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Towards the development of an *Eruca sativa* crop with improved nutritional and flavour qualities by investigating the relationship between phytochemical and sensory attributes of breeding lines cultivated in different environments and identification of molecular markers for sugars.

Thesis submitted for the degree of Professional Doctorate in Sustainable Food Quality for Health (DAgriFood)

Department of Food and Nutritional Sciences

By

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Declaration

I confirm that the work presented in this thesis is my own and that the use of all the literature from other sources has been properly and fully acknowledged.

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ABSTRACT

Eruca sativa, also known as 'salad' rocket, is a ready-to-eat leafy salad of the *Brassicaceae* family that is gaining popularity. It contains various important phytochemicals such as glucosinolates, flavanols, vitamins, and minerals that are thought to benefit human health. 'Salad' rocket is known for its distinct sensory characteristics, such as hot, pungent, peppery, and bitter; sometimes not widely accepted by many consumers. Numerous factors such as genetics (crop and human), stresses (abiotic and biotic), seasons, and cultivation practices influence the sensory attributes such as taste and flavour of 'salad' rocket, resulting in inconsistent nutritional 'quality'. Due to growing demand for rocket crops, growers and producers are increasingly under pressure to provide supermarkets and consumers with consistent high 'quality'. Moreover, the ability of human taste receptors to assess sensory attributes is highly subjective. However, it is known that sugars play a key role in determining the overall taste and flavour of fruit and vegetables as they can mask other tastes, such as bitterness.

The overall aim of the present study was to identify molecular markers for sugars in a mapping population of *E. sativa* for increased consumer acceptance while maintaining the health benefits associated with the crop. Instruments such as high-performance liquid chromatography, liquid chromatography-mass spectrometry, and inductively coupled plasma optical emission spectroscopy were used to measure sugars, organic acids, glucosinolates, and sulphur content present in the 'salad' rocket. Sensory analysis was carried out using two trained panels, differing in genotype for the TAS2R38 bitter taste receptor that was associated with the perception of a bitter taste for glucosinolates. Three objectives were set to achieve the overall aim of the present study.

Firstly, to measure the abundance of phytochemicals (sugars, organic acids, and GSLs) present in an F3 mapping population of 141 recombinant inbred lines of 'salad' rocket (*Eruca sativa*) grown at two separate locations: Italy and the UK and to understand the influence of environmental conditions on the accumulation of phytochemicals present. Secondly, to identify the quantitative trait loci responsible for the accumulation of primary metabolites that will be utilised in the future breeding programme of *E. sativa* for targeted nutritional 'quality'. Finally, to understand the sensory perceptions of 'salad' rocket on human genotype by investigating the relationship between environmental factors and phytochemical (sugars, glucosinolates, and sulphur) constituents on selected six lines of *E. sativa*, on the first and second cut with two-time points (day 0 and day 5). The six lines (21, 25, 68, 72, 112, and 130) were chosen based on their high or low abundance of glucosinolate content from a previously developed mapping population.

The results from the first objective suggested a clear influence of the growth environment on the accumulation of phytochemicals with the UK-grown plants showing a two-fold higher total sugar concentration compared to Italian-grown plants. Other phytochemicals such as total organic acids and total glucosinolates did not show any statistically significant differences between the trial locations, however, individual glucosinolate and organic acid varied significantly ($p \leq 0.05$). In the second objective, a total of 20 quantitative trait loci were identified across the two trials, with 13 quantitative trait loci identified from the UK trial and 7 quantitative trait loci from the Italian trial. Here, we presented a first linkage and quantitative trait loci map for metabolites such as sugars, organic acid, and glucosinolates using a mapping population of 141 F3 recombinant inbred lines of *E. sativa*. The linkage map was constructed using 285 high-quality single nucleotide polymorphism markers having a map length of 889.2 cM, distributed onto 18 linkage groups covering all 11 chromosomes. The results from the third objective showed a significant difference

($p \leq 0.05$) in phytochemical content and sensory attributes, which were influenced by both locations and selected six lines (21, 25, 68, 72, 112, and 130). The second cut of UK-grown leaves showed a 3.5-fold higher total sugar concentration compared to the first cut. Total glucosinolates and sulphur contents were higher in the Italian trial and were positively correlated with sensory attributes such as bitterness and pepperiness. Sugars were higher in the UK-grown trial and were positively correlated with a sweet taste. Furthermore, individuals with PAV/PAV TAS2R38 diplotypes showed a reduced perception of the subtle flavour component of rocket leaves compared with AVI/AVI diplotypes. Lines 68, 112, and 130 were positively correlated with sensory attributes such as pepperiness, pungency, and sweetness, while lines 21, 25, and 72 were positively associated with green flavour, green aroma, and moistness.

The results from the present study highlighted the components important for determining the taste/ flavour of *E. sativa*. Lines 68, 112, and 130 could be used as the potential candidates in a breeding programme for those who prefer their rocket 'hot', 'peppery', and 'sweet', while lines 21, 25, and 72 for those who prefer 'mild' rocket. This information will enable breeders to select specific cultivars to cater for the specific consumer groups that have known sensory profiles. Combining the knowledge of genetic and chemical information will help to breed a 'salad' rocket for increased consumer acceptance while maintaining the maximum health benefits associated with the crop.

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Chapter 2: Text, tables and figures produced by Manik Puranik with additional comments and guidance by Prof Carol Wagstaff and Dr Luke Bell. Field trials at both locations were carried out manually, by planting each seedling into the prepared bed at both locations and later harvested by Manik Puranik with the help of Dr Luke Bell, Dr Jake Jasper, and Dr Lucy Turner. Washing, rinsing, spinning, and packaging steps for the leaves of all six lines of rockets were carried out by Manik Puranik, Dr Luke Bell and Dr Martin Chadwick.

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Chapter 4: Text, tables, and figures produced by Manik Puranik with additional comments and inputs provided by Prof Carol Wagstaff and Prof Lisa Methven. Sugar, sulphur, and GSL extractions were performed by Manik Puranik and sugar analysis on HPLC was performed by Manik Puranik with the additional guidance of Dr Martin Chadwick. Sulphur analysis was performed by Dr Martin Chadwick on the ICP-OES instrument and GSL analysis was performed by Dr Luke Bell on LC-MS. All the statistical analysis on sugar, sulphur, and GSL data was performed by Manik Puranik with additional guidance from Dr Luke Bell and Dr Martin Chadwick.

Sensory analysis such as recruitment of panellists, training sessions and assessment of panellists was performed by Dr Luke Bell, Manik Puranik, and Dr Martin Chadwick. Sample preparations for assessment criteria were prepared by Manik Puranik. Sample serving during the trials was performed by Manik Puranik with additional help from Dr Martin Chadwick. Application for ethical approval was performed by Dr Luke Bell with the help of Prof Lisa Methven. Data generated in the sensory study were analysed and interpreted and all the statistical analysis was performed by Manik Puranik with the guidance of Prof Lisa Methven and Dr Luke Bell.

Chapter 5: All text by Manik Puranik with additional comments and inputs by Prof Carol Wagstaff.

ABBREVIATIONS

A	Aroma
AE	Aftereffects
AFLP	Amplified fragment length polymorphism
ANOVA	Analysis of variance
AVI	Alanine Valine isoleucine
BTL	Butyl GSL
CA	Controlled atmosphere
CIM	Composite interval mapping
cM	Centimorgan
CMS	Cytoplasmic male sterility
CRISPR	Clustered regularly interspaced short palindromic repeats
D0	Day 0 (intake)
D5	Day 5 (postharvest shelf life)
DAD	Diode array detector
DGTB	Diglucothiobeinin
DH	Doubled haploid
DMB	Dimeric-4-mercaptobutyl glucosinolate
DNA	Deoxyribose nucleic acid
DW	Dry weight
F	Flavour

GAL	Glucoalyssin
GBC	Glucobrassicin
GBT	Glucoberteroin
G x E	Genotype x environment
GIB	Glucoiberin
GER	Glucoerucin
GKR	Pentyl GSL
GNP	Gluconapin
GNT	Gluconasturtiin
GPCR	G-protein coupled receptor
GPJ	Glucoputranjivin
GRA	Glucoraphanin
GRM	Glucorucolamine
GSL	Glucosinolate
GSV	Glucosativin
GTP	Glucotropaeolin
HEX	Hexyl GSL
HPLC	High-performance liquid chromatography
HSD	Honest significant difference
ICIM	Inclusive composite interval mapping
ICP-OES	Inductively coupled plasma-optical emission spectroscopy

ISSR	Inter-simple sequence repeat
ITC	Isothiocyanate
LC-MS	Liquid chromatography-mass spectrometry
LEDs	Light-emitting diodes
LOD	Logarithm of the odds
MAP	Modified atmosphere packaging
MCA	Multiple component analysis
MF	Mouthfeel
MIM	Multiple interval mapping
MQM	Multiple QTL mapping
NIL	Near isogenic line
NGB	Neoglucobrassicin
NPBT	New plant breeding technology
PAV	Proline alanine valine
PC	Principal Component
PCA	Principal Component Analysis
ppb	Parts per billion
PRO	Progoitrin
PTC	Phenylthiocarbamide
PROP	6-n-propyl-thiouracil
QDA	Quantitative descriptive analysis

QTL	Quantitative trait loci
RADP	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RH	Relative humidity
RI	Refractive index
RIL	Recombinant inbred line
RNA	Ribose nucleic acid
ROS	Reactive oxygen species
RTE	Ready-to-eat
SIM	Single interval mapping
SIN	Sinigrin
SMA	Single marker analysis
SNP	Single nucleotide polymorphism
SSD	Single seed descent
SSR	Single sequence repeat
T	Taste
TCA	Tricarboxylic acid
1-MCP	1-methylcyclopropene
4HGB	4-hydroxyglucobrassicin
4MGB	4-methoxyglucobrassicin
4MP	4-methylpentyl GSL

CHAPTER 1

Introduction

1.1. Background

1.1.1. Minimally processed leafy salad as a pre-packed ready-to-eat food

The world's population is projected to increase to 10 billion by 2050. The overall agricultural productivity must increase by at least 70% by the year 2050, to feed the growing population (Godfray *et al.*, 2010; Voss-Fels *et al.*, 2019). So far, to improve food production and food security, various approaches such as optimising plant regime, sustainable farming practices, traits introgressing etc., have been proposed, of which optimising the plant breeding and biotechnology approach is the most promising (Ansarifar *et al.*, 2020). The increase in agricultural productivity must be achieved despite the change in global climate with limited resources, hence, breeding new varieties with higher crop yields will be an absolute necessity to fulfil future needs and food security (Witcombe *et al.*, 2013).

Stating a phrase quoted by Dr Howard-Yana Shapiro, "It is not so much a question of more food, it is more a question of better food" (Bell and Wagstaff, 2017). Historically, agricultural strategies have been to breed crop varieties for increased yields, but this came at the cost of nutritional quality in some instances (Benbrook, 2009; DeFries *et al.*, 2015). Recently, due to consumer awareness and demand for healthy food, breeders have begun focusing on creating new and nutritionally dense varieties (Francisco *et al.*, 2017). In recent years, the consumer has become more oriented towards low-energy foods with low-fat content, at least in some demographics, but at the same time pays

attention to the presence of bioactive compounds derived from plant-based food (Toscano *et al.*, 2019).

In today's busy lifestyle, consumers increasingly feel that they do not have enough time to prepare meals and prefer convenient ready-to-eat (RTE) food products. Minimally processed leafy vegetables are considered one such RTE food, which is sold as RTE salad is gaining more attention worldwide as these do not need further processing and are ready for direct consumption (Lemoine *et al.*, 2007; Cavaiuolo *et al.*, 2015). Furthermore, RTE leafy salads could retain the nutrient levels as these do not need further processing such as cooking (Wagstaff, 2014). They enable consumers to meet a '5-a day' target conveniently as they fit the criteria of being both healthy and convenient (Atkinson *et al.*, 2013a). Minimally processed RTE salads are often sold in supermarkets, fast food outlets, restaurants, and food vending areas of airports or are served to passengers during flights (Ansah *et al.*, 2018).

