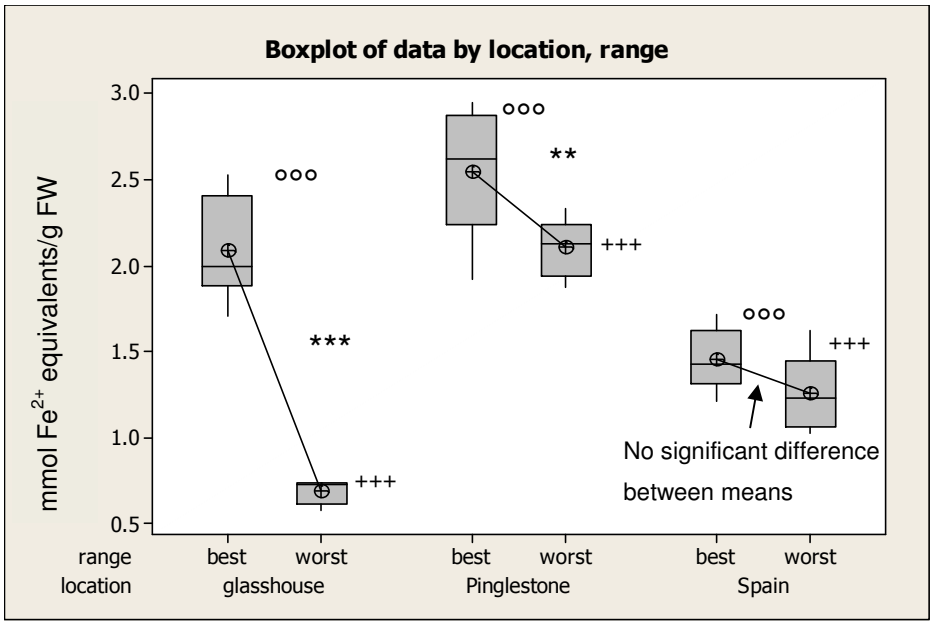


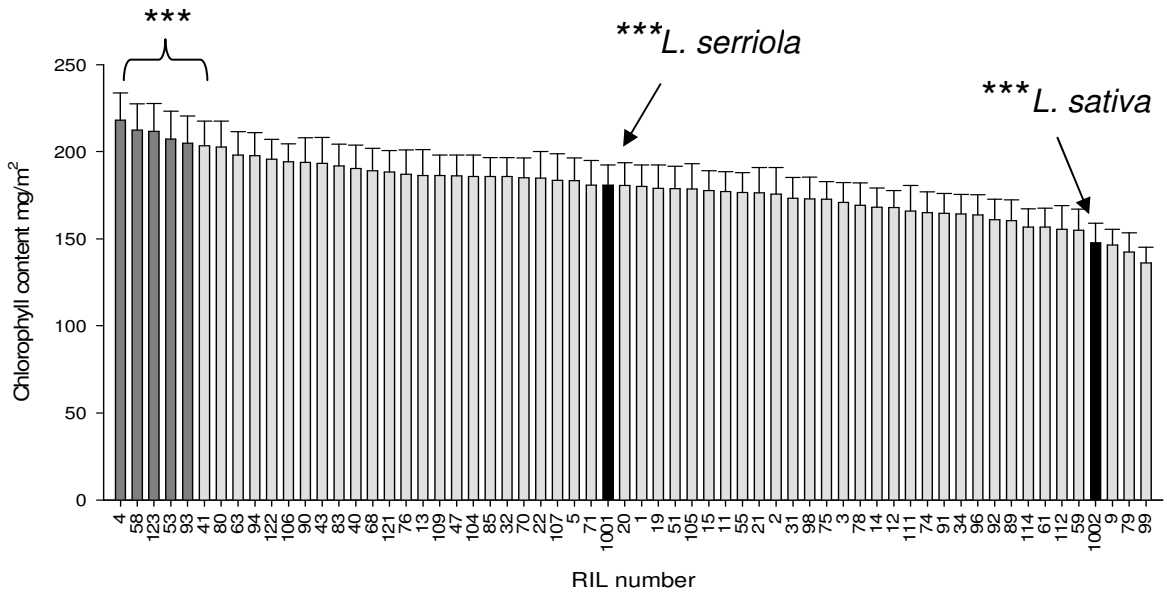
Fig 4.12 Extreme lines grown in three different environments. Averages of the glasshouse five highest and five lowest extreme lines for the antioxidant trait are displayed. The lines were grown in the glasshouse and in two different field sites, in the UK (summer) and in Spain (winter). The effects of different environmental conditions on the selected lines on their antioxidant potential are shown as reduced difference between the blocks of extreme values. One-way ANOVA detected a significant difference between the averages of the highest and averages of the lowest lines grown in the glasshouse ($F_{1,74}$=63.09;***P<0.001) and for those grown at Pinglestone farm in the UK ($F_{1,62}$=9.71; **P<0.01); no significant difference was found for the averages of the extreme lines grown in Spain. One-way ANOVA analysis of averages of the three groups of best lines grown in the three different environments showed a significant difference between environmental groups ($F_{2,88}$ =145.66; °°°P<0.001); the groupings by environment of the lowest lines were also significantly different ($F_{2,90}$ =298.41; $^{+++}$P<0.001).

4.11 Total chlorophyll and total carotenoid contents vary considerably between the lines

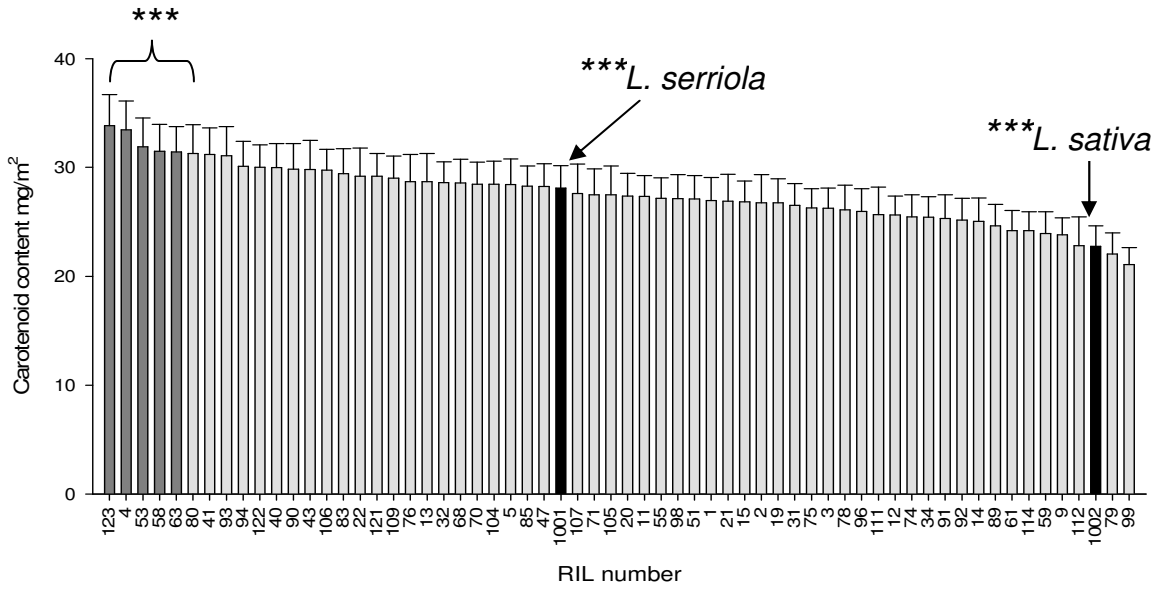
The RILs were sampled for chlorophyll and carotenoid content by using the standard DMF (Di-Methyl Formamide) pigment extraction method and spectrophotometric assay. A set of samples was grown in the glasshouse at University of Southampton in a randomised pattern of nine biological replicates organised into three blocks.

A number of lines produced significantly higher pigment levels than *L. serriola* and *L. sativa* (Fig 4.13). This allowed for the selection of lines with extreme values. The results also confirm initial findings that the wild parent, *L. serriola*, had significantly higher concentrations of both chlorophyll and carotenoids than the cultivar

Numerous progeny lines produce considerably higher levels of chlorophyll and carotenoids than the commercial cultivar. The distribution resembles a Gaussian curve.



(a)

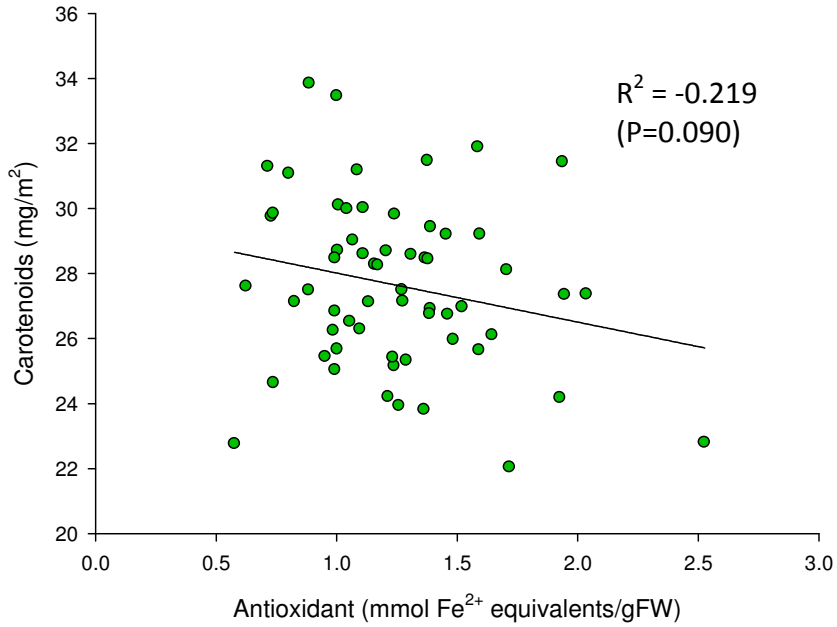


(b)

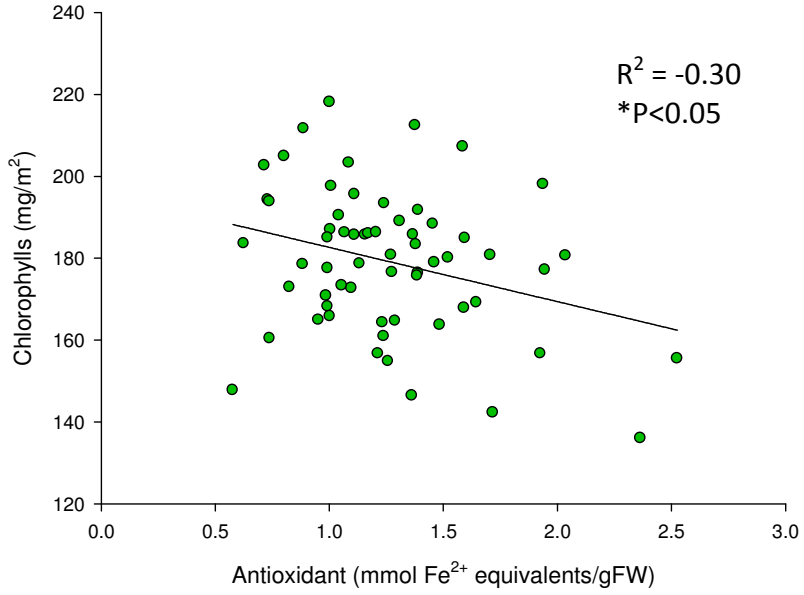
Fig 4.13 Chlorophyll and carotenoid contents of the RIL population and their parent lines. The lines were grown in the glasshouse and organised in three blocks of three randomised replicates of each line per block (nine replicates in total for each line). Average of the total chlorophyll content for each line (a) average of the total carotenoid content for each line (b) are shown. The parent lines are indicated with black arrows and represented in black. ANOVA analysis and Tukey 95%

Simultaneous Confidence Interval post hoc tests showed that the five highest chlorophyll lines (indicated in dark grey) contained significantly higher levels than both the parent lines ($F_{6,57}$=16.05; ***P<0.001) (a), and that the five highest carotenoid lines (shown in dark grey) contained significantly higher levels than both the parent lines ($F_{6,57}$=13.03; ***P<0.001) (b).

Interestingly, carotenoid and chlorophyll traits did not correlate with the antioxidant trait (Fig 4.14) whilst levels of chlorophylls a and b were highly correlated with carotenoid levels, as expected (Fig 4.15). Furthermore, lines 99 and 112, which contained the highest levels of antioxidant potential, fell in the low end of the scale for both pigment contents (Fig 4.9 and Fig 4.13a and b) (Table 4.6).

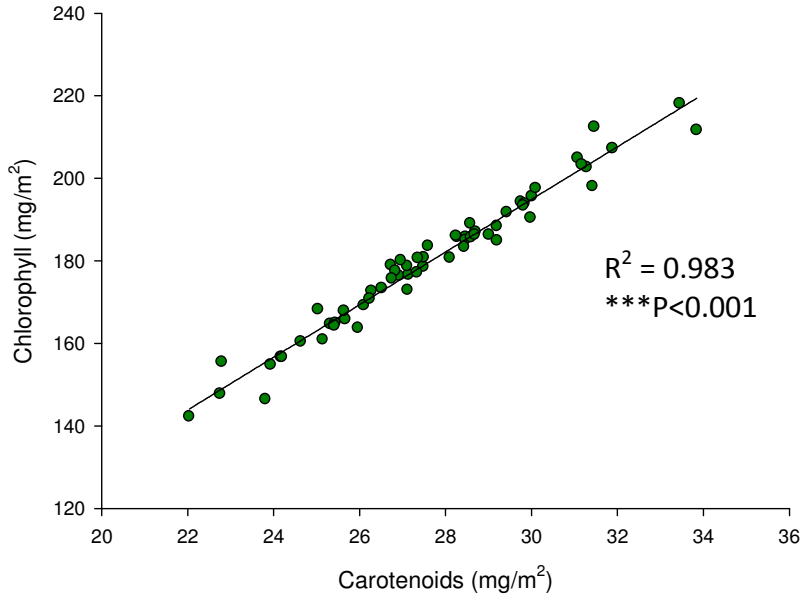


(a)

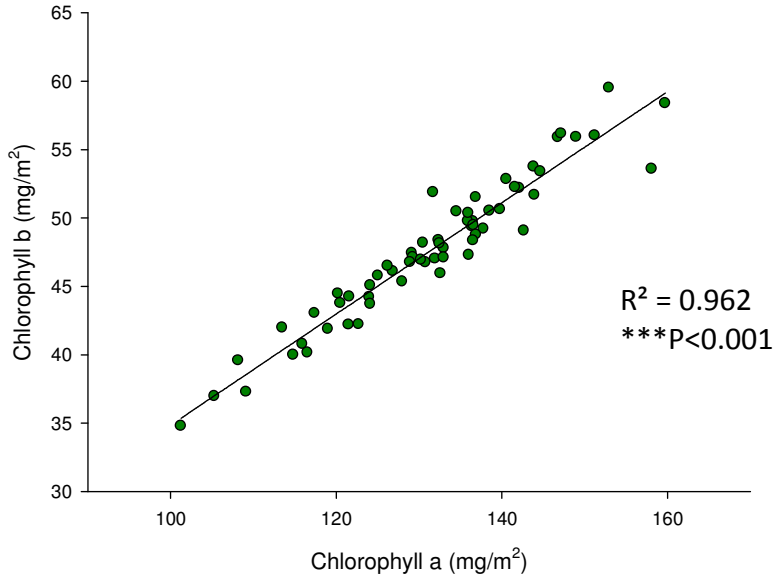


(b)

Fig 4.14 Correlation coefficient of antioxidant and pigment trait means of the mapping population. Pearson's analysis established that there is no correlation between antioxidants and carotenoids (a), however, it found a negative correlation between chlorophyll and antioxidants (*P<0.05) (b).



(a)



(b)

Fig 4.15 Pearson's correlation coefficient of chlorophyll and carotenoid trait means of the mapping population. RIL chlorophyll and carotenoids were highly correlated (***P<0.001) (a); chlorophylls a and b were also highly correlated (***P<0.001) (b).

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Table 4.6 Carotenoid and chlorophyll values for the highest and lowest lines. *The complete mapping population was grown in the glasshouse. Nine replicates per line, organised in a randomised pattern, were sampled by DMF. Total carotenoid and total chlorophyll values are displayed for the five lines with the highest and lowest levels of the complete mapping population.*

	RIL	**Total Carotenoid content (mg/m^2)**	**Total Chlorophyll content (mg/m^2)**
Highest	123	33.85±2.85	211.66±15.98
	4	33.46±2.65	218.11±15.70
	53	31.89±2.66	207.24±15.90
	58	31.47±2.50	212.44±15.04
	63	31.43±2.33	198.06±13.48
	L. serriola (1001)	28.11±2.06	180.74±11.55
Lowest	59	23.93±1.00	154.80±12.15
	9	23.81±1.56	146.43±9.01
	112	22.80±2.65	155.48±13.55
	79	22.04±1.93	142.25±11.20
	99	21.07±1.57	136.04±9.09
	L. sativa (1002)	22.76±1.87	147.76±11.16

4.12 Discussion

4.12.1 Antioxidant phytonutrient levels greater in wild lettuce

The cultivated lettuce *L. sativa* (cv Salinas) and its wild relative *L. serriola* (acc UC96US23) of the Compositae (Asteraceae) family present very different morphologies. For instance, *L. serriola* plants develop protective thorns, the leaves have an elongated slim shape and they generally have a rather bitter taste, whereas leaves of the commercial cultivar, *L. sativa,* have been bred to develop smooth wider leaves and are much sweeter and delicate in taste. These taste and aspect inequalities suggested that there may be differences in other phenotypic aspects such as in the phytonutrient content of the leaves. Thus, the antioxidant nutritional content of the two parent lines was explored to establish potential differences in the antioxidant and vitamin compounds and to guide subsequent analyses on the Recombinant Inbred Lines (RILs). Potential genotypic variability would then offer the possibility of improvement of this trait by selective breeding in the quest for a nutritionally rich as well as appetizing commercial lettuce leaf.

The total antioxidant content in lettuce leaves is composed of a number of different metabolites in different proportions, such as carotenoids and polyphenols (Lako et al. 2007;Llorach et al. 2008;Mou 2005;Mou & Ryder Edward 2004;Nicolle et al. 2004;Niizu & Rodriguez-Amaya 2005;Oh et al. 2009;Serafini et al. 2002). Thus, differences in the total antioxidant potential, vitamin C, vitamin E and carotenoid contents were investigated in the parent lines, *Lactuca sativa* and *L. serriola*.

Firstly, owing to the modern day trend of using baby leaves in packaged salads, the antioxidant potential in differently aged leaves from the same *L. sativa* plant was compared (Fig 4.1). The results obtained showed that younger leaves have significantly higher levels of total antioxidants than their older counterparts, which agrees with past findings that the younger parts of a plant can produce or concentrate considerably greater quantities of

phytonutrients than their older counterparts (Drewnowski & Gomez-Carneros 2000;Fahey, Zhang, & Talalay 1997;Wink M. 1999). These differences in the distributions of micronutrients depend on the physiology of leaf growth, where, as the plant grows, the leaf cells start to expand and elongate rather than continuing to divide. Following from this experiment, all subsequent leaf harvests were carried out on young leaves of similar ages, i.e. at circa 1month post germination.

The total antioxidant potential was then investigated in the parent lines, *L. sativa* and *L. serriola* (Fig 4.2). The results of the FRAP analysis revealed inequalities in the antioxidant potential between the two parent lines, suggesting that significant differences in antioxidant potential exist between closely related species. Interestingly, the wild species, contained significantly higher levels of antioxidants than the cultivated type, which could be explained by traditional agricultural practices breeding out certain antioxidants such as certain bitter phenolics and terpenes, whilst focusing on crops which were higher yielding, germinated faster, were softer or lacked thorns, generally tasted sweeter and had a more delicate flavour. Drewnowsky and Carneros (2000) and Zandstra et al (2007) explain this sort of trade-off has happened in a number of other crop species such as sweet corn (*Zea mays*), onion (*Allium cepa*) and tomato (*Lycopersicum esculentum*).

Carotenoid and chlorophyll pigment contents were investigated in the *L. sativa* and *L. serriola* parents as past studies have shown these metabolites to be relevant antioxidant compounds in lettuce leaves (Li & Kubota 2009;Mou 2005;Nicolle et al. 2004;Schreiner 2005). Preliminary investigations using the non-destructive, remote sensing methods GER and CCM (data not shown) and the standard chemical extraction and spectral analysis method, DMF, showed that the wild relative produced significantly higher levels of total carotenoids and of chlorophylls a and b. The total carotenoid and total chlorophyll contents in the wild species were almost 20% higher than in the cultivar (Table 4.2). Interestingly, in contrast to the

results on differences in total antioxidant content in young and old leaves, the pigment content did not change with leaf age, until the leaf was at a senescing stage (Fig 4.5). Furthermore, the effects of storage on carotenoid and chlorophyll content were investigated in *L. sativa* and *L. serriola* stored at 4℃ in the dark. Results showed that these pigments do not decline appreciably within the first 12 days of shelf life. This is partly in accordance with Ferrante et al. on lamb's lettuce who noted significant chlorophyll and carotenoid reduction was only observed after eight days of storage in (Ferrante 2009) . Pigment loss is one of the first visible signs of senescence in types of plants such as Brassicaceae (Lefsrud et al. 2007), whereas in lettuce visible signs of senescence are leaf browning and loss of turgor, an effect of polyphenolic compound production. These polyphenols are the result of the mixing of previously compartmentalised enzymes and substrates due to membrane disruption which determines cell death (O'Beirne & O'Kenny 2010;Wagstaff et al. 2007). The decrease in carotenoid content only becomes apparent when the leaves are at a very late stage of senescence, in accordance with O'Beirne and Kenny who even noted an increase in carotenoids and phenolic compounds during storage of fresh cut produce. These results, together with the fact that total antioxidant potential did not correlate with carotenoid content (Fig 4.14) gave an indication that whilst pigments represent a significant portion of the total antioxidant content of Lactuca leaves, they do not constitute the major proportion, a result which is in accordance with Nicolle et al (2004).

Subsequent, in-depth investigations by HPLC analysis, carried out at Campden BRI, revealed that the predominant carotenoids in both lines were zeaxanthin, lutein and β-carotene (Fig 4.7). This is partly in accordance with Mou who analysed 52 lettuce genotypes, including crisphead (perhaps the most similar to the Salinas cultivar studied here), leaf, romaine, butter, primitive, latin and stem lettuces, and wild species, and found lutein and β-carotene to be predominant in lettuce varieties (2005). In this study another xanthophyll, i.e. zeaxanthin, was also found to constitute a major part of the total carotenoid content, in agreement with Nicolle et al. (2004), who analysed five green cultivars of lettuce - butter lettuce, batavia lettuces and

oak leaf lettuce. Interestingly, these results are in slight disagreement with Sommerburg who found that the major carotenoid compounds present in lettuce were cryptoxanthins, neoxanthins, violaxanthins, lutein and α-carotene but found no zeaxanthin metabolites (1998). However, they do not specify the type of lettuce cultivar studied. These differences may in fact be due to varietal differentiation, or even to environmental variation.

The individual carotenoid metabolites found in this study were statistically comparable in both Lactuca accessions, however, there was a consistent tendency for the wild relative to produce more of each compound than the cultivar (Fig 4.7; Table 4.2). This is in agreement with another study by Mou et al who compared three different varieties of wild lettuce (*L. serriola*, *L. saligna* and *L. virosa*) to 52 commercial varieties and found that the wild varieties consistently contained higher levels of carotenoids than the cultivars analysed (2004).

To further the investigations of the micronutrient content of the Lactuca parent lines, vitamins C and E were analysed, as a number of studies have found these to be important antioxidants in lettuce leaves (Garcia-Closas et al. 2004;Nicolle et al. 2004;Oh et al. 2009). Vitamin E was found to be much higher in *L. serriola* than in *L. sativa,* a greater than 12-fold difference was noted. Previously, vitamin E has been enhanced in lettuce by overexpressing a cDNA encoding γ-tocopherol methyltransferase (Cho Eun 2004), however, a novel less contentious way of increasing vitamin E in lettuce would be through introgression of genes from its wild relative.

Conversely, no significant difference was found in vitamin C levels between the parent lines (Fig 4.8; Table 4.2). However, there was an interesting trend for the vitamin C levels to be higher in the cultivar than in the wild relative. This is in agreement with the literature, which suggests that artificial selection by traditional agricultural practices may have inadvertently been promoting a decrease in certain important antioxidant phytochemicals (such as bitter polyphenols, on the basis of taste) thus inducing the plant to increase its

vitamin C levels in an attempt to substitute for the lack of these antioxidant compounds (Morris & Sands 2006). The authors note that this type of unintentional selection for traits has occurred several times in the past, in the quest for high agronomic yield, easy and consistent processing and disease and pest resistance. An interestingly high amount of vitamin C levels has also been noted by Llorach et al, where, among five different lettuce cultivars, iceberg, romaine, red oak leaf, continental and lollo rosso, the highest levels of vitamin C were detected in the (green) continental cultivar (2008).

Overall, the wild lettuce presented a richer nutritional phenotype as it contained significantly higher levels of most nutrients than the commercial cultivar (Table 4.2). Total antioxidant and total carotenoid contents, chlorophylls a and b and vitamin E content were significantly higher in *L. serriola*, whereas vitamin C was comparable in both subspecies. Of the antioxidant phytonutrients studied here, carotenoids in particular seemed to be present in the highest proportion in both subspecies. These data suggest that the wild relative could contribute genes for higher levels of total antioxidants, carotenoids, including vitamin A precursors, and vitamin E in a number of Recombinant Inbred Lines (RILs) increasing the potential to improve the nutritional quality of *L. sativa*.

4.12.2 Phytonutrients in the mapping population show transgressive segregation

Following these initial findings, antioxidants, carotenoids and chlorophyll contents have been analysed in a set of 60 RIL lines originating from a cross between *L. sativa* var. Salinas and *L. serriola*. The cross was carried out at University California Davies as part of Genbank and The Compositae Genome Project to identify the genes involved in pathogen recognition, resistance signal transduction, defence responses, and disease resistance (McHale et al. 2009;Rauscher et al. 2009). The RILs have also been analysed for the identification of seed and seedling traits (Argyris et al. 2005)

and for traits linked to shelf life (Zhang 2006;Zhang et al. 2007). Further RILs have been developed from a cross between the Salinas cultivar and the romaine cultivar Valmaine for the study of resistance genes to lettuce dieback disease (Simko et al. 2010). However, this is the first study to investigate lettuce antioxidant nutritional traits in the Salinas cv – wild relative *L. serriola* mapping population.

The complete mapping population was firstly grown in the glasshouse to minimise variation due to environmental effects. This investigation allowed for the identification of the extreme lines for the antioxidant trait i.e. the five lines containing the highest levels (112, 99, 20, 11 and 63) and the five containing the lowest levels (89, 90, 106, 80 and 107) of antioxidants (Figs. 4.9 and 4.10 and table 4.1).

The cross between the two parent lines gives rise to a high frequency of recombinants with greater levels of antioxidants than the commercial cultivar, *Lactuca sativa* (cv Salinas). From a commercial point of view, the identification of these extreme lines is interesting as they contain significantly higher levels of antioxidants than a typical supermarket green lettuce cultivar, such as salinas.

A number of RILs produced higher levels of antioxidants than both parent lines, suggesting that the genes controlling the antioxidant potential have an additive effect, indicating a quantitative trait and potential for selectively breeding higher antioxidant levels in lettuce cultivars. Therefore, existing genetic variability provided by *L. serriola* wild lettuce offers the possibility of improvement for this trait.

Past studies have investigated the effects of different environmental conditions on selected traits of RIL populations of other crops, for instance β-carotene levels in melon RILs (Cuevas et al. 2008), tomato volatiles (Tieman et al. 2006) or drought resistant traits in sorghum RILs (Tao et al. 2000), in the quest for finding robust QTL. However, none to date have focused on the

nutritional qualities of lettuce RILs. Thus, the complete mapping population was grown under different climatic conditions to assess the environmental effects on their antioxidant potential. Replicating standard commercial practice, the lines were grown in July in Southern UK and in March in South-Eastern Spain. Surprisingly, a comparison between the field grown lines revealed that the lines grown in the UK produced on average between a quarter and a third more antioxidants than those grown in Spain (Fig 4.11 and 4.12). An explanation for this may be due to the greater light irradiation intensity and to the longer summer daylight hours. The effects of different environmental conditions on the antioxidant production of lettuce was studied by Oh et al, who discuss the enhanced health benefits of the rise in antioxidant production due to light intensity increases (2009).

Unfortunately due to adverse climatic and storage conditions, the reliability of the complete mapping population was dramatically affected in both field sites. However, a boxplot of averages of the data taken from the three growing environments illustrates the effects of different environmental conditions on the antioxidant potential of the selected lines and suggest it would be possible to identify robust QTL and markers for future breeding and improvement for antioxidant potential in lettuce (Fig 4.12).

From the analysis of the field-grown extreme lines, it emerged that under ideal, stress-reduced environmental conditions (such as in the glasshouse) the lines showed considerable difference between extreme lines in their antioxidant production, whilst under different environmental conditions, such as in the field, this difference was considerably reduced. There was nevertheless a significant difference in total antioxidant production between the five lines with the highest levels grown in the summer in southern UK and the five lines with the lowest levels, however the difference between the two groups of extreme lines was reduced (Fig 4.12) in comparison to those grown in the glasshouse. Furthermore, this difference between the two environmental groups of extreme lines was not significant for the lines grown

in southern Spain during the winter, where the antioxidant production of these lines became comparable.

There was also a significant difference between the three groups of five 'worst' lines grown in different environments: the two groups of field grown samples both produced significantly higher levels than the glasshouse worst lines. This was an indication that the lines on the lower end of the scale could be stimulated to produce higher antioxidant contents. Thus, the differences between the extreme lines were reduced due to environmental agents: the lines on the lower end of the scale responded to external stimuli and did not have a high baseline production of antioxidants, as did the lines in the higher end of the range in the glasshouse. These 'worst' lines seemed to produce a basal level of antioxidants but needed a stimulus to produce higher amounts.

4.12.3 Light and temperature variations affect the antioxidant potential of the parent lines

The effects of the abiotic condition of reduced temperature and light irradiation conditions on the total antioxidant production of the Lactuca parent lines were therefore investigated further. A trial was carried out in which a set of *L. sativa* and *L. serriola* plants were grown in the glasshouse during summer months under standard glasshouse sunlight levels (average irradiation level: 494.19 µmol/s/m^2/µA; average temperature: 25.5℃), whilst another set were grown under lower levels of light to simulate 'shady' conditions (average level: 27.56 µmol/s/m^2/µA; average temperature: 22.9℃) (Fig 4.3). as expected, the results showed a significant increase in total antioxidant potential for leaves grown under a higher sunlight level and higher temperatures (Fig 4.4 a and b). Furthermore, *L. serriola* responded better to light intensity variations: the effect of light irradiation intensity was four-fold in the wild parent, whilst in the cultivar it only slightly increased. This increase in total antioxidant potential would be explained by a boost in the levels of a number of antioxidants: carotenoids, anthocyanins and phenols. These results are in agreement with other studies (Andrew 2006;Caldwell &

Britz 2009;Li & Kubota 2009) showing that higher light intensity leads to higher oxidative stress for the plant tissue thus to a higher risk of oxidative damage. Many carotenoids play an important role in the prevention of UV damage, by protecting the photosynthetic membrane from excess energy. Therefore, the greater effects of different irradiation intensity in the wild parent could be explained by the higher pigment levels in *L. serriola*.

4.12.4 Pigment variations in the RILs show considerable transgressive segregation

Initial studies on the parent lines found that a large portion of the phytonutrient qualities of lettuce was due to their carotenoid content (Fig 4.7). Significantly higher levels of carotenoids were found in the wild parent, in accordance with Mou (2004) thus, chlorophyll and carotenoid contents were analysed in the complete mapping population. This, again, allowed for the identification of extreme lines, i.e. the five lines with the highest levels (63, 58, 53, 4 and 123) and five lines with the lowest levels (99, 79, 112, 9, 59) lines (Fig15 and Table 4.2). The averages of the five lines on the higher end of the scale were significantly greater than the averages of the five lines on the lower end.

These extreme lines were then compared to the highest and lowest lines for the antioxidant trait. Correlation analyses indicated that the total carotenoid content did not correlate significantly with the total antioxidant content (Fig 4.14a), in agreement with other studies which claim that whilst carotenoids are important antioxidants in lettuce, the greatest proportion of the total antioxidant content is constituted by polyphenols (Llorach et al. 2008;Nicolle et al. 2004;Romani et al. 2002).

The lines with the highest and lowest pigment levels did not match the highest and lowest lines for the antioxidant trait which also indicates that these phytonutrients may not be contributing a significantly great proportion of the total antioxidant potential. These results are in accordance with Nicolle

et al who state that carotenoids are an important part of the antioxidant potential of lettuce, but they do not contribute the major proportion to the total antioxidant potential of lettuce (2004).

43% of the RILs produced higher levels of carotenoids than both parent lines, this suggests that the genes controlling the carotenoid phenotype have an additive effect, indicating a potential for selectively breeding higher carotenoid levels in cultivated lettuce. Therefore, existing genetic variability imparted by the wild parent *L. serriola* offers the possibility of improvement for these specific traits by selective breeding.

4.13 Conclusions

1. *Lactuca sativa* and *L. serriola* presented marked phenotypic and nutritional differences;

2. Younger leaves produced higher levels of total antioxidant content;

3. The wild parent produced higher total antioxidant, carotenoid, chlorophyll and vitamin E levels than the cultivar;

4. Carotenoids in particular constituted the highest proportion of the nutrients investigated, and a breakdown of the carotenoid content showed that the predominant compounds were lutein, zeaxanthin and β-carotene;

5. The recombinant inbred line (RIL) mapping population showed transgressive segregation for the total antioxidant content as well as for the pigment levels;

6. The findings suggest that the increase of antioxidant and of pigment levels in lettuce cultivars through introgression of genes from its wild relative would be feasible.

CHAPTER – 5 QUANTITATIVE TRAIT LOCI FOR ANTIOXIDANT PHYTONUTRIENTS IN THE RILs

5.1 Introduction

Analyses and comparisons of the cultivated lettuce (*Lactuca sativa,* cv. Salinas) and its wild relative (*Lactuca serriola,* acc.UC96US23) revealed significant differences between their nutritional contents. Total antioxidant potential, total carotenoid content and chlorophyll a and b contents were all significantly higher in the wild species than in the cultivar. These traits were then investigated in 60 informative ninth generation Recombinant Inbred Lines (RIL) derived from a cross between the two Lactuca species. From a full mapping population of 113 lines, the 60 most informative lines were selected. These lines, previously selected by using MapPop (Vision et al. 2000) and Genoplayer (www.compgenomics.ucdavies.edu/genoplayer/), had nearly as many breakpoints as a population of 90 RILs and were thus as informative. The samples were grown in the glasshouse at Southampton University and on two field sites, at the Vitacress farms in Hampshire (UK) and near Águilas (Murcia, Spain) in a randomised pattern of nine biological replicates per line, organised into three blocks of three replicates per line each. Large phenotypic variation and transgressive segregation were observed for the chosen traits in the mapping population grown in the glasshouse, however, due to extreme environmental circumstances, the field samples were not comparable (refer to chapter 4).

Most quality traits show continuous variation which is influenced by their genotype and by environmental conditions. The genetic variation of such traits can be attributed to the combined action of many genes, which can be mapped on the genome with genetic markers (Quantitative Trait Loci, or QTLs). A genetic map developed at University California Davis, containing 1334 AFLP (Amplified Fragment Length Polymorphism) and SSR (Simple Sequence Repeat) markers was used for QTL analysis (http://www.cgpdb.ucdavis.edu/database) of the complete mapping

population. The data obtained from the trait analyses were thus studied for the identification of significant QTLs underlying the traits analysed in the mapping population. QTL analysis was run using the WinQTL Cartographer 2.5 software on the data provided by the total antioxidant potential analysis of the RILs to ascertain which alleles were involved in antioxidant and pigment production and which chromosomes, or Linkage Groups (LG), underlie those locuses. A composite interval mapping method was used to increase the resolution and reduce background marker effect (Wang 2007;Zeng 1994). The Log of Odds (LOD score) threshold level was set at 3.7; the threshold value was estimated by permutation analysis for each trait using 1000 iterations (Churchill & Doerge 1994).

Aims

1. To identify significant QTL for antioxidant and pigment compounds in Lactuca species;

2. To analyse transgressive segregation of nutritional properties of an interspecific lettuce cross;

3. To localise potential hotspots for these nutritional properties.

5.2 QTL analysis reveal four different loci underlie Lactuca's antioxidant potential

Previous analyses on the total antioxidant potential of the parent lines revealed differences between the wild lettuce, *L. serriola*, and the cultivar, *L. sativa*. The wild relative consistently produced higher levels of antioxidants (Chapter 4) (Figs. 4.2, 4.4 and 4.9). The complete RIL mapping population of 60 lines and their parent lines was then sown in the glasshouse and on two field sites: in the UK and in Spain, during the summer months and winter months, respectively. Nine replicates were sown per line, randomly organised into three groups (A, B and C) with three replicates per line. The complete mapping population was tested for total antioxidant potential by using the FRAP assay. The antioxidant analyses revealed significant variation between the lines which allowed for the selection of the 10 extreme lines (Fig 5.1; Table 5.1). Transgressive segregation was also observed for the antioxidant trait as seven lines produced higher levels of antioxidants than either parent line, with two lines (99 and 112) producing significantly higher levels than the wild parent *L. serriola* (Fig 5.1 and 5.2).

Quantitative Trait Loci analysis was carried out on the phenotypic data obtained from the glasshouse trial, using the WinQTL Cartographer 2.5 software, on the results from the antioxidant analyses on the complete mapping population grown in the glasshouse during summer 2006. The analysis revealed that the major alleles for total antioxidant biosynthesis lie on chromosomes 3, 7 and 9, at 30.93cM, 91.49cM and 30.60cM, of the respective chromosomes (Fig 5.3). From the map it was also possible to determine the loci's origin in the parent lines: the alleles for increasing the antioxidant traits mapping to LGs 3 and 9 both originated from the wild *L. serriola* parent, whilst the alleles on LG 7 derived from the *L. sativa* parent. Furthermore, the genotypes for the increased and decreased traits in the extreme lines corresponded to the correct parent. A total of 52.9% of phenotypic variance (R^2) was explained by the QTL (Table 5.2). 22.54%,

11.79% and 18.57% were explained by the QTLs on LGs 3, 7 and 9 respectively.

A QTL analysis was also carried out for the antioxidant phenotypic data from the RIL mapping population grown in the field at the Vitacress Pinglestone farm in the UK. Two significant QTL were detected, one on LG 3 and a second on LG 4, explaining 20.90% and 23.92% of the variance respectively.

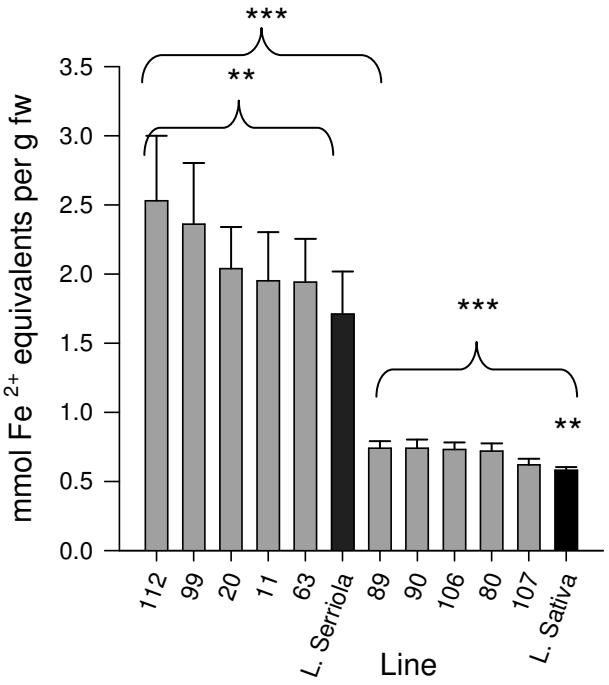


Fig 5.1 Antioxidant potential of the mapping population extreme lines and their parent lines. The complete mapping population was grown in the glasshouse. The parent lines and the five RILs with the highest and the five RILs with the lowest antioxidant values are displayed. *Lactuca sativa* and *L. serriola* are highlighted in black. One-way ANOVA showed a significant difference between the five lines in the high end of the range and the cultivar *L. sativa* ($F_{5,65}$ =2.91;**P=0.01). Averages of the five lines with the highest antioxidant levels pooled together and averages of the five lines with the lowest values pooled together were also significantly different ($F_{1,65}$=63.09;***P<0.001). Antioxidant values for lines 99 and 112 were also significantly higher than lines 11, 63, and 1001 (*L. serriola*) (and 20 from 112). Whilst, there was no significant difference between the individual five lines in the low end of the range.

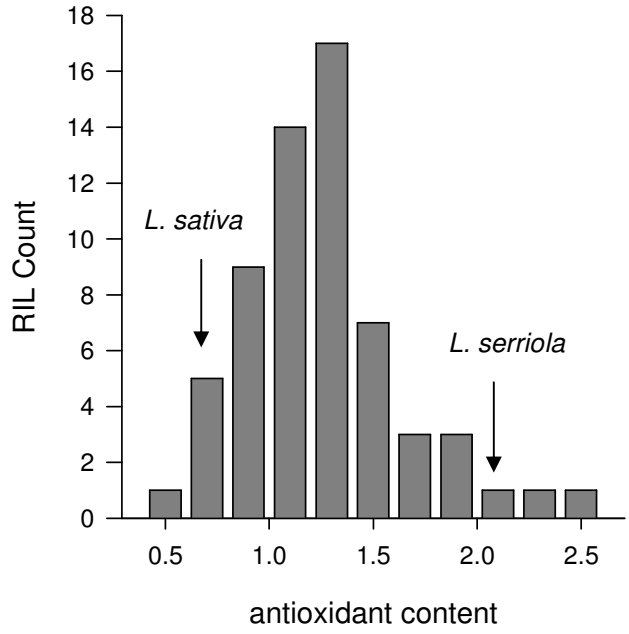
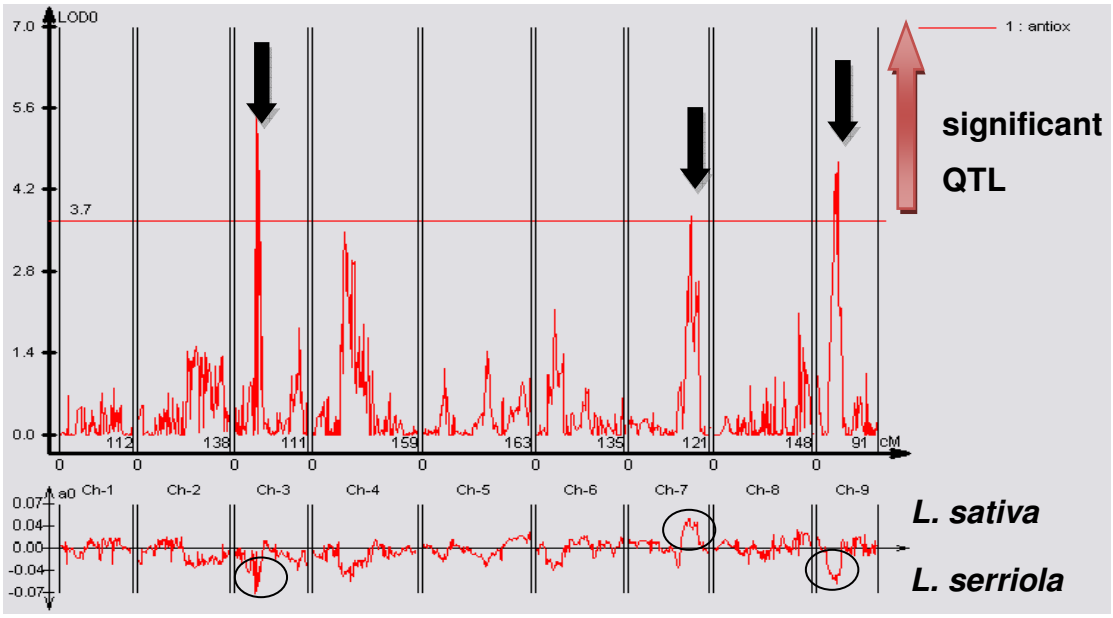
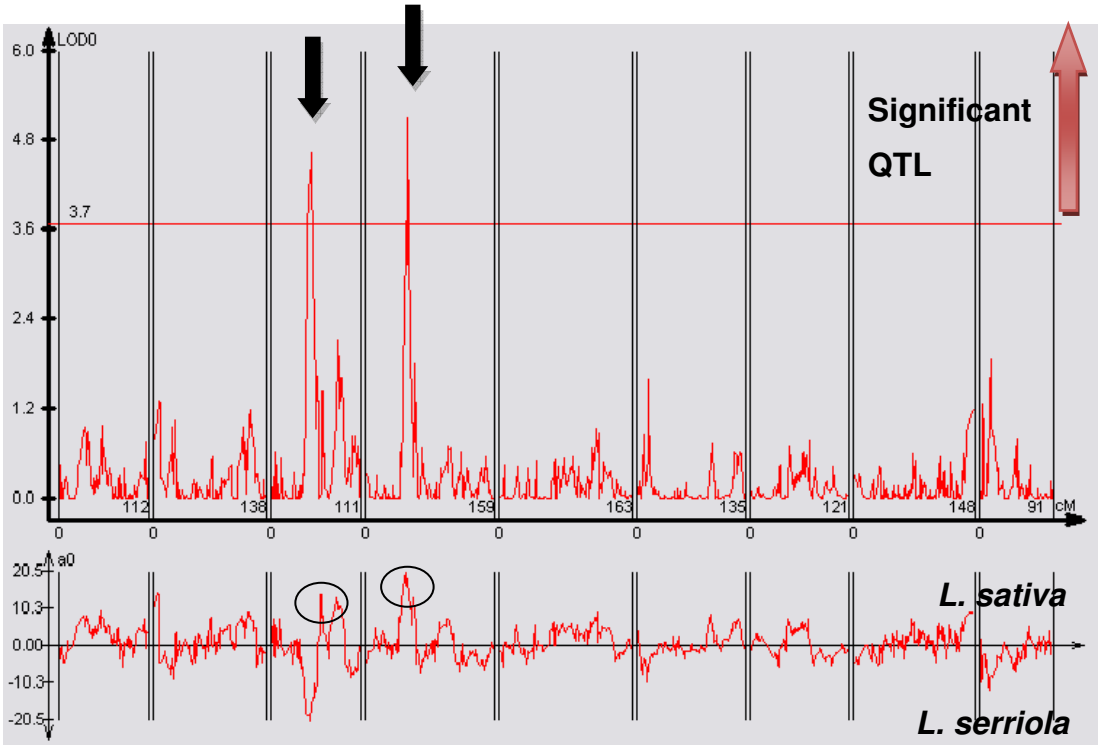


Fig 5.2 Frequency distributions of the antioxidant content of the complete mapping population. The mean values of the parent lines, *L. sativa* and *L. serriola*, are indicated by the arrows. The lines were grown in the glasshouse and organised into three blocks of three randomised replicates of each line per block (nine replicates in total for each line).



(A)



(B)

Fig 5.3 Quantitative Trait Locus (QTL) map for total antioxidant potential in the RIL mapping population. The lines were grown in the glasshouse (A) and in the field, in the UK (B). QTL cartographer plot for antioxidant content index of the F_9 RIL mapping population. The LOD score is indicated on the upper Y axis. The X axis represents the nine linkage groups of the F_9 recombinant inbred line map arranged

end-to-end and their length in cM. The horizontal red line (LOD = 3.7, P<0.05) represents the significant threshold value for the trait value. The lower graph in the plot shows the additive effect of the parental allele that causes an increase in the trait value. Positive values indicate the cultivar (*L. sativa*) allele increased the trait values and negative values indicate wild relative (*L. serriola*) allele increased the trait values.

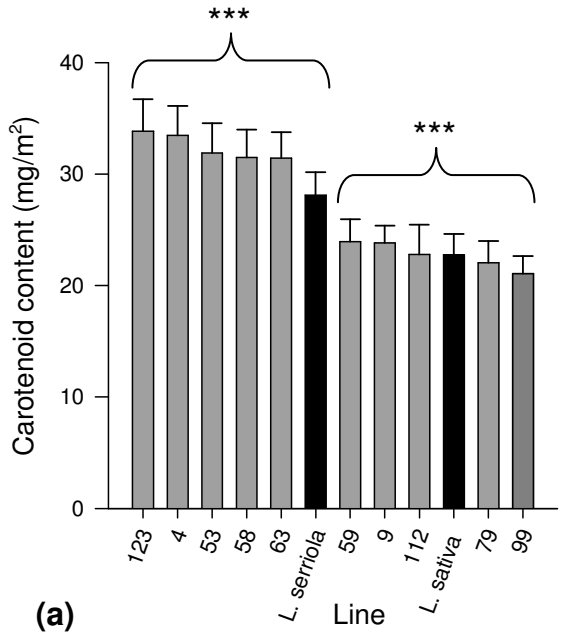
5.3 QTL analyses of chlorophyll and carotenoid contents reveal four different loci underlie Lactuca's pigment traits

The mapping population of 60 lines and their parent lines were organised in the glasshouse into three blocks of three replicates per line. Analyses of carotenoid and chlorophyll contents were carried out using the chemical extraction method DMF and subsequent spectrophotometric reading. the results showed large variation between the lines, as well as significant differences between *L. sativa* and *L. serriola* (Chapter 4) (Figs. 4.5, 4.6 and 4.15). Considerable transgressive segregation was also observed for these traits as numerous lines (43%) were found to produce significantly higher levels of chlorophyll and carotenoids than both parent lines. Ten extreme lines were also selected for these traits (Fig 5.4 and 5.5; Table 5.1).

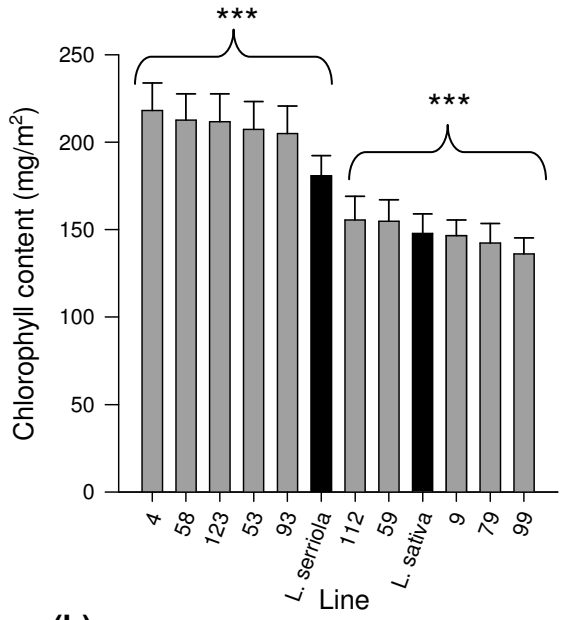
QTL analyses were run using the WinQTL Cartographer 2.5 software, on the phenotypic data provided by the pigment analyses of the RILs and their parent lines, to pinpoint the location of the alleles involved in chlorophyll and carotenoid production (Fig 5.6). The analyses determined that the alleles underlying total chlorophyll synthesis are located on LG 3 (at 35.39cM), and 7 (at 97.45cM). Furthermore, when the mapping was carried out for chlorophylls a and b separately, chlorophyll a also mapped to LG 9 (at 51.54cM) and chlorophyll b showed a second significant QTL on LG 3 (at 106.92cM). The genes responsible for total carotenoid biosynthesis were linked to chromosomes 3 (at 34.53cM), 7 (at 97.45cM) and 9 (at 51.54cM) (Fig 5.7; Table 5.2).

From the map it was also possible to determine the loci's origin in the parent lines: the loci for both pigment traits mapping to LG 3 and 9 derived from the *L. sativa* parent for each trait, except for the second QTL on LG3 for chlorophyll b, which originated from the wild *L. serriola*. Those alleles located on LG 7 originated from the *L. serriola* parent for both traits.

The percentages of phenotypic variance explained by each of the QTL found are displayed in Table 5.2. Phenotypic variance explained was 63.15% in total for chlorophyll a; 65.08% in total for chlorophyll b; 42.62% for total chlorophyll and 68.63% for total carotenoids.



(a)



(b)

Fig 5.4 Chlorophyll (a) and carotenoid (b) values of the five lines with the highest and the five with the lowest levels of the selected traits for the mapping population. The values of the parent lines are shown in black. One-way ANOVA analysis revealed a significantly higher value for the pooled mean values of the five lines with the highest carotenoid contents compared to the five lines with the lowest carotenoid content ($F_{1,89}$ =168.70; ***P<0.001). Pooled together, the chlorophyll content of the five lines with the highest levels was also significantly higher than the content of the five lines with the lowest values ($F_{1,89}$ =214.59; ***P<0.001).

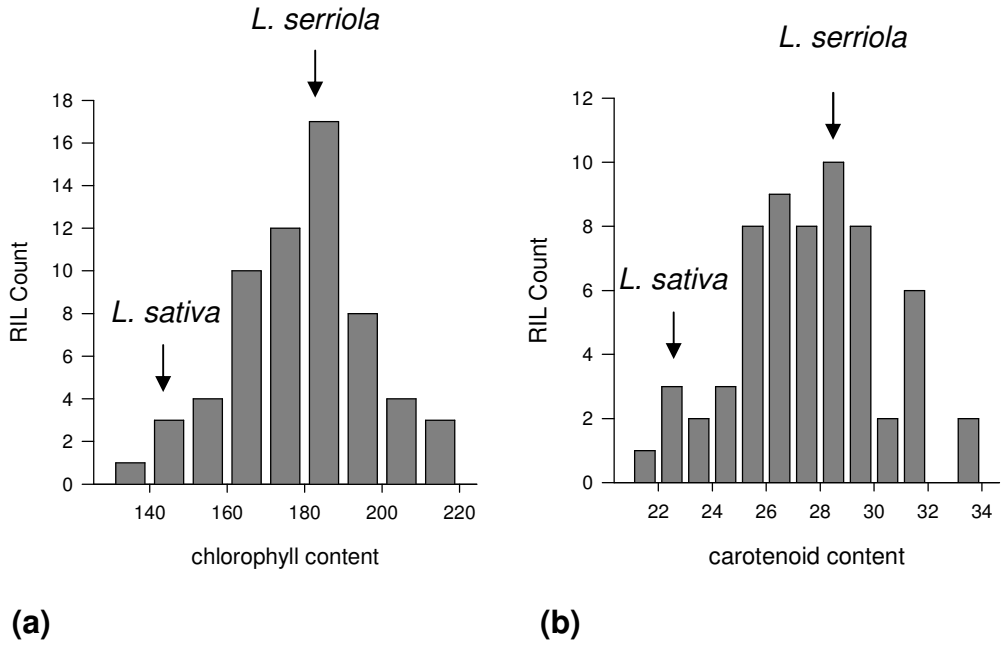
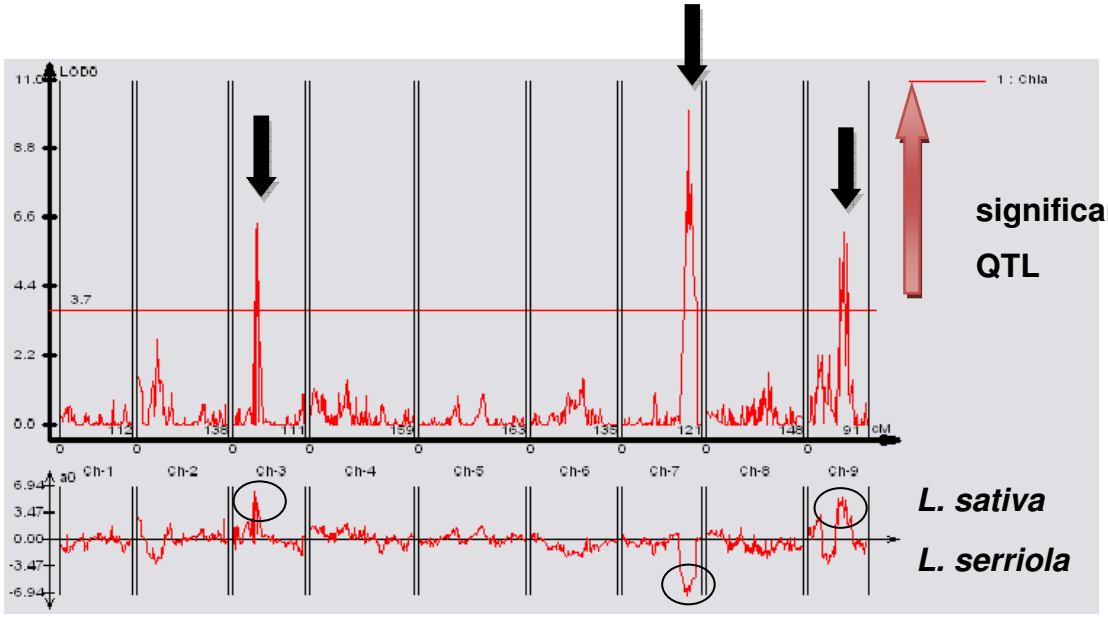
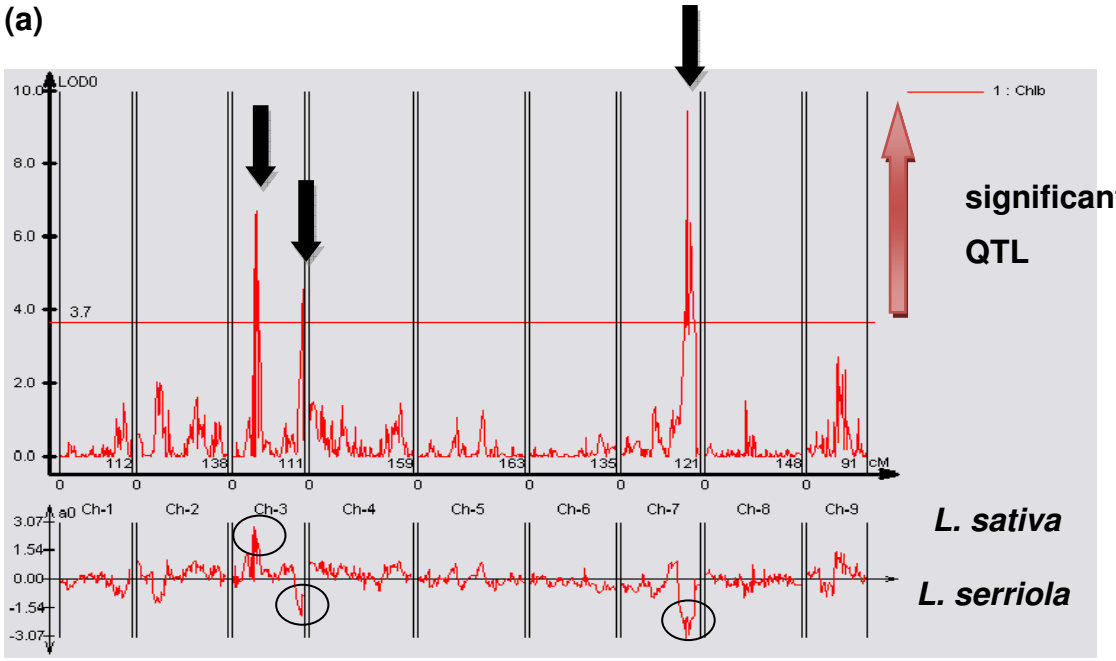


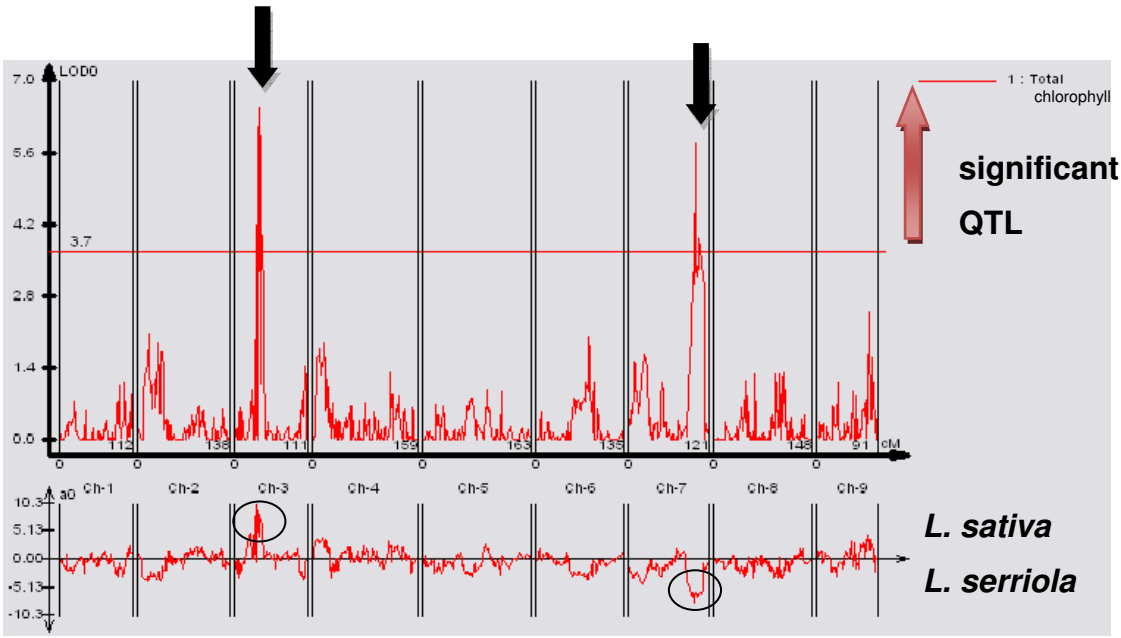
Fig 5.5 Frequency distributions of selected traits of the RIL mapping population. (a) chlorophyll content and (b) carotenoid content. The mean values of the parent lines, *L. sativa* and *L. serriola*, are indicated by arrows. The lines were grown in the glasshouse and organised into three blocks of three randomised replicates of each line per block (nine replicates in total for each line). The black arrows show where the parent lines fall on the distribution of the mapping population.


LOD0
11.0
8.8
6.6
4.4
2.2
0.0
3.7
112
138
111
159
163
135
121
148
91
cM
1 : Chla
significant
QTL
aQ
Ch-1
Ch-2
Ch-3
Ch-4
Ch-5
Ch-6
Ch-7
Ch-8
Ch-9
6.94
3.47
0.00
-3.47
-6.94
L. sativa
L. serriola

(a)


LOD0
10.0
8.0
6.0
4.0
2.0
0.0
3.7
112
138
111
159
163
135
121
148
91
cM
1 : Chlb
significant
QTL
aQ
Ch-1
Ch-2
Ch-3
Ch-4
Ch-5
Ch-6
Ch-7
Ch-8
Ch-9
3.07
1.54
0.00
-1.54
-3.07
L. sativa
L. serriola

(b)



(c)

Fig 5.6 QTL map for chlorophyll traits in the RIL mapping population. QTL cartographer plots for chlorophyll a (a), chlorophyll b (b) and total chlorophyll (c) content index of the F_9 RIL mapping population. The LOD score is indicated on the upper Y axis. The X axis represents the nine linkage groups of the F_9 recombinant inbred line map arranged end-to-end and their length in cM. The horizontal red line (LOD = 3.7, $P<0.05$) represents the significant threshold value for the trait value. The lower graph in the plot shows the additive effect of the parental allele that causes an increase in the trait value. Positive values indicate the cultivar (*L. sativa*) allele increased the trait values and negative values indicate wild relative (*L. serriola*) allele increased the trait values.

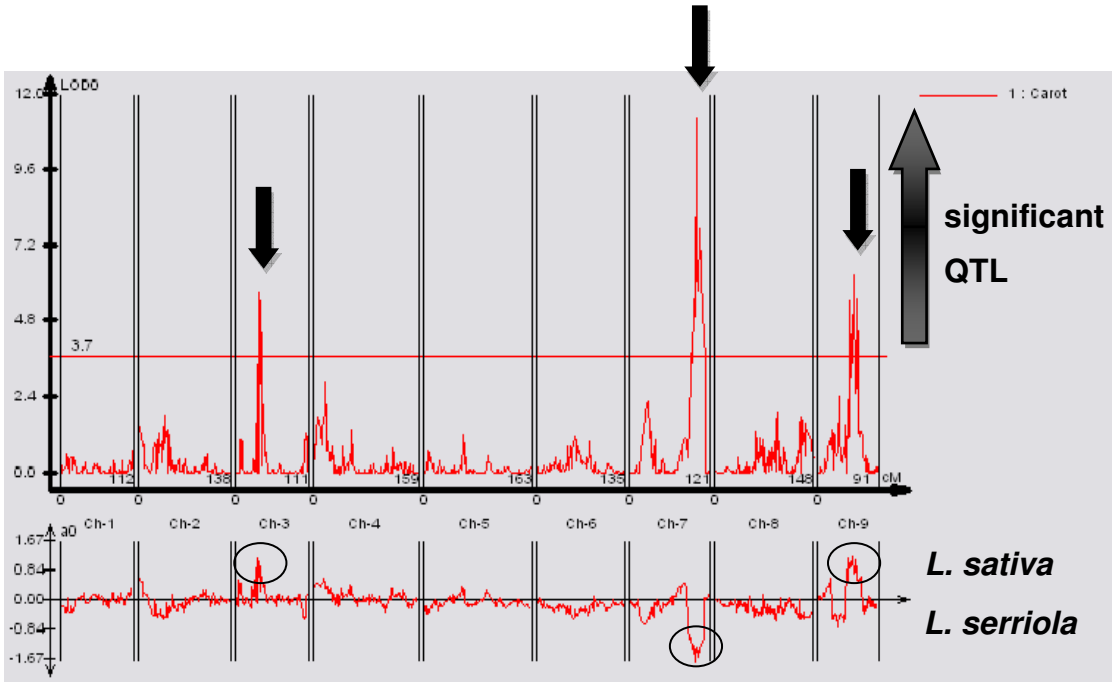


Fig 5.7 QTL map for the carotenoid trait in the RIL mapping population. The LOD score is indicated on the upper Y axis. The X axis represents the nine linkage groups of the F_9 recombinant inbred line map arranged end-to-end and their length in cM. The horizontal red line (LOD = 3.7, P<0.05) represents the significant threshold value for the trait value. The lower graph in the plot shows the additive effect of the parental allele that causes an increase in the trait value. Positive values indicate the cultivar (*L. sativa*) allele increased the trait values and negative values indicate wild relative (*L. serriola*) allele increased the trait values.

Table 5.1 Mean and range values for measured traits of the complete mapping population. *The mapping population was grown in the glasshouse and on two field sites. Parent line values and RIL line values for minimum, maximum and mean values of the selected traits are displayed.*

Traits	**Site**	**Parents**			**RILs**		
		L. serriola	***L. sativa***	**mean**	**Min**	**Max**	**Mean**
Antioxidants	**Glasshouse**	1.71±0.31	0.58±0.24	1.14	0.62±0.04	2.53±0.35	1.58
(mmol Fe^{2+}	**Spain**	1.21±0.13	1.14±0.02	1.17	1.14±0.10*	1.59±0.14*	1.36*
eq/g FW)	**Pinglestone**	2.52±0.27	1.96±0.07	2.24	1.96±0.19*	2.72±0.25*	2.33*
Chlorophyll	**Glasshouse**	180.74±11.55	147.76±11.16	164.25	136.04±9.09	218.11±15.70	177.08
(mg/m^2)							
Carotenoids	**Glasshouse**	28.11±2.06	22.76±1.87	25.43	21.07±1.57	33.85±2.85	27.46
(mg/m^2)							

*only the ten extreme lines from the glasshouse study were sampled in the field sites

Table 5.2 QTLs detected by composite interval mapping for all leaf traits assessed in the RIL mapping population grown in the glasshouse (UK).

Trait[a]	Position[b] (cM)	Marker[c] and number		UK glasshouse trial Leaf no.[d]	LOD[e]	Additive[f]	Variance (%)(R^2)[g]
ANTIOX	LG3: 30.93	1A02-270	(52)	n/a	6.1775	-0.0731	22.54
	LG7: 91.49	E54/M48-F-145	(81)	n/a	3.7611	0.0481	11.79
	LG9: 30.60	LE3066	(28)	n/a	4.7054	-0.0569	18.57
CHL A	LG3: 35.39	E45/M48-F-147	(65)	4	6.4392	5.6976	18.3
	LG7: 97.45	LK1426	(88)	4	10.0803	-6.8750	30.36
	LG9: 51.54	1A18-075<N>	(56)	4	6.1433	5.6500	14.49
CHL B	LG3: 35.39	E45/M48-F-147	(65)	4	6.7128	2.6196	21.54
	LG3: 106.92	LE0473	(129)	4	4.5767	-1.9056	12.07
	LG7: 97.45	LK1426	(88)	4	9.4669	-3.0540	31.47
TOT CHL	LG3: 35.39	E45/M48-F-147	(65)	4	6.4754	9.0968	24.66
	LG7: 97.45	LK1426	(88)	4	5.7930	-7.7814	17.96
CAROT	LG3: 34.53	LK1225	(62)	4	5.7402	1.2058	17.29
	LG7: 97.45	LK1426	(88)	4	11.2324	-1.6617	36.13
	LG9: 51.54	1A18-075<N>	(56)	4	6.3042	1.2567	15.21
				UK field trial			
ANTIOX	LG3: 49.43	L2598	(76)	n/a	4.637	-20.5356	20.90
	LG4: 50.55	LE189	(83)	n/a	5.1211	20.4386	23.92

[a] Trait abbreviation: ANTIOX, antioxidant potential (mmol Fe^{2+} equivalents per g FW) analysis was carried out for both the full set of RILs grown in 3 blocks in the glasshouse; CHL A, chlorophyll a content (m/m^2); CHL B, chlorophyll b content (m/m^2); TOT CHL, total chlorophyll content (m/m^2); and CAROT total carotenoid content (m/m^2).

[b] Position indicated by the linkage group number.

[c] Markers that are the nearest marker to the QTL.

[d] Leaf no. indicates at which leaf development stage the QTL was detected. n/a, not assessed for the individual trait.

[e] LOD indicates the logarithm of odds score.

[f] Additive effect indicates which parental allele causes an increase in the trait value. Positive values indicates that the cultivated (*L. sativa*) allele increases the trait value and negative values indicate that the wild-type (*L. serriola*) allele increases the trait value.

[g] The variance indicates the proportion of phenotypic variance in the mapping population explained by the detected QTL.