Several studies in the literature have highlighted that prolonged intake of leafy vegetables has a beneficial impact on human health, however, much of the world's population does not consume enough of them to receive these benefits (Martinez-Sanchez *et al.*, 2008; Aires *et al.*, 2011; Bell *et al.*, 2015). Despite government initiatives for the '5-a-day' campaign in the UK and in the US, diets in Western countries generally lack fruits and vegetables as people are not consuming enough, leading to premature deaths (Bell and Wagstaff, 2014). Studies from Cox *et al.* (2012) and Bell *et al.* (2015) proposed to breed a more nutritionally dense variety while maintaining sensory and visual acceptance, as achieved for the Beneforte variety in Broccoli by using advanced screening, and plant breeding.

1.1.2. Pre-packed RTE salad market

The demand for nutritionally rich convenient food is gradually increasing in both developed as well as developing countries (Saini *et al.*, 2017). The market has been rapidly growing and offering consumers a convenient and appealing product rich in not only nutrition but also taste, flavour, and texture. Recently, the consumption of RTE leafy green salads has increased in Europe with a turnover of about 600 million Euros (Arienzo *et al.*, 2020). Kanter World Panel (2022) reported that in the last decade the number of prepared salads purchased has doubled in the UK from a spend of £820 million to £1167 million per annum showing an increase in the consumption of RTE leafy salads. In Italy, the consumption of the RTE fresh cut has increased by more than 200 %, over 10 years suggesting that the turnover of the RTE market in 2011 was about $862 million with a 4.4 % increase (Saini *et al.*, 2017; Ansah *et al.*, 2018). One of the largest retail chains in Sweden reported the sale of RTE prepacked mixed salad bags increased from 600,000 in 2005 to nearly 40 million bags in 2016 (Söderqvist, 2017). Moreover, RTE minimally processed leafy salads are becoming more popular and profitable due to the high demand for healthy and convenient food and due to their softer textures and attractive presentation (Martínez-Sánchez *et al.*, 2012).

1.1.3. Beneficial effects of RTE leafy salads on heath

Consumption of fruits and vegetables is an effective way to maintain and improve health (Poiroux-Gonord *et al.*, 2010; Saini *et al.*, 2017). Convenience is the key factor that leads a consumer to choose minimally processed RTE salads over whole head salads, however, the nutritional quality is the most important criterion for choosing these due to increased perception of preserving health by choosing a healthy diet (Barrett *et al.*, 2010; Poiroux-Gonord *et al.*, 2010; Saini *et al.*, 2017). Health-promoting phytochemicals such as flavonoids, carotenoids, phenolics, GSLs, vitamins and

minerals are abundantly present in RTE leafy vegetables (Martinez-Sanchez *et al.*, 2008). Various authors in the literature reported the bioactive phytochemicals present in the vegetables are responsible for reducing the risk of hypertension, coronary heart disease, stroke, diabetes mellitus (type 2), certain eye diseases, dementia, osteoporosis, asthma, chronic obstructive pulmonary disease, and rheumatoid arthritis (Björkman *et al.*, 2011; Francisco *et al.*, 2017; Saini *et al.*, 2017).

1.1.4. Agronomic practices related to RTE leafy salads

1.1.4.1. Preharvest factors affecting the quality and nutritional content of RTE leafy salads

Plants are claimed to have unlimited sources of phytochemicals that are thought to be significantly influenced by the growing environment and plant genetics (Sudha and Ravishankar, 2002). RTE leafy vegetables are a source of health-beneficial phytochemicals, however, the nutritional quality and shelf life of these products can significantly be influenced by preharvest factors such as cultivar selection, cultivation practices, environmental conditions [temperature, relative humidity (RH), light intensity and rainfall], maturity at harvest as well as postharvest handling (Frezza *et al.*, 2010; Björkman *et al.*, 2011; Bhandari and Kwak, 2015; Lee *et al.*, 2017; Caruso *et al.*, 2019; Simko, 2019; Koukounaras *et al.*, 2020). Preharvest production practices and factors such as cultivation practices (open field or greenhouse), water supply, soil/soilless culture, temperature, and mechanical damage (such as cutting, wounding, etc.,) may affect the postharvest quality and result in the rejection or downgrading of produce at the point of the sale (Clarkson *et al.*, 2005; Carlos de Freitas *et al.*, 2009; Frezza *et al.*, 2010; Acikgoz, 2011; Toscano *et al.*, 2019).

In crops, environmental stresses such as temperature (low or high), salinity, drought etc., may reduce the overall photosynthetic capacity (Ashraf and Harris, 2013) influencing the accumulation of metabolites (Steindal *et al.*, 2015; Petretto *et al.*, 2019; Jasper *et al.*, 2020). Temperature stress is the most common stress experienced by plants around the globe which could alter and affect crop yield and growth. Photosynthesis is highly sensitive to high temperature, where heat stress causes membrane disruption, particularly of the thylakoid membranes, which inhibits the activity of membrane-associated electron carriers and enzymes, resulting in a reduced rate of photosynthesis (Ashraf and Harris, 2013). Furthermore, plants when exposed to high temperature or heat stress showed reduced chlorophyll biosynthesis. On the other hand, when subjected to a low temperature/cold stress, it limits their productivity and compromises quality (Ferrante and Maggiore, 2007). Many of the plant species in nature get damaged due to freezing temperatures (ranging between 0 to -15 °C), however, when exposed to chilling temperatures ranging between 0 and 15 °C, plants survive due to their cold acclimation. Several dysfunctions at the cellular level due to the cold stress could result in disruption of membranes, reactive oxygen species (ROS) accumulation (due to reduced enzyme activity), protein denaturation etc. (Yuanyuan *et al.*, 2009; Sami *et al.*, 2016; Pareek *et al.*, 2017). To cope with the low temperature/cold stress, the plant uses a mixture of strategies such as stress avoidance or stress tolerance resulting in the accumulation of osmolytes such as soluble sugars (Browse and Xin, 2001). Moreover, when subject to cold stress, not all soluble sugars (sucrose, glucose, and fructose) play a similar role during metabolism. For example, soluble sugars such as sucrose and glucose relate to osmoprotectant function and act as a substrate for cellular respiration, whereas fructose seems to relate to the synthesis of secondary metabolites and does not act as an osmoprotectant. A study by Akula and Ravishankar (2011) suggested that temperate plants adapted to variation by adjusting their metabolism towards the synthesis of cryoprotectant molecules to withstand cold tolerance. It has also been reported that

under natural conditions, in the winter season, soluble sugars increase when plants are subjected to low temperatures, in contrast to in the spring season where sugars decline when plants are de-acclimating (Yuanyuan *et al.*, 2009). Studies in the literature reported a higher accumulation of soluble sugars in vegetables such as spinach (Yoon *et al.*, 2017), kale (Steindal *et al.*, 2015), and leaves of cabbage seedlings (Sasaki *et al.*, 1996) when subjected to cold stress (9 °C).

Therefore, the selection of the most appropriate cultivar for RTE leafy salads is of primary importance to have a superior quality, reduced storage losses and high nutritional contents (Fadda *et al.*, 2016). Along with the preharvest factors, cultivation practices also influence the quality and nutritional content of RTL leafy salad.

1.1.4.2. Agricultural practices impacting the quality of RTE leafy salads

Modern agriculture is divided into two main production systems: conventional and alternative (controlled environment with or without soil). To preserve the quality of fresh produce, the adoption of the most suitable cultivation practices is essential (Mahajan *et al.*, 2017). Cultivation practices varied between countries and individual growers (Bell *et al.*, 2020b). Fresh leafy salads are grown and harvested under a wide range of climatic and geographical conditions. These are grown using various agricultural inputs and technologies, such as on-farm or in a protected environment (Gil *et al.*, 2014). A protected/controlled environment is usually where crops are grown indoors (tunnels or glasshouses; increasingly indoor farms that rely wholly on artificial temperature control and light). With regard to quality assurance, a protected environment (greenhouse production) has more advantages over open-field production. In the controlled environment, the parameters such as light, temperature, humidity, atmospheric CO_2, precise water

supply, fertilisation dose, etc., can be optimised and therefore, the fresh produce is not exposed to sudden changes in climatic conditions (Rouphael *et al.*, 2012). Other advantages of a controlled environment are yield could be increased, off-season production could be allowed, and stresses (biotic and abiotic) could be controlled resulting in a higher accumulation of phytonutrients (Bian *et al.*, 2015). This may be the reason most of the leafy vegetables used for the fresh-cut industry are grown in a protected cultivation environment in Europe (Mahajan *et al.*, 2017). One such example is from Philips Research Laboratories (2018) in Eindhoven (The Netherlands), where light-emitting diodes (LEDs) have been introduced to improve the qualities such as taste, yield, vitamin C, and reduced nitrate concentrations of fresh produce such as lettuce, arugula, etc. A previous study reported a higher accumulation of phytochemicals such glucosinolates (GSLs) and their hydrolysis product, isothiocyanates (ITC) in accessions of *Eruca sativa* and *Diplotaxis tenuifolia* when grown under a controlled environment at different temperatures such as 20 °C, 30 °C, and 40 °C (Bell *et al.*, 2015; Jasper *et al.*, 2020) which suggests cultivating practices affect the accumulation of phytochemicals (Guijarro-Real *et al.*, 2018). Although a study on rocket species reported a higher accumulation of GSLs and health-related ITCs, yield and productivity reduced significantly (Jasper *et al.*, 2020). Controlled environment crop production has many advantages over an open field, however, the overall cost of greenhouse production and infrastructure to provide such a facility is expensive to run. Some studies reported for a few crops, field-grown produce showed more accumulation of phytochemicals as compared to those when grown in the greenhouse (Rouphael *et al.*, 2012). The present study on 'salad' rocket reported a three-fold higher accumulation of total sugar concentration when grown in the field grown conditions of the UK environment as compared to the polytunnel grown condition in Italian environment.

1.1.4.3. Impact of maturity and harvests on the nutritional quality of RTE salads

Leafy vegetables are characterised as very perishable commodities, with a high rate of respiration and water loss. Depending upon the final destination of fresh produce, the desired quality attributes, and their tolerance to withstand handling and processing operation (postharvest), a wide range of possibilities are practised during harvesting (Gil *et al.*, 2012). Once harvested, leaves still carry out metabolic processes such as photosynthesis, respiration, or light-dependent biological process (Ruiz de Larrinaga *et al.*, 2019). Maturity is the stage at which a commodity has reached a sufficient stage of development for growth. If the commodity is harvested before or after its maturity index, it could impact postharvest quality and shelf life (Gil *et al.*, 2012).

Different variables associated with harvesting such as the stage of maturity, season, and time of the day to harvest, may influence the accumulation of phytonutrients in leafy salads (Jones *et al.*, 2006; Ansah *et al.*, 2018). It is, therefore, necessary to identify the appropriate maturity stage for better quality phytonutrient retention in fresh produce (Weston and Barth, 1997; Kader, 2002a; Gil *et al.*, 2012; Ansah *et al.*, 2018). It is recommended to harvest leafy vegetables at the optimal maturity stage, not only for their nutritional value but also for economic benefits for the producers. Furthermore, the optimum climatic conditions at harvest and the time of the day influence the development of desired flavours, texture, and colour of fresh produce (Turner *et al.*, 2021a). Fresh produce is usually harvested early in the morning when the temperature is cooler to reduce the respiration rate for better quality (Prusky, 2011; Ansah *et al.*, 2018) which is also practised in the present study. Rocket leaves in the present study were harvested manually by cutting leaves with a knife 2-3 cm above the ground, allowing the crop to re-grow and produce more leaves as suggested by Koukounaras *et al.* (2007a).

Harvesting and handling cause severe stress conditions, resulting in water loss and variations in nutrient and hormone content, inducing the early onset of senescence observed in pak choy leaves when stored at 20 °C (Able *et al.*, 2005; Büchert *et al.*, 2011). As a result, a loss of the superficial green colour of the product was observed, which decreases the commercial approval of fresh produce. Moreover, senescence accelerates the loss of sugars leading to a loss of nutritional quality (Able *et al.*, 2005). Rapid senescence is the major postharvest problem in the rocket, which is expressed as the yellowing of leaves (Koukounaras *et al.*, 2009).

1.1.4.4. Postharvest stress factors affecting the quality and nutritional content of RTE leafy salads

Appearance, sensory quality (texture, taste, and aroma), nutrient content and longer shelf life are the major factors that affect the postharvest quality of fresh produce (Kader, 2002c). Once harvested, fresh produce is removed from its reserves (such as carbohydrates, water, and nutrient supply), and no further improvement in the quality could be achieved. Both quantitative and qualitative losses occur during the postharvest handling system of perishable goods (from harvesting, through handling, processing, packaging, storage, and transportation to the final delivery of the fresh produce to the consumer). Postharvest handling causes stress conditions on fresh produce resulting in cell weakening, membrane leakage, loss of nutritional quality etc. (Ansari and Tuteja, 2015). Two of the most important means for maintaining the quality of fresh produce during postharvest handling are minimizing mechanical injury and managing temperature. Mechanical damage during harvesting, processing, bruising from vibration during transport etc., could lead to increased electrolyte leakage and a higher rate of respiration. This hastens senescence and can accelerate the loss of water, thus increasing the susceptibility to decay-causing pathogens

(Saini *et al.*, 2017). By storing a commodity at a low temperature, respiration could be reduced, senescence could be delayed, thus extending the storage life and nutritional quality.

Plants also produce adenosine triphosphate by oxidising reduced sugars (glucose, fructose, and galactose) through respiration. High respiration in leafy salads is associated with increased oxidative stress, which results in reduced postharvest quality (Martínez-Sánchez *et al.*, 2008). A higher respiration rate also indicates a more active metabolism which can result in a more rapid loss of acids, sugars, and other components that determine flavour quality and nutritive value of leafy salads (Martínez-Sánchez *et al.*, 2012) and loss of saleable weight (Prusky, 2011). Temperature management (keeping temperature low) and RH (high up to 100%) are the most important tools for maintaining the quality and safety of fresh produce as it slows down plant metabolic processes, such as respiration, ethylene production, and enzyme activity (Kader, 2013), however, this can vary between cultivars and species.

It is suggested that the postharvest quality of fresh produce is maintained usually for a period of one to four days from harvest to processing, packaging, and transport (Wagstaff, 2014), however, it is crop-dependent. Any changes in these parameters will directly affect the perception of quality and may lead to rejection by the consumer. Thus, it is particularly important to maintain ‘quality’ all the time for repeated purchases by the consumer to reduce food waste. A newspaper article from The Independent (2017), reported that around 40% of bagged salads-equivalent to 178 million bags (37,000 tonnes) get thrown away every year due to loss of quality (such as appearance, taste and flavour) and not meeting consumer expectations.

1.1.4.5. Shelf life of RTE leafy salads

Leafy salads are generally characterised as very perishable products due to their high respiration rate and need to consume either within a few days after harvest or subject to preservation methods to extend the shelf life. Leafy crops are high in water content and are subject to wilting, shrivelling, and mechanical damage. They can be easily attacked by bacteria and fungi, resulting in the deterioration of fresh produce with changes happening to texture, colour, flavour, and nutritive value. The freshness of RTE leafy food also gets compromised during postharvest processing such as cleaning, washing, and packaging which causes water loss, microbial growth, the rupture of cell membranes, increasing respiration and ethylene production resulting in reduced shelf life (Danza *et al.*, 2015). To preserve the quality and to extend the shelf life of RTE leafy salads, various postharvest techniques such as pre-cooling, hydro-cooling, vacuum cooling, controlled atmosphere (CA), packaging, modified atmospheric packaging (MAP), use of 1-methylcyclopropene (1-MCP) (to reduce ethylene production), ultraviolet C treatment, use of oxalic acid, etc., are practised in industries (Watada *et al.*, 1996; Costa *et al.*, 2006; Lemoine *et al.*, 2007; Jia *et al.*, 2009; Yuan *et al.*, 2010; Char *et al.*, 2012; Cefola and Pace, 2015; Pinela *et al.*, 2016). A study by Cantwell *et al.* (1998) reported the shelf life of all leafy greens is best maintained at 0 °C with 90% of product volume being marketable at 0 °C for 21 days, however, when stored at 10 °C, marketability is reduced to 70% after seven days. This was further supported by a study on perennial wall rocket leaves reporting that the visual quality was retained for almost 15 days when stored at 4 °C (Martínez-Sánchez *et al.*, 2006a). Once opened, RTE leafy green salads can be stored at a refrigerated temperature lower than 8 °C for a maximum of two days (Arienzo *et al.*, 2020). At supermarkets and other places, RTE salads are usually stored in open refrigerated cabinets typically having temperatures around 4 °C, with a shelf life of 7 to 14 days (Martínez-Sánchez *et al.*, 2006a). Storing the RTE minimally processed leafy salads at this temperature is the most critical factor in

maintaining the quality and shelf life of fresh produce. Temperature near 0 °C with a high RH of 95% (Cantwell *et al.*, 1998) and proper packaging is highly recommended to retain the quality and shelf life of *E. sativa* (Koukounaras *et al.*, 2007a).

1.1.5. Accumulation of primary (sugar, sulphur, and organic acid) and secondary (glucosinolate) metabolites due to various abiotic stresses

All lifelong crops are frequently exposed to environmental (biotic as well as abiotic) stresses both in natural as well as agricultural conditions and may limit crop production by up to 70% (Boyer, 1982). However, crops do adapt and acclimate to these environmental stresses and thus survive. A crop's response to abiotic stresses is both elastic (reversible) as well as plastic (irreversible). Abiotic stresses such as water stress, salinity, temperature (low and high), light intensity, and nutrient imbalances may cause cell weakening, membrane leakage, flavour loss, textural changes and internal browning of postharvest produce (Ansari and Tuteja, 2015) that significantly affect plant growth, development, and productivity (Rosa *et al.*, 2009; Ashraf and Harris, 2013; Zhang *et al.*, 2018; Al-Huqail *et al.*, 2020; Saddhe *et al.*, 2021). Stresses either biotic or abiotic may affect the regulation of the biosynthetic pathway that is involved in the production of bioactive compounds (Singh *et al.*, 2015; Francisco *et al.*, 2017). A few of the primary (sugars, organic acids, and sulphur) and secondary (GSLs) metabolites accumulated due to various stresses are discussed below.

1.1.5.1. Sugars

Plants are both autotrophic as well as photosynthetic organisms that produce and consume sugars (Rosa *et al.*, 2009). Sugar performs multiple roles such as providing energy, carbon transport

molecules, and signalling molecules as well as a source of materials from which plants make proteins, lipids, and polysaccharides (Halford *et al.*, 2011). Soluble sugars (sucrose, glucose, and fructose) play an important role in maintaining the overall structure and growth of plants (Rosa *et al.*, 2009) where they act as a nutrient as well as regulators (Sami *et al.*, 2016). Sugars represent the energy source for maintaining the basal metabolism of cells in leafy salads (Cavaiuolo *et al.*, 2015). The most abundant free sugars in plants are sucrose, maltose, glucose, and fructose. Sucrose and maltose are disaccharides whereas glucose, fructose, and galactose are monosaccharides (Figure 1.1).

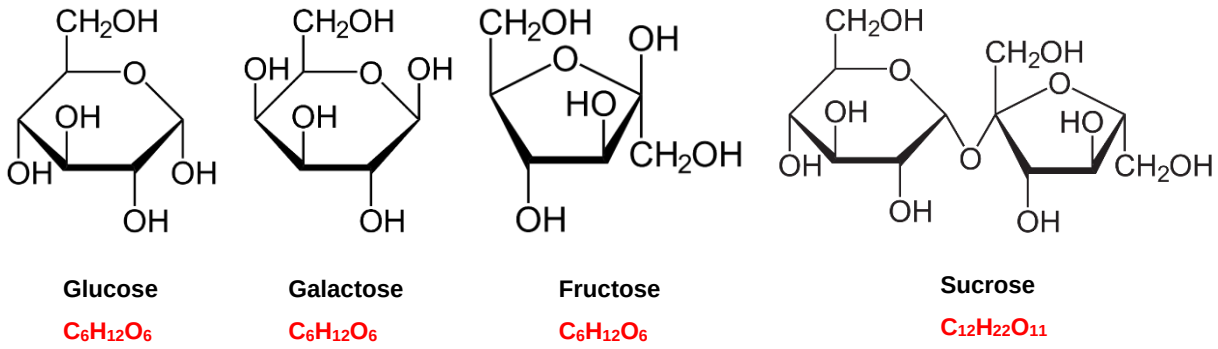


Figure 1. 1. Various structures of mono- and disaccharides.

In all green plants, photosynthesis is the most fundamental and intricate physiological process that produces the sugars which govern growth and development. Photosynthesis takes place at the chloroplast of the mesophyll cell of a leaf where both light-dependent and independent reactions of photosynthesis occur. Chloroplasts present in plant cells are sensitive to different environmental stress such as salinity, drought, temperature, and varying light intensity. Damage at any level caused by stress may reduce the overall photosynthetic capacity of a green plant (Ashraf and Harris, 2013).

Environmental stress may lead to a significant decrease in the efficiency of photosynthesis in the source tissues (leaves), thus reducing the supply of sugars to sink tissues. This affects physiological and biochemical changes which occur in plants to sustain respiration and other metabolic processes. During stress, cells sense changes in the ratio between sucrose and hexoses and feed this information to the signalling pathway, which furthermore affects enzymes involved in both synthesis and cleavage of sucrose. The three main enzymes i.e. invertase (EC 3.2.1.26), sucrose synthase (EC 2.4.1.13), and sucrose phosphate synthase (EC 2.4.1.14) are recognised for affecting the accumulation and metabolism of soluble sugar (Liu *et al.*, 2020).

Abiotic stress triggers sucrose catabolic enzymes such as invertase and sucrose synthase (Saddhe *et al.*, 2021) which modulates the source-sink activities. Sucrose on degradation by enzyme invertase produces glucose and fructose, whereas sucrose synthase enzyme produces uridine 5 diphosphate glucose and fructose, which changes the concentration of soluble sugars within the cell. The products obtained after the cleavage of sucrose by sucrose synthase are available for many metabolic pathways such as energy production, synthesis of complex carbohydrates and production of primary metabolites (Stein and Granot, 2019).

Soluble sugars are sensitive to environmental stresses such as drought, salinity, extreme temperatures, oxidative stress, ROS, ultraviolet-B radiations, heavy metals, flooding, and atmospheric pollutants. Various studies in the literature suggested that abiotic stresses such as drought, salinity, low temperature, and flooding can increase the soluble sugar levels in plants (Figure 1.2), whereas high light irradiance, heavy metals, nutrient shortage, and ozone result in low

sugar levels (Gupta and Kaur, 2005; Rosa *et al.*, 2009; Sami *et al.*, 2016; Cocetta *et al.*, 2018; Toscano *et al.*, 2019).

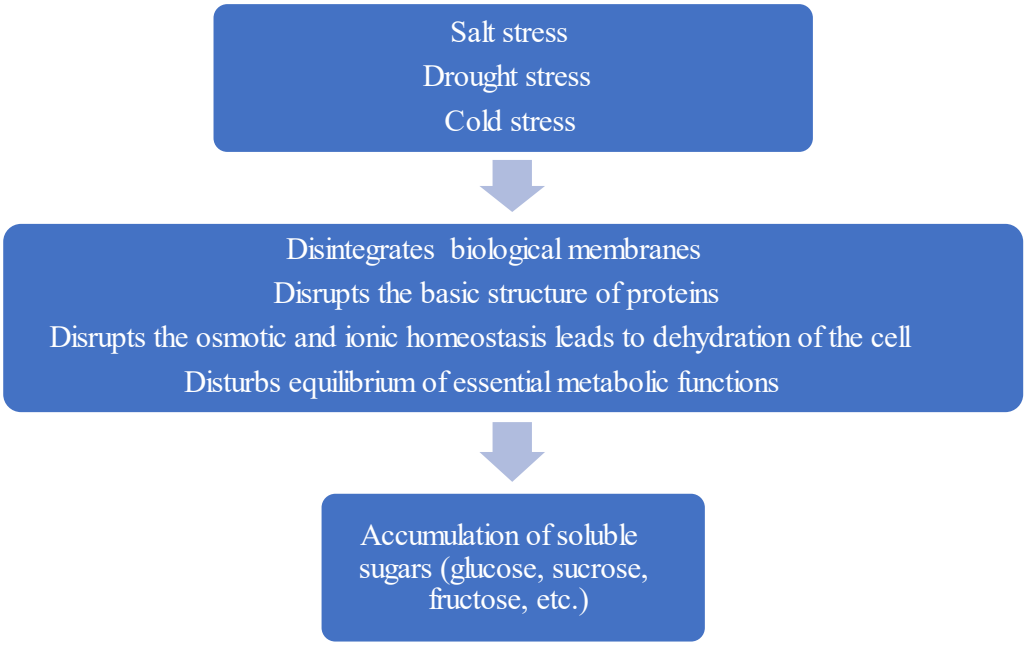


Figure 1. 2. Abiotic stresses influence the accumulation of sugars in the plant, a figure acquired from Sami and Hayat (2018).