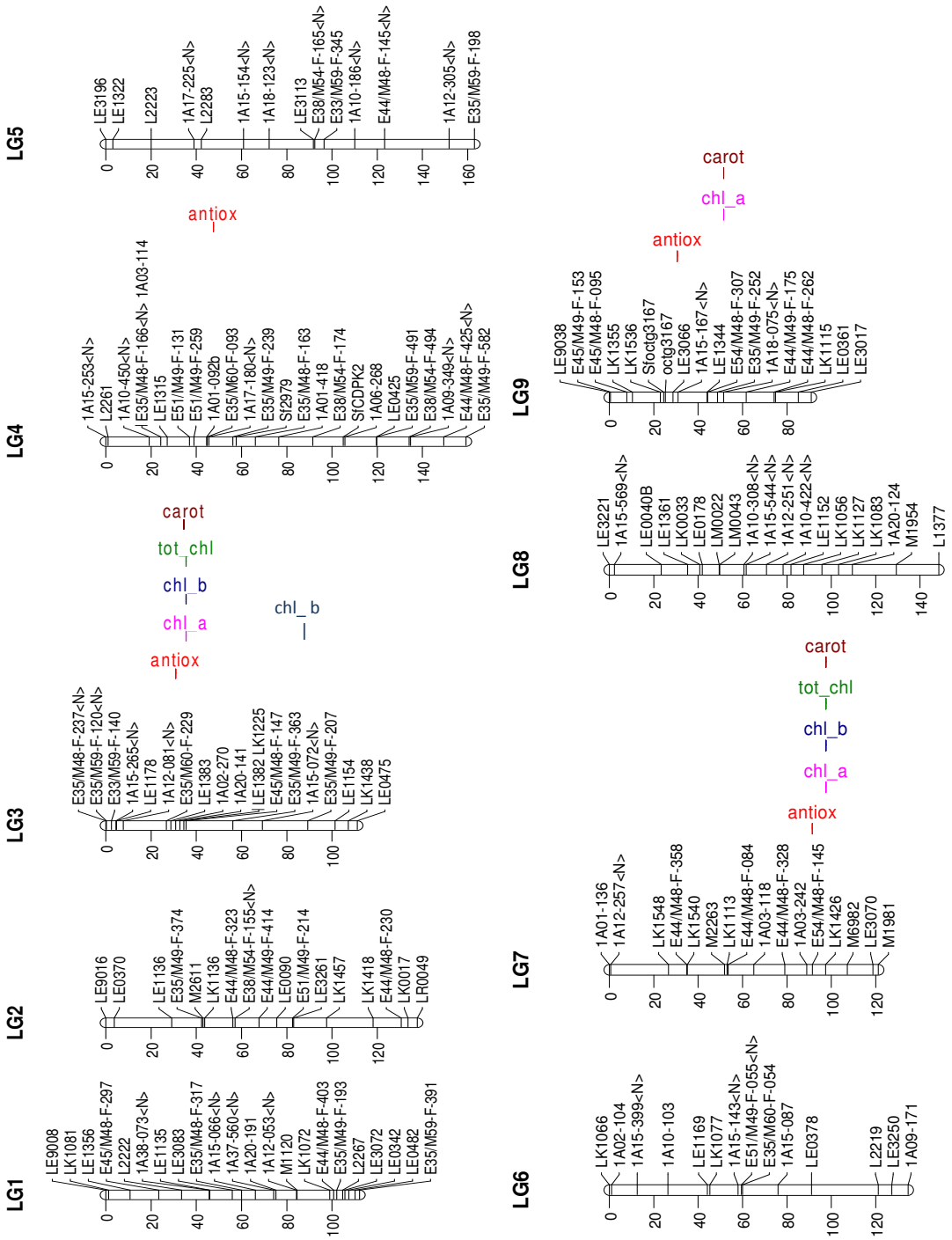


Fig 5.8 QTL map for antioxidant, chlorophyll and carotenoid attributes, based on an interspecific RIL population originating from a cross between a cultivated and a wild lettuce. The QTL distributions are detected by composite interval mapping (CIM). The positions of the markers are indicated on the right of each linkage group and an indication of the locations is given at fixed tick intervals of 20cM on the left. The QTL for the traits of interest are indicated in colour. For trait abbreviations, see table 5.2.

5.4 Discussion

The cultivated lettuce *Lactuca sativa* (cv Salinas) and its wild relative *Lactuca serriola* (acc UC96US23) present very different morphologies and phenotypes reflecting their genetic divergence. Previous comparisons between the two species have shown that the cultivated lettuce has significantly lower antioxidant, chlorophyll and carotenoid content compared to its wild relative. Subsequent analysis of the total antioxidant potential and of the pigment content (carotenoids and chlorophylls) in the mapping population of 60 F_9 Recombinant Inbred Lines (RILs) developed from a cross between *L. sativa* and *L. serriola* has shown great variability and transgressive segregation. Thus the variations between the parent lines were passed on to the mapping population which allowed for QTL mapping of these traits to their nine chromosomes, or linkage groups (LG). The objective of the present study was to identify QTL for the traits of interest and to locate the alleles responsible for some of Lactuca's phytonutrient biosynthesis on its nine chromosomes.

In contrast to the relatively abundant information on antioxidant and pigment QTL in other species, such as antioxidant traits in tomatoes and raspberries; or chlorophyll traits in sunflower, a related Compositae species (Kassim et al. 2009;Poormohammad Kiani et al. 2008;Rousseaux et al. 2007), there is no previous work that focuses on antioxidants and pigments in lettuce species. A number of QTL studies have been carried out on Lactuca varieties, however, most studies have focused on resistance to pathogens and traits relating to environmental adaptations, such as studies on downy mildew resistance in *L. saligna* (another species of wild lettuce) (Jeuken 2002), shelf-life in the *L. sativa* and *L. serriola* mapping population (Zhang et al. 2007) and on root architecture and deep soil water exploitation in the same RIL population (Johnson 2004). Several other studies have focused on QTLs for other nutritional traits in other plant species, such as vitamin C in various fruit types (Davey et al. 2006), however, the present study was the

first to investigate QTL for lettuce antioxidant and pigment phytonutrient contents.

5.4.1 QTLs for antioxidant potential co-locate with those for carotenoids and chlorophyll

5.4.1.1 QTLs for total antioxidant potential

Following the initial finding that the wild lettuce, *L. serriola*, produces around a third more antioxidants than the cultivated line, *L. sativa*, antioxidants have been analysed in each RIL. This investigation allowed for the identification of extreme lines, i.e. the five lines producing the highest (112, 99, 20, 63 and 11) and lowest (90, 89, 80, 106 and 107) antioxidant levels (Fig 5.1). The RILs together with their parent lines were grown in the glasshouse to minimise variation due to environmental effects. A number of lines produced higher levels of total antioxidant potential than even the wild parent, two of these lines, 99 and 112, were significantly higher than the wild parent. This indicated transgressive segregation for this trait, a characteristic which is often ubiquitous in plant hybrids, particularly in crosses between inbred populations, the major cause of which has been attributed to complementary gene action (Rieseberg et al. 2003;Rieseberg et al. 1999).

QTL analysis carried out for the antioxidant trait mapped alleles related to antioxidant production to LGs 3, 7 and 9, with a strong QTL on LG 3 explaining 22.54% of the phenotypic variance (Fig 5.3 and Table 5.2). The alleles mapping to LG 7 originated from *L. sativa*, whilst, interestingly, QTLs on LGs 3 and 9 originated from the parent line *L. serriola*. Interestingly, a genotypic comparison of the highest and lowest scoring RILs with the QTL results showed that the lines corresponded to the genotypic results of the parent lines. This would indicate a potential for the reintroduction of these genes from the wild parent into domesticated varieties as a way to increase the antioxidant quality of commercial lettuce.

A total of 52.9% of phenotypic variance was explained by the QTL (Table 5.2). Thus 47.1% of the phenotypic variance was left unexplained and are thus due to alleles which were not found to be significant but which nevertheless play a role in antioxidant production. An indicative QTL on LG 4 would have been found with a significance threshold level set slightly lower (LOD=3.5 instead of 3.7), with an allele originating from the wild parent. For instance, other studies have set the LOD threshold at lower levels such as 3.0 or even 2.0 (Paterson et al. 2003;Poormohammad et al. 2008). Another way of enhancing the sensitivity of the QTL mapping analysis would be to increase the mapping population line number (Zhang 2006) as only large effect QTL will be found with small sample sizes so very large sample sizes are required to detect QTLs with moderate to small effects (Falconer & Mackay 1996). Interestingly, a significant QTL was found on LG 4 in the field grown population, which was not detected in the glasshouse trial, indicating a locus due to an environmental effect.

5.4.1.2 QTLs for carotenoids and chlorophyll

A proportion of the antioxidant potential in lettuce is due to its pigment content. Thus previous studies examined the carotenoid and chlorophyll levels of the complete mapping population. Initial analyses found higher levels of carotenoids in the wild parent, therefore, chlorophyll and carotenoid contents were then analysed in the RILs. This allowed for the identification of the extreme lines (the five with the highest pigment content: 63, 58, 53, 4, 123; and the five with the lowest levels 99, 79, 112, 9, 59 and *L. sativa*) (Fig 5.6 and 5.7). Almost half of the RILs produced higher pigment levels than both parent lines, showing considerable transgressive segregation for these traits. This suggests that the genes controlling the carotenoid phenotype have an additive effect, indicating a potential for selectively breeding higher carotenoid levels in cultivated lettuce.

QTL analysis was carried out to locate the alleles involved in carotenoid and chlorophyll biosynthesis. Significant QTLs were found on LGs 3 and 7 for

total chlorophyll content and on LGs 3, 7 and 9 for carotenoid content (Fig 5.7). A breakdown of the QTL analysis in chlorophylls a and b also showed a significant QTL on LG 9 for chlorophyll a and a second QTL on LG 3 for chlorophyll b which interestingly had not been detected when chlorophylls a and b were analysed as one trait, indicating a smaller effect locus.

The QTL results for the total chlorophyll trait were comparable to those reported by Zhang et al (2007) who found significant QTLs on LGs 3, 4 and 7 for the same RIL mapping population grown in the field at Pinglestone, near Winchester, UK, and a further QTL on LG 9 for those grown in Portugal. In this study, chlorophyll analysis has been subdivided between total chlorophyll, chlorophyll a and chlorophyll b. The results reported here for chlorophyll content indicate that this trait maps to LGs 3, 7 and 9, thus similar to Zhang's results, with the exception of LG4. These results indicate a constitutive nature, independent of the environment, of the QTLs for chlorophyll found on LGs 3, 7 and 9 and a smaller effect QTL on LG 4, which may vary with different environmental conditions. Further analysis revealed that total chlorophyll mapped to regions of LGs 3 and 7 which were very close to those mapped by Zhang's group although the markers correlated to those regions were not the same ones. Encouragingly, the same parental alleles were found to underlie each QTL trait value: the cultivated parent, *L. sativa*, increased the trait value on LG 3 and the wild parent, *L. serriola*, increased the trait value on LG 7.

QTL for the chlorophyll b trait mapped only to LGs 3 and 7, whereas, QTLs for chlorophyll a were also found on LG 9. Whereas Zhang et al reported that only when the RILs were grown in Portugal did a QTL map to LG 9. Whilst chlorophyll a on LGs 3 and 7 were mapped to a region very close to that found by Zhang, the QTL on LG 9 reported here does not lie close to Zhang's (Zhang's: 9.9-12.6cM; this study: 51.54cM).

68.63% of the phenotypic variance was explained by the QTLs found for the carotenoid trait thus only 31.37% of the variance was left unexplained and

due to alleles which were not found to be significant but nevertheless played a significant role in carotenoid production. Similarly, for chlorophyll a, b and total chlorophyll traits the respective unexplained phenotypic variance were 36.85%, 34.92% and 57.38% which were probably due to smaller effect alleles mapping to other segments of the genome, such as were suggested by Rousseaux et al in tomato antioxidants (2007).

5.4.2 QTL Hotspots

Two distinct QTL clusters (hotspots) have been identified on LGs 3 and 7 relating to antioxidant and pigment traits (Fig 5.8). These hotspots may be due to chance linkage of traits or to a single QTL controlling multiple traits because of related biosynthetic pathways, such as were found by Zhang et al (2007) for developmental characteristics and postharvest performance. QTLs for antioxidants, chlorophylls and carotenoids on LGs 3 and 7 are located very closely to each other on the genome (between 5 and 6 cM), thus a larger mapping population may provide more accurate relative locations, however, the minimum length which can be mapped has been reported to be no less than 3cM. The maximum number of QTLs associated with each trait is three, in line with the trends observed by Kearsey and Farquar who reported four as a maximum level (1998). Similar QTL clusters have been reported in studies regarding leaf traits (Rae et al. 2004), rice seed traits (Xu et al. 2004) and fruit quality traits (Causse et al. 2002). These QTL hotspots can have important implications for plant breeding programmes as the selection of a QTL region with an ideal genotype for a specific trait could simultaneously improve several other traits. However, for other QTL hotspots where both desirable and undesirable traits cluster together, fine mapping would be required to assess whether there are multiple QTLs or a single QTL with pleiotropic effects. The results presented in this chapter indicate that the selection for an increase in the total antioxidant trait would result in the decrease of the pigment traits.

5.4.3 Concluding remarks

In summary, a number of points can be stated following this study. A total of 16 QTLs with significant effects were detected for the five traits, distributed on LGs 3, 4, 7 and 9 (Table 5.2). Phenotypic variance explained by the QTLs ranged from 12% to 36%, explaining a total of: 52.9% for the antioxidant trait (glasshouse grown); 44.82% for the antioxidant trait of the field grown lines; 68.63% for the carotenoid trait; and 42.62% for the total chlorophyll trait. Interestingly, significant QTLs were reported on LGs 3, 7 and 9 for all of the selected traits, however alleles on the same LG mapped to the opposite parents for the antioxidant and for the pigment traits. A number of elite lines have been found for each of the traits of interest, with one line in particular, line 63, which brings favourable alleles for both total antioxidant and total carotenoid contents together.

Growth environment and seasonal variation can constitute an important factor in phytonutrient regulation and thus in QTL detection (Kassim et al. 2009;Rousseaux et al. 2007). It was possible to confirm the QTL by repeating the analysis across different environments with the field trial carried out in the UK. Furthermore, QTL were fine mapped by minimizing experimental variance, analysing a large number of replicates and maximising the information provided by the population by using the most informative lines. Studies have reported a lower number of observed QTL in glasshouse trials than in field trials (Rousseaux et al. 2007). These findings suggest even more QTL may be detected in the investigation of field-grown RILs across different seasons. Furthermore, other small QTL effects may also be detected if the population size were increased. Future work could also focus on confirming the identified QTLs on Near Isogenic Lines (NIL) or Heterozygous Inbred Families (HIF) deriving from a single RIL segregating a single QTL region in an inbred background that is a mixture of the two parent lines (Borevitz & Chory 2004).

Numerous studies have proposed that polyphenolic compounds are major contributors to the total antioxidant content of fruit and vegetables (Kahkonen et al. 1999;Kang & Saltveit 2002;Llorach et al. 2008;Lotito & Frei 2006;Nicolle et al. 2004;Rice-Evans et al. 1997;Trichopoulou & Vasilopoulou 2000), and an obvious question that arises from these results for QTL mapping of total antioxidant traits and pigment traits is which other compounds are present in lettuce leaves that confer antioxidant potential. Future work would focus on the identification of QTL for total polyphenolic traits in the mapping population.

Overall, these results demonstrate the feasibility of improving lettuce leaves' nutritional quality through an increase in their antioxidant and carotenoid content through breeding of lines produced by crossing a lettuce cultivar with a wild relative.

5.5 Conclusions

1. Four significant QTLs were identified for the antioxidant trait;

2. Three significant QTLs were identified for the carotenoid trait and three for chlorophylls a and b;

3. Two significant QTLs were identified for the total chlorophyll trait, when chlorophylls a and b were analysed together;

4. The origins from the parent lines were determined for all of the genes of the traits of interest;

5. Two distinct QTL clusters (hotspots) have been identified on LGs 3 and 7 relating to antioxidant and pigment traits.

CHAPTER 6 – MOLECULAR ANALYSES OF THE MAPPING POPULATION AND THEIR PARENT LINES

6.1 Introduction

Previous studies showed that the wild species, *L. serriola* (acc. UC96US23), produced significantly greater levels of total antioxidant content, carotenoids and chlorophylls a and b, than the cultivar *L. sativa* (cv Salinas). Further analyses of these phytonutrients in the Recombinant Inbred Lines (RIL) mapping population originating from a cross between these Lactuca species showed considerable variation between the lines and, perhaps most importantly, transgressive segregation for the carotenoid trait (Chapter 4). Subsequent QTL analyses revealed the genetic location of loci underlying the alleles for these traits and their origins in the parent lines (Chapter 5). These results showed potential for increasing the total antioxidant and carotenoid traits in Lactuca cultivars. Therefore, genes involved in the production of key compounds in the carotenoid biosynthetic pathway were assessed both in the parent lines and in the RILs by SNP genotyping. In particular, the focus was on the genes involved in lutein and β-carotene production, as these were shown to be present in significant amounts in both parent lines in comparison to other nutrients (results from Campden Chorleywood Food Research Association, Chapter 4).

From the literature it is clear that a number of other phytonutrients than carotenoids contribute significantly to the total antioxidant potential of lettuce leaves (Davey et al. 2007;Kang & Saltveit 2002;Liu et al. 2007;Llorach et al. 2008;Mou 2005;Nicolle et al. 2004). As well as a number of vitamins and minerals, several polyphenols also play an important part in the total antioxidant content of leaf material (Nicolle et al. 2004). Nicolle et al. state that the polyphenolic content contributes up to 70% of the total antioxidant content of lettuce leaves. Due to their importance in contributing to the total antioxidant content, genes involved in the production of key compounds of the polyphenol pathway were also analysed in depth with PCR-based Single

Nucleotide Polymorphism (SNP) genotyping assay. With the SNP assay variation at a nucleotide level can be assessed and, more importantly, these polymorphisms in the genome sequence of individuals in a population constitute the most abundant molecular markers and are widely distributed throughout the genome (Agarwal et al. 2008).

Aims

1. To identify Single Nucleotide Polymorphisms (SNP) between the parent lines, *Lactuca sativa* and *L. serriola*, for the genes involved in carotenoid and polyphenol biosynthesis;

2. To investigate the SNPs found in the parent lines, in the RIL mapping population;

3. To characterise potential genetic markers related to the antioxidant trait;

4. To relate the SNP results to the QTL findings in Chapter 5.

6.2 Amplification of polyphenolic biosynthesis genes in the parent lines

Candidate genes involved in the polyphenol biosynthesis were sourced from the literature. Based on the availability of Expressed Sequence Tags (ESTs), sets of forward and reverse oligos were designed for: phenylalanine ammonia-lyase (PAL 1, 2, 3 and 4); hydroxycynnamoyl transferase (HC/BT); shikimate *p*-hydroxycinnamoyl transferase and *p*-hydroxycinnamoyl-CoA: quinate (HCT); *p*-coumarate 3-hydroxylase (C3H); 4-coumarate-CoA ligase (4CL); and cinnamate 4-hydroxylase (C4H) (Chapter 2; Table 2.2) for amplification and sequencing of the parent lines and then of the mapping population.

These genes were chosen as they are activated early on in the polyphenol biosynthetic pathway. Phenylalanine Ammonia-Lyase is involved in the biosynthesis of cinnamic acid, found upstream in the pathway. Cinnamate 4-hydroxylase and 4-coumarate-CoA ligase are synthesised just upstream of an important branch-point in the pathway, leading to a number of different phenolic acids, such as the *p*-coumaroyl shikimic or quinic acids and the caffeic and ferulic acids. One step further downstream, *p*-coumarate 3-hydroxylase is involved in the formation of caffeoyl shikimic acid and caffeoyl quinic acid (Fig 6.1).

The most abundant phenols derive from phenylalanine via the elimination of an ammonia molecule to form the cinnamic acid. This reaction is catalysed by Phenylalanine Ammonia Lyase (PAL). The point of control is the initiation of transcription of mRNA encoding for PAL which in turn stimulates the synthesis of phenolic compounds. However, it is a complex process: as there are multiple PAL encoding genes some of which are expressed only in specific tissues or under certain environmental conditions

Sets of forward and reverse primers were designed for: Phenylalanine Ammonia-Lyase (PAL 1, 2, 3 and 4), shikimate *p*-hydroxycinnamoyl transferase (HCT), *p*-hydroxycinnamoyl-CoA: quinate (HCT), *p*-coumarate 3-

hydroxylase and (C3H), 4-coumarate-CoA ligase (4CL) and cinnamate 4-hydroxylase (C4H) by searching for 21-24bp in the most homologous regions, with a reasonable G-C content (40-50%) and for a T_m ≥60°. The online software, Primer3, was used for oligo design and primers were then ordered from Invitrogen Ltd.

All primers for phenylalanine ammonia-lyase, hydroxycynnamoyl transferase and shikimate *p*-hydroxycinnamoyl transferase, *p*-hydroxycinnamoyl-CoA: quinate amplified well (Fig 6.2). Quantification of the amplified products was as follows: PAL1, HC/BT2 (and 40S16) c. 200bp; PAL2 c. 400bp; PAL4 and HCT2 c. 600bp; and PAL3 c. 800bp.

Three extra primer sets were also designed for the amplification of p-hydroxycinnamoyl-CoA quinate and shikimate p-hydroxycinnamoyl transferase (C3H, C4H and 4CL). The investigation of these gene fragments would be an interesting area for future study (data not shown).

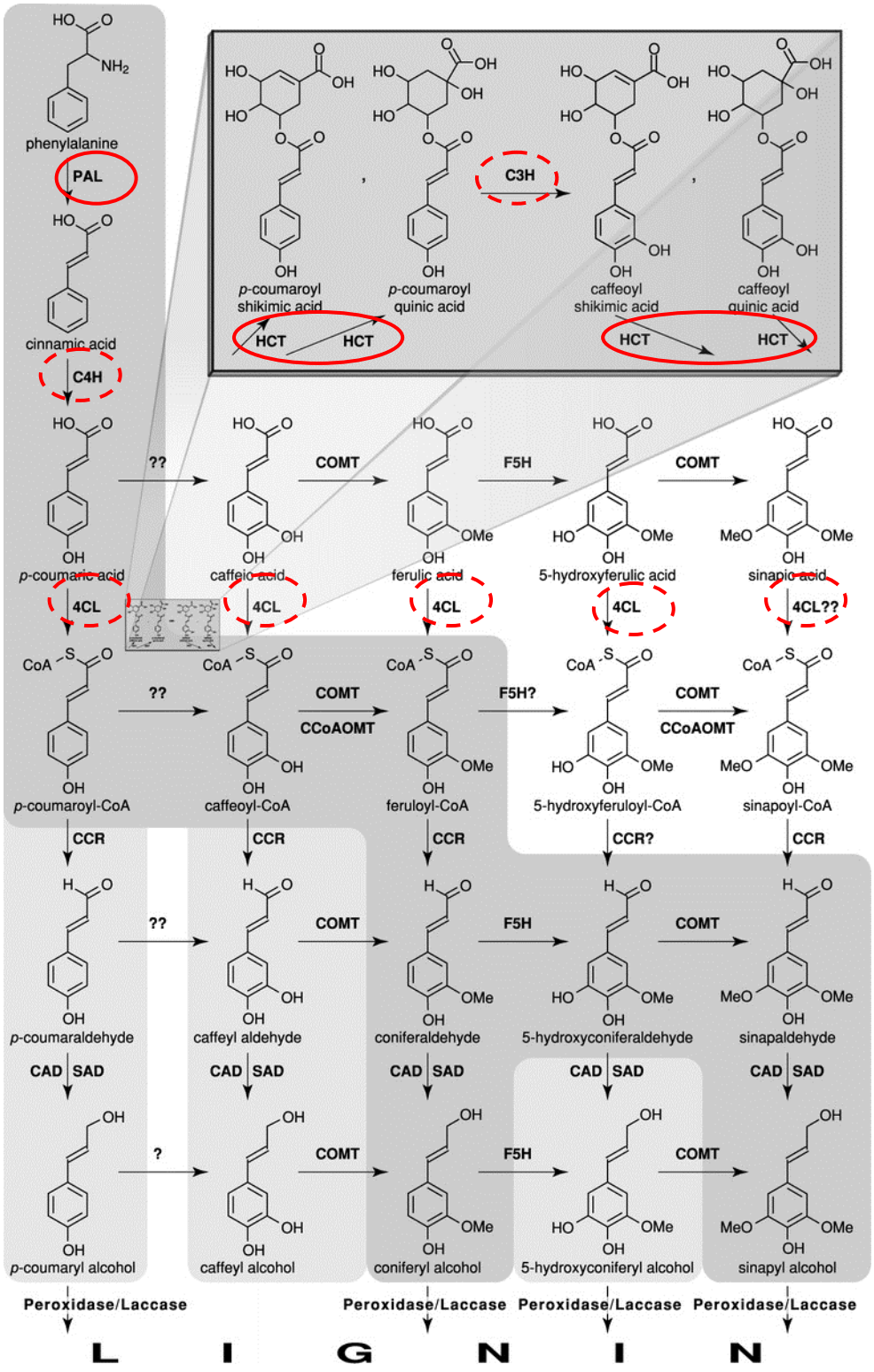
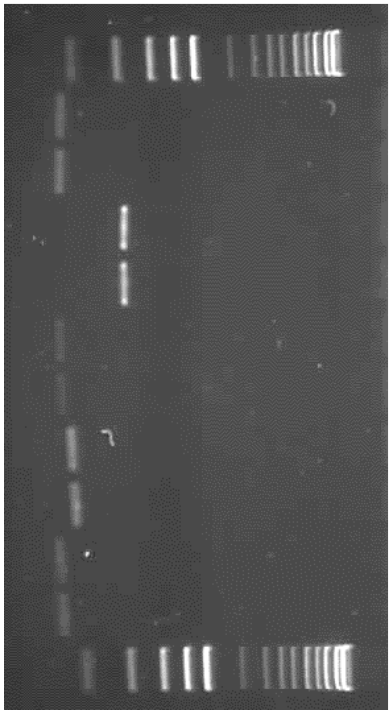


Fig 6.1 the polyphenolic biosynthetic pathway. The enzymes Phenylalanine Ammonia-Lyase (PAL 1, 2, 3 and 4), shikimate *p*-hydroxycinnamoyl transferase (HCT), *p*-hydroxycinnamoyl-CoA: quinate (HCT), *p*-coumarate 3-hydroxylase and (C3H), 4-coumarate-CoA ligase (4CL) and cinnamate 4-hydroxylase (C4H) are circled in red. Fig reproduced from Boerjan et al. (2003).



Size standards

PAL1 + *L. sativa*

PAL1 + *L. serriola*

PAL2 + *L. sativa*

PAL2 + *L. serriola*

HC/BT2 + *L. sativa*

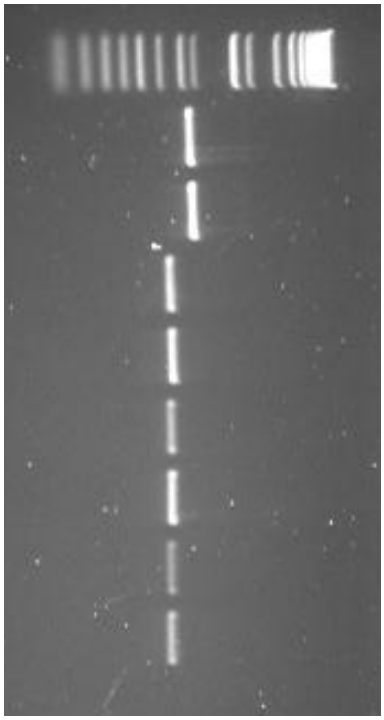
HC/BT2 + *L. serriola*

HCT + *L. sativa*

HCT + *L. serriola*

40S16 + *L. sativa*

40S16 + *L. serriola*



Size standards

PAL3 + *L. sativa*

PAL3 + *L. serriola*

PAL4 + *L. sativa*

PAL4 + *L. serriola*

HCT2 + *L. sativa*

HCT2 + *L. serriola*

HCT3 + *L. sativa*

HCT3 + *L. serriola*

Fig 6.2 Amplification of gene fragments involved in the biosynthesis of polyphenol compounds from the parent lines. A number of different primer sets were designed and tried out for phenylalanine ammonia lyase (PALs 1, 2, 3 and 4); hydroxycinnamoyl transferase (HC/BT2) (a); and p-hydroxicinnamoyl Co-A: quinate shikimate/p-hydroxycinnamoyl transferase (HCT, HCT2, HCT3). Amplification was carried out on the parent lines, *Lactuca sativa* and *L. serriola*.

6.3 SNP analysis for polyphenolic biosynthetic pathways in the parent lines

Amplified products were submitted to the Dundee Sequencing Service (http://www.dnaseq.co.uk/) for Single Nucleotide Polymorphism (SNP) detection. Sequences were visualised using the Chromas Pro (version 1.41) software and sequence alignment between the two parent lines was carried out manually. Unfortunately, after numerous attempts at sequencing the parent line gene fragments with each of the amplicons designed, the only definite SNPs detected were for the gene fragments amplified using HCT3 (Fig 6.3) and PAL4 primer sets (Fig 6.4) as the other primer sets did not produce sequences of high enough quality. SNPs were detected and confirmed by reverse complement checking of the chromatogram results of the amplified forward and reverse strands (CRC check). Eight SNPs were detected for the p-hydroxicinnamoyl Co-A: quinate shikimate/p-hydroxycinnamoyl transferase (HCT) gene fragment, and four for the phenylalanine ammonia-lyase (PAL) gene fragment. SNPs were detected and checked manually by reverse complementing (CRC check) of the resulting chromatogram. Chromatogram sections are shown for relevant SNPs.

(A)

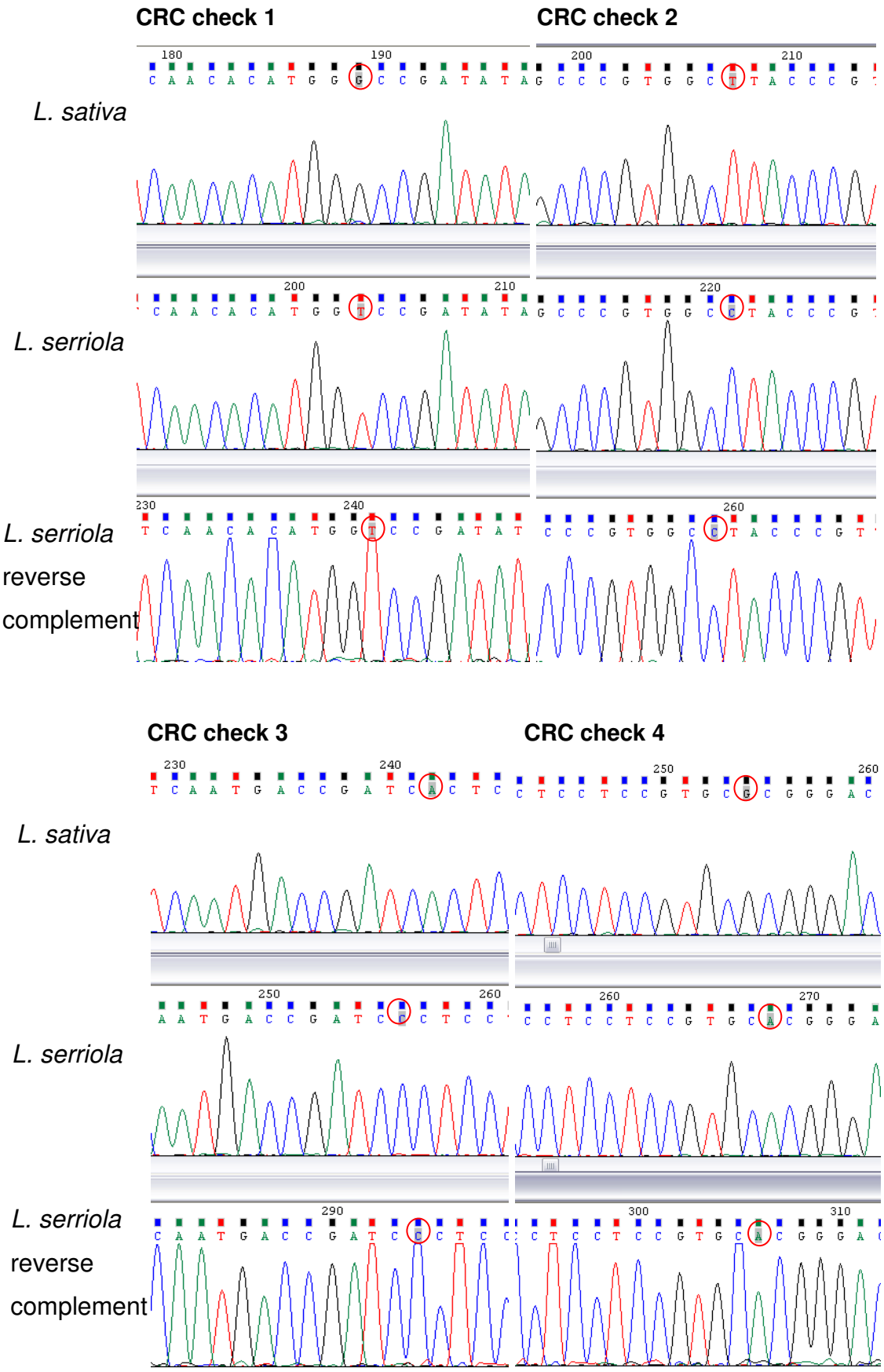
HCT3

<u>αα sequence:</u>

YPLVITQVTRFKCGGVSLGCGVHHTLSDGFSSLHFINTWADIARGLPVAIPPFNDRSLLRARDPPTPMFD

HVEYHPPPSLITPPENHKSPASTTILRLTLDQINDLKSKGKGDGSVYHX

(B)


CRC check 1
CRC check 2
L. sativa
L. serriola
L. serriola reverse complement
CRC check 3
CRC check 4
L. sativa
L. serriola
L. serriola reverse complement

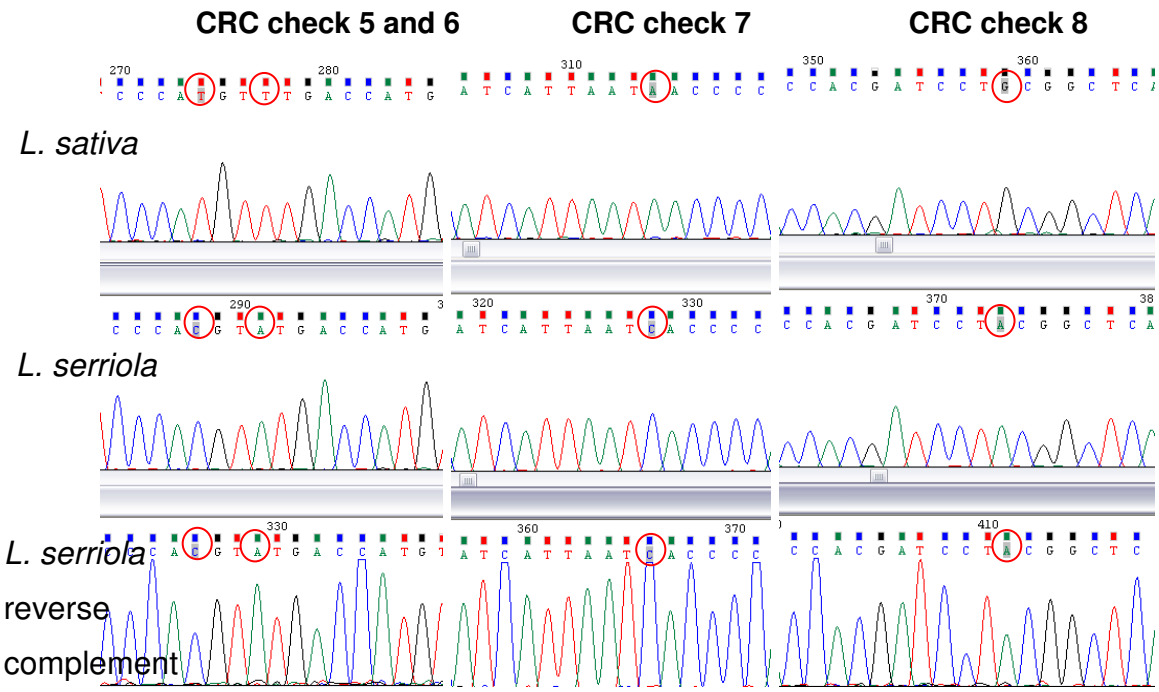


Fig 6.3 Sequence comparisons and SNP detection of the parent line haplotypes for HCT polyphenol compounds. (A) p-hydroxicinnamoyl Co-A: quinate shikimate/p-hydroxycinnamoyl transferase (HCT) gene fragments from the parent lines were aligned and compared for SNP detection. *L. sativa* sequenced using the forward (F) primer is shown in black; below is the *L. serriola* F strand; below that is the *L. serriola* strand sequenced with the reverse (R) primer. Primer designs for subsequent single base extensions of the RILs are shown below the parent line sequences. **(B)** SNPs were detected and checked manually by reverse complementing (CRC check) of the resulting chromatogram. The sequence of the p-hydroxicinnamoyl Co-A: quinate shikimate/p-hydroxycinnamoyl transferase amino acid sequence encoded by the gene fragment is also displayed. Primer design for Single Base Extension (SBE) of the RILs are shown.