To avoid stresses, plants develop a range of adaptive strategies to survive, however response to specific stress can vary with the genotype (Rosa et al., 2009; Ashraf and Harris, 2013). Furthermore, it is important to understand various mechanisms controlling different metabolic pathways.

1.1.5.1.1. Analytical instruments to measure sugars

There are various extraction methods and analytical tools that have previously been used in numerous studies to identify sugars in leafy salads: Villatoro-Pulido *et al.* (2013) used gas

chromatography-mass spectrometry to measure sugars in *E. sativa* accessions and identified that glucose as the primary photosynthetic product and was the predominant sugar, representing > 70% of the total soluble carbohydrates. Other sugars identified were sucrose, fructose, galactose, arabinose, and mannose, which were found in lower concentrations. A study by Bell *et al.* (2017a) used capillary electrophoresis to identify sugars in seven accessions of *E. sativa*. Other authors (Ayaz *et al.*, 2006a; Pinela *et al.*, 2016; Thavarajah *et al.*, 2016) have used high-performance liquid chromatography (HPLC) to identify fructose, glucose, and sucrose as the major soluble sugars in kale and watercress leaves. Furthermore, fructose, glucose, and sucrose in kale have been identified as masking agents for the bitter taste of certain GSLs (Groenbaek *et al.*, 2016). Helland *et al.* (2016) used Dionex ICS 5000 ion chromatography system to identify sugars in swede and turnip vegetables. Beck *et al.* (2014) used the Dionex series 300DX ion chromatograph to quantify sugars in brassica vegetables.

1.1.5.2. Sulphur

Sulphur is as important as nitrogen and is an essential macronutrient. The plant requires sulphur for both growth and development and is ranked fourth after nitrogen, phosphorous and potassium. It is also important in improving crop productivity, quality, and plants' tolerance to abiotic stress (Zenda *et al.*, 2021). The total sulphur content in plant tissues ranged from 0.3 to 7.6% (Zhao *et al.*, 2008). The requirement of sulphur can vary with plant families with members of *Brassicaceae* being found to be most sulphur dependent. Sulphur enters the biological systems of the soil through microbial activities involving the mineralisation of organic matter (Prasad and Shivay, 2018). Plants accept sulphur only as sulphate anions (SO_4^{2-}) from soil or as a fertiliser by the roots. It reduces to form sulphur-containing amino acids (cysteine and methionine) to synthesise protein

and other compounds such as glutathione (which acts as an antioxidant). Furthermore, it reduces to form secondary metabolites such as alliins and GSLs in Brassicas, which play important physiological roles and protect plants against environmental stresses and pests (Wang *et al.*, 2020; Zenda *et al.*, 2021). It is due to its potential defensive mechanism against pests, the good nutritive potentiality to crops and its relative immobility in the soil-plant system, which makes sulphur seeks the most attention. Excess sulphate is transported to the leaves and stored in vacuoles. Using sulphur as a fertiliser for crops can improve nitrogen uptake efficiently and help in protein development (Mazid *et al.*, 2011). Sulphur is also used in the synthesis of thioredoxins (protein-containing sulphur) where it regulates chloroplastic enzymes which are used in photosynthesis such as fructose 1, 6 bisphosphatase (EC 3.1.3.11). Sulphur is also used in the synthesis of acetyl co-enzyme [needed for the tricarboxylic acid cycle (TCA) or Krebs cycle], which takes place in mitochondria from pyruvate (through glycolysis of glucose) by catalysing through three enzymes: thiamine pyrophosphatase (TPP), lipoic acid, and Coenzyme A. Furthermore, sulphur is used in cellular resistance to oxidative stress that happened due to abiotic stress such as dehydration, drought, heat, and frost damage where amino acid cysteine helps to protect the cell. Finally, sulphur affects crop yield, taste, and aroma of cruciferous vegetables.

Epidemiological studies revealed that crops of the *Brassicaceae* family contain numerous phytochemicals that are thought to benefit human health (Bell *et al.*, 2018; Abukhabta *et al.*, 2020). These include sulphur-containing GSLs, and their hydrolysis products, particularly ITCs, and sulphur-containing volatile compounds. GSL compounds are abundantly present in both genera of the rocket, which has gained significant popularity amongst consumers (Bell and Wagstaff, 2014), however, the application of supplementary sulphur to rocket crops is not yet explored in the literature.

1.1.5.3. Organic acids

Organic acids such as citric, malic, fumaric, and succinic are produced in plant cells as an intermediate product in an energy metabolism TCA pathway (mitochondria) and glyoxylate cycle (occurs in specialised peroxisome in plant cells called glycosomes, where fats are converted into carbohydrates through acetyl-CoA). Organic acids are involved in various metabolic pathways in plants including energy production, carbon storage, biosynthesis of amino acids, regulating osmotic pressure, pH homeostasis, stress resistance as well as in the C_4 photosynthetic pathway as an intermediate connecting CO_2 uptake and fixation (Ludwig, 2016; Huang *et al.*, 2021). Citrate and malate which are the conjugate base of citric and malic acid accumulate under developmental stages and environmental conditions, for example through the effect of cultural practices, irrigation, drought, high temperature etc. (Zhang and Fernie, 2018; Huang *et al.*, 2021).

Citric acid is a six-carbon molecule synthesised by citrate synthase where condensation of oxaloacetate with acetyl-CoA produces intermediate citrate. In the TCA cycle, various other intermediates are also produced such as succinate, fumarate, and malate. Once citrate is synthesised, it is transported to the cytosol, where it can be utilised by cells immediately or stored in the vacuole to maintain the cytosolic pH (Tahjib-Ul-Arif *et al.*, 2021). Furthermore, the concentration of endogenous citrate has been increased in various parts of the plants such as the leaf, shoot, root, and tuber when subjected to stresses such as salinity, drought, heat, and heavy metal in various plant species (Tahjib-Ul-Arif *et al.*, 2021).

Malic acid or malate is another major four-carbon molecule synthesised as an intermediate in the TCA cycle and is later stored in the vacuole. It plays a key role not only in metabolic pathways

such as photosynthesis and respiration but also in the defence functions of plants. Furthermore, it plays a considerable role in the functioning of guard cells by mediating the opening and closing of stomata. Low-temperature stress can induce an increase in malate content in some crops (Sun *et al.*, 2019). Malic acid regulates a good osmotic adjustment and thus responds to the osmotic stress caused by low temperature by regulating NADP-malic enzyme activity.

1.1.5.4. Glucosinolates

Environmental stresses such as temperature (high and low), humidity, light intensity, the supply of water, minerals, CO_2, and pathogen attack, influence plant growth and the production of secondary metabolites. Furthermore, secondary metabolites contribute to odour, flavour, taste, and colours in plants (Bennett and Wallsgrove, 1994). Of the many secondary metabolites, GSLs are the N and S-containing compounds found in the *Brassicaceae* family that plays a vital role in human health. Moreover, GSLs are chemically stable under normal conditions.

GSLs are the plant's secondary metabolites classified into three chemical classes: aliphatic, aromatic, or indolic side chains (R) attached to glucose. The aliphatic, aromatic, and indolic GSLs are derived from the amino acid precursor methionine, tryptophan, and phenylamine, respectively (Cartea and Velasco, 2008). Their chemical structure diverges accordingly to species, and cultivar, even within varieties of the same species (Aires *et al.*, 2012).

Rocket belongs to the *Brassicaceae* family, and vegetables belonging to this family are particularly rich in GSLs (Wagstaff, 2014). Epidemiological studies have shown that consumption of *Brassicaceae* vegetables that contain GSLs, and their degradation products are linked to reduced

incidences of several cancers such as prostate, colon, bladder, lung, and potentially breast cancers (Björkman *et al.*, 2011). GSLs are synthesised by the plant as a part of its defence mechanism against pests and diseases. Upon disruption either by pathogen attack or by physical damage in plant tissue, GSLs are degraded by the enzyme myrosinase (EC 2.2.3.1) to produce bioactive products such as thiocyanates, ITCs, and nitriles. The ingestion of GSLs without active plant myrosinase still leads to the formation and absorption of bioactive breakdown products by enzymes from gut microflora, however, their bioavailability is lower as compared to active myrosinase (Francisco *et al.*, 2017).

High growth temperature (40 °C) increases the accumulation of GSL, however, it retards germination, growth, regrowth, and survival of rocket plants (Jasper *et al.*, 2020). Their study further revealed a high accumulation of GSLs when leaves were harvested for second cuts due to wound response. A higher accumulation of GSL is associated with higher pungency and hotness and reduced consumer liking (Bell *et al.*, 2020b). Cooler temperature accumulates lower concentration of GSLs due to cold stress, with less bitterness and hotness perceptions resulting in more likely to be preferred by consumers, however, losing the beneficial effect of GSLs on health. Moderate cold stress or controlled cold stress could result in a higher accumulation of secondary metabolites which could be a strategy to be considered for increasing the presence of health-related compounds. For example, when vegetables like tomato and watermelon were subjected to moderate temperature stress, it resulted in a higher accumulation of phenolic compounds (Rivero *et al.*, 2001). Postharvest processing such as handling, storage, and distribution also exerts stress on leaves which significantly increases the accumulation of GSLs (Bell and Wagstaff, 2017). Furthermore, water stress could also improve the nutritional value of the plant. Limited water

availability or in other words, mimicking the drought-like conditions on the Mediterranean plant like *Eruca sativa,* could result in a significant increase in GSL concentration (Ogran *et al.*, 2021).

1.1.5.4.1. Analytical instruments to identify GSL

Various extraction methods and analytical instruments have been previously used in literature to identify individual GSLs in *Brassicaceae* vegetables (Pasini *et al.*, 2011; Helland *et al.*, 2016; Jasper *et al.*, 2020). Depending upon the availability and affordability of analytical instruments at the workplace, numerous studies in the literature used different analytical instruments to identify and quantify GSLs. For example, LC-MS was used by (Bell *et al.*, 2015; Jasper *et al.*, 2020) to identify GSLs in 'salad' and 'wild' rocket species. A high-performance liquid chromatography diode array detection was used by (Guo *et al.*, 2011; Pasini *et al.*, 2011; Mølmann *et al.*, 2015; Helland *et al.*, 2016) in brassica vegetables such as Brussel sprouts, broccoli, rocket, and swede root, whereas, authors such as (Thomas *et al.*, 2018; Molmann *et al.*, 2020) used ultra-performance liquid chromatography to identify and quantify GSLs in swede root bulbs and broccoli vegetables.

1.1.6. Sensory characteristics of RTE leafy salads

1.1.6.1. Sensory attributes influencing leafy salads

The demand for RTE salads is growing very rapidly due to their health benefits and nutritional content. The appearance is the main factor that affects consumers to choose the fresh produce to purchase at the first instance, however, consumer satisfaction in terms of organoleptic characteristics such as aroma, taste, and texture makes them repeat the purchase (Kader, 2000; Francis *et al.*, 2012). The quality of RTE leafy salads is defined by sensory characteristics which include appearance, aroma, firmness, and taste and these parameters must be preserved during

postharvest shelf life for consumer acceptance (Cavaiuolo *et al.*, 2015). A study by Barrett *et al.* (2010) reported an interesting relationship between consumer acceptance and the colour, flavour (taste and aroma), texture, and nutritional content of fruit and vegetable. For RTE salads, taste plays a crucial role (Chadwick *et al.*, 2016). Francis *et al.* (2012) in their study reported the quote that 'we eat with our eyes' and if they attract us then only, we put it into our mouth. However, the truth is many of the health-beneficial compounds are bitter tasting. Given a choice, all humans are drawn to sweeter-tasting food and there is considerable evidence that taste is often reported to be the main driver of liking (Cox *et al.*, 2012).