(A)

PAL4

L. sativa R CCGACAACAATGCAAGCACAT
L. serriola R CCGACAACAATGCGAGGACAT
L. serriola F CCGACAACAATGCAAGGACAT

αα sequence:

*LRRGPWKPCQIRRRCH*QVLDLLSFEEDHGSHARSDGGAINKC*TFFRWVRLLWSWGGR*EVRRCK

TMGQGRRRR*LCLGGVSKG*RWC*GT*LWLPRSQNGCRSNPGGGC*FLRAWX

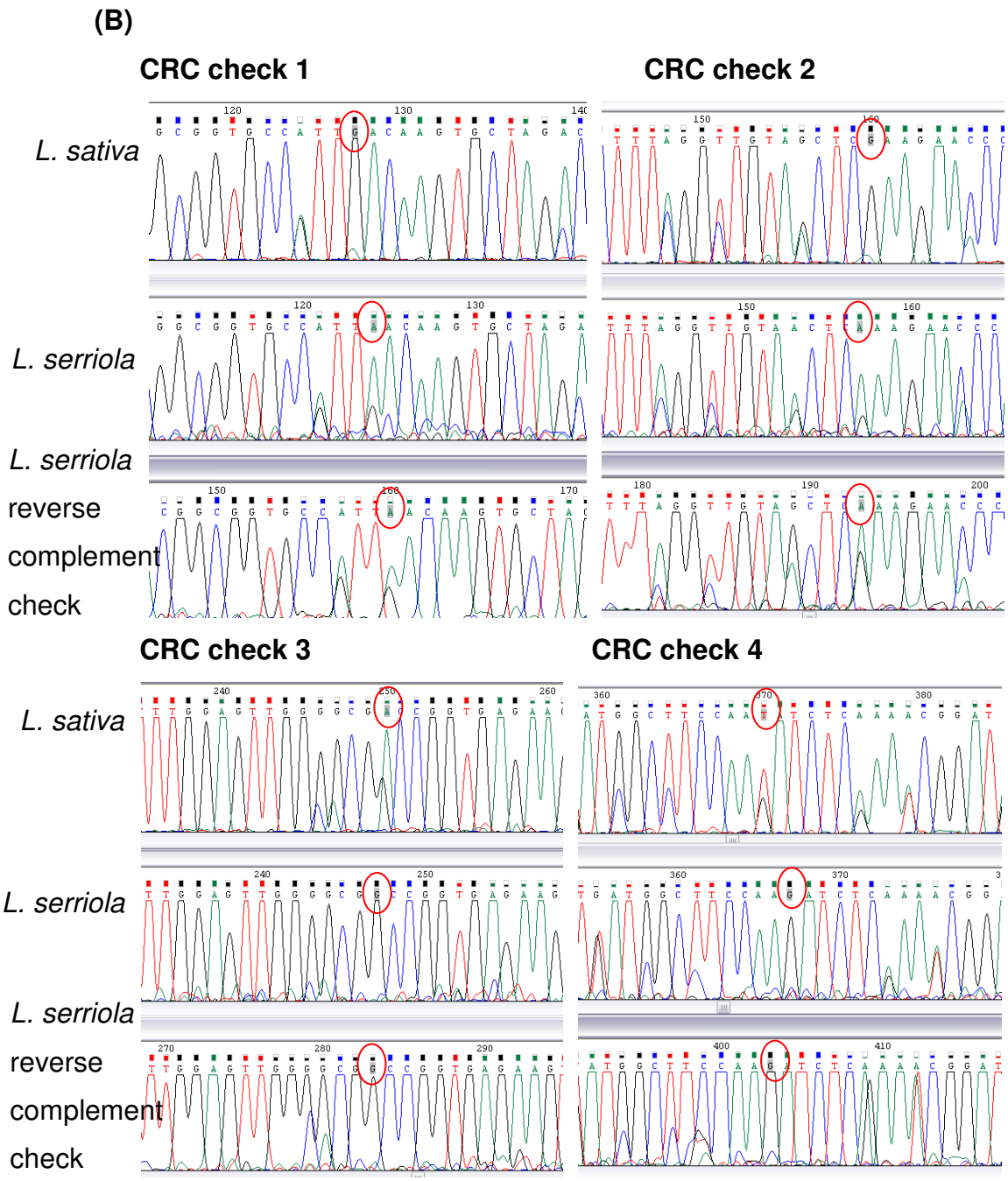


Fig 6.4 Sequence comparisons and SNP detection of the parent line haplotypes for PAL polyphenol compounds. (A) Phenylalanine ammonia-lyase (PAL) gene fragments from the parent lines were aligned and compared for SNP detection. The *L. sativa* sequence was carried out using the reverse (R) primer and is shown above the other sequences; below is the *L. serriola* R strand; below that is the *L. serriola* strand sequenced with the forward (F) primer. Primer designs for subsequent single base extensions of the RILs are shown above the aligned sequences. The sequence of the phenylalanine ammonia-lyase amino acid sequence encoded by the gene fragment is also displayed. Primer design for Single Base Extension (SBE) of the RILs are also shown.

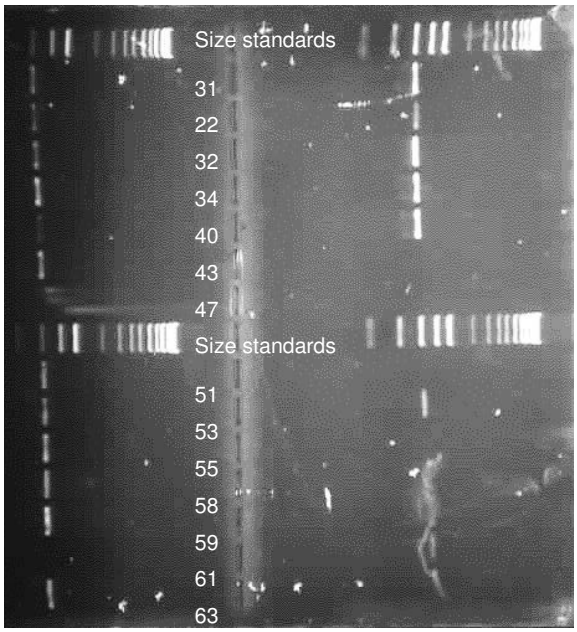
6.4 Amplification and sequencing of polyphenol biosynthesis genes in the Recombinant Inbred Lines

SNPs found in the parent lines were subsequently investigated in the Recombinant Inbred Lines (RILs). Successful amplification of the p-hydroxycinnamoyl Co-A: quinate shikimate/p-hydroxycinnamoyl transferase and the phenylalanine ammonia-lyase gene fragments were carried out using the same PAL4 and HCT3 primer sets as for the parent lines (Fig 6.5).

cDNA from the RILs was used to investigate polymorphisms in the mapping population. cDNA from all the RILs was amplified and the amounts were quantified using a Nanodrop. Oligos were designed for Single Base Extension (SBE) (Table 6.1) based on the results from the SNP analyses carried out on the parent lines. Sequencing and alignment of each line were carried out at the John Innes Genome Centre, using VectorNTI Advance software (by David Baker) (Fig 6.6 and 6.7). The resulting sequences were compared to the parent line sequences to identify the origins of the SNPs. For the HCT gene, the same polymorphisms found in the parent lines were identified in the RILs. Unfortunately, the parent line SNPs for PAL4 were not identified in the RIL sequences as they could not be sequenced.

Furthermore, evaluation of the antioxidant extreme lines and the discovered SNPs, showed that there is no clear association between these high or low lines and the parental nucleotide for this gene fragment. The high antioxidant level lines 99, 20, and 11 shared the same SNPs as *L. sativa*, whilst the high antioxidant lines 63 and 112 shared the same polymorphism as *L. serriola*. The low antioxidant lines 107 and 80 shared the same SNP as *L. sativa* and line 89 the same as *L. serriola*.

(A)



Size standards
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X
Size standards
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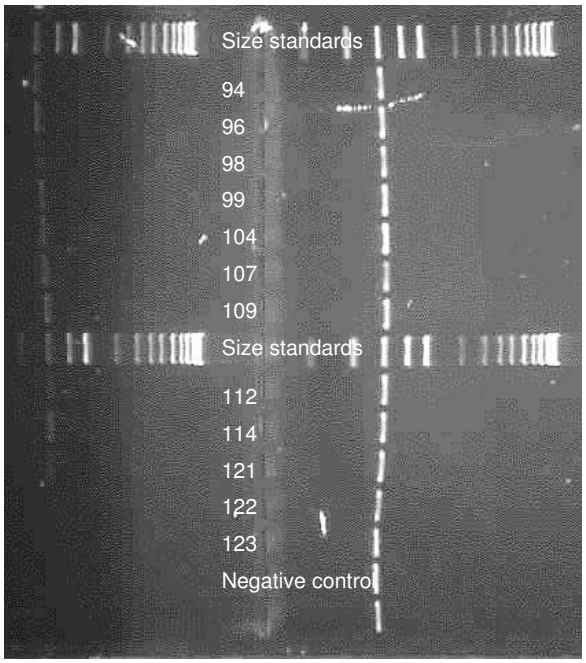
20
21

(B)



Size standards
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Size standards
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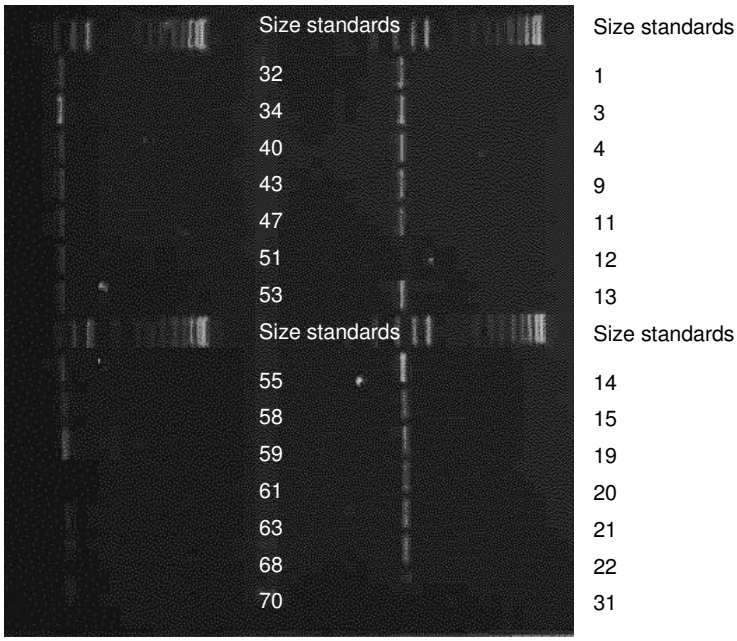
93

(C)



(D)

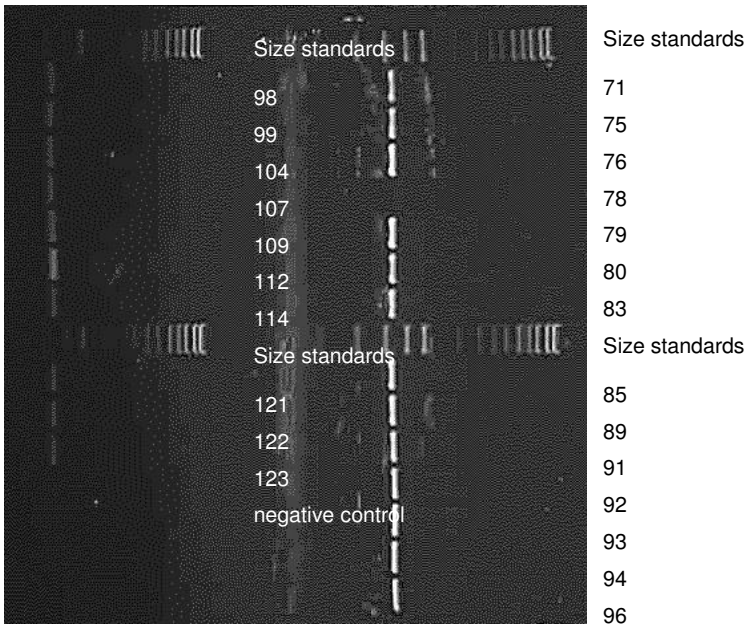
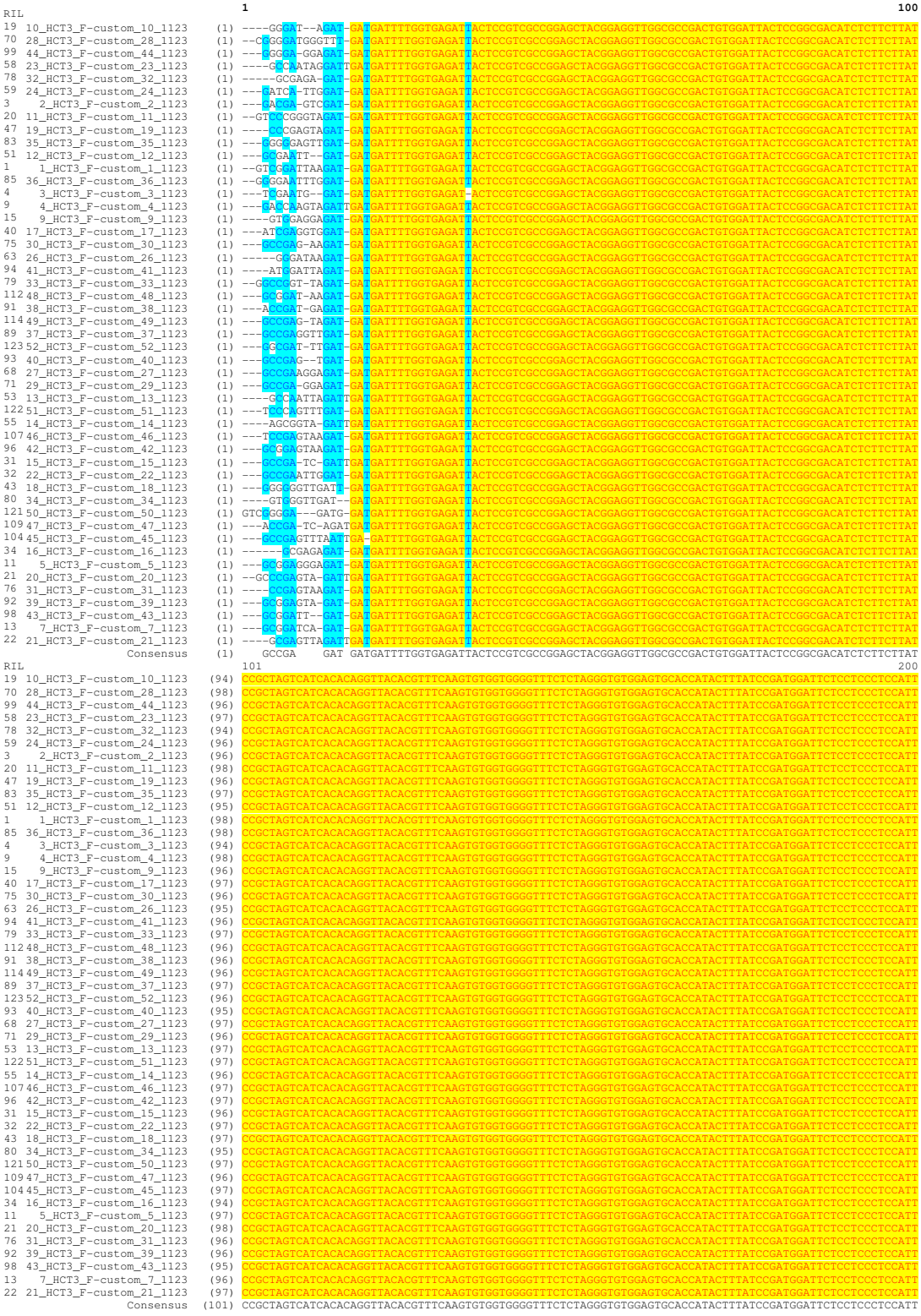


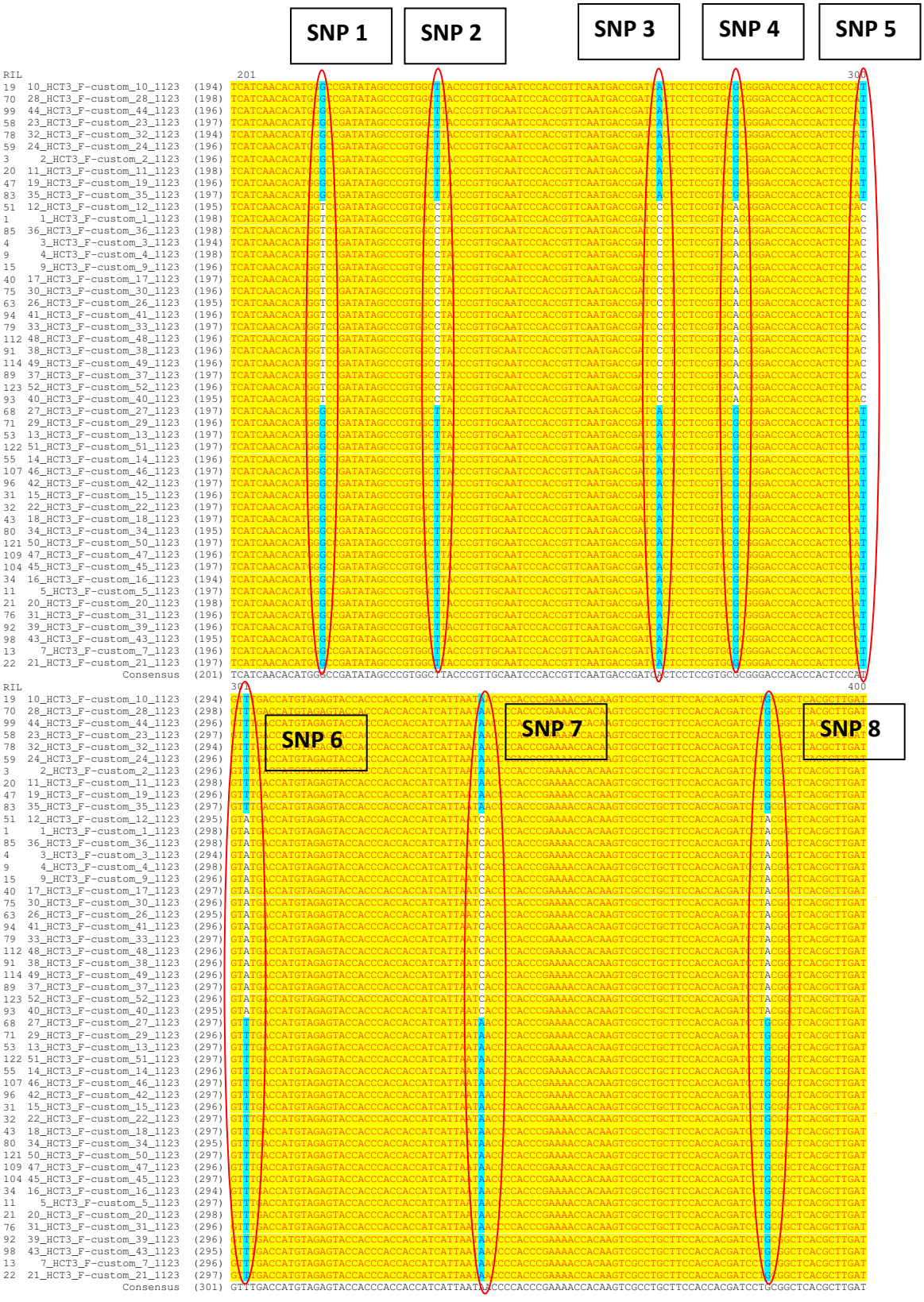
Fig 6.5 Amplification of gene fragments involved in the biosynthesis of polyphenol compounds from the mapping population cDNA. HCT3 oligos were used to amplify RIL cDNA fragments of p-hydroxicinnamoyl Co-A: quinate shikimate/p-hydroxycinnamoyl transferase **(A)** and **(B)** and PAL4 oligos to amplify phenylalanine ammonia lyase gene fragments **(C)** and **(D)**.

Table 6.1 Primer design for Single Base Extension of the RILs. *Oligos were designed for the extension of p-hydroxicinnamoyl Co-A: quinate shikimate/p-hydroxycinnamoyl transferase (HCT3) and phenylalanine ammonia-lyase (PAL4) gene fragments in the RILs following the detection of SNPs in the parent lines.*

HCT3	**SNP1**	$(T)_4$-AAGAGGAGGGAGGTAAAGTAGTTGTGTACC **T_m 62°C;** (G/T)30bp (w/T-tail 34); MW: 9425 Daltons; 46.67%GC
	SNP2	$(T)_8$-GGCTATATCGGGCACCG **T_m 52°C** (T/C)17bp(w/T-tail 25) MW: 5212 Daltons; 64.71%GC
	SNP3	$(T)_{12}$-AACGTTAGGGTGGCAAGTTACTGGCTAG **T_m 61°C** (A/C)28bp(w/T-tail 40) MW: 8710 Daltons; 50%GC
	SNP4	$(T)_{16}$-GCCCTGGGTGGGTGAGGGT **T_m 60°C** (T/C)19bp (NB: check 6 is only another 3bp ahead) (w/T-tail 35) MW: 5958 Daltons; 73.68%GC
	SNP5,6,7	$(T)_{20}$-CATCTCATGGTGGGTGGTGGTAGTAATTA **T_m 60°C** (A/C)29bp(w/T-tail 49) MW: 9020 Daltons; 44.83%GC
	SNP8	$(T)_{24}$-CGGACGAAGGTGGTGCTAGGA **T_m 58°C** (G/A)21bp(w/T-tail 45) MW: 6577 Daltons; 61.9%GC
PAL 4	**SNP1**	$(T)_{28}$-CTAGGCTGCCGCCACGGTAA **T_m 58°C** (G/A)20bp(w/T-tail 48) MW: 6104 Daltons; 65%GC
	SNP2	$(T)_{32}$-CGATCTGGAAGAAATCCAACATCGAG **T_m 58°C** (G/A)26bp(w/T-tail 58) MW: 6169 Daltons; 45%GC
	SNP3	$(T)_{36}$-CGAAACCACAACCCCGC **T_m 52°C** (A/G)17bp(w/T-tail 53) MW: 5079 Daltons; 64.71%GC
	SNP4	$(T)_{40}$-CAACTCCTTGAACCACTACCGAAGGTT **T_m 60°C** (T/G)27bp(w/T-tail 67) MW: 8189 Daltons; 48.15%GC

HCT3 alignment results




SNP 1
SNP 2
SNP 3
SNP 4
SNP 5
SNP 6
SNP 7
SNP 8

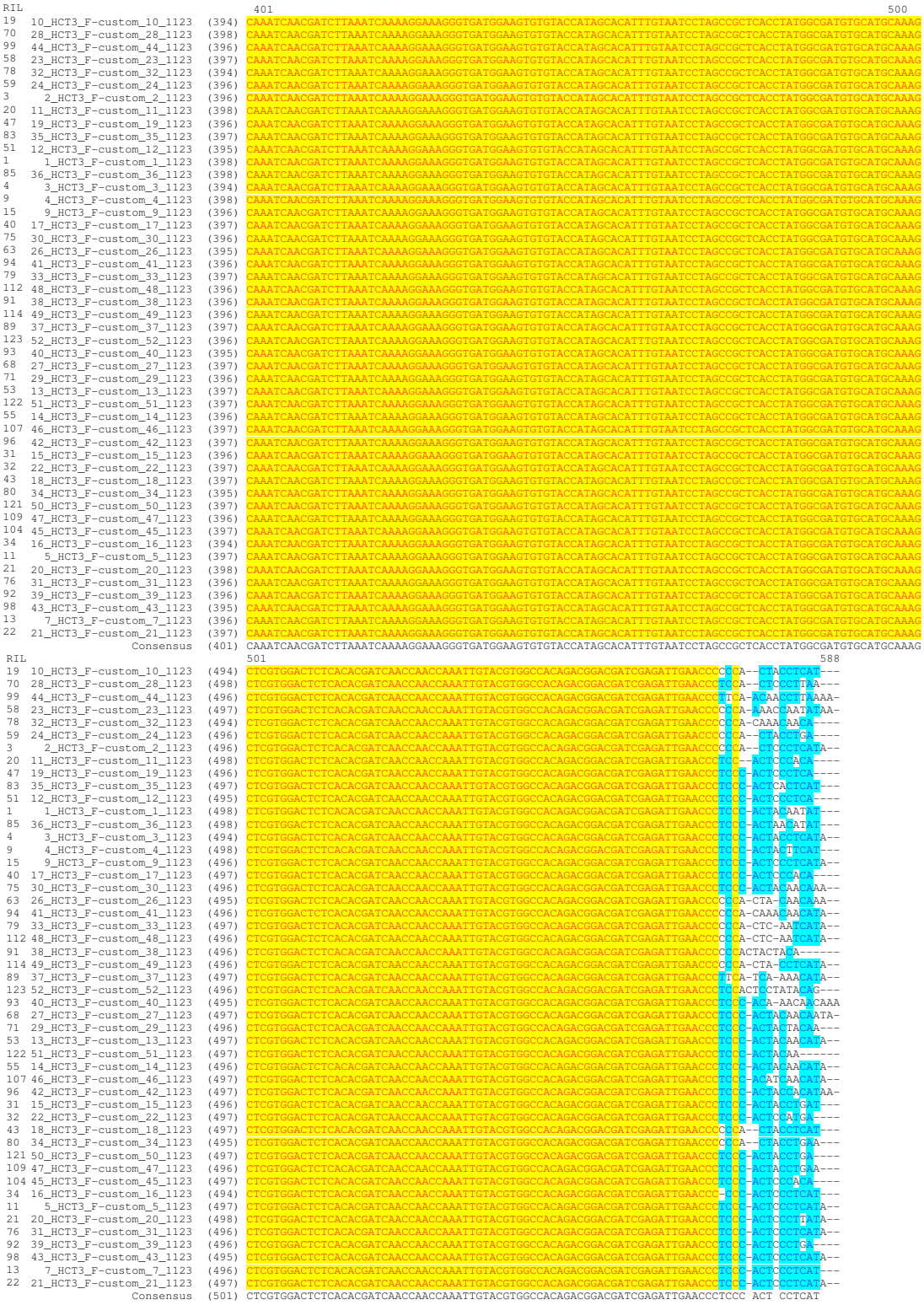
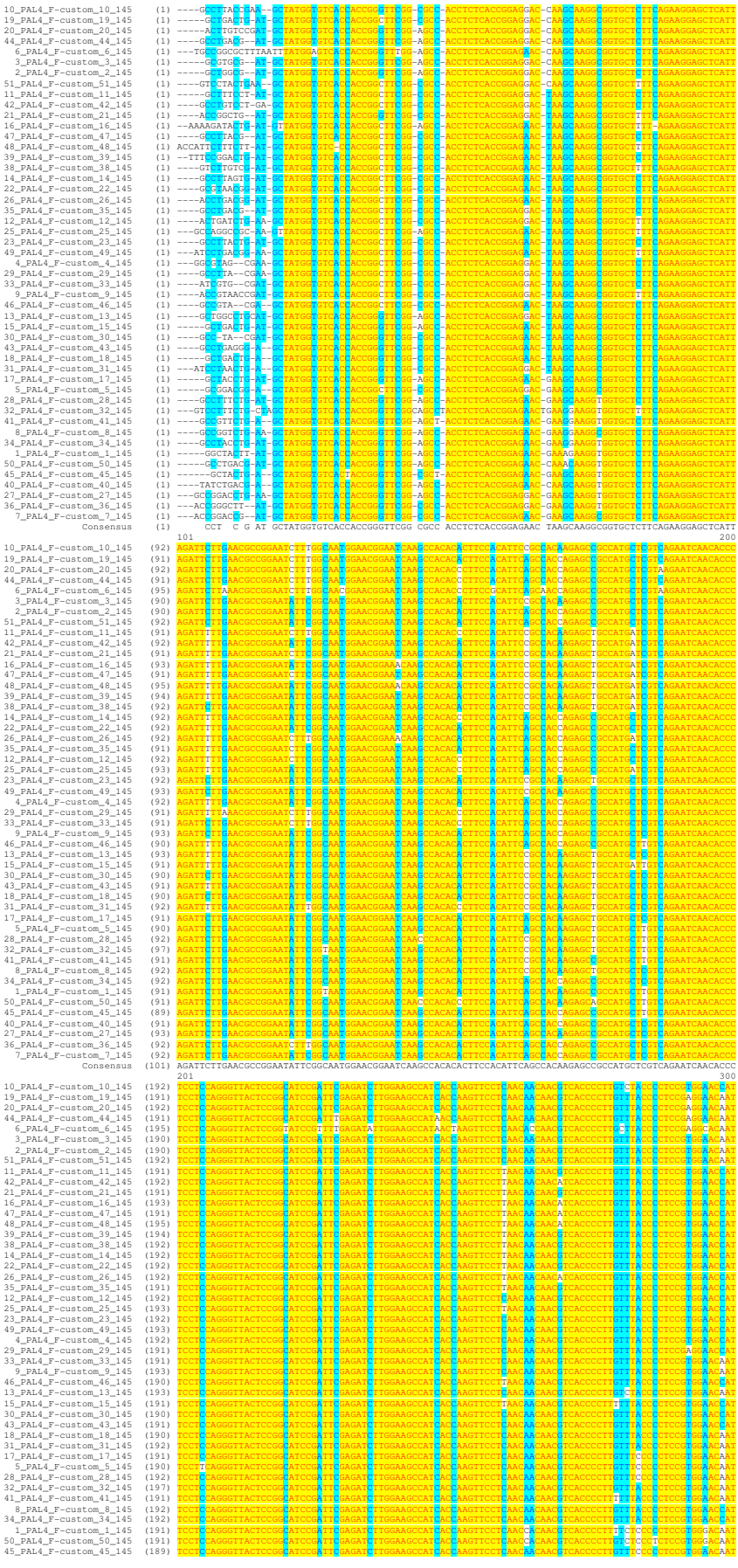
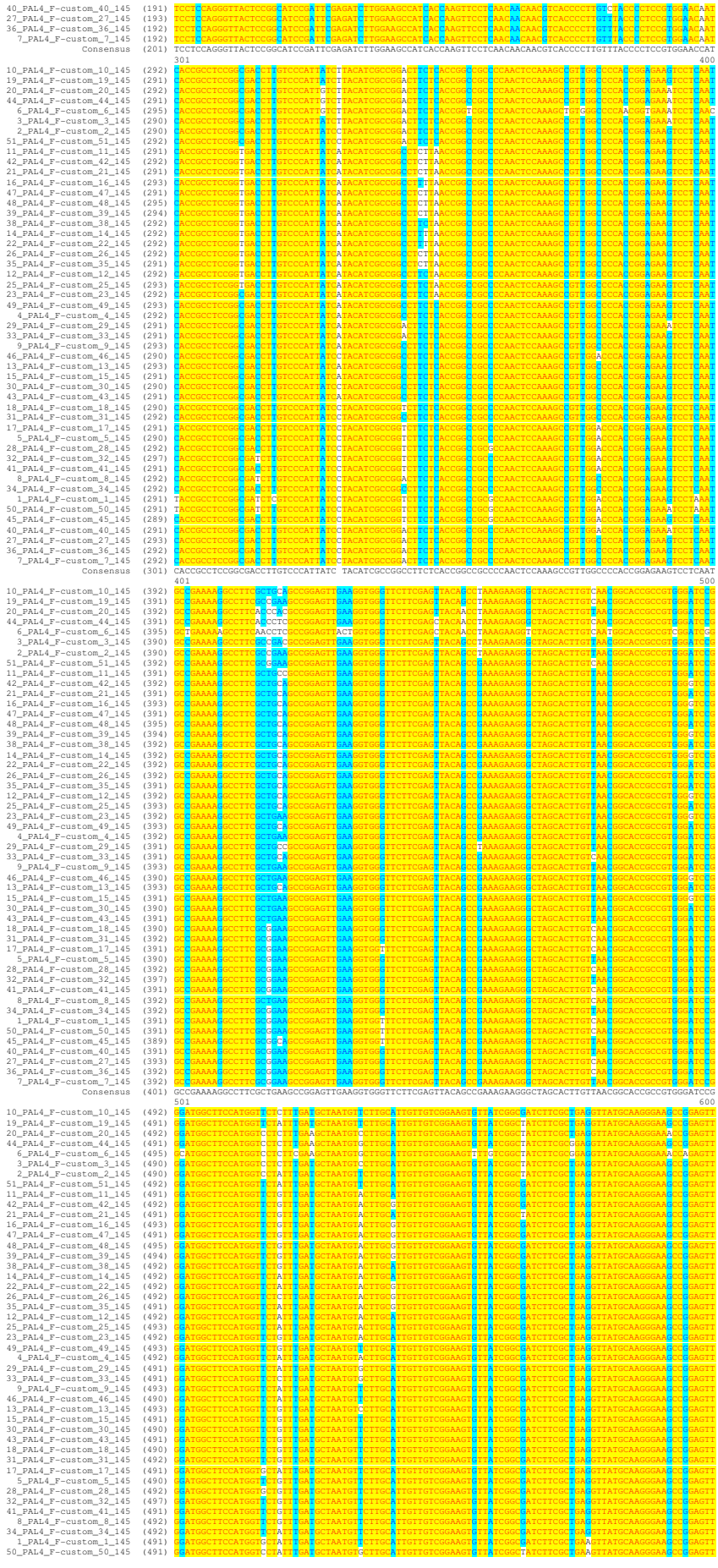


Fig 6.6 Sequence alignment for the HCT gene fragments from the Recombinant Inbred Lines. RIL gene fragments encoding p-hydroxicinnamoyl Co-A: quinate shikimate/p-hydroxycinnamoyl transferase (HCT) were sequenced and aligned for the detection of SNPs.

PAL4 alignment results





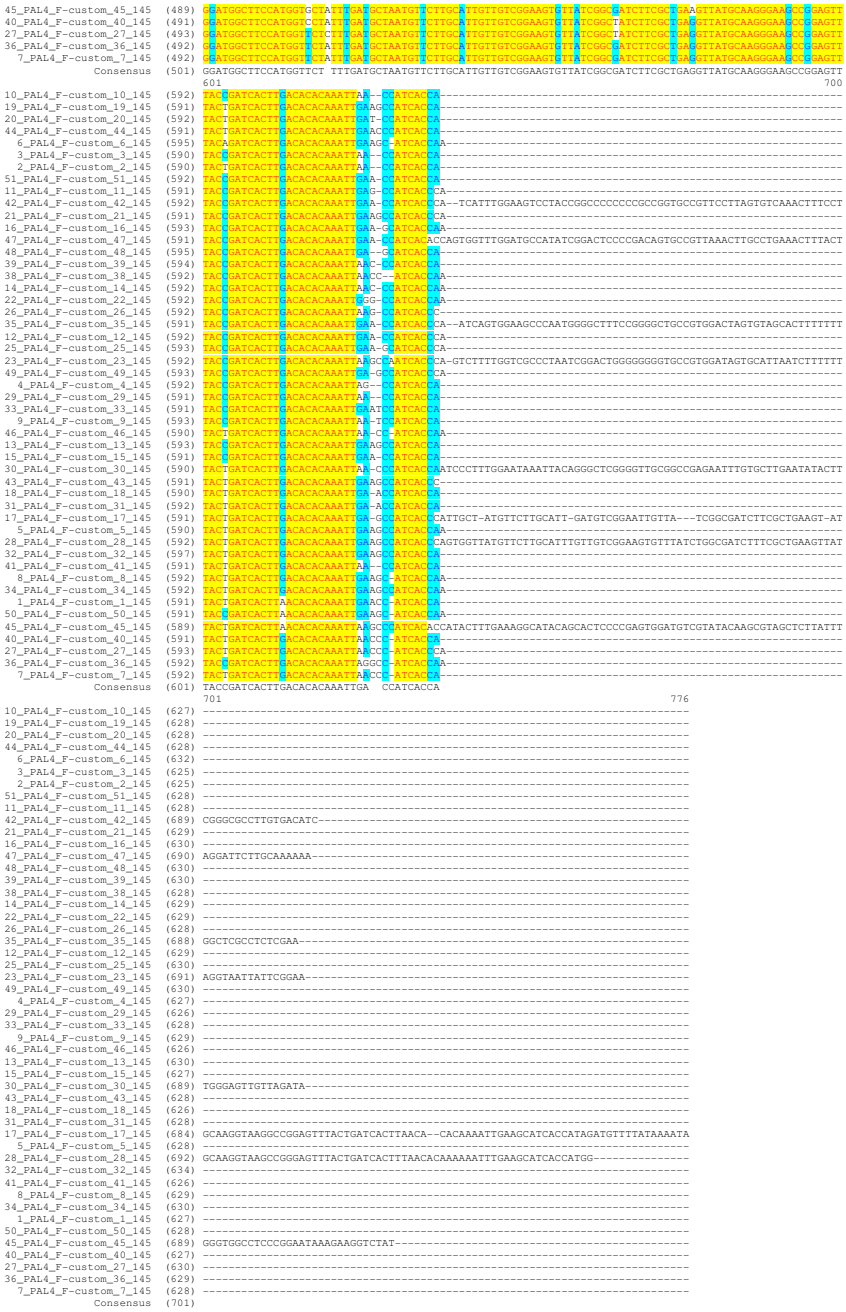


Fig 6.7 Sequence alignment for the PAL4 gene fragments from the Recombinant Inbred Lines. RIL gene fragments encoding phenylalanine ammonia-lyase (PAL) were sequenced and aligned for the detection of SNPs.

6.5 Expression analysis of polyphenol genes

Real Time PCR was carried out on *L. sativa* and *L. serriola* genes encoding the enzymes Phenylalanine Ammonia-Lyase (PAL 1, 2, 3 and 4), hydroxycinnamoyl transferase (HC/BT), *p*-hydroxycinnamoyl-CoA: quinate (HCT). Real time PCR analysis was conducted according to Pfaffl's method to quantitate differences in mRNA expression between the parent lines' production of enzymes involved in the polyphenol biosynthesis (Pfaffl 2001). Expression levels in *L. sativa* were considered as the control and *L. serriola* as the treatment to investigate the relative expression levels of the genes encoding these enzymes. No significant difference in the expression of the selected genes between the two parent lines was found.

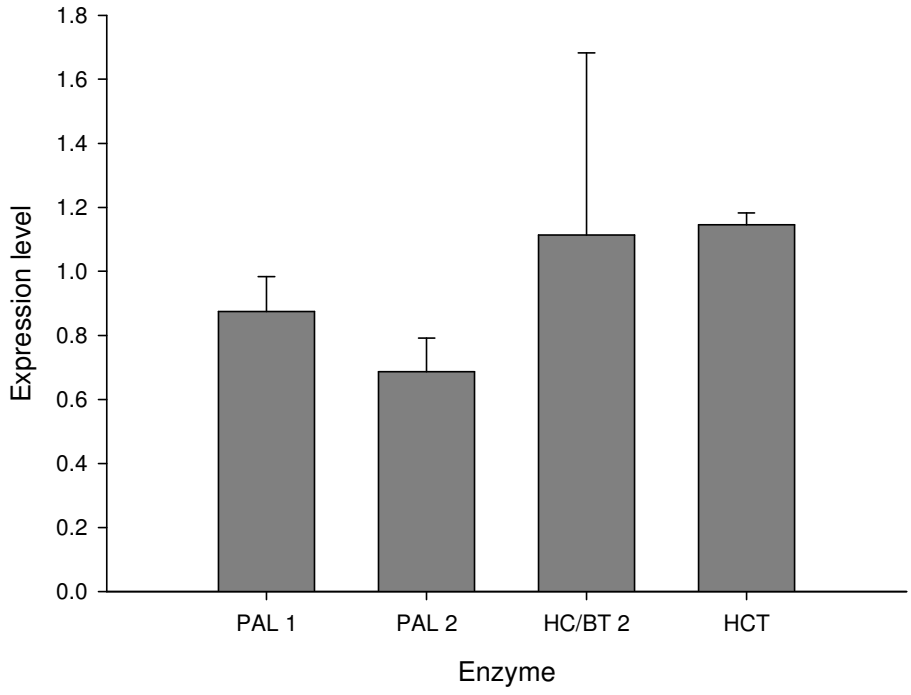


Fig 6.8 Expression levels of polyphenol enzymes in *L. serriola* and in *L. sativa*. Expression levels of Phenylalanine Ammonia-Lyase (PAL 1 and 2), hydroxycinnamoyl transferase (HC/BT2), *p*-hydroxycinnamoyl-CoA: quinate (HCT) were investigated through real time PCR.

6.6 Amplification of carotenoid genes in the parent lines

Candidate genes involved in the carotenoid biosynthetic pathway were chosen based on the literature (Cunningham & Gantt 1998;Grotewold 2006). These were Lycopene ε-cyclase, a gene involved in the cyclisation of lycopene, as this represents a branch point in the pathway leading to either lutein or astaxanthin, two carotenoid end products; and β-carotene hydroxylase, an enzyme involved in the biosynthesis of the precursors to both lutein and zeaxanthyn (Fig 6.9).

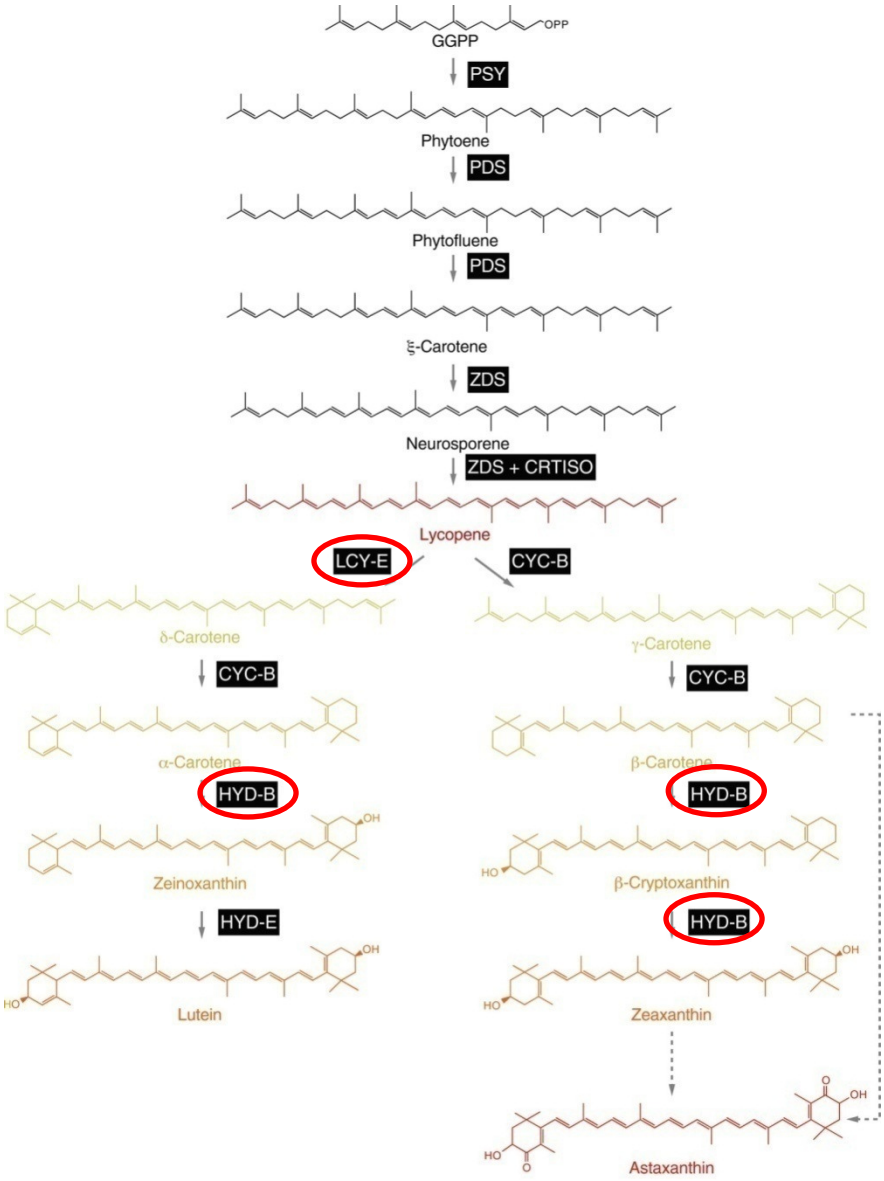


Fig 6.9 Biosynthetic pathway of some major carotenoid pigments (reproduced from Grotewold, 2006). Lycopene represents an important branch point in the carotenoid biosynthetic pathway, leading to either α- carotene and lutein end products or to β- carotene and the xanthins.

EST (Expressed Sequence Tag) sequences for these genes were retrieved through the UC Davis web site: http://cgdb.ucdavis.edu/sitemap.html.

The associated EST contig (QG_CA_contig1411) for lycopene ε-cyclase, formed by the alignment of *L. sativa* and *L. serriola* ESTs (Fig 6.10), was aligned to the Arabidopsis genomic sequence for the same gene (from http://www.arabidopsis.org/blast/index.jsp) to find the possible locations of introns using http://www.abi.ac.uk/emboss/align/.

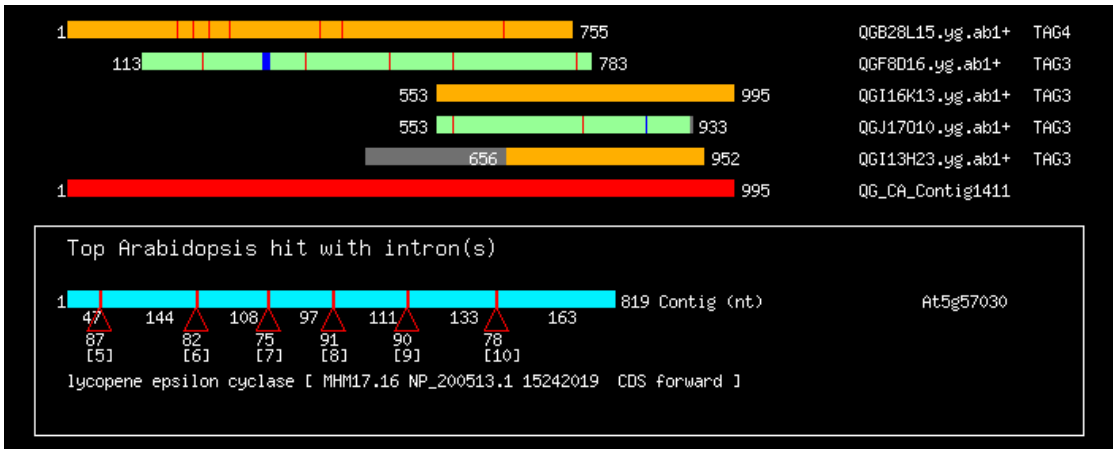


Fig 6.10 Samples of EST contigs for Lycopene ε-cyclase in *L. sativa* and *L. serriola*. Lettuce ESTs for this gene were retrieved from the UC Davies website for their Compositae Genome Project Database (http://cgpdb.ucdavis.edu/sitemap.html). Green bars represent the *L. serriola* ESTs, orange bars represent *L. sativa*, the red bar represents the composite of all EST information and the blue bar represents the alignments with Arabidopsis genomic sequence for lycopene ε-cyclase (Fig reproduced from the CGPDB website: http://cgpdb.ucdavis.edu/ and http://compgenomics.ucdavis.edu/).

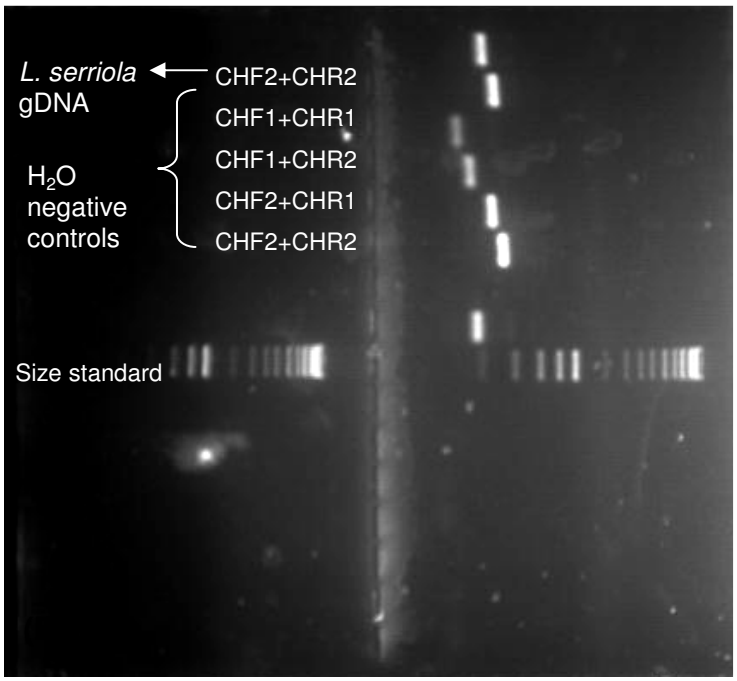
The EST contig database was searched for β-carotene hydroxylase using the same procedure described above for the Lycopene ε-cyclase. The resulting contig sequence (QG_CA_contig7453) was aligned to the Arabidopsis genomic sequence for the same gene. Two sets of forward and reverse primers were designed for each gene using Primer 3 online software

(http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and ordered from Invitrogen Ltd (Table 2.1).

The four primers were used in different combinations to amplify cDNA synthesised from mRNA extracted from the lettuce parent lines. The results were used to search for SNPs between the parent lines and the amplified cDNA was sent off to be sequenced at the Dundee Sequencing service.

cDNA amplified well for all CH and LC primer sets (amplified fragments ranged between 200bp and 400bp, i.e. amounts ranged between 20 and 40ng). However gDNA fragments did not amplify, possibly due to the presence of introns, therefore only the cDNA was sequenced (Fig 6.11).

(A)



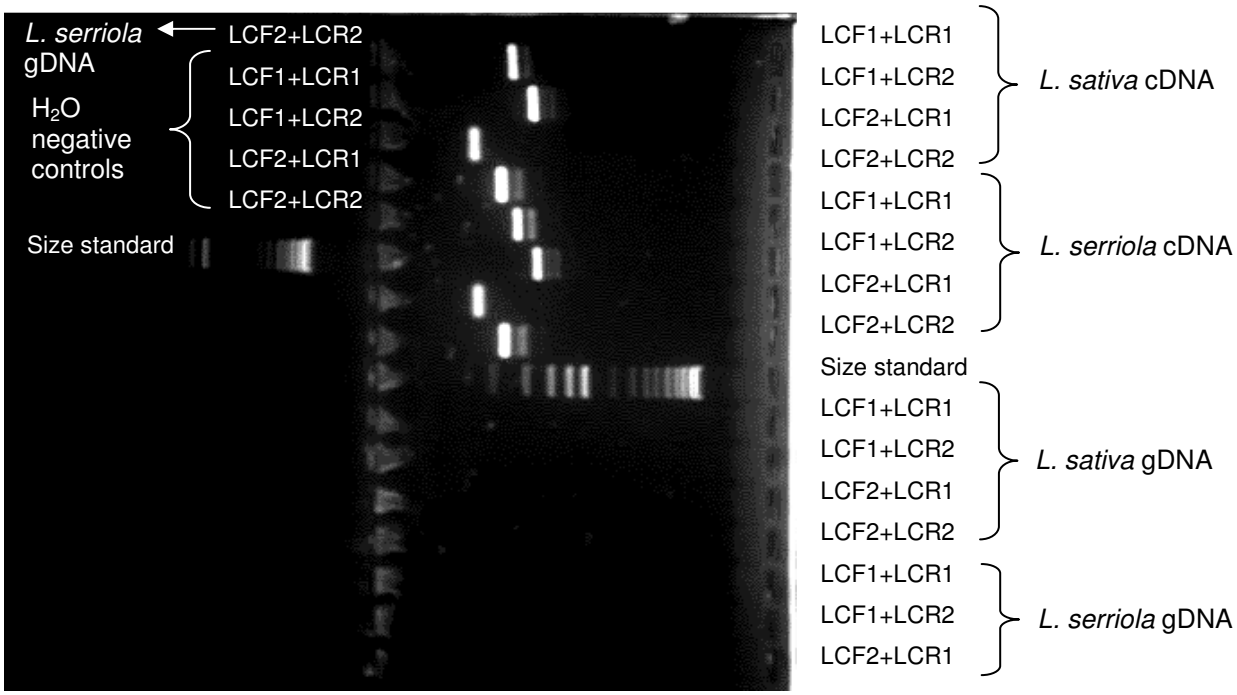


Fig 6.11 Amplification of genes involved in the biosynthesis of carotenoid compounds. Amplified products for β-carotene hydroxylase (CH) **(A)** and lycopene ε-cyclase (LC) in parent line cDNA and gDNA **(B)**.

6.7 SNP analysis for carotenoid biosynthetic pathways in the parent lines

The amplified cDNA products were submitted to the Dundee Sequencing Service (http://www.dnaseq.co.uk/). Sequences were visualised using the Chromas Pro (version 1.41) software and sequence alignment between the two parent lines was carried out manually. Unfortunately, following numerous attempts at sequencing with the various working amplicons designed, no definite and unambiguous SNPs were detected between the parent lines for the selected carotenoid gene fragments (a possible polymorphism was detected close to the CH primer sequence, however it looked more like a wrong base calling of the Chromas analysis) (Fig 6.12).

CH

L. sativa F ATGGCCGCATGAAGCTCTATGGCATGCTTCTTTATGGCACATGCATGAGTCACACCA

L. serriola F ATGGAGATTGGGCGAGATGGCCGCATGAAGCTCTATGGCATGCTTCTTTATGGCACATGCATGAGTCACACCA

TAAACCCCGAGAAGGCCCCTTCGAGCTCAACGACGTGTTCGCGATTATAAACGCCGTTCCGGCGATTGCGTTA

TAAACCCCGAGAAGGCCCCTTCGAGCTCAACGACGTGTTCGCGATTATAAACGCCGTTCCGGCGATTGCGTTA

CTGAACTACGGCTTCTTCCATAAAGGAATATTTCCCGGCCTCTGTTTCGGCGCTGGGCTTGGGATAA

CTGAACTACGGCTTCTTCCATAAAGGAATATTTCCCGGCCTCTGTTTCGGCGCTGGGCTTGGGATAA

CGGTGTTTGGAATGGCGTACATGA (reverse complement to R primer)

CGGTGTTTGGAATGGCGTACATGA

αα sequence:

PHEALWHASLWHMHESHHKPREGPFELNDVFAIINAVPAIALLNYGFFHKGIFPGL
CFGAGLGITVFGMAYMX (EMBOSS Transeq results from EMBL-EBI web site)

Fig 6.12 Sequence alignments and SNP investigation for gene fragments involved in β-carotene hydroxylase (CH) biosynthesis in *L. sativa* and *L. serriola*. The β-carotene hydroxylase amino acid sequence encoded by the gene fragment is also displayed.

6.8 Discussion

The candidate gene approach and SNP genotyping in lettuce studies in the past have mostly focused on the identification of single nucleotide polymorphisms in disease resistance genes, such as the RGC2 gene cluster in *L. serriola* (Van Eck et al. 2008;McHale et al. 2009); dieback resistance genes (*Tvr1*) (Simko et al. 2009) or resistance to corky root (Dufresne et al. 2004). The present study was the first to investigate polymorphisms in genes involved in antioxidant phytonutrients in the Lactuca species.

The aim of this study was to investigate genetic variations linked to functionality in the sequences of genes involved in phytonutrient biosynthesis of the Lactuca species. The Lactuca genome has not yet been fully sequenced, as opposed to other plant species, such as *Arabidopsis thaliana*. Thus, once candidate genes had been selected for the chosen traits, publicly available Expressed Sequence Tags (ESTs) from the Compositae Genome Project (http://compgenomics.ucdavis.edu/) (Heesacker et al. 2008) were chosen that displayed polymorphism between the parent lines and contained sequence similarities to Arabidopsis in order to be able to amplify and sequence sections of the genes of interest.

PCR amplicons were therefore designed to detect Single Nucleotide Polymorphisms (SNPs) between the parent lines and then in the RIL mapping population using oligos designed from the EST sequences, to look for variations that may have a functional consequence, as suggested by Borevitz and Chory (2004). Lee et al state, in fact, that sequencing and analysis of ESTs remain a key implement for the discovery of novel genes and a primary factor in genome annotation (2005).

Amplification of cDNA, rather than gDNA, allowed for the analysis of functional genes, without intron fragments, which meant that the SNPs discovered had a greater potential of being variations that bring a phenotypic difference between the lines. In fact, SNPs within the coding regions can

either be non-synonymous and resulting in an aminoacid sequence change (Sunyaev et al. 1999) or synonymous which does not alter the amino acid sequence but can modify mRNA splicing and still cause phenotypic changes (Richard & Beckmann 1995).

6.8.1 SNPs in the polyphenolic biosynthetic pathway

Previous studies have found strong correlations between plant phenolic content and total antioxidant contents in plants (Nicolle et al. 2004;Schlesier et al. 2002). Nicolle et al have also shown that polyphenols can contribute up to 70% to the total antioxidant potential of lettuce. Notable levels of polyphenolic compounds have been reported in lettuce, comparable to other important sources of these compounds such as onions, apples, tea and broccoli (Llorach et al. 2008). Thus polyphenol genes were selected for SNP detection in the complete mapping population.

A total of 12 SNPs were found for both the p-hydroxicinnamoyl Co-A: quinate shikimate/p-hydroxycinnamoyl transferase (HCT) and phenylalanine ammonia lyase (PAL) gene fragments in the parent lines (Fig 6.3 and 6.4). Eight polymorphisms were detected in the HCT gene fragments amplified, sequenced and aligned, and four polymorphisms were identified for the PAL gene fragments. These genetic variations were then investigated in the RIL mapping population (Fig 6.6 and 6.7). The SNP genotyping of the RILs was carried out successfully for the HCT gene fragment and the same SNPs were identified as in the parent lines. However, after numerous attempts, the results were not as clear-cut for the PAL SNP genotyping of the RILs.

Following the sequence alignment of the mapping population carried out for the amplified HCT gene fragments, it was possible to identify the origin of the polymorphisms in the parent lines and thus of the HCT gene fragment analysed. Unexpectedly, analysis of the antioxidant extreme lines showed that both parents were contributing the analysed gene fragments to the five highest lines for the total antioxidant potential trait analysed previously.

Three of the highest total antioxidant potential lines (99, 20 and 11) presented the same nucleotide variations as the *L. sativa* parent, whilst lines 63 and 112, also in the highest level range, presented the *L. serriola* parent nucleotides for all SNPs identified. Likewise with the lines: 107 and 80 which were the same as *L. sativa*; whilst 89 had the same nucleotide polymorphism as *L. serriola*. There are three possible explanations for these results: a) the nucleotide changes do not determine changes in aminoacid sequences due to genetic code degeneration; b) the nucleotide changes could lead to aminoacid changes but the polypeptide functions remain unmodified, thus the biochemical characteristics remain similar (*synonymous* changes, sometimes also called silent mutations, or if a different polypeptide sequence is produced they would be *nonsynonymous* changes); c) there is a change in aminoacid sequence as well as function but these are not determinant to the overall phenolic antioxidant phenotype.

Further investigations are necessary, such as polyphenol analyses in the complete mapping population, to be able to relate the SNP genotyping results for the HCT gene fragment. Further attempts at sequencing and SNP detection using different PAL oligos as well as the C3H and 4CL primers already designed would also be important steps in furthering the analysis of polyphenol functional genes in Lactuca species. Cloning could also be used as a method for increasing the amounts of amplified products. These results could then be followed by marker assisted selection strategies for the selective increase of polyphenol antioxidants in lettuce cultivars. Therefore, this study provides an initial step for defining the genetic control of HCT in lettuce leading to the development of varieties with enhanced phytonutrient polyphenol antioxidant content. Future analyses would also point at investigating enzymes further down the biosynthetic pathway, closer to the point of synthesis of the compounds of interest.

6.8.2 Expression levels of polyphenol genes of interest

Interestingly, the quantitative measurements carried out using real time PCR found no significant difference in expression of the PAL, HC/BT and HCT genes between the parent lines. This could be due to the environmental conditions at time of harvest, as they may not have been stimulating the production of certain polyphenols which can often be found in greater quantities in the wild parent, such as light irradiation intensity or temperature differences.

6.8.3 SNP investigations in the carotenoid biosynthetic pathway

The selected carotenoid gene fragments were amplified successfully for the parent line cDNA (Fig 6.11). However, unfortunately, following a number of attempts, it was only possible to sequence and align the β-carotene hydroxylase (CH) gene fragment in the parent lines (Fig 6.12), whilst no sequences were attained for the lycopene ε-cyclase (LC) gene fragment. Furthermore, no single nucleotide polymorphisms were detected following sequencing of the CH gene fragments. These genes were therefore not investigated further in the mapping population at this point, as the EST data available for these gene fragments was rather more limited than for the polyphenol genes.

6.9 Conclusions

1. Eight single nucleotide polymorphisms were detected in the p-hydroxicinnamoyl Co-A: quinate shikimate/p-hydroxycinnamoyl transferase (HCT) gene fragments from the parent lines;

2. Four polymorphisms were detected in the phenylalanine ammonia-lyase (PAL) gene fragments from the parent lines;

3. The HCT polymorphisms were also identified in the recombinant inbred lines;

4. Both parent lines were contributing to the gene fragments to the highest and lowest lines.

CHAPTER 7 – GENERAL DISCUSSION AND CONCLUSIONS

Lettuce is a major horticultural crop with a worldwide production of over 21million tons in 2004 and which accounts for $2.06billion in farm value in the USA. In the UK (in 2009) home production marketed 117,3 thousand tonnes and imports in the UK were 155,1 thousand tonnes, with a total supply of 266,7 (DEFRA horticultural statistics 2010). Salad leaves contribute significantly to the recommended intake of fresh fruits and vegetables as lettuce remains one of the preferred vegetable crop species in Europe and the United States.

However, throughout hundreds of years of domestication, many crop species, such as lettuce, have been genetically manipulated with the purpose of increasing favourable traits and decreasing unfavourable traits through artificial selection. In the past these efforts were achieved through traditional agricultural practices without knowledge of which genes were responsible for the selected traits and without knowledge of the phytonutrient contents that differentiate cultivated lettuce varieties from their wild relatives. This selection process, for instance, has occurred in the very widespread and popular green lettuce cultivar iceberg (also known as Salinas or saladin).

The present study aimed to investigate the antioxidant nutritional content of common green lettuce cultivars and of a wild relative to be able to ultimately improve lettuce's nutritional content through the expansion of its gene pool. Firstly, the study focused on placing lettuce cultivars (*Lactuca sativa*) on a scale of antioxidant potential in comparison to other salad leafy crops and to a wild relative (*L. serriola*); antioxidant qualities were investigated further through a breakdown of the individual phytonutrients which compose the total antioxidant content. The decline in antioxidant and phytonutrient content with harvest procedures and during storage were also investigated and a human intervention trial was carried out to relate the storage investigation results to their bioavailability. Lastly, the underlying genotypic differences

were investigated in depth through quantitative genetics (QTL) and genotypic polymorphism (SNP) analyses.

The antioxidant contents of lettuce cultivars, *Lactuca sativa* sp, were firstly compared to other leafy crop varieties commonly consumed raw in salads and sandwiches (chapter 3). Spinach (*Spinacia oleracea,* Emilia cultivar, Pop Vriend, Holland), rocket (*Eruca sativa,* Shamrock Seeds Selection, standard variety), red cos and green cos (both *Lactuca sativa*), were analysed and compared for total antioxidant potential with the intention of placing cultivated lettuce on a nutritional scale in terms of its antioxidant potential. Green leafy varieties, such as green cos and tango, consistently produced lower levels of total antioxidant potential than other varieties of leafy salad crops. Furthermore, the antioxidant content correlated positively with the darkness of the leaf colour. As suggested by Mou et al., studies carried out on lettuce samples showed that β-carotene and lutein were highly correlated with chlorophyll and suggest that carotenoid content could be selected indirectly through chlorophyll or colour measurement (2005). The results reported in this study were in agreement with a number of studies reporting lower antioxidant potential in green lettuce varieties than in numerous other leafy salad crops (DuPont et al. 2000;Liu et al. 2007;Llorach et al. 2008;Proteggente et al. 2002).

The study then focused on tracking changes in antioxidant potential following differences in harvest procedures and establishing their decline with storage. Harvesting is generally carried out in the early hours of the morning so that the leaves are then processed at the factory and ready to be despatched before the end of the day. Previous studies have found that harvesting at the end of the day rather than during early morning hours, correlated positively with the shelf life of salad leaves (Clarkson et al. 2007) and suggested changes in lettuce quality, in terms of weight losses, colour, brightness, decay and freshness, depending on the time of harvest (Moccia et al. 1998). Thus, a similar trial was carried out in this study to investigate whether

harvesting in the evening had an effect on the antioxidant potential of different qualities of salad leaves.

Green cos, red cos, spinach and rocket leaves were harvested at four different times during the span of 24 hours; 01:00, 07:00, 13:00 and 19:00. The time of harvest had a significant impact on the antioxidant potential of fresh red cos leaves. Red cos had significantly higher levels of antioxidants when harvested in the afternoon or in the evening (i.e. at times 13:00 or 19:00). These higher levels of antioxidants could be due to an increase in the pigment levels owing to the light irradiation they receive during the day, as suggested by Oh et al. (2009). The results also showed a significant knock-on effect in rocket and green cos after seven days of storage, where the decline in antioxidant potential for leaves harvested earlier in the morning was significantly greater than for those harvested in the afternoon.

The leaves investigated for the storage study were tango, green cos and lollo rosso. A general tendency was noted where leaf varieties with the higher initial antioxidant levels, such as lollo rosso, lost a large proportion of their antioxidant content soon after harvest, whilst leaves with a lower antioxidant level starting level, such as green cos and tango, kept their status for a few days longer. Vitamin C levels in tango leaves declined rapidly from the first days of storage, whilst carotenoid and chlorophyll contents in *L. sativa,* Salinas cv, and in the wild lettuce, *L. serriola*, remained constant for the first 12 days of storage, or until they reached the senescence stage.

After establishing the antioxidant qualities of green lettuce varieties and their decline with storage time, the bioavailability of lettuce antioxidants in human blood plasma and the difference in bioavailability after consumption of fresh versus stored lettuce was ascertained. Green cos was chosen as the lettuce cultivar to be consumed by the volunteers as it is one of the most commonly consumed varieties, which also showed a reduction in antioxidant levels soon after harvest. The intervention trial results demonstrate that consumption of fresh lettuce significantly increases the total antioxidant

potential found in blood plasma and illustrates how it increases with time after consumption before declining again. A three-fold increase from the antioxidant baseline was noted 90min postprandial before declining again. Interestingly, consumption of lettuce samples stored for one week did not rise significantly in the blood plasma samples. This study is in accordance with Serafini et al who noted that consumption of stored, chopped lettuce did not increase blood antioxidant levels significantly (2002b).

Further work would focus on investigating the individual metabolites present in the blood after fresh lettuce consumption. The bioavailability of carotenoids after consumption of both fresh and stored lettuce would be an interesting study, as carotenoids in lettuce did not decline appreciably for the first 12 days (Chapter 4). The bioavailability of polyphenolic antioxidants, for instance, has been investigated in a number of studies after consumption of other varieties of fruit and vegetables (Manach et al. 2004;Mithen et al. 2000). Studies have shown that polyphenols such as quercetin contribute significantly to the antioxidant bioavailability in blood. The absorption of quercetin glycosides, for instance, the most abundant flavonol in fruit and vegetables, also present in significant amounts in lettuce (Nicolle et al. 2004), have been shown to range between 20-50%, depending on the glucose moiety as an important determinant which enhances absorption (Manach et al. 2004; Hollman 2000). Hollman also reports that flavonols are extensively metabolised as only 1-2% are excreted with an intact flavonoid backbone (only 0.1-1.4% of ingested dietary quercetin was excreted as unchanged quercetin in urine). Interestingly, quercetin glycosides seem to have a range of absorption rates, with peak values being reached between <0.5 to 9 hours, depending on the type of glycoside. Whereas, excretion of the glycosides is slow, the compounds seem to accumulate in blood plasma throughout the day, with elimination half-lives reported to be as long as 24 hours.

Phytochemical composition variations can also often be seen between individuals of the same plant population as well as between populations.

Many of these metabolites play specific roles in allowing adaptation to specific ecological niches. It has been estimated that 25-30% of the genes in *Arabidopsis* encode enzymes of metabolism. This range of chemical properties poses a challenge to the analytical tools both for profiling multiple metabolites in parallel and for quantification of the selected ones (Oksman-Caldentey et al. 2004). One of the key challenges is also finding an optimal balance between accuracy and coverage of metabolite measurements due to the fact that metabolites have very different chemical natures such as temperature, pressure and time, which can influence their extractability in various solvents.

Further bioavailability studies could also investigate different lettuce qualities, such as a red-leaf cultivar, which may have higher antioxidant bioavailability levels. Shorter storage time may also be interesting to explore, as it would give more precise indications on the time point at which the bioavailability of stored lettuce antioxidant levels ceases to be significant.

The morphologies of *L. sativa* cultivars and its close wild relative (and possible ancestor) *L. serriola* (acc. UC96US23) differ considerably which suggested possible inequalities in phytonutrient composition between the two species. Therefore, the antioxidant compounds of the *Lactuca sativa* cultivar Salinas and of *L. serriola* were analysed in depth by comparing their total antioxidant contents and individual phytonutrients (chapter 4). The wild species contained almost three times the amount of total antioxidant content as the cultivar and almost 20% higher levels of carotenoid and chlorophyll contents. Carotenoid contents were analysed further by investigating the predominant compounds present in both lettuce species. In agreement with other studies on several lettuce cultivars, the main carotenoids found in the parent lines were zeaxanthin, lutein and β-carotene (Mou 2005;Nicolle et al. 2004).

A complex trait such as the antioxidant nutritional qualities of leaves is regulated by a number of different genes and should be dissected into a

number of component traits. Comparing phenotypic information with the QTL analysis of the data would make it possible to improve the understanding of the genetic basis of nutrition in lettuce leaves.