RTE leafy salads are excellent sources of fibre, vitamins, minerals, phenolics, and GSLs, however, GSLs are bitter-tasting compounds (Björkman *et al.*, 2011). As bitterness could be offset by perceptions of sweetness, Bell *et al.* (2018) in their study hypothesised that cultivars' tastes could be modified by manipulating sugar-GSL ratios for consumer acceptance. To encourage more people to consume leafy salads to benefit health, more research is needed to offset the bitterness either by breeding varieties having a low content of bitter-tasting compounds or by raising the sugar content. Few other studies proposed a similar strategy to modify/increase the sugar profile to counteract the perception of bitterness by breeding cultivars while retaining all the vital phytochemicals beneficial to health (Schonhof *et al.*, 2004; Chadwick *et al.*, 2016; Simko, 2019).

Growers and producers are increasingly under pressure to provide supermarkets and consumers with a consistent 'quality' (taste, flavour, and appearance) product. This is near impossible to achieve the above 'quality' every time as crops are significantly affected by climatic, environmental, biotic, and genetic factors (Bell and Wagstaff, 2019). Taste and flavour are two

complex components that are influenced by the cultivation environment and season (Bell *et al.*, 2020b). From the previous studies, it was revealed that taste and flavour differ markedly according to genotype and environment in vegetables such as rocket (Bell *et al.*, 2015) as well as in celery (Turner *et al.*, 2021b). To date, no single cultivar is known to have stable sensory characteristics in multiple growth conditions. Therefore, understanding the genotypic responses to environmental conditions and abiotic stresses is essential for consistency and consumer acceptance.

1.1.6.2. Interaction between sweetness and bitterness

It is widely known that sweetness reduces the perception of bitterness (Bell *et al.*, 2017a; Bell *et al.*, 2018) and several studies have observed that free sugars (e.g., glucose, fructose, galactose, maltose, raffinose, sucrose, etc.) in abundance, reduces the intensity of bitterness in *Brassicaceae* crops. Previous studies reported a negative relationship between bitter-tasting GSLs contents and consumer acceptance (Van Doorn *et al.*, 1998). In scientific literature it is generally accepted that GSLs and ITCs contribute toward distinctive tastes and flavour, however, only specific GSLs impart bitter taste while many ITCs impart pungency to *Brassicaceae* crops (D'Antuono *et al.*, 2009; Bell *et al.*, 2018).

The perceived bitterness in leafy salads is the reason for the consumer to reduce the intake of such vegetables in their diet, however, it is known that sugars can mask the bitter taste of certain GSLs (Groenbaek *et al.*, 2019). The bitter taste of leafy salad is considered a barrier to buying these RTE leafy salads as most consumers dislike bitter and strong-tasting vegetables, however, reducing the content of bitter-tasting GSLs in these leafy salads is not the solution due to its benefits related to health (Wilkie *et al.*, 2013; Beck *et al.*, 2014).

Sugars along with sweetness also represent the energy source for maintaining the basal metabolism of cells in leafy salads (Cavaiuolo *et al.*, 2015) and after harvest, sugar is essential for keeping cells alive to ensure for longer shelf life (Bulgari *et al.*, 2017). Therefore, crops like kale, increase their cytosolic sugar contents to prevent ice formation and cell damage, when the temperature reaches freezing, which might be the reason for the sweetness of kale during frost (Steindal *et al.*, 2015).

1.1.6.3. Sensory characteristics and perception of bitter taste receptor (TAS2R38)

Humans possess five basic tastes: salty, sour, sweet, umami, and bitter, which are involved in the detection of desirable components in foods. Food preference and choice are determined by several factors, of which taste has been reported as one key factor in food perception (Shen *et al.*, 2016). Salt (high concentration), sour, and bitter tastes are involved in defensive eating. The bitter taste is generally thought to have evolved to protect humans from the consumption of toxic compounds (Beckett *et al.*, 2014). Salt and sour receptors are channel-type receptors whereas sweet, umami, and bitter are detected by G-protein coupled receptors (GPCRs). Sweet and bitter tastes are sensed through the binding of the GPCRs which are located within papillae on the tongue. Two classes of GPCRs have been identified in the taste receptor cells: sweet and umami - TAS1R family and bitter compounds - TAS2Rs (Beckett *et al.*, 2014). There are just two T1R receptors involved in sweet perception (T1R2/T1R3), however, 25 T2R receptors are responsible for bitter molecules.

The perception of basic tastes, mouthfeel sensation and aroma contribute to the sensory profile (Francis *et al.*, 2012). There are many genes responsible for the ability to perceive taste, aroma, and flavour. Aroma compounds contribute to flavour either directly, or indirectly through retro

nasal stimulation in the nose during chewing (Francis *et al.*, 2012). TAS2R38 is the most studied bitter taste receptor gene in the literature (Calo *et al.*, 2011; Feeney, 2011; Gorovic *et al.*, 2011) with bitter-tasting compounds such as GSLs and ITCs being linked to the gene *hTAS2R38* (Meyerhof et al., 2010). The thiourea group (N-C=S) within GSL and ITC is predominately responsible for the bitter taste (Shen *et al.*, 2016). The TAS2R38 bitter taste receptor gene detects a compound with thiocyanate moiety present in phenylthiocarbamide (PTC) and 6-n-propyl-2-thiouracil (PROP) (Calò *et al.*, 2011). PTC and PROP are the two most common compounds used as a marker for bitter taste research. PTC/PROP tasting phenotypes are divided into two categories: 'non-tasters' who are blind to bitterness, and 'tasters' who find PTC/PROP bitter. Tasters are further divided into 'medium tasters' and 'super-tasters'. Due to the genetic recombination, three common diplotypes are present within the human population: PAV/PAV (proline alanine valine) homozygotes which are categorised as 'supertasters', PAV/AVI heterozygotes 'medium tasters', and AVI/AVI (alanine valine isoleucine) as 'non-tasters' (Bell *et al.*, 2017b). Individuals with the 'PAV/PAV' genotype perceive bitter taste very intensely due to the presence of a functional copy of gene TAS2R38, which constitutes 25% of the population in the European population. Individuals with the 'PAV/AVI' genotype lack one functioning copy of TAS2R38 and so perceive the bitterness to lower intensity while the individuals with the 'AVI/AVI' genotype completely lack any functional copy of the gene and are therefore considered 'bitter blind', however, this is only true for some bitter compounds. Recently, PROP has now become more common in laboratory studies, as compared to PTC as PTC possesses a slightly sulphurous odour, which has been reported for its toxicity (Beckett *et al.*, 2014).

The perceived bitterness in leafy salads is the reason for the consumer to reduce the intake of such vegetables in their diet. *Brassicaceae* vegetables have a chemoprotective effect, and the

consumption of these vegetables in the UK is low due to their bitter taste (Shen *et al.*, 2016). Several studies have indicated that bitter-tasting GSLs and ITCs compounds present in Brassica vegetables may play a key role by reducing the intake by consumers who are sensitive to bitterness, as they can activate the TAS2R38 taste receptor (Fenwick *et al.*, 1983; Van Doorn *et al.*, 1998; Schonhof *et al.*, 2004; Pasini *et al.*, 2011).

1.2. 'Salad' rocket (*Eruca sativa*) as RTE salad crop

There are two predominant cultivated rocket species across the globe: 'wild' or 'perennial' rocket (*Diplotaxis tenuifolia*) and 'salad' or 'annual' garden rocket (*Eruca sativa*) (Hall *et al.*, 2012c; Bell and Wagstaff, 2019). 'Salad' rocket is a minor crop and follows the C_3 photosynthetic pathway, however, 'wild' rocket uses C_3-C_4 pathways which may influence the response of leaves during storage (Hall *et al.*, 2013). The common names of 'salad' rocket are rucola, rucoli, arugula, colewort, roquette, etc., and is a part of the same *Brassicaceae* plant family with the genus *Eruca* (Figure 1.3). 'Salad' rocket originated from the Mediterranean and Western Asian region extending as far as Pakistan in the Indian subcontinent. Currently, it is cultivated all around the world, however, remains most popular in the Mediterranean region. Being a fast-growing crop with an efficient root system, it is capable of withstanding severe drought conditions which makes this crop an important food source for arid areas (Garg and Sharma, 2014). In Asian countries, the *Eruca* crop is cultivated for both oilseeds as well as fodder purposes (Garg and Sharma, 2014). The oilseed crop has antioxidant, and antimicrobial properties and serves to inhibit the proliferation of tumour growth. Moreover, 'salad' rocket is a minimally processed RTE salad, and the market is growing fast due to its convenience, nutrition, and easy accessibility (Hall *et al.*, 2012c) with 40 million

bags consumed in the UK annually (information provided by Dr Shaw, Bakkavor, UK). Leaves are sold in bags as loose, or as a part of a leafy salad mixture with other crops (Bell *et al.*, 2020b).

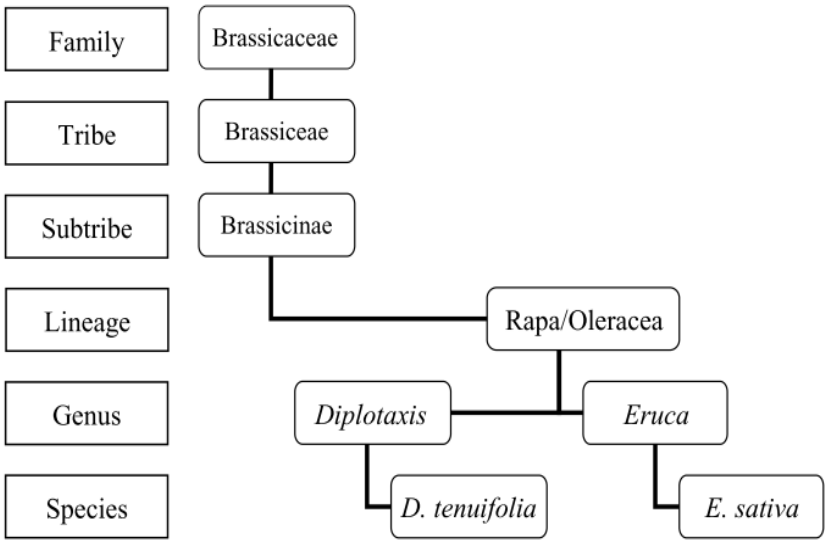


Figure 1. 3. Classification of perennial wall rocket (*Diplotaxis tenuifolia* (L.) DC.) and annual 'salad' rocket (*Eruca sativa* Mill.). Figure acquired from Hall *et al.* (2012c).

'Salad' rocket is high in biologically active compounds such as ascorbic acid, carotenoids, fibres, polyphenols, and GSLs (Martínez-Sánchez *et al.*, 2006b; Hall *et al.*, 2012a; Bell and Wagstaff, 2014). GSLs and their hydrolysis products, ITCs are potentially linked to the prevention of certain diseases and some types of cancer (Bell and Wagstaff, 2014; Tripodi *et al.*, 2017). As compared to other leafy salads such as kale (10.7 mg. g^{-1} DW), wild rocket (11.2 mg. g^{-1} DW), watercress (5.0 mg. g^{-1} DW), etc., 'salad' rocket contains a higher concentration of total GSLs (15.5- 20.5 mg. g^{-1} DW). GSLs contribute to pungent flavour by the formation of ITCs through the action of enzyme myrosinase on cutting and chewing rocket leaves (Bennett *et al.*, 2007) and share a peppery taste and distinct aroma (Bell and Wagstaff, 2014). According to D'Antuono *et al.* (2009), many of the

intact GSLs have a bitter taste, with some ITCs producing a burning sensation in the mouth and other enzymatic degradation products sharing unpleasant sulphur or rotten cabbage taste and smell.

When consumed, both GSLs and ITCs are thought to reduce the risk of carcinogenesis or heart disease (Spadafora *et al.*, 2016), however, much of the population do not consume enough to get benefits. Recommended daily intake of fresh salad is in a range of 70-200 g (D'Antuono *et al.*, 2009). To get maximum health benefits, it is suggested that rather than consuming more rocket leaves, it would be more sensible to increase the nutritional content by developing a cultivar through advanced screening and plant breeding methods (Bell *et al.*, 2015). Recently, a Beneforté broccoli cultivar has been developed through a selective breeding method to increase glucoraphanin/sulforaphane (GSLs) content in broccoli to benefit health (Traka *et al.*, 2013).