In quantitative genetics studies, the parent lines of a mapping population must have differing phenotypes for the traits of interest, as QTL can only be mapped if polymorphism is observed in the segregating population. Total antioxidant content, chlorophylls a and b, and carotenoid traits identified in the parental experiments described in Chapter 4 showed significant inequalities between the parental *L. sativa* and *L. serriola* lines.

The phytonutrient data on the wild and cultivated Lactuca species suggested that the wild relative could contribute genes for higher levels of total antioxidants, carotenoids, and vitamin E, thus increasing the potential to improve the nutritional quality of *L. sativa*. These inequalities between the parent lines prompted the antioxidant phytonutrient investigations in a mapping population of 60 informative ninth generation Recombinant Inbred Lines (RIL) derived from a cross between *L. sativa* and *L. serriola*. From a full mapping population of 113 lines, the 60 most informative lines were selected. These lines, previously selected by using MapPop (Vision et al. 2000) and Genoplayer (www.compgenomics.ucdavies.edu/genoplayer/), had nearly as many recombinations as a population of 90 RILs and were thus as informative.

Total antioxidant and pigment levels were thus investigated in the mapping population and considerable transgressive segregation due to complementary gene action was observed for all of the selected traits. A number of lines produced higher levels of total antioxidant content than both parent lines and almost half of the RILs produced significantly higher pigment levels than the wild parent. These results indicated the existence of genetic variability offered by the wild lettuce which could be exploited for nutritional improvement of the lettuce cultivar Salinas. Following the antioxidant and

pigment analyses of the complete mapping population, it was then possible to utilise the data to map QTL for these traits.

In this study, a total of 16 QTLs were found for the selected traits (Chapter 5). Interestingly, all significant QTL mapped to the same linkage groups: 3, 7 and 9 (except for a QTL on LG 4 in the field grown lines). However, the genes responsible for the total antioxidant trait and those for the pigment traits on each linkage group (LG) derived from opposite parents, indicating an additive effect of the genes responsible for the total antioxidant content. Interestingly, QTL on LGs 3 and 9 derived from *L. serriola* and those on LG 7 from *L. sativa*, whilst for the pigment traits, those on LGs 3 and 9 derived from *L. sativa* and those on LG 7 from *L. serriola*. Two QTL clusters (or hotspots) were identified in this study which could be regarded as two potential QTL with pleiotropic effects. The QTL mapped to the same region for all pigment traits and to very close regions of the chromosomes for the pigment traits and the antioxidant traits. This indicates a possible linkage between the genes responsible for the biosynthesis of these traits or even a likely regulation of these traits by the same group of genes and a similar physiological pathway. This association would be interesting for further exploration, as the selective increase of one trait in a hotspot would have the potential to bring an increase of the other trait. However, in the present study, the QTL for the selected traits derived from opposite parents, thus the selective increase of one trait could potentially bring the decrease of the other. Co-locations of traits, such as growth traits (leaf area, chlorophyll fluorescence, growth rate, weight epidermal cell area and number and stomatal density and index), have been reported in other studies on the same lettuce RILs (Zhang 2006) and in an Arabidopsis RIL population (El-Lithy et al. 2004). The identification of QTL hotspots can have important implications for plant breeding programs as the selection of an ideal genotype for one QTL region could simultaneously improve several other traits. However, for QTL clusters where both desirable and undesirable traits map together, fine-mapping using Near Isogenic Lines (NILs) would determine whether there are multiple QTL or a single QTL with pleiotropic

effects. In the case of the results presented here, the selective increase of one trait (total antioxidant content) has the potential to result in the increase of deleterious antioxidants as well (e.g. bitter compounds), thus further metabolic profiling of the population and subsequent QTL analyses would be necessary.

The detection of significant QTL depends on a number of factors as well as the genetic variation between the population parent lines. Factors such as population size, the heritability of the trait, the efficacy of the trait evaluation method, gene by environment interaction, the reliability of the position of the markers on the chromosomes, size effect of individual QTL and the accuracy of the genotype information are all important in the identification of significant QTL (Kearsey 1998). The ability of QTL analysis is determined by the size of the standard error of the mean of each line relative to the size of individual gene effects. Thus, large numbers of genotypes and of replicates are important in the identification of small effect QTL. The more individuals scored, the smaller the difference that can be detected between two means of genotypes can be recognized as being significantly different (Kearsey 1996). In this study, nine replicates of 60 RILs grown in the glasshouse were analysed for antioxidant phytonutrients and a total of 16 QTLs were found. Even though only 60 RILs were used in this study, they had been selected due to their number of recombination points, thus providing a population which had nearly as many recombinant breakpoints as a population of c. 90 RILs. The population used in this study proved to be effective in the detection of significant QTL in this study, but also in previous studies on shelf life properties (Zhang 2006). Further work on data obtained from field trials allows for the confirmation of robust QTL and possibly for the identification of new QTL dependent on genotype by environment interactions. A QTL analysis was run on the data and two significant QTL were detected on LGs 3 and 4. The QTL on LG 4 may be a smaller effect environmental QTL, as it had not been detected in the glasshouse samples. Genotype by environment interactions are crucial in plant improvement programmes for the industry,

thus future work would need focus on trait analyses and QTL mapping in field experiments.

The total antioxidant content of leaf material is a complex trait which follows from the joint action of a number of different metabolites and thus of an even greater number of different genes. Further research would continue to focus on analysing a breakdown of the metabolites that compose the total antioxidant content of a lettuce leaf, such as phenolic acids, which would make it possible to improve the understanding of the genetic basis of leaf antioxidant nutritional content.

Furthermore, owing to the high density of markers positioned in the lettuce genetic linkage map, the confidence interval for significant QTL used in this study is of 10cM, a relatively short distance in comparison to other studies which generally range between 10 to 30 cM. Within this chromosomal distance, several hundreds of genes may be present, for instance, an average of 440 genes in a 10 cM interval has been estimated for Arabidopsis (Salvi & Tuberosa 2005). Thus, an increase in mapping resolution would provide a more accurate measure to reduce the number of genes associated with the QTL.

The development of NILs for gene cloning at the two regions identified as hotspots, on LGs 3 (between 30.93 cM – 35.39 cM) and 7 (between 91.49cM – 97.45 cM) could be used to pinpoint a more precise location of the genes involved in the biosynthesis of the trait of interest. To date, QTL cloning has been successfully carried out in a number of studies, such as in Arabidopsis (Werner et al. 2005), rice (Doi et al. 2004) and tomato (Frary et al. 2000;Fridman et al. 2004).

Another method utilised in the present study for the investigations of genes involved in the biosynthesis of plant compounds was the study of candidate genes. The exploration of Single Nucleotide Polymorphisms was applied to the investigations of genes encoding enzymes involved in the biosynthetic

pathway of carotenoid and polyphenolic compounds. The availability of an extended Expressed Sequence Tag (EST) database developed at the University California Davis provided useful information for SNP investigations of genes involved in these antioxidant phytonutrient investigations. A number of polymorphisms were detected in the parent lines and subsequently in the mapping population. Further work would see the application of this method on additional EST sequences involved in the biosynthesis of important lettuce polyphenols. Further SNP analyses would also aim to investigate enzymes further down the biosynthetic pathway, closer to the point of synthesis of the compounds of interest.

A final development of the project would need to be applied to validate the role of the identified candidate genes and their effects on the desired phenotypes. The functional testing of the candidate gene can be performed by over-expressing or down-regulating the target gene through transformation or new, less contentious, technology, such as RNAi or TILLING. TILLING, for instance, has recently been applied in wheat for crop improvement (Slade et al. 2005).

In conclusion, a number of different methods can be applied for the selective increase of antioxidant compounds in different varieties of lettuce. Pre-harvest and time of harvest methods can be applied, such as increasing light and temperature exposure by harvesting at different times of the day or of the year. Oh et al suggest that mild environmental stresses or modifications have no adverse effects on the overall growth of lettuce, suggesting that it is possible to use these to successfully improve the phytochemical content and hence the health-promoting quality of lettuce with little or no adverse effect on its growth or yield (2009). Furthermore, the results reported in the bioavailability study have shown that to be able to make certain nutritional claims, green lettuce must be eaten before storage for one week since harvest. This has important implications for the producer as well as for the consumer, as lettuce, especially when produced abroad, may often be

several days old by the time it reaches the table, thus its antioxidant content may have decreased substantially before consumption.

Pre- and post-harvest methods will result in a plant which potentially produces antioxidants at its highest potential. However, phytonutrient investigations on the mapping population generated from a cross between *L. sativa* (cv Salinas) and *L. serriola*, have shown that it was possible to exploit existing genetic variability provided by the wild parent, to generate a number of lines which produce significantly higher levels of antioxidant phytonutrients than even the wild parent, through transgressive segregation. In this study, a number of such lines have been identified for total antioxidants, chlorophyll and carotenoid levels and could be exploited commercially. In particular, line 112 was found to produce significantly high levels of antioxidants and, interestingly, in past studies on shelf life traits on the same population, the same line was found to be the best line in terms of shelf life. Line 112 could therefore be marketed directly as a natural resource, a less contentious method than genetic modification of a Lactuca cultivar.

Flavour, texture and taste would also need to be taken into consideration. Thus an important factor to breeding a marketable, nutritionally enhanced plant would need to take into account sensory factors and food preferences, by finding a balance between increased levels of phytonutrients and an increased level of bitterness. One way this could be achieved is by increasing the low molecular weight phenols, e.g. the less bitter flavonoids (Noble 1994), to make the plant more nutritious, and it would also be possible to compensate for this increase in bitterness by decreasing tannin levels (which have a higher molecular weight and are more bitter) to equilibrate taste.

Overall this study was an all-round complete investigation of lettuce antioxidant nutritional qualities as it started with the investigations of their inter- and intra- specific differences, how they decline with storage, their potential for improvement through breeding with more nutritious wild relatives

and the study was completed by an intervention study investigating the lettuce antioxidant bioavailability in humans.

APPENDIX 1

LIST OF PHYTONUTRIENTS, VITAMINS AND MINERALS, THEIR SOURCES IN GREEN LEAFY VEGETABLES, AND THEIR ESTABLISHED OR PROPOSED BENEFICIAL PROPERTIES ON HUMAN HEALTH.

CVD: Cardiovascular Disease; CAD: Coronary Artery Disease (Kader et al. 2001(1);Nicolle et al. 2004(3);Rowland 1999(2)).

Phytochemical groups	**Examples**	**Green leafy vegetables**	**Established or proposed beneficial properties**
Carotenoids	Carotenes:		
	Lycopene	Leafy greens, e.g. spinach and lettuce	Antioxidants Cancer, heart disease, male infertility[2]
	β-carotene	Lettuce	Cancer [2]
	α-carotene	Green leafy vegetables	Tumour growth[2]
	Lutein		Antioxidant Blocks cellular damage by free radicals, (Sommerburg et al. 1998) macular degeneration[2]
	Xanthophylls: zeaxanthyn	Spinach	Macular degeneration[2]
Terpenes	Monoterpenes limonene		Cancer[2]

Phenolic compounds	Aromatic substances		
	Dicaffeoyl tartaric acid (also known as chicoric acid)		Antioxidants[1]
	Chlorogenic acid		Antioxidants[1]
	Phenolic acids:		
	Caffeic acid	Mustard (Cruciferae) lettuce	Antioxidants[1], cancer, cholesterol[2],
	Ferulic acid	Lettuce	cancer, cholesterol[3]
	Flavonoids: Anthocyanins (also known as glycosides) and anthocyanidins	All vegetables	Heart disease, cancer, diabetes, cataracts, blood pressure, allergies[2]
	Flavones	Spinach	Cancer, allergies, heart disease[2]
	Flavonols	All vegetables	Antioxidants[12] Heart disease, cancer, capillary protectant[2]
	Flavanones		Cancer, Cardiovascular disease protection, immune modulation[12]
	Proanthocyanins (tannins)		Cancer[2]
	Catechins (flavan-3-ols)(flavanols)		Antioxidants , Platelet aggregation, cancer, CHD[12]

Phytoestrogenes	Isoflavonoids (isoflavones)		Breast and prostate cancers, anti-oestrogenic effects, effects on serum lipids[1]
	Coumarins		Cancer?
	Lignans	Some cruciferous vegetables	Colon and prostate cancers, antioxidant and anti-oestrogenic effects[1]
Nitrogen containing compounds (sulphur compounds)	Alkaloids Cyanogenic glycosides Glucosinolates: Glycosides Sinigrin Progoitrin Glucobrassicin	Cruciferous vegetables (e.g. watercress)	Induction of phase 2 *enzymes*[1], lung cancer[2] (Hecht et al. 2004), cholesterol, blood pressure, diabetes[2]
	Isothiocyanates (ITCs)	Cruciferous vegetables	Prostate cancer (Chiao et al. 2004;Conaway et al. 2002)
Folates (folicin or folic acid)	Vitamin B_9	Dark green leafy vegetables, including many crucifers, e.g. spinach, mustard greens	Birth defects? cancer, CVD, CAD nervous system[2]
Vitamins	Vitamin C (ascorbic acid)	Green leafy vegetables, certain crucifers (including watercress)	Antioxidant Prevents scurvy, aids wound healing, healthy immune system, CVD[2]

	Vitamin E (α-tocopherols and tocotrienols)	Green vegetables, e.g. spinach, watercress and lettuce	Antioxidant Heart disease, LDL-oxidation, immune system, diabetes, cancer[2], atherosclerosis? Parkinson's disease (Etminan et al. 2005)
	Vitamin A (retinol) (carotenoids)	Green vegetables, e.g. spinach, watercress and lettuce	Antioxidant Night blindness prevention, chronic fatigue, psoriasis, heart disease, stroke, cataracts[2]
	Vitamin K	Crucifers, green leafy vegetables	Synthesis of pro-coagulant factors, osteoporosis[2]
Terpenes – essential oils	monterpenes and sesquiterpenes	Some cruciferous vegetables	(food flavouring)
Auxins	Indoles	Most cruciferous vegetables	Block tumour production
Omega 3	polyunsaturated fatty acids (PUFAs) stearidonic acid (SDA) alanyl-glutamine (ALA)	Some green leafy vegetables (including watercress, (Pereira et al. 2001)	CHD, hypertension, type 2 diabetes, rheumatoid arthritis, ulcerative colitis, Crohn disease and chronic obstructive pulmonary disease (Simopoulos 1999), inflammatory and autoimmune diseases and cancer (Ursin 2003)
Organosulphides			Phase II enzyme induction, effects on serum lipids and platelet aggregation, cancer prevention[1]

Non starch polysaccharides (NSP)	Soluble (e.g. Pectins and Gums)	All vegetables	Colon and breast cancer, diverticular disease[1]
	Insoluble (celluloses)		
Dietary fibre	NSP		
	Soluble (e.g. pectins, gums)		
	Insoluble (celluloses)	All vegetables	Diabetes, heart disease, colon and breast cancer, diverticular disease[12]
Minerals	Calcium	Cooked vegetables	Osteoporosis, muscular, skeletal, teeth, blood pressure[2]
	Potassium	Cooked green leafy vegetables	Hypertension (blood pressure), stroke, arteriosclerosis[2]
	Magnesium	Spinach	Osteoporosis, nervous system, teeth, immune system [2]
	Selenium		Liver cancer (Ganther 1999;Schrauzer 2000), thyroid hormone metabolism (Hotz et al. 1997;Ruz et al. 1999)
	Iodine		Thyroid hormone metabolism (Hotz et al. 1997;Ruz et al. 1999)

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APPENDIX 2

TOTAL ANTIOXIDANT AND VITAMIN C ANALYSES OF DIFFERENT LEAFY SALAD CROPS FOLLOWING STANDARD COMMERCIAL POST-HARVEST PROCEDURES

Three experiments were carried out aiming to reconstruct common processing procedures used in salad industries: post-harvest chilling, washing in distilled water and chlorine washes. The post-harvest chilling trial, conducted on spinach (Lazio Variety, PopVriend) and on tango lettuce samples, showed that delaying the chilling procedure from the standard 1 hour to 4 or 8 hours post-harvest (in the tango lettuce trial) or to 2, 4, 8 or 20 hours (in the spinach trial) does not seem to affect the sample's total antioxidant or vitamin C contents. There were also no knock-on effects on antioxidant potential or vitamin C content in the chill-delayed tango samples which were tested after being kept for 10 days in the dark at 4ºC (simulating a typical consumer's storage conditions). Moreover, the four and eight hour chill-delayed samples showed a tendency for antioxidant status to increase on the tenth day. This may be caused by the delay in chilling promoting an increase in polyphenols which are produced in response to the damage caused by chopping.

A set of green cos and red cos was tested for antioxidant content after being washed in plain, distilled water and no significant difference was detected between the washed and the non-washed samples. A separate set of tango samples were washed in a chlorine dilution, to simulate industrial chlorine washes of lettuce. This treatment also did not have a significant effect on antioxidant and vitamin C contents. There was even a tendency for vitamin C to increase after washes in chlorinated water in comparison to the control washes.

Total antioxidant contents were tested during storage in tango lettuce samples to quantify its decay during storage in the dark at 4ºC. The purpose

of this study was to mimic the storage effects of a common household fridge on a common green lettuce cultivar. Samples were washed and tested one, four, seven and ten days after being kept in a common household fridge. Half of the tango lettuce samples were also washed in chlorinated distilled water, to analyse the effects of chlorine washes on the antioxidant content, which is commonly carried out in the lettuce industry. Antioxidant analyses showed that decay is not appreciable within the first week to ten days of shelf life. In fact, tango lettuce seemed to visually spoil before any significant nutrient loss occurred. These findings matched Gil et al's results on fresh cut fruit, who found that minimal processing had almost no effect on the main antioxidant components, even though it accelerates a reduction in visual quality (2006).

Procedures, results and figures are detailed below:

a. ***Antioxidant levels of green cos and red cos are not affected by washing procedures***

Total antioxidant potential analysis was carried out on commercial green cos and red cos samples to establish whether post-harvest processing such as washing had an effect on their antioxidant potential. Commercial samples of green cos and red cos were collected from the Vitacress Ltd factory at Saint Mary Bourne.

Samples were washed and bagged using a twin-tub washing machine for one minute on minimum speed, then spun dry for 20 seconds. Leaves were bagged, sealed and kept for one day in the fridge at 7°C, before being opened and tested for antioxidant potential. Six biological replicates and three technical replicates for each biological replicate were sampled for total antioxidant potential, using the FRAP assay.

Comparison between previously washed and un-washed green cos and red cos leaves showed no significant difference in antioxidant potential (Fig 1).

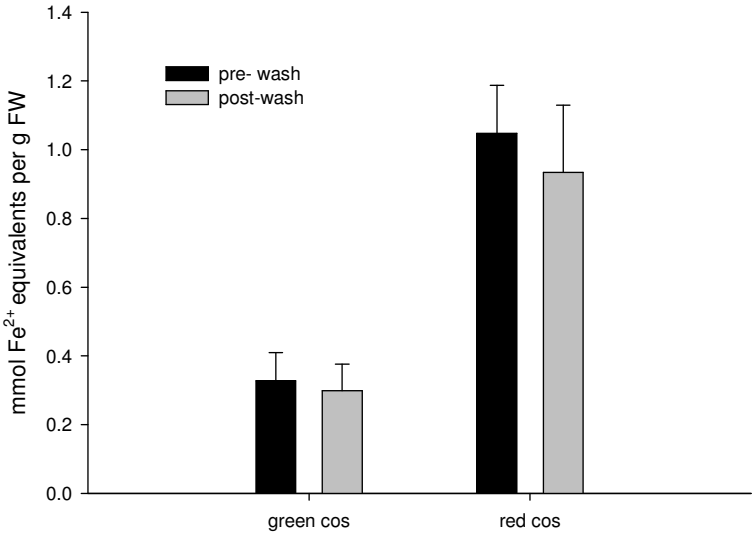


Fig 1 Effects of washing procedures on total antioxidant levels of commercial green cos and red cos. Two-way ANOVA showed significant differences between cultivars ($F_{1, 20}$ = 28.49, P<0.000); however, the differences between treatments were not significant.

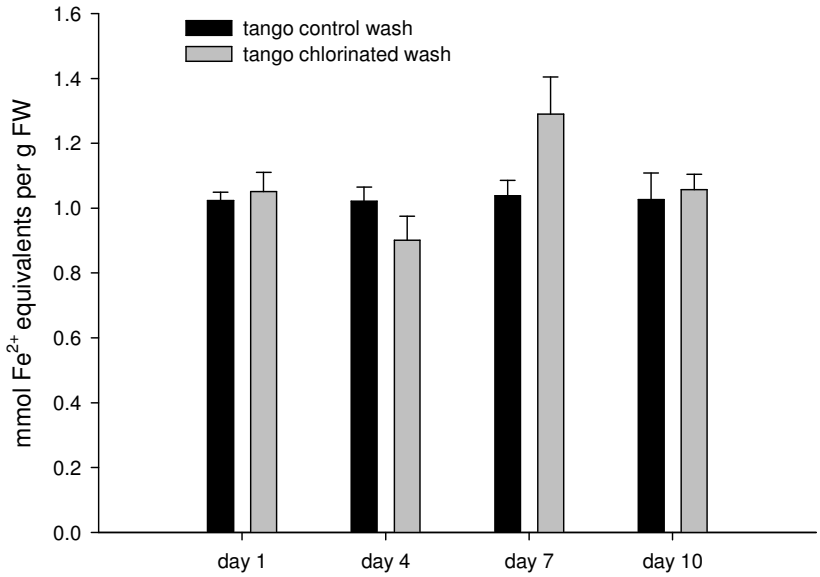
b. Chlorine washes do not affect the total antioxidant potential or vitamin C levels of tango lettuce

A total antioxidant potential and vitamin C analysis were carried out on commercial tango baby leaf samples to establish whether the post-harvest processing treatment of washing in chlorinated water had an effect on the selected traits.

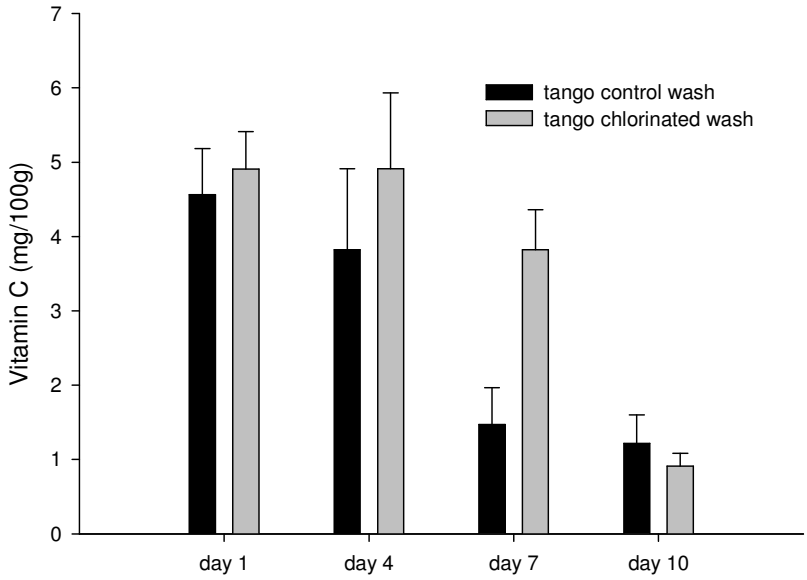
Samples were washed and bagged using a twin-tub washing machine for one minute on minimum speed, then spun dry for 20 seconds. Half of the samples were washed in two litres of distilled water and half were washed using distilled water and chlorine in a 1:40 dilution. Leaves were then bagged and sealed, and kept for one, four, seven and ten days in the fridge at 7°C, before being opened and tested for antioxidant potential. Antioxidant potential during shelf life was analysed using the FRAP assay. Six biological

replicates and three technical replicates for each biological replicate were sampled. Vitamin C levels were tested with HPLC at Leatherhead Food International.

Tango leaves washed in chlorinated water showed a trend towards higher levels of antioxidant potential and vitamin C levels than leaves washed in non-chlorinated water (Fig 2), however the difference was not significant. Interestingly, vitamin C levels started dropping after day four.



(A)



(B)

Fig 2 Chlorinated and non-chlorinated wash comparison of antioxidant potential and vitamin C levels in commercial tango lettuce stored for up to 10 days. 50% of the samples were washed in distilled water and 50% in distilled water with chlorine, in a 1:40 dilution. Two-way ANOVA showed no significant difference between treatments (A). However, a significant drop in vitamin C levels was detected between days 4 and 7 for the control wash and between days 7 and 10 for the chlorine wash ($F_{1, 8}$ = 34.13, P=0.000) (B).

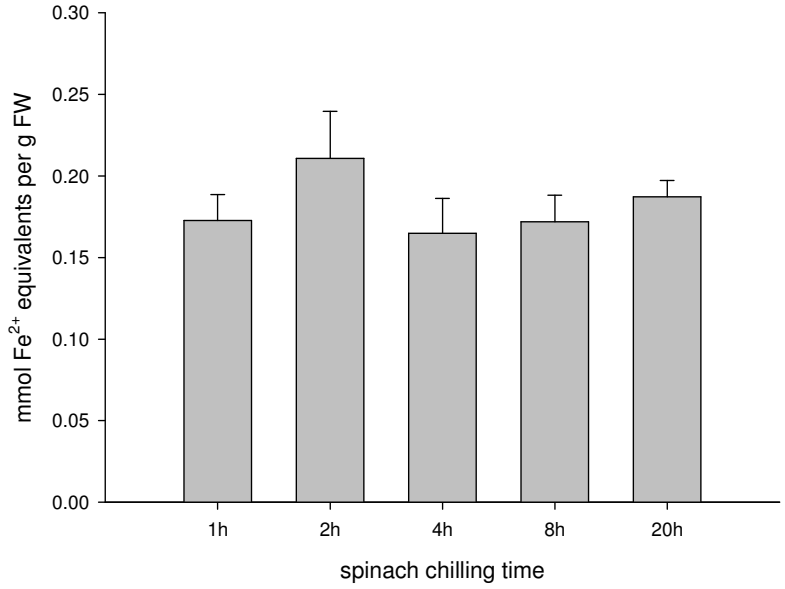
c. Delaying vacuum chilling of salad leaves has little effect on spinach total antioxidant and vitamin C levels

Commercial baby leaf spinach (Lazio Variety, PopVriend) samples grown in Portugal and harvested during the day when the temperature was above 15℃, were chilled at different times post-harvest. The treatments included: standard vacuum-chilling to 3℃ within one hour, or chilling after two hours, after four hours, after eight hours and after twenty hours. Three biological replicates and three technical replicates for each treatment were sampled for total antioxidant potential, using the FRAP assay.

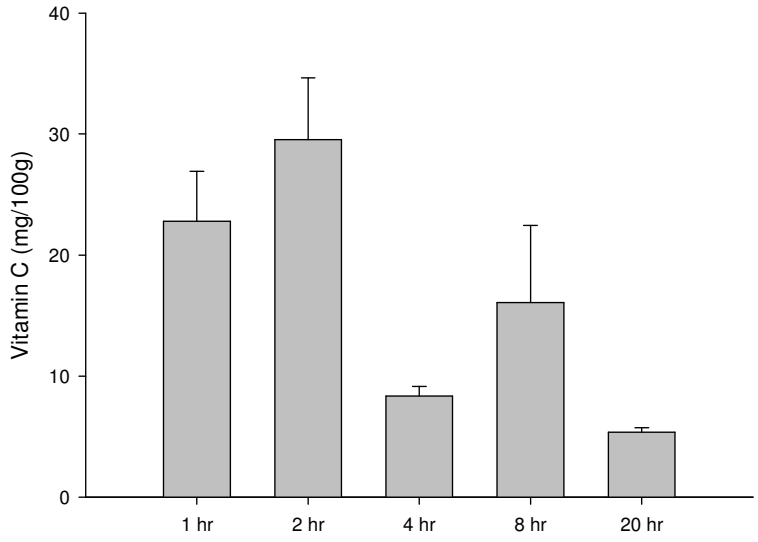
A FRAP analysis was carried out between commercial spinach (Lazio variety, PopVriend) baby leaves which had been chilled at different time delays post-harvest to establish whether chilling delays had an effect on antioxidant potential. The chilling was carried out within the commercial standard one hour, for the first batch, and then different batches were chilled after two, four, eight or twenty hours post-harvest.

Comparison between baby spinach leaves kept at ambient temperatures of 15℃ or higher then chilled to 3℃ at different times post-harvest (1, 2, 4, 8 and 20hours) showed no significant difference in antioxidant potential (Fig 3 A).

There was a trend in the spinach chill-delay samples for vitamin C to decline with longer delays in chilling, with levels remaining fairly high only during the first two hours (Fig 3 B). However, the levels did not seem to decline in a continuous manner.



(A)



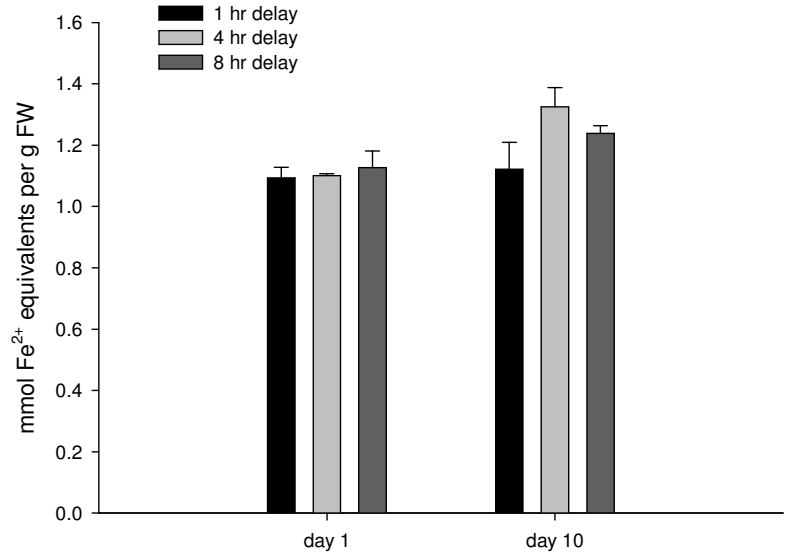
(B)

Fig 3 Total antioxidant and vitamin C levels of spinach chilled at different times postharvest. FRAP analysis for total antioxidant potential (A) and HPLC analyses of vitamin C (B) of commercial spinach baby leaves chilled one hour, two hours, four hours, eight hours or twenty hours post-harvest. Three biological replicates were carried out for each treatment and three technical replicates for each biological replicate. One-way ANOVA found no significant effects on antioxidant potential due to delays in chilling time. A significant difference between the 2 hour delay and the 4 and 20 hour chill-delays was found for vitamin C content ($F_{4, 10} = 5.91$, $P=0.01$).

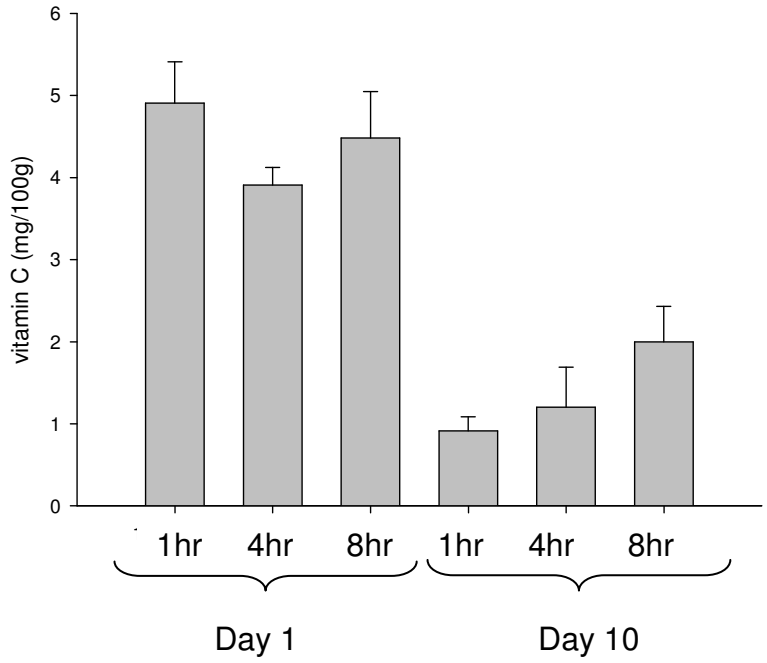
d. Delaying chilling of tango leaves has little effect on total antioxidant and vitamin C levels

An analogous trial was subsequently conducted on commercial tango baby leaf lettuce samples grown in the UK, at the Vitacress Pinglestone farm. The first batch of leaves were chilled within the commercial standard one hour post-harvest, a second batch was chilled after four hours and a third after eight hours. Samples were washed and bagged using a twin-tub washing machine for one minute on minimum speed, then spun dry for 20 seconds. Leaves were then bagged and sealed, and kept for one and ten days in the fridge at 7°C, before being opened and tested for antioxidant potential. Samples were analysed on day one as well as on the tenth day of shelf life, after being kept at 7ºC, to check on any possible knock-on effects of the changes in chilling process. Three biological replicates, consisting of approximately five leaves each, were carried out for each treatment and three technical replicates were carried out for each biological replicate.

A delay in chilling commercial tango baby leaves produced no evident changes in total antioxidant production. The antioxidant analyses carried out on day ten confirm these findings and also show that there is no knock-on effect for the delays in chilling (Fig 4 A). They do, interestingly, show a difference in antioxidant potential between day one and day ten, for each delay in chilling, with an increase in antioxidant potential on day ten. Delays in postharvest chilling did not significantly affect vitamin C content in tango lettuce (Fig 4 B).



(A)



(B)

Fig 4 Effects of chill delay on short and long term antioxidant and vitamin C levels in tango lettuce. Total antioxidant analysis using FRAP were carried out on commercial tango baby leaves chilled at different hours after harvest. Measurements were taken at two time points: day one and day ten post harvest. Two-way ANOVA showed no significant effects due to delays in chilling (A). HPLC analyses of vitamin C in tango lettuce were carried out after different chill-delay treatments. No significant differences due to delays in chilling (B) were detected.

APPENDIX 3

THE TROLOX EQUIVALENT ANTIOXIDANT CAPACITY (TEAC III)

The Trolox Equivalent Antioxidant Capacity (TEAC III) assay was used as comparative method to measure total antioxidant potential, as first described by Re *et al* (1999) with minor modification (Prior et al. 2005;Schlesier et al. 2002).

The assay is based on the oxidisation of 2,2'-azino-bis-(3-ethilbenz-thiazoline-6-sulfonic acid) (ABTS) to the radical cation, $ABTS^{\bullet+}$ (blue-green colour), by potassium persulfate, and measuring decolourisation with a spectrophotometer, at wavelength 734 nm, following the addition of the antioxidant sample or standard. The $ABTS^{\bullet+}$ turns dark blue-green and loses colour when added to the sample. The assay distinguishes between lipophilic and hydrophilic compounds, it is therefore necessary to carry out 2 separate assays in parallel where the ABTS is re-diluted in 18MΩ H_2O or phosphate buffer saline (PBS) (pH 7.4, 5mM) (for the hydrophilic compounds) and in ethanol 100% (for the lipophilic ones). Serial dilutions of Trolox, a vitamin E analogue, diluted in PBS (pH7.4, 5mM) and in ethanol 100%, were used as standards.

a. Materials and methods

Leaf samples were ground with pestle and mortar in liquid nitrogen, spun down at 13K rcf for 3 minutes in an elution tube (Quiashredder™) and eluted sap was collected and kept on ice until needed. $ABTS^{\bullet+}$ was prepared by mixing 7mM ABTS with 2.45mM Potassium persulfate, in a ratio of 50:50. The mixed reagents were wrapped in foil and kept for 12 - 24h in the dark, at room temperature, until the reaction was complete and the absorbance stable (the radical is stable for more than two days when stored in the dark at room temperature). Serial dilutions of ethanol or PBS solutions of known Trolox concentration in the range of 0.5-2.5mM were prepared to use as

standards. $ABTS^{\bullet+}$ was then diluted with H_2O or PBS, for analysis of hydrophilic antioxidants, and with Ethanol for analysis of lipophilic antioxidants, to an absorbance of 0.7 $\pm$ 0.02 at 734 nm. 1ml $ABTS^{\bullet+}$ was then vortexed with 100µl of antioxidant solution for 45 sec. Solutions (1µl for aqueous solutions; 5µl for the ethanol solutions) were measured immediately after 1min at 734nm on a Nanodrop ND-1000 spectrophotometer.

b. Sample analysis of TEAC III measurements

A calibration curve for the Trolox was plotted and used to convert sample results to Trolox equivalents in mmol/g FW using Excel 2003 for Windows and the percentage of antioxidant activity was calculated using the molar extinction coefficient, expressed as percentage antioxidant activity of lipophilic or hydrophilic antioxidant activity, as described by Re et al (Re et al. 1999;Smith et al. 1985;Wiechelman et al. 1988) (Fig 5).