1.2.1. *Eruca sativa* and its genetic diversity

E. sativa is a diploid plant species having 11 pairs of chromosomes (2n = 22) and the life cycle for flowering begins in spring and ends with seed production in late spring/early summer (Tripodi *et al.*, 2017). *E. sativa* is an annual, fast-growing crop with a growing height as tall as 100 cm (Garg and Sharma, 2014). The lower leaves are petiolate, but the upper leaves are almost sessile. Leaves of *E. sativa* are dark green and leaf size increased up to 20 cm long having white/cream flowers (Hall *et al.*, 2012c). These crops are generally cool season with optimum temperature varying between 14 - 21 °C, depending upon the variety. They generally prefer deep, well-drained, fertile, sandy, or silty loam soils having neutral pH (approximately 6.5).

This species is generally self-compatible but sometimes prefer out-breeding with varying degree of self-incompatibility depending upon the cultivars (Bell and Wagstaff, 2019). The self-incompatibility could be overcome by bud-pollination and reducing the ambient temperature during flowering (Bell and Wagstaff, 2019). Bud-pollination can be performed manually where the pollen is applied to the stigmas of the plants before the flowers open. There is various physiological and chemical manipulation previously been reported to overcome self-incompatibility in different plants: bud pollination, delayed pollination, heat treatment, use of mentor pollen and chemical treatment (Sun *et al.*, 2005). Of the different techniques previously proposed, chemical treatment was the most favoured one due to its effectiveness and labour efficiency. Sun *et al.* (2005) in their study, used gibberellin to break the dormancy and promote cell elongation, to overcome the self-incompatibility in *E. sativa*.

E. sativa has many vital phytochemicals such as GSLs, ITCs, flavanols, vitamins, and minerals with GSLs and ITCs (hydrolysis product of GSL) are considered as imparting taste and flavour. As compared to other brassicas e.g., *B. oleracea* species, rocket has only come to prominence within the last 25 years (Bell and Wagstaff, 2019).

1.2.1.1. Improvement of genetic diversity

Rocket species can hybridise with members of respective genera through sexual reproduction, ovary culture, embryo culture, and protoplast fusion (Hall *et al.*, 2012b). Due to an elevated level of genetic similarity between 'salad' and 'wild' rocket, intergeneric hybridisation has been possible for this species. Crosses with *Diplotaxis* species have been performed, however, resulting in no viable outcome/progeny. Previously, rocket has been propagated through somatic embryogenesis

and zygotic embryos (Garg and Sharma, 2014) with *Brassica oleracea* to introduce cytoplasmic male sterility (CMS) (Bell and Wagstaff, 2019). As compared to other Brassicas, studies on the CMS process on rocket crop is not enough. An article by Budahn *et al.* (2018) reported on the potential use of CMS *E. sativa* species for breeding, however, the study is still ongoing. The CMS lines of *E. sativa,* in the breeding process, could be developed through intergeneric hybridization using CMS *Brassica oleracea* and then proceed further through recurrent backcrossing (Nothnagel *et al.*, 2016). Their study proposed that as both *Brassica* and *Eruca* genera belong to the same *Brassicaceae* family and breeding such a line could help to produce uniform F1 seeds, however, this is not cost-effective.

Recently, Bell *et al.* (2020a) presented a first *de novo* reference genome sequence and annotation for *Eruca* species, where I was the co-author on this paper (see appendix 1.1). Earlier, Wang *et al.* (2014) sequenced the mitochondrial genome, where their study determined that *E. sativa* is more closely related to *Brassica oleracea* and *Raphanus sativus* than to *Arabidopsis thaliana*.

1.2.2. Importance of *Eruca sativa* to human health

E. sativa is a member of the *Brassicaceae* family and is consumed worldwide in the human diet due to its taste, aroma, and trigeminal attribute. It has been indicated in different epidemiological studies that diet and cancers are interlinked (Cartea and Velasco, 2008). Studies in literature have reported that the consumption of such vegetables that contain GSLs and ITCs is associated with a reduction in the risk of several types of cancers (such as prostate, breast, lung, etc.,), cardiovascular disease, and diabetes (Björkman *et al.*, 2011; Pasini *et al.*, 2011; Neugart *et al.*, 2018).

GSLs are the secondary plant metabolites produced by all members of the *Brassicaceae* family and are the specialised plant defence compounds that help the plant to protect itself from a range of insect herbivores and pathogens. The main biological role of GSLs is to provide a chemical signal which results in both defensive and attractant signals (Hall *et al.*, 2015). This defence system only gets initiated when the enzyme myrosinase comes in direct contact with GSLs. Within the plant cell, GSLs are present in the vacuole and are separated from the enzyme myrosinase which is present in the cytoplasm (Jin *et al.*, 2009). Upon cell disruption (mechanical wounding/cutting/damage by insect) GSL gets hydrolysed by endogenous enzyme myrosinase to yield glucose, sulphates, and an unstable intermediate, which rearranges spontaneously to produce several degradation products including ITCs, nitriles, and other minor products in plant tissue (Kim and Ishii, 2007; Jin *et al.*, 2009) (Figure 1.4). ITCs are largely responsible for the characteristic hot and pungent flavour and have been shown to have cancer-chemo preventive activity and anti-carcinogenic effects in laboratory animals (Kim and Ishii, 2007).

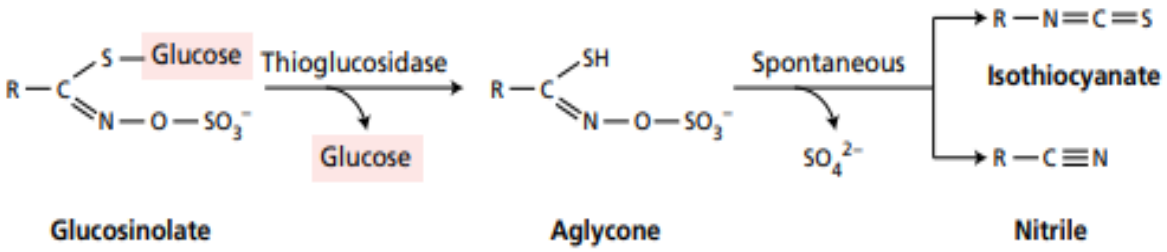


Figure 1. 4. Hydrolysis of glucosinolate to an isothiocyanate, where R represent various alkyl or aryl substituents. Figure acquired from Taiz and Zeiger (2010).

1.3. Rocket mapping population and molecular breeding

1.3.1. Mapping population

Depending upon the mating system of the species, several types of mapping populations exist for a particular crop. Breeding lines must be selected based on multiple traits (Yan and Fregeau-Reid, 2008). Selection of the breeding parents for crosses is one of the most challenging tasks for plant breeders. Once the parental lines are selected, breeders test the mapping population by planting them in multiple locations and weather (Ansarifar *et al.*, 2020). There are different ways of deriving mapping populations where the hybridisation of two parental lines/ genotypes has significant variations for a trait of interest. The different types of mapping populations produced are F2 generation (2^{nd} generation plants), back cross (crossed back to the parental line), doubled haploid (DH), recombinant inbred lines (RILs) and near-isogenic lines (NILs) (Figure 1.5) and more recently multi-parent advanced generation intercross population was constructed for crop species (Kumar, 1999; Collard *et al.*, 2005; Singh and Singh, 2015b; Xu *et al.*, 2017).

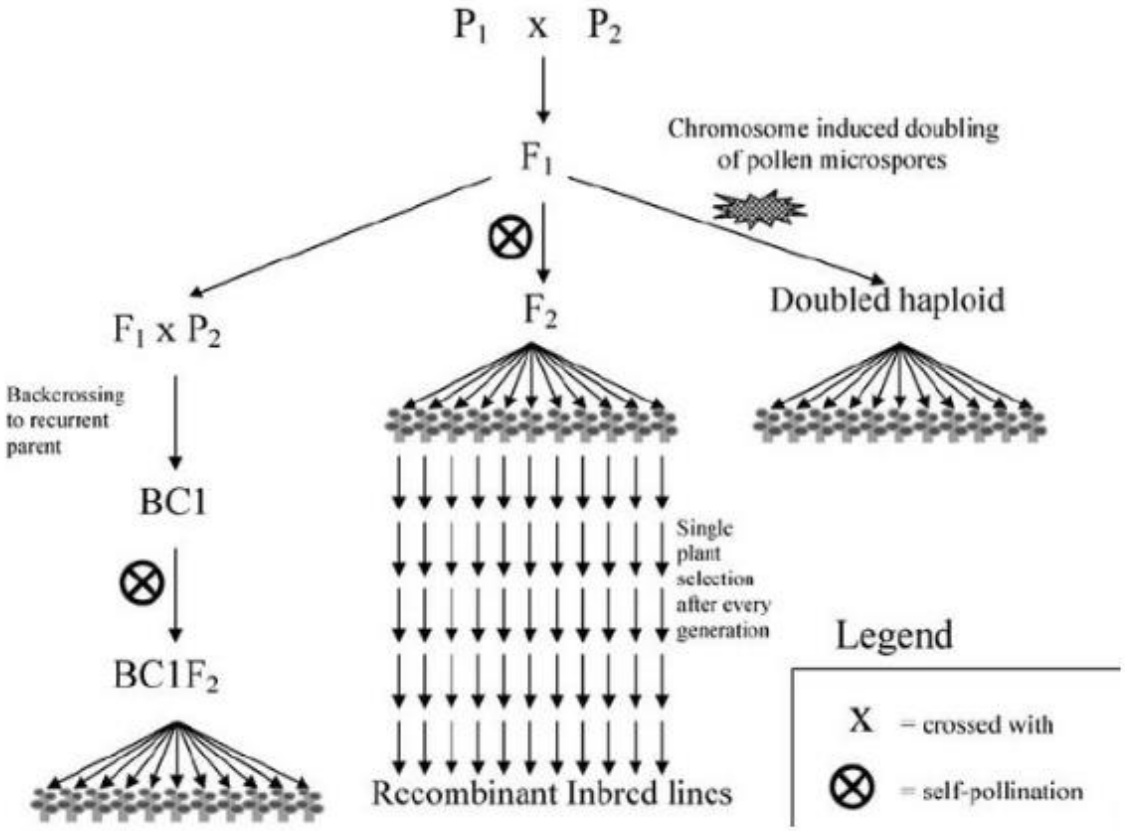


Figure 1. 5. Diagram of several types of mapping populations for self-pollinating species, a figure acquired from Collard *et al.* (2005).

RIL and DH populations are considered permanent mapping populations, as they produce homozygous lines. Several different populations may be used for mapping within a given species with each population type having various advantages and disadvantages discussed below (Table 1).

Table 1. 1. Commonly used biparental populations with few advantages and disadvantages

Mapping Population	Advantages	Disadvantages
F2	Rapid construction with minimum efforts, estimation of both additive and dominant effects.	Lower power due to one meiotic cycle, limited recombination, and temporary nature.
Back cross	Utility for introgressing specific genes, less time required to develop.	Impossibility of estimation of dominant effects, time requirement, temporary nature.
DH	Rapid construction, immortality, and easy replication over locations and years to produce homozygous lines.	Limited recombination, expensive, and the impossibility of estimating dominant effects. Only possible in species that are responsive to tissue culture.
RILs	An abundance of recombination, immortality, and easy replication over locations and years, produces homozygous lines	The impossibility of estimation of dominant effects requires time to develop usually five to eight generations, difficult in crops having high inbreeding depression.
NILs	Immortal population, used for fine mapping	Many generations are required to develop, with increased cost, time and effort, and linkage drag.

1.3.1.1. Recombinant inbred line (RIL)

RIL offer certain advantages over the other mapping population as these are permanent populations. A RIL population is developed by using the single seed descent method (SSD) from the F2 generation. An SSD is a modified form of the bulk method where a single seed is randomly selected from each plant in the F2 generation. The selected seeds are mixed (bulked) and sown to have the next generation (F3) and so on. It can be continued till F7 generations achieve homozygosity (99.23%), thus useful for repeated trials at multiple locations and across years. RILs are extremely informative in terms of gene combinations and recombinant events. In RILs, many lines outperformed the inbred parental line for all traits, however, developing a RIL population is time-consuming and costly, as they require a minimum of five to seven generations of selfing to be homozygous which is considered one of the major disadvantages.

1.3.2. Molecular breeding technique for constructing a linkage and quantitative trait loci (QTL) map

The use of molecular markers to increase the efficiency of selection has been known for a long time, as it allows selection to take place at an early stage, however, only recently has it become a reality for *E. sativa*. Various studies in the literature have proposed that molecular markers are particularly useful in identifying invisible traits at an early stage e.g., disease resistance, which otherwise takes a long time to appear (Tuvesson *et al.*, 2007; Collard and Mackill, 2008; Brown *et al.*, 2014).