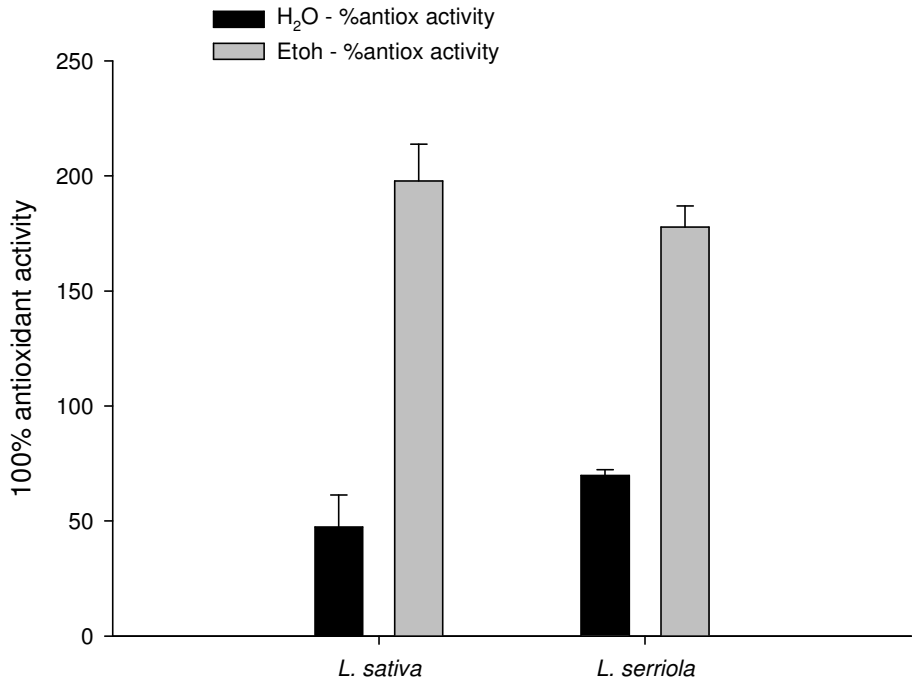


Fig 5 *L. sativa* and *L. serriola* antioxidant capacity. The antioxidant capacity was assessed using the Trolox Equivalent Antioxidant Capacity assay.

APPENDIX 4

PROTEIN EXTRACTION AND ASSAY

The protein assay is based on a Biuret reaction which reduces Cu^{+2} to Cu^{+1}.The BCA then forms a complex with Cu^{+1} turning the assay purple. The assay can then be measured in a spectrophotometer at absorbance 560nm .

a. Extraction

Three to four whole lettuce plants, approximately 5g FW were ground in liquid nitrogen, using pestle and mortar, transferred to a falcon tube and kept in liquid nitrogen (until needed). Two separate methods of extraction were carried out. Part of the ground tissue was spun down at 13K rcf for 3 minutes, in an elution tube and eluted sap was collected, kept on ice or in liquid nitrogen and then directly assayed for protein content. Part of the powdered tissue was suspended in a solution labelled as STOCK 1, made up of 25mM $NaPO_4$ containing 2mM EDTA, pH 7.2, and made up to 100ml, in a 3:1 (volume:weight) ratio of buffer to tissue. Water, 90ml, was added to 10ml of STOCK 1 and brought up to a concentration of 2mM by adding 400µl 0.5M EDTA. This solution was then stored as STOCK 2 and kept at 4ºC (solution keeps 3-4 days). 5ml of STOCK 2 were then added to 120µl of protease inhibitor cocktail and 5ml H_2O (33ml protease inhibitor cocktail per g tissue). 24µl 500mM DTT was added to 400mg of ground tissue into an eppendorf tube, 1200µl extraction buffer was added to bring final concentration of DTT to 10mM. The mix was left on ice for 10min then centrifuged at high speed (10K *g*) at 4ºC for 10min. 1200µl cold acetone was added to 200µl supernatant and kept on ice for 10min then centrifuged at high speed for about 10min. Supernatant was then discarded and pellet re-dissolved in 1% SDS (final volume 100µl). The remaining supernatant was stored at -20°C.

b. Assay

Dye reagent was prepared by mixing 20ml Bicinchloronic acid with 0.4ml BCA protein assay reagent B, 50:1 parts. 20ml of dye reagent were used for a full plate and 200µl of dye reagent. Serial dilutions of Bovine Serum Albumin (BSA) standards were prepared in the range of 0.0 to 1.0 mg/ml H_2O. These were then aliquoted and frozen. 10µl of BSA standard were added to a 96-well microtitre plate and three technical replicates of each sample and standard were carried out. For the standards and those wells with 10µl of sample, a further 10µl of 1% aqueous SDS was added. For the wells with 5µl of sample, 15µl of 1% aqueous SDS was added. For the wells with 1µl of sample 19µl of 1% aqueous SDS was added, so that each well contained 20µl of liquid. 200µl of the freshly prepared mixed dye reagent was finally added to each sample and standard. The plate was then incubated at 60ºC in a drying oven for 45-60min, and optic density (OD) was measured at 560nm on the spectrophotometer.

c. Protein levels in lettuce parent lines as determined by different assay methods

A protein assay was carried out on the two parent lines to use as a potential standard to correlate all of the results from the antioxidant assays. The assay was carried out after using two separate methods of extraction: the standard protein extraction method and a sap extraction using an elution tube, which could be employed as a single extraction method suitable for all of the assays carried out on the RILs.

The sap extraction method, whether kept on ice or in liquid nitrogen, resulted in a higher protein level for both parent lines (Fig 6). In every extraction method used, the *L. serriola* parent line has a significantly higher protein level than the *L. sativa*.

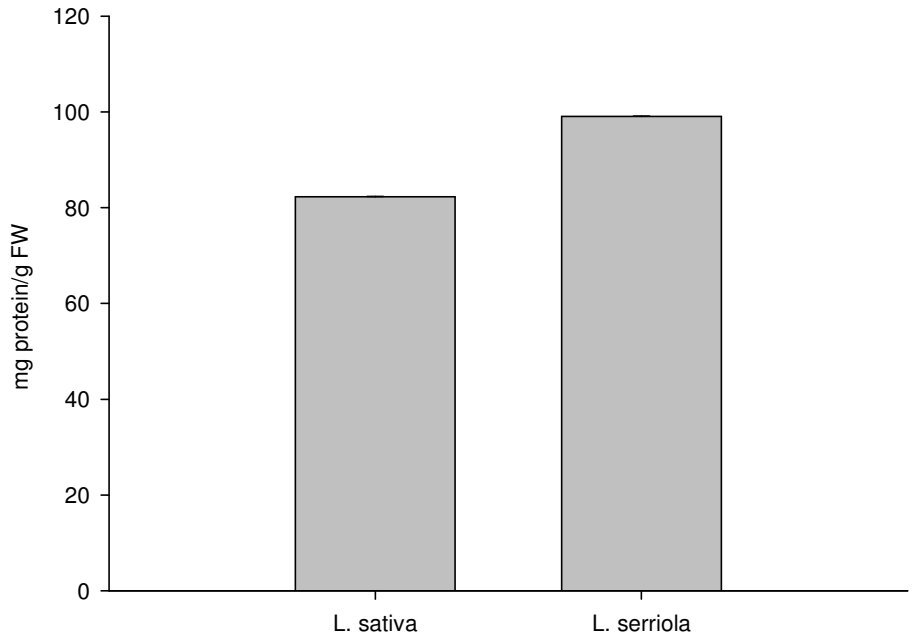


Fig 6 Protein levels in *L. sativa* and *L. serriola*. One biological replicate and three technical replicates were used for each treatment.

d. Protein analysis of the RILs shows considerable variation between and within the same lines

Protein quantification of the RILs was carried out using the sap extraction method. The purpose of this assay was to use the protein level of each line as a possible standard to relate to the other assays carried out on the lines.

The results showed significant variation between each line. However, they also showed considerable variation within each biological replicate, thus protein levels were not used as a standard with which to relate all subsequent assays (Fig 7).

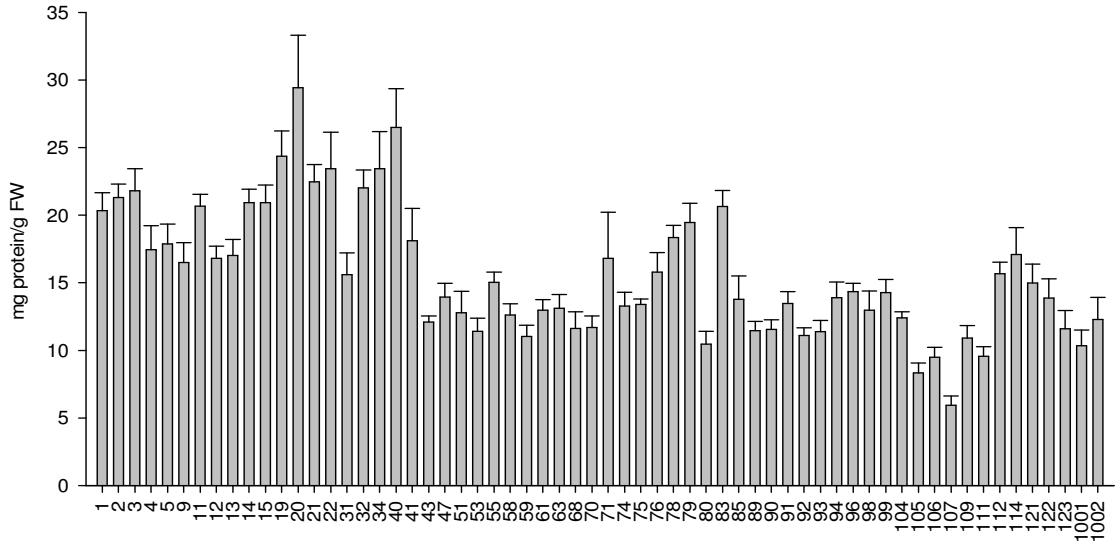


Fig 7 Protein levels in the RILs. The RILs were organised in three blocks of three randomised replicates of each line per block. Nine biological replicates and three technical replicates were used for each sample.

e. QTL analysis of the RIL protein levels detected no significant QTL

QTL analysis was carried out for protein content, however no significant QTL were identified (Fig 8).

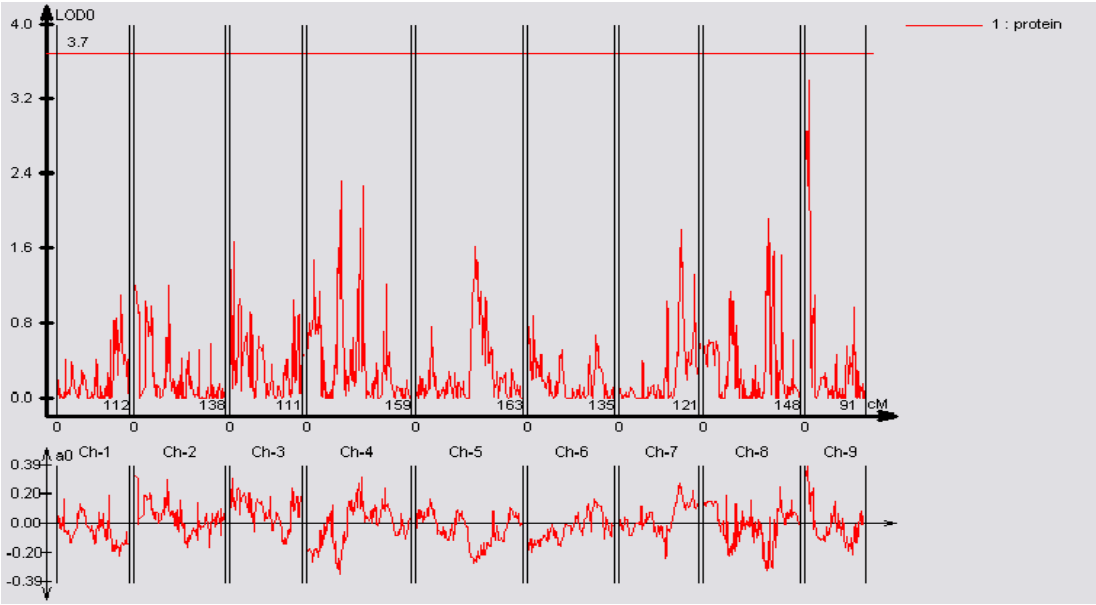
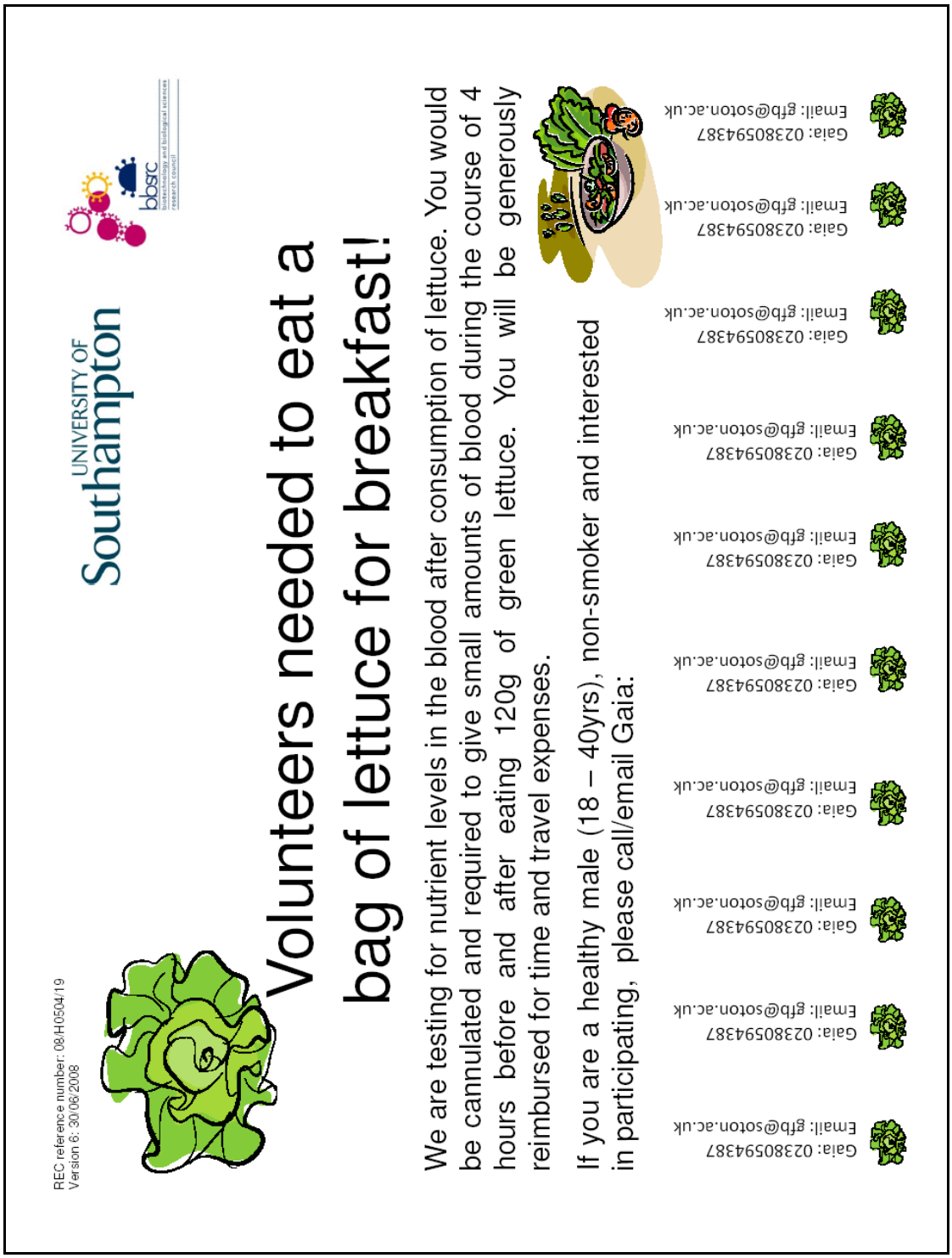


Fig 8 QTL cartographer plot for protein content index of the F_9 RIL mapping population. The LOD score is indicated on the upper Y axis. The X axis represents the nine linkage groups of the F_9 recombinant inbred line map arranged end-to-end and their length in cM. The horizontal red line (LOD = 3.7, P<0.05) represents the significant threshold value for the trait value. The lower graph in the plot shows the additive effect of the parental allele that causes an increase in the trait value. Positive values indicate the cultivar (*L. sativa*) allele increased the trait values and negative values indicate wild relative (*L. serriola*) allele increased the trait values.

APPENDIX 5

INTERVENTION TRIAL DOCUMENTS, REPORTS, FORMAL LETTERS AND ADVERTISEMENTS

a. Advertisement material (version 7) (PowerPoint)



b. Local press advertisement material and email

Local press advertisement material and email

Volunteers needed to eat a bag of lettuce for breakfast!

We are looking for volunteers to eat a bag of lettuce and be tested for nutrients in the blood.

We are testing for antioxidant levels after consumption of red cos. You would be required to give blood 7 times during the course of 5 hours before and after eating 120g of lettuce.

If you are a healthy, Caucasian male (20 – 35yrs), non-smoker and interested in participating, please call Gaia on: 02380594387 or Mark on: 02380594342.

SUSSED classified:

Volunteers needed!

We are looking for volunteers to eat a bag of lettuce and be tested for antioxidants in the blood. If you are a healthy, Caucasian male (20 – 35yrs), non-smoker and interested in participating, please call Gaia on: 02380594387 or email: gfb@soton.ac.uk.

Daily Echo:

Volunteers needed!

We are looking for volunteers to eat a bag of lettuce and be tested for antioxidants in the blood. If you are a healthy male (18 – 35yrs), non-smoker and interested in participating, please call Gaia on: 02380594387 or email: gfb@soton.ac.uk. You will be reimbursed generously for your time and travel expenses.

REC reference number: 08/H0504/19 Version 1 25/03/2008

c. Protocol (for the REC committee): Intervention Trial (version 3)

Effects of lettuce ingestion on postprandial antioxidant phytonutrient levels in human blood plasma.

REC number: 08/H0504/19

R&D No: RHM NUT0053

Aims

The aim of this project is to determine how the ingestion of fresh and stored baby salad leaves influence the levels of total antioxidants in human blood plasma.

A secondary research objective would be to identify the individual antioxidant phytonutrients which compose the total antioxidant content absorbed by humans.

Background

Preliminary analyses on the effects of storage on lettuce leaves have shown only a small reduction in antioxidant potential during the first four days of storage. The objective of this study would be to relate these results to the bioavailability of stored lettuce in human blood plasma. The study will use baby leaf lettuce (whole not chopped) as initial analysis suggests nutrient stability is greater in leaves which are kept intact and harvested when young. Metabolomics analyses would then potentially identify additional antioxidants absorbed by the body.

A number of studies have been carried out examining the effects of postprandial blood antioxidant status, however, a single previous study examined the effects of eating fresh chopped and stored chopped lettuce and found that eating lettuce stored for three days under modified atmosphere packaging (MAP) had no benefit on plasma antioxidant levels (Serafini et al., 2002). However, Serafini and his colleagues carried out their research on both male and female volunteers, whereas it is normally advisable to work on male volunteers only, due to hormonal fluctuations in female metabolism.

Furthermore, Serafini used chopped leaves of an unspecified variety of lettuce. Plant metabolites start to decline soon after harvest, further chopping of leaf material would result in further declines in certain metabolites, as well as increases in certain polyphenols (Kang and Saltveit, 2002), thus introducing other elements of variation to the samples.

The proposed study would test antioxidant levels in male volunteers after ingestion of intact baby leaf lettuce samples of the red cos variety. The choice of using baby leaves also follows from the modern trend for bagged baby leaf salads, a product which seems to maintain nutrient levels more efficiently than chopped whole heads.

No other study has focused on the postprandial effects of packaged and stored baby leaf lettuce on human blood antioxidant status. Therefore, the proposed study would expand our understanding of the true nutritional value of modern convenience packed salad, and provide critical information about the bioavailability of individual phytonutrients.

Methods and overview of the planned research

Subject selection and recruitment

The following approaches will be used to attract twenty-four volunteers:

1. Advertisements in University of Southampton publications and in the Southampton general press.
2. E-mail messages to staff in the School of Medicine and School of Biological Sciences.
3. Posters within the University of Southampton and Southampton General Hospital (SGH).

The advertisements will invite a personal, telephone or e-mail enquiry. After a brief discussion, volunteers who appear to be suitable will be sent the project information sheet (also explaining why after an initial blood test they may still not be able to be part of the study) and invited to attend an appointment at the SGH.

Participants will be healthy Caucasian (or similar ethnic background) men 20 to 35 years of age, non-smokers, with body-mass-index >20kg/m^2 and <25kg/m^2, with fasting plasma triglyceride concentration less than 2.5mM and cholesterol less than 8mM (lettuce antioxidants have been shown to have an effect on rat cholesterol levels) (Nicolle et al., 2004) .

Those not fulfilling the inclusion criteria will be those who have diagnosed type 1 or type 2 diabetes mellitus or are receiving anti-coagulant therapy; those who take vitamin supplements; vegetarians

or vegans; and subjects who exercise daily and are unwilling to suspend these activities for the duration of the study.

Subjects will also be asked to complete a brief general health questionnaire and they will be shown the samples and the quantities they would have to eat.

Suitable volunteers will be invited to a short interview (about 20 minutes) one week later in order to discuss the project, to assess their general health and suitability, and to record their height and weight. The project will be discussed in detail during the interview and written consent obtained. They will have a week to decide and once their consent has been received, they will be asked to attend the Wellcome Trust Clinical Research Facility, at the SGH on one occasion of approximately 6 hours for the postprandial studies, for which they will be reimbursed £40 for time and travel.

Study design

Participants will take part in one of two studies in random order in which they will be given 120g of fresh or stored baby leaves of *Lactuca sativa* (red cos variety).

The protocol for each study is as follows. Subjects will be asked not to consume any fruit and vegetables 24 hours prior to the study, and to fast until the start of the study on the following morning (about 12 hours). On arrival at the Wellcome Trust Clinical Research Facility, SGH, participants will be requested to remain resting on a bed as much as possible. Periods when the subjects are not at rest will be recorded. A venous cannula will be placed in a forearm vein and a baseline blood sample collected (15ml). The participants will then consume a breakfast composed of a packet containing 120g of either stored or fresh lettuce leaves, they will be allowed free access to water but will not be allowed to eat anything else throughout the study. Venous blood samples (15ml each time) will be drawn at 0, 0.5, 1, 2, 3, 4, 5 hours (please refer to the diagram below: Fig. 1). Subjects will also be asked to complete a brief general health questionnaire both before and during the sampling.

Plasma will then be isolated from the blood by centrifugation and stored at -80°C. Total antioxidant content will be measured using the FRAP assay. And a metabolomics analysis will be carried out (by HPLC) to identify phytonutrients (phenolics, carotenoids, vitamin C) present in the blood samples after lettuce ingestion.

In parallel to the study, *in vitro* analysis of lettuce samples, fresh and stored will be carried out for total antioxidant potential, phytonutrient and metabolomics analyses. (Total antioxidant potential and phytonutrient analyses will be carried out at the School of Biological Sciences, Boldrewood, University of Southampton, and Metabolomics will be carried out at the Umea Plant Science Centre or similar centre of excellence for metabolomics).

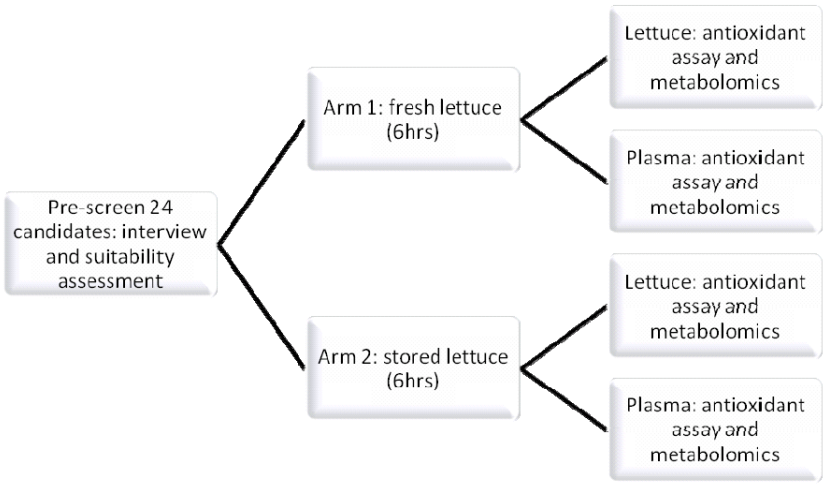


Fig 1 Study design. After initial pre-screening and candidate selection, each volunteer will undergo a study in which seven blood samples will be harvested once every hour (twice in the first hour) over a total period of six hours per session. These samples will then be analysed for total antioxidant content and for individual phytonutrients (metabolites).

Risk assessment

Low risk.

Statistical methods

The effect of lettuce consumption on postprandial changes in blood antioxidant levels and metabolite content will be assessed by 2 and 3-way repeated measures ANOVA with Bonferroni's post hoc correction for multiple comparisons between groups.

Study timeline

Due to the considerable amount of time required for the preparation and submission of the required papers for the proposal of an intervention study involving human subjects, the study will start no sooner than spring 2008 and will be concluded by autumn 2008.

References

KANG HM, SALTVEIT ME (2002). Antioxidant Capacity of Lettuce Leaf Tissue Increases after Wounding. *J. Agric. Food Chem.*, **50:** 7536-7541.

NICOLLE C, CARDINAULT N, GUEUX E, JAFFRELO L, ROCK E, MAZUR A, et al. (2004). Health effect of vegetable-based diet: lettuce consumption improves cholesterol metabolism and antioxidant status in the rat. *Clinical Nutrition*, **23:** 605-614.

SERAFINI M, BUGIANESI R, SALUCCI M, AZZINI E, RAGUZZINI A, MAIANI G (2002). Effect of acute ingestion of fresh and stored lettuce (Lactuca sativa) on plasma total antioxidant capacity and antioxidant levels in human subjects. *British Journal of Nutrition*, **88:** 615-623.

PI Signature:

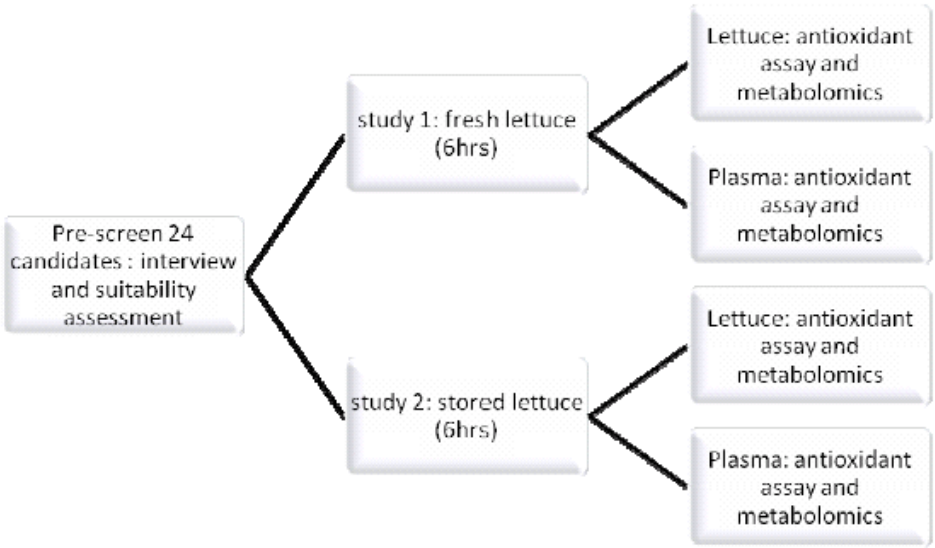
d. Diagram of protocol (version 2) (for the ethics committee)

UNIVERSITY OF Southampton

Effects of lettuce ingestion on postprandial antioxidant phytonutrient levels in human blood plasma

REC reference number: 08/H0504/19

Diagram of protocol:



e. WTCRF application form

WELLCOME TRUST CLINICAL RESEARCH FACILITY
Application form

To enable your application to be considered running within the WTCRF, please complete this form. To assist with the review process, please ensure that all sections of the application form are completed and note that apart from study number, all fields are MANDATORY. Missing details (ESPECIALLY THOSE RELATED TO FUNDING) will result in delay processing your application. *NB Studies undertaken for financial gain will be subject to recovery of costs, including overheads.*

If you have any queries, please telephone: 023 8079 4989

Study Title: **Bioavailability of lettuce antioxidants**	**WTCRF Study number:**
Name of Clinical Principal Investigator Dr Saul Faust **E-mail:** s.faust@soton.ac.uk	**Address:** Southampton University Wellcome Trust Clinical Research Facility, Mailpoint 218, Level C West WIng, Southampton Univeristy Hospital NHS Trust, Tremona Road, Southampton SO16 6YD, U.K. **Tel No:** 02380794989
PI's SUHT Directorate/Division	WTCRF
PI's University Division	IIR
Name of Study Chief Investigator: Gaia Francesca Biggi **E-mail:** gfb@soton.ac.uk	**Address:** 62 Bassett Crescent East, School of Biological Sciences, University of Southampton, Southampton, Hampshire, SO16 7PX **Tel No:**02380594387

Please risk assess clinical cover required for this study (see Application guidance notes):	**Low (a)** X		**Med (b)**		**High (c)**	
If study is rated Med or High, please name the clinician responsible for this study:						
Planned start date: Mid-March 2008	**Estimated study duration:** 12 months					

Funding source: (please circle)	**Research Council or Govt**	**Charity**	**Investigator-led, commercially supported**	**Commercial**	**UKCRN Network (specify)**	**Other (please specify)**
Name of funding body: BBSRC and Vitacress Salads Ltd						
Value of funding: (please state total value) £15,000 (Vitacress)	**Confirmation of funding received?** Yes / No					

Name of sponsor: (SUHT) / University / Other (please specify)	
Protocol devised by: Gaia Francesca Biggi	Data owned by: Gaia Francesca Biggi, Mark Dixon and Gail Taylor
Has your project been peer reviewed? Yes (external) / (Yes) (internal) / No	If yes, by whom? PhD supervisors; BBSRC
Has ethics approval been agreed? Yes / (No)	LREC number: 08/H0504/19 MREC number:

Which WTCRF facilities does your study require? (please tick):

Environmental lab		Sleep lab		High dep bed		Single room	
Endoscopy / bronch suite		Cat III lab		Adult consult room	X	Paed consult	
Physiology lab		Nutrition kitchen		Adult bed		Paed cot or bed	
Bod Pod		Temp controlled consult room		Adult treatment		Paed treatment	

Which other WTCRF rooms or facilities will your study require? Healthy volunteers having blood tests half-hourly for the first hour, then hourly for the following 5 hours. Please advise the most appropriate space requirement.
We need to test the participants for fasting plasma triglyceride concentration and for cholesterol levels (physiology lab?)

Please specify amount of WTCRF nursing assistance required below in hours per visit:

	Total		Hrs/visit
Adult (days)	204 total for the study	Paediatric (days)	0
Adult (nights)	0	Paediatric (nights)	0
Adult (weekends)	0	Paediatric (weekends)	0

How many nursing hours does this represent PER WEEK?28

Please specify below what sort of nursing skills your study will require:
Phlebotomy

Planned number of subjects who will attend the CRF: 24

How many visits per patient? 1-2

Please enclose copies of the following with your application:

	Y/N	Details or Comments
1. Copy of completed protocol	Y	I am enclosing an amended version of the protocol

2.	Copies of patient information sheet and consent form	Y	
3.	Copy of Research Ethics Committee submission	N	I will be sending the ethics form in when I get it back
4.	Copy of completed grant application form	N	
5.	SUHT R&D number provided	Y	

f. Participant information sheet (version 3)

UNIVERSITY OF Southampton

School of Biological Sciences

62 Bassett Crescent East, Southampton, SO16 7PX

Bioavailability of Antioxidants in Lettuce

(Ethical submission No: 08/H0504/19)

Participant Information Sheet

Southampton NHS
University Hospitals NHS Trust

wellcome trust

Bioavailability of Antioxidants in Lettuce

Thank you for expressing an interest in taking part in this study. Before you decide, you need to understand why the research is being done and what it would involve for you. Please take time to read the following information carefully. You may talk to others about the study if you wish.

Below is a description of the study and your part in the project. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

It is up to you to decide. We will describe the study and go through this information sheet, which we will then give to you. You will then be presented with a consent form for you to sign, which confirms your willingness to participate in the study, after having been informed of all aspects of the trial that are relevant to your decision to participate. Part B of the form seek consent to store samples for potential future testing but each such future study will need to be reviewed and approved by a Research Ethics Committee. Should you wish to do so, you are free to withdraw at any time.

What is the purpose of the study?

Many plant compounds have been shown or have been proposed to aid in the prevention of a number of diseases by acting as antioxidants, compounds which help in the protection against different types of cancer, in helping to reduce cholesterol levels, helping to prevent age-related eye diseases or diabetes.

Lettuce is one of the top vegetables consumed but is often over looked in terms of its nutritional contributions. Therefore, the purpose of this project is to determine how the ingestion of fresh or stored baby lettuce leaves influence the levels of total antioxidants in human blood and how these nutrients decline in time. The project also aims to identify the individual antioxidant phytonutrients (i.e. nutrients found in plants) which compose the total antioxidant content absorbed by humans.

Your involvement in the project

If you decide to take part in the study, you will be required to attend the Wellcome Trust Clinical Research Facility (WTCRF) facility on one occasion, for a maximum period of 6 hours.

You will take part in one of two blind trials (where you will not know which treatment group you are in), in which you will be given fresh or stored lettuce baby leaves (*Lactuca sativa*) of the green cos variety. You will be reimbursed £40 for your time and travel.

What will I have to do?

The procedure will be as follows: it is vital that you do not to have anything to eat or drink (except water) on the mornings of your appointments. You must not eat fruit or vegetables for 24 hours before the trial begins, as the nutrients from these would compromise the final results. You will be required not to carry out any strenuous exercise activities (such as cycling) the day before and on the days of your appointments. You will also be required not to take any medicines and vitamins for the duration of the study and for two weeks prior to the commencement of the study.

On arrival at the WTCRF, at the Southampton GH, you will be requested to remain resting on a bed or chair as much as possible. A venous cannula (needle and butterfly) will be placed in your forearm vein and a baseline blood sample collected (15ml, the equivalent of approximately 30 teaspoons). You will then consume a breakfast composed of a packet containing 200g of either stored or fresh lettuce leaves, you will be allowed free access to water but will not be allowed to eat anything else throughout the study. Venous blood samples (15ml each time) will be drawn 7 times over a total period of 5 hours. You will also be asked to complete a brief general health questionnaire both before and during the sampling (Fig. 1). You will then be offered a snack such as sandwiches, chocolates, crisps and fruit juices prior to being sent home after the study visit.

What are the possible disadvantages and risks of taking part?

You may feel some mild discomfort during cannulation; however, the risks involved are very low and you will be closely monitored to ensure that the fasting does not highlight any underlying health problems.

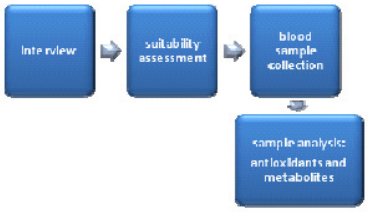


Fig. 1 Outline of the project. After the initial interview, your suitability will be assessed (Body Mass Index, BMI, should be between 19kg/m^2 and 25kg/m^2, with fasting plasma triglyceride concentration less than 2.5mM and cholesterol less than 8mM). During the study (sample collection 1) you will be cannulated (i.e. a needle will be inserted in your forearm) and blood will be drawn 7 times over a period of 7 hours, before and after lettuce ingestion. The blood will then be analysed for antioxidants at the University's School of Biological Sciences and then sent off to another centre to be analysed for individual nutrients.

Confidentiality

Your confidentiality will be safeguarded during and after the study. Your blood samples will be stored at the University of Southampton and coded so that only authorised persons will have access to them, i.e. researchers, sponsors, regulatory authorities and R&D audit. All of the blood samples will be retained for a year and then disposed of securely (procedures for handling, processing, storage and destruction of your data match the Caldicott principles and/or the Data Protection Act 1998). All data collected will be kept in accordance with the Trust's policy.

You will have the right to check the accuracy of the data held about you and to correct any errors.

What will happen if I don't want to carry on with the study?

You will be free to withdraw from the study at any time and the blood samples collected from you will be destroyed, but we will need to use the data already collected up to your withdrawal.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions (contact number). If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure (or Private Institution). Details can be obtained from the hospital.

What if something goes wrong?

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you have concerns about any aspect of the way you have been approached or treated during the course of this study you may wish to contact the hospital's Patient Advice and Liaison Service (PALS) on 023 8079 8498, email PALS@suht.swest.nhs.uk or write to PALS, C Level, Centre Block, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD.

What will happen to the results of the research study?

The results obtained will then be published in a scientific journal. They will also constitute a section of a PhD thesis. A report will be available to the participants explaining the project findings. You will not however, be identified in any report or publication, unless you have given your consent.

Who has reviewed the study?

All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee to protect your safety, rights, wellbeing and dignity. This study has been reviewed and given favourable opinion by the Southampton and Southwest Hampshire Research Ethics Committee.

Please also note that, at the start of the trial, you will also be given a copy of a signed consent form to keep.

Study funding

The study is partly funded by Vitacress Salads Ltd and partly by the BBSRC (Biotechnology and Biological Sciences Research Council).

Further information and contact details

Should you require additional information regarding any of the following, please do not hesitate to contact the principal investigator, the co-investigators or search for information in one of the links below.

1. General information about research.
2. Specific information about this research project.
3. Advice as to whether you should participate.
4. Who you should approach if unhappy with the study.

Principal investigator:
Gaia Biggi BSc MSc
gfb@soton.ac.uk
Tel: 02380594387

Co-investigators:
Dr Mark Dixon
dixon@soton.ac.uk
Prof Gail Taylor
g.taylor@soton.ac.uk

WTCRF: http://wtcrf.soton.ac.uk

Southampton University Hospitals Trust: http://www.suht.nhs.uk/index.cfm?articleid=1134

g. Email enquiry reply

Dear ...,

Thank you for expressing an interest in this study.

The actual study will commence in July at the WTCRF (in the General Hospital), but we would require your attendance on two separate occasions: the first (in June) for a quick general chat about the study and the second, in July, for the actual sampling. During this second visit, you will be cannulated, a first small amount of blood sample will be drawn, then you will be given the lettuce to eat and blood will be drawn again another 6 times, once every hour. You will then be offered some lunch (sandwiches, crisps etc) and something to drink before leaving, as you will not be allowed to eat or drink anything except water during the actual sampling. You will be reimbursed £40 for time and travel.

We require non-smokers, with body-mass-index >20kg/m^2 and <25kg/m^2. Please note that we will not be able to include participants with diagnosed type 1 or type 2 diabetes mellitus or who are receiving anti-coagulant therapy; you will not be allowed to take vitamin supplements (but only for a week prior to the study); we will also not be able to include vegetarians or vegans; and you will not be allowed to exercise for the 3 days prior to the study.

During the first visit (in June), you will be asked to complete a brief general health questionnaire and will be shown the samples and the quantities you would have to eat, and you will be explained the study in detail.

I am attaching the Information Sheet which explains everything in more detail and what your involvement in the study would be.

I hope you are still interested in participating. If you have any questions, please don't hesitate to contact me again or, if you prefer, we can arrange for you to come and meet me directly here at Boldrewood.

Best regards,

Gaia

Version 1 REC: 08/H0504/19

h. Email to confirm and to set the dates for each participant

Dear all,

The dates have been set for commencement of the Lettuce study at the General Hospital. The trial will start at 8am on the following days: the 16th, 17th, 23rd and 24th of July. I would like to ask you if you have any preferences and, if so, could you please let me know your order of preference.

Could you also please send me: your correspondence address, your age, and your height and weight measurements in cm and Kg (for BMI calculations).

Blood pressure measurements will take place on the day, at the trial site, and lunch will be provided (free) at the WTCRF centre (at the Hospital) after the sampling has taken place.

A formal letter of invitation will be sent to you, once we have organised the sampling days.

Please let me know if you are still interested in participating and if you would like to meet up some time before the trial starts to have a chat about the study and to give you a chance to answer any further questions you may have (please note I shall be away between the 1st and the 8th of July). Or, if you prefer, we can meet directly on the trial days.

Thank you,

Best regards,

Gaia

i. Letter of invitation to the participants (version 2)

UNIVERSITY OF Southampton

School of Biological Sciences
University of Southampton
Bassett Crescent East
Southampton, SO16 7PX

15/07/2008

Study title: Bioavailability of Lettuce Antioxidants

Dear **(participant)**,

Thank you for agreeing to take part in our study.

An appointment has been made for you at **(time/date)** in the Welcome Trust Clinical Research Facility (WTCRF) at Southampton General Hospital. If you enter the hospital through the main entrance, turn right; continue past the lift, the WTCRF is located on your left. On arrival at the WTCRF, please ask a member of the green team.

Please remember you must not eat any fruit or vegetables for 24 hours before the trial begins and you must not to have anything to eat or drink (except water) on the mornings of your appointments as the nutrients from these would compromise the final results. You will then be given a bag of fresh or stored lettuce for you to eat. Please also remember not to carry out any strenuous exercise activities (such as cycling) the day before and on the days of your appointments. You will be provided with something to eat before being sent home after your study visit.

Please avoid taking any other medicines and vitamins for the duration of the study and for 2 weeks prior to commencing the study. If you do need to take any medications, please make a note of them. If you are unclear about any of these instructions please do not hesitate to contact me. You will be closely monitored during the study visit to ensure that the fasting does not highlight any underlying health problems.

If you have any problems with this appointment or develop a cold or infection shortly before this appointment please telephone 02380594387 to arrange an alternative appointment. If you need to call on the day of your appointment please call the WTCRF directly on 02380794989.

Thank you again for your participation in this study.

Gaia Biggi BSc MSc

Tel: 023 80594387

Email: gfb@soton.ac.uk

j. Final email to all the participants

Hello everyone,

I would just like to remind you, before the study starts, not to eat fresh fruit, vegetables or chocolate, and not to drink tea, coffee, cocoa or red wine for the previous 24 hours, and not exercise for the previous 2 days. You must not to have anything to eat or drink (except water) on the mornings of your appointments as the nutrients from these would compromise the final results. Please don't take any vitamin supplements or medicines (but that would have been from the previous 2 weeks anyhow). If you do need to take any medications, please make a note of them.

To get to the Welcome Trust Clinical Research Facility (WTCRF) at Southampton General Hospital: if you enter the hospital through the main entrance, walk past the shops and turn right; continue past the lift, the WTCRF is located on your left. On arrival at the WTCRF, please ask a member of the green team.

We start at 8am and should be done by 12, when you will be provided with some lunch before leaving. You are allowed to drink plenty of water throughout the study.

In case you don't remember your allocated date, please find attached a spreadsheet with details of who is due in on which day.

If you have any problems with your appointment or develop a cold or infection shortly before the appointment please telephone 023 80594387 to arrange an alternative appointment. If you need to call on the appointment days (16th, 17th, 23rd and 24th) please call the WTCRF directly on 023 80794989.

Thanks again for your participation.

See you all soon,

Gaia

k. Consent form (version 2)

UNIVERSITY OF Southampton

Consent Form

Thank you for reading the information about our research project. If you would like to take part, please read and sign this form

Study Identification Number: REC reference number: 08/H0504/19

Name of Researcher: Gaia Francesca Biggi

Contact details of research team: email gfb@soton.ac.uk; tel 02380594387

Participant Identification Number:

Title of Project: Bioavailability of Lettuce Antioxidants

Part A: *Consent for the current study*

(samples to be destroyed unless part B completed)

PLEASE INITIAL THE BOXES IF YOU AGREE WITH EACH SECTION:

1. I confirm that I have read the information sheet dated 01/02/2008, version 2, for the above study, have had the opportunity to ask questions, understand why the research is being done and any possible risks which have been explained to me. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time by contacting Gaia Biggi without giving any reason and without my medical care or legal rights being affected. If I withdraw I understand that any unused donated blood will be disposed of, in the case of linked anonymised samples (i.e. samples that are fully anonymous to the researchers who receive them, but contain codes that would allow to link them back to identifiable individuals). ☐
3. I understand that sections of any medical notes may be looked at by responsible individuals from Southampton General Hospital or from regulatory authorities where it is relevant to my taking part in this study. I give permission for these individuals to have access to my records. I understand that the information will be kept confidential. ☐
4. I understand that I will be informed, through my family doctor, if any of the results of the medical tests done as part of the research are important for my health. ☐
5. I agree to take part in the above study to collect a sample of blood to look at antioxidant levels after lettuce consumption. ☐
6. I have informed the researchers of my participation in any other research study. ☐
7. I know how to contact the research team if I need to. ☐

Version 2 03/04/2008 REC: 08/H0504/19

UNIVERSITY OF Southampton

Samples gifted for storage and use in future studies

PART B *Linked anonymised samples* (samples that are fully anonymous to the researchers who receive them, but contain codes that would allow to link them back to identifiable individuals)*:*

8. Provided that specific study protocols have been reviewed and approved by the local research ethics committee. I indicate my consent for the samples to be stored by Gaia Biggi and Prof Gail Taylor at the University of Southampton (potentially for many years) for the following types of studies. I understand that these studies are not for the purpose of directly benefitting my health:

   I give permission for the samples to be used for investigations of nutritional properties relating to the consumption of lettuce. ☐

9. I want/do not want (**delete as applicable**) to be told the results of this test. I understand I can change my mind about this later. ☐

10. In the case of linked anonymised samples, I give permission for a member of the research team to look at my medical records, to obtain information on medical history. I understand that the information will be kept confidential. ☐

Name of patient	Date	Signature
Name of person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 for patient, 1 for researcher, 1 to be kept with university notes

Version 2 03/04/2008 REC: 08/H0504/19

I. Volunteer details and questionnaire form

University of Southampton

Volunteer details and questionnaire

Name _____ Date _____

Age _____ DOB _____

BMI _____

What you have eaten in the past 24 hours (yesterday)

- Breakfast:
  _____
  _____
- Lunch:
  _____
  _____
- Dinner:
  _____
  _____

Activities carried out during the past 3 days (including walking/cleaning/cycling etc):

_____

_____

_____

Blood group: _____

Triglycerides: _____

Medications: _____

REC reference 08/H0504/19 version 1 23/06/2008

University of Southampton

Ethnic background

(Please tick)

- White
  - ☐ British
  - ☐ Irish
  - ☐ Scottish
  - ☐ Irish traveller
  - ☐ Welsh
  - ☐ Other European white background:_____
  - ☐ Other Caucasian Background (e.g. Middle Eastern or North African countries):_____
- Asian or Asian British
  - ☐ Indian
  - ☐ Pakistani
  - ☐ Bangladeshi
  - ☐ Other Asian background: _____
- Mixed
  - ☐ White and Asian
  - ☐ Other mixed background: _____
- Other ethnic background:_____

m. Fresh Produce Journal article

Science in the bag

SCIENTISTS FROM the University of Southampton, in collaboration with Vitacress Salads, are undertaking a trial to assess the health benefits of eating bagged lettuce.

Previous studies involving other vegetables and fruit have shown a number of plant compounds to have properties that aid prevention of certain diseases. Less information is available for lettuce, while consumption of bagged salads has increased significantly in recent years, with Vitacress Salads often producing 1.5 million bags of salad for the UK market each week

By acting as antioxidants, plant compounds have shown, or been proposed to show, benefits in preventing certain forms of cancer, in reducing cholesterol levels, helping to prevent age-related eye diseases and in prevention of diabetes.

The purpose of the study is to determine how the ingestion of 'fresh from the field' and 'supermarket bagged' baby lettuce leaves influence the levels of total antioxidants in human blood and how these nutrients decline in time. The project also aims to identify the nutrients found in plants – individual antioxidant phytonutrients – which compose the total antioxidant content absorbed by humans, pinpointing which plant chemicals are important.

Volunteers will take part in one of two blind trials, in which they will be given fresh or bagged baby lettuce leaves of the green cos variety for breakfast. Blood samples will then be taken over several hours and analysed at the University of Southampton's School of Biological Sciences, to assess the impact of lettuce eating.

Dr Graham Clarkson from Vitacress said: "We at Vitacress are passionate about the health-giving properties of our salads, from watercress and wild rocket down to the not-so-humble lettuce. We are particularly keen to see proof that eating our freshly prepared and bagged leaves is just as good as cutting them from your own garden." ○

n. Progress report form

NHS
National Patient Safety Agency

National Research Ethics Service

ANNUAL PROGRESS REPORT TO MAIN RESEARCH ETHICS COMMITTEE
(For all studies except clinical trials of investigational medicinal products)

To be completed in typescript and submitted to the main REC by the Chief Investigator. For questions with Yes/No options please indicate answer in bold type.

1. Details of Chief Investigator

Name:	Gaia Biggi
Address:	School of Biological Sciences, 62 Bassett Crescent East, University of Southampton, Boldrewood Campus, Southampton, Hampshire, SO16 7PX
Telephone:	023 80594387
Email:	gfb@soton.ac.uk
Fax:	023 80594459

2. Details of study

Full title of study:	Effect of lettuce ingestion on postprandial antioxidant phytonutrient levels in human blood plasma
Name of main REC:	Southampton and South West Hampshire Research Ethics Committee (B)
REC reference number:	08/H0504/19
Date of favourable ethical opinion:	09/04/2008
Sponsor:	SUHT R&D

3. Commencement and termination dates

Has the study started?	***Yes*** / *No*
If yes, what was the actual start date?	16/07/2008
If no, what are the reasons for the study not commencing? What is the expected start date?	
Has the study finished?	***Yes*** / *No* *If yes, complete and submit "Declaration of end of study" form, available at www.nres.npsa.nhs.uk*

Annual progress report (non-CTIMP), version 3.2, dated January 2007

If no, what is the expected completion date? *If you expect the study to overrun the planned completion date this should be notified to the main REC for information.*	
If you do not expect the study to be completed, give reason(s)	

4. Site information

Is this a study requiring site-specific assessment (SSA) and ethical approval of each site and local Principal Investigator? If yes, how many UK research sites have been recruited Has the Site Specific Information Form (SSIF)* been submitted to the local REC for each local Principal Investigator? **or Part C or Annex D of the former MREC application form if submitted prior to 1 March 2004*	*Yes / **No*** *Proposed in original application:* *Actual number recruited to date:* *Yes / No /Not applicable*
Is this study "SSA-exempt"? * If yes, how many UK sites are currently involved in facilitating this research? ** or was previously designated as a "no local investigator" or "no local researcher" study*	***Yes** / No* 2 sites (the WTCRF at the Southampton General Hospital and the Southampton University School of Biological Sciences)
Do you plan to increase the total number of UK sites proposed for the study? If yes, how many sites do you plan to recruit? *In the case of studies requiring SSA, all sites must be approved by the main REC as part of the favourable opinion.*	*Yes / **No***

5. Recruitment of participants

*Number of participants recruited:	*Proposed in original application: between 20 and 24* *Actual number recruited to date:22*
*Number of participants completing trial:	*Proposed in original application: between 20 and 24* *Actual number completed to date:19*
*Number of withdrawals due to: (a) lack of efficacy (b) adverse events **(c) self-withdrawal** **(d) non-compliance** Total number of withdrawals: 3	

Have there been any serious difficulties in recruiting participants?	***Yes*** */ No*
If Yes, give details:	
Do you plan to increase the planned recruitment of participants into the study? *Any increase in planned recruitment should be notified to the main REC as a substantial amendment for ethical review.*	*Yes /* ***No***

** In the case of international trials, please provide separate figures for UK and non-UK participants.*

6. Safety of participants

Have there been any *related* and *unexpected* Serious Adverse Events (SAEs) in this study?	*Yes /* ***No***
Have these SAEs been notified to the Committee? *If no, please submit details with this report and give reasons for late notification.*	*Yes / No /* ***Not applicable***
Have any concerns arisen about the safety of participants in this study? *If yes, give details and say how the concerns have been addressed.*	*Yes /* ***No***

7. Amendments

Have any substantial amendments been made to the trial during the year?	*Yes /* ***No***
If yes, please give the date and amendment number for each substantial amendment made.	

8. Other issues

Are there any other developments in the study that you wish to report to the Committee? Are there any ethical issues on which further advice is required?	*Yes /* ***No*** *Yes /* ***No*** *If yes to either, please attach separate statement with details.*

9. Declaration

Signature of Chief Investigator:	
Print name:	Gaia Biggi

Date:	07/08/2008

o. End of study report form

NHS
National Patient Safety Agency

National Research Ethics Service

DECLARATION OF THE END OF A STUDY
(For all studies except clinical trials of investigational medicinal products)

To be completed in typescript by the Chief Investigator and submitted to the Research Ethics Committee that gave a favourable opinion of the research ("the main REC") within 90 days of the conclusion of the study or within 15 days of early termination. For questions with Yes/No options please indicate answer in bold type.

1. Details of Chief Investigator

Name:	Gaia Biggi
Address:	School of Biological Sciences, 62 Bassett Crescent East, University of Southampton, Boldrewood Campus, Southampton, Hampshire, SO16 7PX
Telephone:	023 80594387
Email:	gfb@soton.ac.uk
Fax:	023 80594459

2. Details of study

Full title of study:	Effect of lettuce ingestion on postprandial antioxidant phytonutrient levels in human blood plasma
Research sponsor:	SUHT R&D
Name of main REC:	Southampton and South West Hampshire Research Ethics Committee (B)
Main REC reference number:	08/H0504/19

3. Study duration

Date study commenced:	16/07/2008
Date study ended:	24/07/2008
Did this study terminate prematurely?	*Yes / **No*** *If yes please complete sections 4, 5 & 6, if no please go direct to section 7.*

4. Circumstances of early termination

What is the justification for this early termination?	

5. Temporary halt

Is this a temporary halt to the study?	*Yes / No*
If yes, what is the justification for temporarily halting the study? When do you expect the study to re-start?	*e.g. Safety, difficulties recruiting participants, trial has not commenced, other reasons.*

6. Potential implications for research participants

Are there any potential implications for research participants as a result of terminating/halting the study prematurely? Please describe the steps taken to address them.	

7. Final report on the research

Is a summary of the final report on the research enclosed with this form?	*Yes / **No*** *If no, please forward within 12 months of the end of the study.*

8. Declaration

Signature of Chief Investigator:	
Print name:	Gaia Biggi
Date of submission:	07/08/2008

p. Final report on the research

06/03/2010

Final report on the research

REC reference number: 08/H0504/19
Researcher: Gaia Francesca Biggi

1. The research has reached its objectives: the main findings are explained below.
2. These findings will be published as part of a PhD final thesis.
3. The participants will be informed of these findings via email.

4. *Bioavailability of lettuce antioxidants: main findings*

The bioavailability of antioxidants in plasma samples of male volunteers was examined after ingestion of intact fresh or stored (for one week at 4°C) baby leaf lettuce samples. Green cos leaves were used as they represent a variety of leaf available in most shops and commonly used as basis for salads or in sandwiches. Consumer sized bags (120g) of baby leaf lettuce such as the commercial varieties commonly found in Europe and the UK were fed to fasting volunteers. Whole baby leaves were used in the present study as plant metabolites start to decline soon after harvest, therefore, further chopping of leaf material would result in further declines in a number of metabolites, as well as increases in certain polyphenols, thus introducing other elements of variation to the samples. The choice of using whole baby leaves also followed from the modern trend for bagged baby leaf salads.

The intervention trial results demonstrate that consumption of fresh lettuce significantly increases the total antioxidant potential found in blood plasma and illustrates how it increases with time after consumption before declining again. A significant rise in antioxidant status in blood plasma occurred soon after lettuce consumption, between 30min postprandial and 90min there was a dramatic and significant rise. A peak in antioxidant potential was found 90 minutes after ingestion after which the levels started to drop off again. The results showed that there was a gradual but significant decline in antioxidant status of blood plasma after 90min and that it was still significantly higher at 120min than at time 0min. Furthermore, whilst the decline from the antioxidant peak at 90min to 150min was significant, the levels

1

at 150min are still greater than at time 0min, thus indicating a gradual decline in antioxidant phytonutrients in the blood (Fig 1).

A three-fold significant increase was noted within 1.5hrs ingestion of fresh lettuce, whereas after ingestion of stored lettuce antioxidants did not rise significantly in blood. Interestingly, in the latter case, the antioxidant potential showed a declining trend at times 60min and 150min, towards levels lower than the initial levels at time 0min.

Previous studies have been carried out examining the effects of postprandial blood antioxidant status after consumption of other types of food or beverages, however, no other study had focused on the postprandial effects of packaged fresh and stored intact baby leaf lettuce on human blood antioxidant status. This study has shown that eating fresh lettuce increases the antioxidant status of blood plasma significantly whereas eating stored lettuce does not induce a significant change in antioxidant levels. Therefore, the study expands our understanding of the true nutritional value of modern convenience packed salad as it underlines the importance of eating sufficient quantities of fresh leaves rather than stored ones.

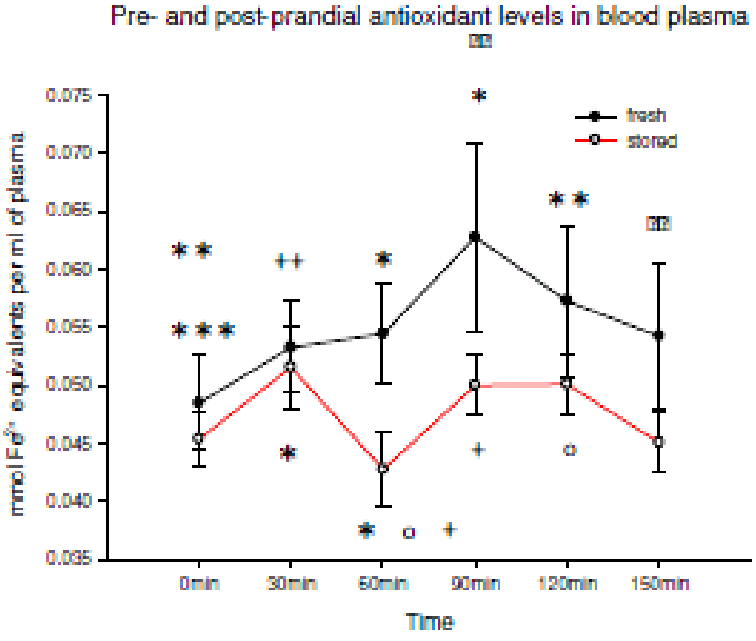
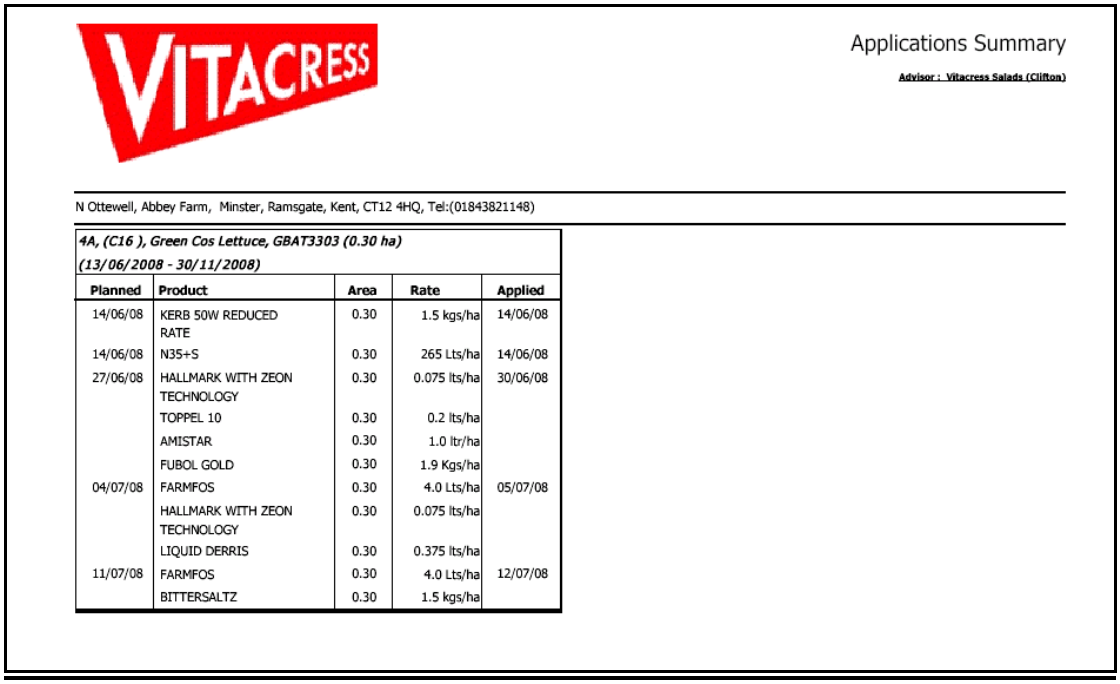


Fig. 1 Antioxidant levels of human blood plasma before and after lettuce consumption.

q. Green cos processing procedure



Applications Summary

Advisor : Vitacress Salads (Clifton)

N Ottewell, Abbey Farm, Minster, Ramsgate, Kent, CT12 4HQ, Tel:(01843821148)

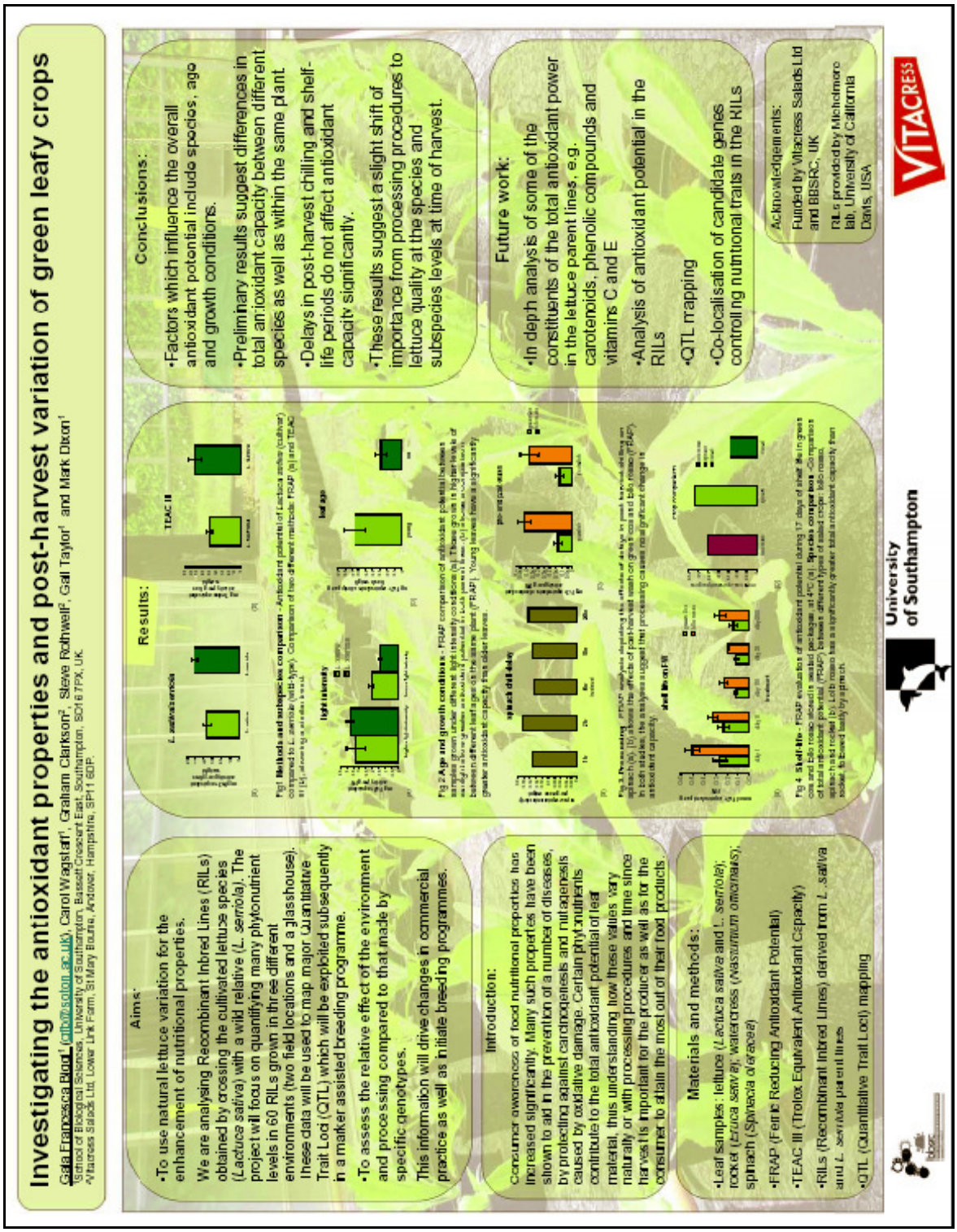
4A, (C16), Green Cos Lettuce, GBAT3303 (0.30 ha)
(13/06/2008 - 30/11/2008)

Planned	Product	Area	Rate	Applied
14/06/08	KERB 50W REDUCED RATE	0.30	1.5 kgs/ha	14/06/08
14/06/08	N35+S	0.30	265 Lts/ha	14/06/08
27/06/08	HALLMARK WITH ZEON TECHNOLOGY	0.30	0.075 lts/ha	30/06/08
	TOPPEL 10	0.30	0.2 lts/ha	
	AMISTAR	0.30	1.0 ltr/ha	
	FUBOL GOLD	0.30	1.9 Kgs/ha	
04/07/08	FARMFOS	0.30	4.0 Lts/ha	05/07/08
	HALLMARK WITH ZEON TECHNOLOGY	0.30	0.075 lts/ha	
	LIQUID DERRIS	0.30	0.375 lts/ha	
11/07/08	FARMFOS	0.30	4.0 Lts/ha	12/07/08
	BITTERSALTZ	0.30	1.5 kgs/ha	

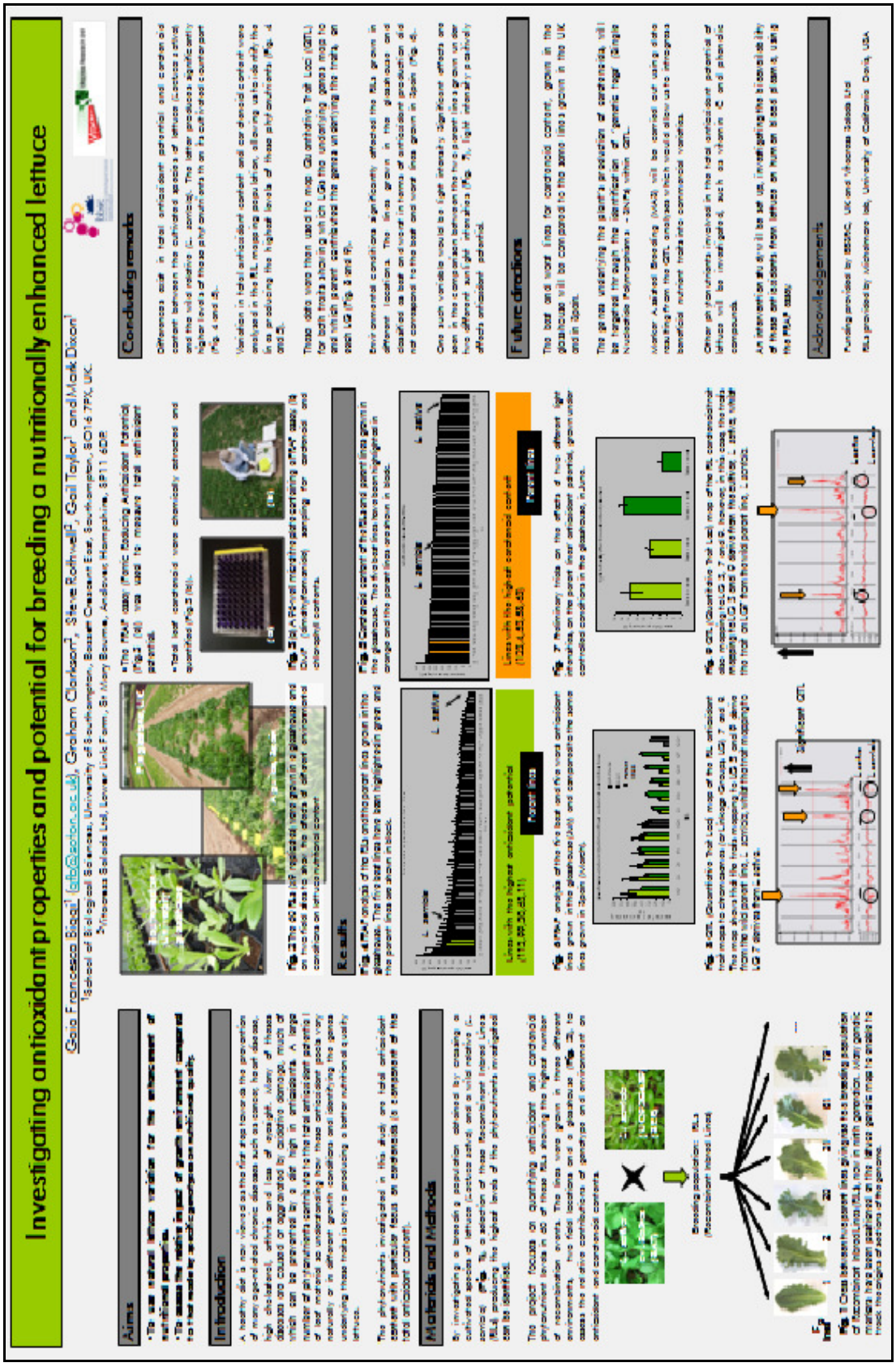
APPENDIX 6

POSTERS

Poster presented at the 2006 Gordon Conference (GRC) on post-harvest Physiology, held at Connecticut College, in the USA.



Poster presented at the Eucarpia Conference in April 2007 held at the University of Warwick UK.



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