Two parental lines with desirable traits of interest were crossed to produce an F1 progeny. A mapping population size of 50 to 250 individuals is considered best for higher resolution (Young, 1994). The F1 population is selfed to generate the F2 population in the glasshouse conditions to achieve prominent levels of germination and survival (Brown et al., 2014). *E. sativa* being an annual crop, it is possible to grow more than one generation each year in the greenhouse to speed up the transition to achieve homozygosity in the lines. In this early generation, DNA analysis could be performed on the plantlets to identify molecular markers and only those lines which are showing the desirable traits were taken forward to the next generation and the rest will be deselected to save resources, time, and labour.

Molecular markers for marker-assisted breeding, is in many instances more expensive and more technically challenging (Brown *et al.*, 2014) and therefore sometimes lead to a lack of adoption. However, this could be overcome by identifying robust QTL by using single nucleotide polymorphism (SNP) or the use of genome selection and utilising it in the breeding programme.

The use of genome selection can enhance the breeding system but will need a training population (phenotype and genotyped closely related selected population) to estimate the effects of all markers in the genome. Genome selection will predict more accurately the genetic variants without needing to wait for progeny test data and could save time up to one-three years.

1.3.2.1. Genetic linkage map

Linkage maps have been utilised in identifying a region on a chromosome that contains inherited markers close to the genes controlling the complex quantitative traits (Collard *et al.*, 2005; Abdurakhmonov and Abdukarimov, 2008). The main objective of genetic mapping is to identify the QTL responsible for phenotypic variation and can be achieved by either using linkage mapping or by association or linkage disequilibrium mapping in plants (Xu *et al.*, 2017). The three main steps which are required for constructing a linkage map are (a) development of a mapping population; (b) identification of polymorphism using DNA markers; and (c) linkage analysis of markers using statistical methods (Collard *et al.*, 2005). The major advantage of using mapping populations such as RILs or DH lines is they produce homozygous lines that can be reproduced at multiple locations/years without genetic changes. If the linkage map is used for QTL studies (which often is the case), then the mapping population must be phenotypically evaluated before QTL mapping (Collard *et al.*, 2005). The genotyping of the population is done by identifying the markers between two parents and screening the entire population. It is critical to have sufficient polymorphism between parents and generally cross-pollinating species possess a higher level of DNA polymorphism. According to Singh and Singh (2015c), molecular markers provide a tool for identifying genomic regions that control traits of interest. The construction of a linkage map is done by analysing many segregating markers. Several types of DNA marker techniques used were

Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeats (SSR), Inter-Simple Sequence Repeats (ISSR) etc., and more recently SNPs a high-throughput marker technique which is more reliable and cost-effective is available to detect markers.

Genetic information on *E. sativa* as such within the published literature is limited. Some molecular marker techniques such as RAPD, ISSR, and AFLP have previously been used to analyse morphological traits of *E. vesicaria* (Egea-Gilabert *et al.*, 2009). Markers such as ISSR and AFLP are relatively robust for screening the mapping populations and useful for discriminating between cultivars, but RAPDs are very unreliable and suffer from a lack of reproducibility and resolution (Karp *et al.*, 1996).

A linkage map is constructed by mapping markers on a chromosome which is measured in centimorgan (cM) by calculating recombinant frequencies. Recombination frequency of one per cent is equal to the distance between markers for which one product of meiosis is recombinant out of a hundred. A mapping function such as Haldane, Kosambi or Morgan is used to convert the recombination frequencies into map units (cM).

Linkage analysis and construction of linkage maps are performed by using software programs such as JoinMap, MapManager or Mapmaker. The linkage between markers is usually calculated using an odds ratio called a logarithm of the odds (LOD). Typically, LOD > 3, is used to construct linkage maps. LOD of 3 between markers represents that linkage is 1000 times more likely than no linkage (Collard *et al.*, 2005). Linked markers are grouped into ‘linkage groups’ which represent a

chromosome. Sometimes it is difficult to obtain an equal number of linkage groups and chromosomes, and this can be overcome by identifying more markers by repeating trials. Finally, once the linkage map is constructed it can then be used during marker-assisted selection in breeding programs, which will make the selection process more efficient.

1.3.2.2. QTL analysis and mapping

QTL is the region within the genome that contains genes associated with a particular quantitative trait. It represents loci or the genomic region that control a quantitative trait, where it could be one gene or cluster of linked genes. QTL analysis is based on the principle of detecting an association between phenotype and genotype of markers (Collard *et al.*, 2005; Singh and Singh, 2015a) where QTL is detected using a suitable statistical tool (Xu *et al.*, 2017). Construction of a linkage map followed by conducting QTL analysis to identify the genomic regions that are associated with the traits of interest is called QTL mapping (Collard *et al.*, 2005).

The main steps involved in QTL mapping are a selection of parents (differ for traits of interest), crossing the parents to develop a suitable mapping population such as RIL or DH lines, and genetic polymorphism study between parents with a suitable molecular marker system (AFLP, RFLP, SSR, SNP's etc.), phenotyping the traits of the mapping population in replicated trials and preferably over locations and years, and detection of QTL using a suitable statistical method (Xu *et al.*, 2017). Markers must cover the entire genome and should be uniformly and densely spread. The RIL and DH populations are considered as best for QTL mapping because of their homozygosity and can be repeated over multiple locations and years (Collard *et al.*, 2005). Furthermore, the RIL and DH populations can be used in varying environments without any genetic changes occurring.

Moreover, once this type of population is derived, the seeds from the individual RIL and DH lines can be transferred between laboratories and used by different researchers for further linkage analysis where additional markers could be added to the existing maps and all the information can be added to a common database (Young, 1994; Kumar, 1999). A biparental mapping has been proven to be useful in many crop breeding, however, the main limitation is only a few recombination events occur during the development of the population that resulted in having a QTL interval of 10-20 cM (Xu *et al.*, 2017). Abdurakhmonov and Abdukarimov (2008) in their article reviewed that the precision of QTL mapping depends on a few things such as genetic variation covered in a mapping population, the size of the mapping population and the number of marker loci. It is essential to collect accurate genotypic as well as phenotypic data. Any experimental errors in collecting the genotypic data can affect the order and distance between the markers within the linkage maps. A reliable or accurate QTL map depends on reliable phenotypic data achieved by replicating phenotypic measurements to reduce the background noise (Collard *et al.*, 2005).

There are many techniques/methods to detect QTL such as single QTL mapping [(single marker analysis (SMA), single interval mapping (SIM)], multiple QTL mappings [(composite interval mapping (CIM), inclusive composite interval mapping (ICIM), multiple interval mapping (MIM), etc.)] which are based on regression analysis, maximum likelihood parameter estimation, or Bayesian model (Collard *et al.*, 2005; Singh and Singh, 2015a). Single QTL mapping detects a single QTL at a time, whereas multiple QTL mapping combines multiple regression analysis with simple interval mapping. The interval mapping technique estimates the effect and position of a QTL between two markers efficiently. Interval mapping assumes that only one QTL affects the quantitative traits of interest and ignores the effects of other QTL on it (Xu *et al.*, 2017), however, quantitative traits are controlled by many loci. To overcome this issue, multiple QTL mapping was

proposed. Multiple QTL mapping is the extension of interval mapping which removes any residual variation caused by other QTL, thereby increasing the power of an individual test (Alonso-Blanco *et al.*, 2006; Xu *et al.*, 2017). QTL cartographer, QTL network, and MapManager QTX are a few of the software packages that are used to perform SIM, CIM or MIM analysis (Collard *et al.*, 2005).

1.4. Aims, Objectives and Hypothesis

Aim (overall)

The overall aims of this study were to:

(A) to understand the influence of genetics and the environment on phytochemical content in a mapping population of *E. sativa* grown at two separate locations: Italy and the UK and to identify the QTL responsible for the accumulation of sugars that may be utilised in breeding *E. sativa* for nutritional quality.

(B) to understand the influence of genetics (crop and human) and environment on the relationship between plant phytochemical content and sensory perception of six lines of *E. sativa* grown at two separate locations: Italy and the UK.

Aims were tested by developing a mapping population of 141 lines of *E. sativa* in conjunction with Elsoms Seeds Ltd. and growing it at two separate locations: Italy and the UK in a randomised block replicated field trials. To date, no robust replicated trials on a mapping population of *E. sativa* have been conducted to measure the effect of preharvest temperatures and growing conditions on the sensory quality as well as the phytochemical accumulations between locations/climates.

Furthermore, it is unknown how genotypes respond to different environmental conditions as these may have broad implications on potential health benefits as well as on the sensory attributes of rocket crops.

Objectives (Chapters 3 and 4) and Hypotheses:

A) The objective of Chapter 3 was to measure the abundance of phytochemical contents (sugars, organic acids, and GSLs) in a mapping population of *E. sativa* grown at two separate locations: Italy and the UK and to construct a QTL map.

A1) It was hypothesised that there would be a significant difference in the accumulation of the phytochemical content (such as sugars, organic acids, and GSLs) between the two locations.

A2) Furthermore, construct a draft linkage and QTL map based on the mapping population of *E. sativa* for sugar and determine the QTL responsible for it.

B) The objective of Chapter 4 was to evaluate the phytochemical content (sugars, sulphur, and GSLs) and investigate the relationship between sensory analysis and human taste receptor genotypes on six lines of *E. sativa* grown in two different locations: Italy and the UK. The six lines of *E. sativa* were selected from the 141 F3 RILs, based on their high and low abundance of GSLs across the two growing locations. Sensory profiling was included for a comparison between the 1st and the 2nd cut on the crops and changes during postharvest shelf life.

B1) It was hypothesised that there would be a significant difference in phytochemical content (sugars, sulphur, and GSLs) between the two locations, for the cuts and the changes during postharvest shelf life.

B2) Furthermore, it was hypothesised that there would be a significant difference in sensory profiling between six lines of *E. sativa* when grown at two locations for the 1st and 2nd cut and changes during postharvest shelf life.

B3) In addition it was hypothesised that there would be a significant difference in sensory perception due to human taste receptor genotypes (TAS2R38) on the six lines of *E. sativa* grown at two locations.

CHAPTER 2

Materials and Methods

2.1. Plant Material

Two elite inbred lines of *E. sativa* ('salad' rocket) were produced through self-pollination for five generations at Elsoms Seeds Ltd. (Spalding, United Kingdom) from 2010 to 2016 by the breeders to obtain near-homozygous lines. Each line was derived from germplasm accessions obtained from the Leibniz-Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK Gatersleben, Germany). For reasons of commercial sensitivity, these two elite inbred lines are referred to as parent B and parent C (coded varieties) and their lineage will not be identified.

2.1.1. The mapping population of *E. sativa*

139 individual lines of F1 *E. sativa* plants were produced from a bi-parental cross of the homozygous parent lines B and C by breeders at Elsoms Seeds (Spalding, UK). The F1 single seed from the cross between two parents (B x C) was grown and then self-pollinated in a controlled glasshouse environment to generate the segregating F2 mapping population. The seeds from the F2 generation were again self-pollinated by using the single seed descent (SSD) method to generate the F3 recombinant inbred lines (RILs) population to achieve homozygosity.

2.1.1.1. Growing locations and conditions

The 139 RILs from the F3 mapping population and the mapping parents (B and C) were grown in two different locations: Italy (September 2017) (41°55'31.1"N 12°08'15.8"E), and the UK (June 2018) (50°40'40.9"N 2°19'34.3"W), respectively (Figures 2.1a and 2.1b). In Rome, Italy, the F3 mapping population was grown in a polytunnel, whereas in Dorchester, UK they were grown in an open field (Figure 2.2a and 2.2b). To avoid the spread of downy mildew, the UK trial was grown in an open field. In the UK, a polytunnel-grown trial could create a humid environment for 'salad' rocket which could exacerbate the growth of a downy mildew disease. In both trials, 10 seeds of each line, per experimental block, were sown in peat blocks, covered with vermiculite, and kept in a vented glasshouse (Elsoms Seeds) for germination by maintaining the optimum temperature (20 - 28 °C) and relative humidity (40 - 45%) for 20 days. Both Parents B and C were included in each trial. Seedlings were transported to the respective trial locations in a temperature-controlled van (10 °C). Upon arrival, seedlings were transplanted by hand into the prepared soil beds. The soil beds were prepared according to standard commercial practices in the respective countries. Seedlings were planted in parallel rows having a soil bed width of 1.5 m (Appendix 2.1). A spacing of 10 cm between the rows was maintained. Individual lines were marked and identified using blue coloured stakes with a label for each row. Each trial comprised a complete randomised block design having three replicate blocks. Each block contained a single plant of 139 F3 RILs and two parents. Each block was surrounded by three guards (commercial *E. sativa* cultivar) plants to provide a buffer against the potential edge effect provided by Elsoms Seeds. A net was spread over plants in the UK trial to protect against fauna as this trial was conducted in an open field (Figure 2.2b).

Figure 2. 1. Map displaying field trial sites (a) Italy: 2017, (b) UK: June 2018.

Figure 2. 2. Field trials (a) mapping population of 141 F3 RILs of *E. sativa* grown in a polytunnel near Rome, Italy; (b) mapping population of 141 F3 RILs of *E. sativa* grown in an open field at Dorchester, UK.

The average daily temperature was 22.4 °C in Italy (September 2017) and 14.9 °C in the UK (June 2018) for a growth period (14 days, post-transplantation). The average daily weather data at each trial location are reported in Table 2.1. In June 2018, crops in the UK received 2.1mm of rainfall

during the growth period and this was on top of the overhead spray irrigation to maintain the trial viability. In the Italian trial, plants received daily overhead irrigation, as per the industry practice.

Table 2. 1. Average daily weather data for *E. sativa* field trials in Italy and the UK.

Trial locations	Average Temp (°C)	Temp max (°C)	Temp min (°C)	Rainfall (mm)	Cloud (%)	Humidity (%)
Italy September 2017	22.4	24.2	20.7	n/a*	21.1	68.2
UK June 2018	14.9	16.0	13.6	2.1	53.1	86.4

* Italy trial was grown in a polytunnel with daily overhead irrigation.

Plants were harvested manually (by hand) in the mornings after 14 days of the growth period in each respective trial. Harvesting rocket leaves at maturity can be done either mechanically or manually by cutting leaves with a knife 2-3 cm above the ground, allowing the crop to re-grow and produce more leaves (Koukounaras *et al.*, 2007a), however, for this set of experiments we used the 1st harvest. Leaves from one plant from each line were harvested from each of the three blocks and were placed in a Ziploc plastic bag (n = 3). These sample leaves were used for DNA extraction and SNP genotyping. Furthermore, leaves from four plants from each line per block were harvested and pooled into a plastic Ziploc bag (labelled with a respective line number) to give a representation of the leaf sample for metabolite analysis. This was done for each block separately, giving pooled samples (n = 3) per line, thus having a total of 423 samples in total. These Ziploc plastic bags (harvested leaves) were immediately placed into crates and stored in a nearby cold room (4 °C) in Italy and a temperature-controlled van (4 °C) in the UK (to reduce the field heat) (Bell *et al.*, 2017c).

Leaf samples from both locations were driven in a temperature-controlled van (4 °C) to the University of Reading, School of Chemistry, Food and Pharmacy (Reading, UK). Samples from Italy took approximately 36 hours, whereas samples from the UK took two hours to reach the University. Upon arrival at the University, sample crates were stored in a 4 °C cold room until further processing for metabolite analysis and DNA extraction. Sample leaves were freeze-dried in batches for several days (in a Vertis Bench-top Series). The dried material was then ground into a fine powder using a Wiley Mini-Mill (Thomas Scientific, Swedesboro, NJ, USA).

2.1.2. Selected six lines of *E. sativa* from the mapping population

The six lines of *E. sativa* were selected from the 139 F3 RIL mapping population based on their high or low abundance of GSL across the two growing locations. The selected lines will be referred as line 21 (low), 25 (low), 68 (high), 72 (low), 112 (high), and 130 (high), respectively. Twenty plants of each line were grown, and these were bulk pollinated in cages within a glasshouse environment at Elsoms Seeds Ltd (Spalding, UK) to produce sufficient seeds for commercial-scale production.

2.1.2.1. Growing locations and conditions

The above-mentioned six lines were grown in two different environments: a polytunnel near Rome, Italy (41°55'31.1"N 12°08'15.8"E) in September 2018, and an open field near Owermoigne, Dorchester, UK (50°40'40.9"N 2°19'34.3"W) in July 2019. In both trials, the seeds of each line were sown in peat blocks, covered with vermiculite, and kept in a vented glasshouse (Elsoms Seeds) for germination by maintaining the optimum temperature (20-28 °C) and RH (40-45%) for 20 days. Seedlings were transported to the respective trial locations in a temperature-controlled van

(10° C) and were planted manually into the prepared soil beds, as per the standard commercial practices followed in the respective countries. The average daily weather data at each trial location are reported in Table 2.2.

Table 2. 2. Average daily weather data for *E. sativa* field trials in Italy and the UK.

Trial location & harvest number	Average Temp (°C)	Temp max (°C)	Temp min (°C)	Rainfall (mm)	Cloud (%)	Humidity (%)
Italy 2018 1st cut	23.1	25.5	20.7	n/a*	22.7	61.4
Italy 2018 2nd cut	20.8	23.0	19.4	n/a*	29.8	69.1
UK 2019 1st cut	16.5	18.3	14.2	19.5	35.5	82.4
UK 2019 2nd cut	16.9	18.5	14.8	10.3	50.4	83.2

* Italy trial was grown in a polytunnel with daily overhead irrigation.

In the Italian trial, the 1st cut leaves were harvested 23 days after sowing and the 2nd cut was harvested 30 days after sowing. However, in the UK trial, the 1st cut was harvested after 26 days after sowing and the 2nd cut was harvested after 37 days after sowing. The difference in harvest days between the two locations was due to the differences in the growing environment. Both trials were surrounded by guard crops of a commercial *E. sativa* cultivar to protect against the edge effect.

In each respective trial (Italy and the UK), leaves were harvested in the morning by hand blade machines and were placed into crates and vacuum cooled using the on-farm facilities. Leaves from the Italian trial were transported in a temperature-controlled van (4 °C) to a processing unit at Alresford Salads, UK. Leaves from the UK trial were stored for two days postharvest in a 4 °C cold store at the same site to match the duration of leaves in transit from Italy. Harvested leaves from each line were hand processed by turbulent washing for one minute followed by rinsing and transferring the leaves in a hand-operated spinner and drying for another one minute according to

the protocol of Jasper *et al.* (2020). A study by Martínez-Sánchez *et al.* (2008) reported that baby leaves when washed and rinsed with cold water, showed a lower respiration rate than any other washing treatment. Washing is followed by spinning/drying to remove excess surface moisture, to avoid creating a microenvironment favourable for the growth of decay-causing pathogens during subsequent storage and distribution (Nicola *et al.*, 2006).

50 g of leaves were taken randomly from crates of each line and placed in microperforated plastic bags and then heat sealed (n = 12 per line). Each 50 g bag constituted a representative biological sample for the subsequent analyses. The sealed bags were sent to the University of Reading, School of Chemistry, Food and Pharmacy (Reading, UK) in a temperate-controlled van (4 °C). Upon arrival at the University of Reading, samples were transferred into a 4 °C cold room. Six bags for each line were used for sensory descriptive analysis and the rest for phytochemical analyses.

For sensory analysis, six bags of each line get divided equally into two parts: three bags were used for 'day 0' (D0) (intake) analysis and the remaining three were stored for five days at 4 °C ['day 5' (D5)] (postharvest shelf life) analysis (Figure 2.3). Day 5 is the typical post-packing shelf life period that bagged rocket leaves are given in the commercial environment before their "best before" date (Jasper *et al.*, 2021). Similarly, for phytochemical analyses, the same procedure was applied for two-time points i.e., three bags of each line were used for 'day 0' (D0) (intake) analysis and the remaining three bags for 'day 5' (D5) (postharvest shelf life) analysis. All samples for phytochemical analyses were stored at -80 °C. Each bag contains approximately 50 g of leaves. Samples of each line, therefore, consist of the following: 1st cut D0 (intake), 1st cut D5 (postharvest

shelf life), 2nd cut D0 (intake), and 2nd cut D5 (postharvest shelf life) for both the trials (Italy and the UK) (Figure 2.3).

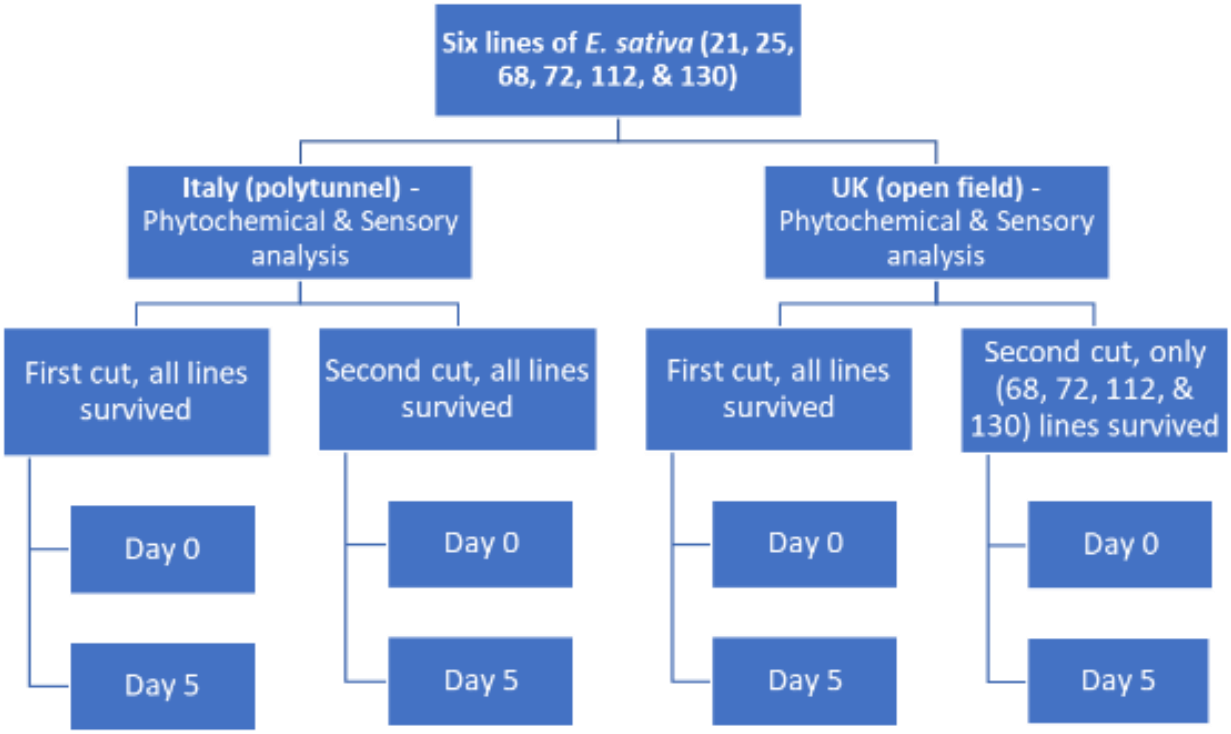


Figure 2. 3. Flow chart for six lines of *E. sativa* grown at two locations for the first cut and second cut (day 0 and day 5) for phytochemical analysis and sensory attributes.

2.2. Sample Preparation

Frozen leaf material was lyophilised in batches for up to a week in a STOKES freeze drier (F. J. Stokes corporation, Philadelphia, 20, PA, USA). Leaves were milled into a fine powder using a Mini Mill (Wiley Mini Thomas Scientific, Swedesboro, NJ, USA). Sample tubes were stored in a cool, dark, dry place until further phytochemical analysis.

2.3. Reagents and Chemicals

All solvents and chemicals used were HPLC and liquid chromatography-mass spectrometry (LC-MS) grade and obtained from Sigma–Aldrich (Poole, UK) and VWR UK Ltd (Lutterworth, UK) unless otherwise stated. 70% nitric acid was procured from Fisher Scientific UK.

2.4. Metabolite Analyses

2.4.1. Sugar and organic acid analysis

2.4.1.1. Sugar and organic acid extraction

Sugars and organic acids were extracted according to the method of Bell *et al.* (2017a) with modifications. Lyophilized leaf powder (0.2 g; n = 3) was suspended in 10 mL of 0.01 M hydrochloric acid in a glass vial. Each sample was stirred by adding a magnetic stirrer for 30 mins at room temperature (20 °C), and the mixture was set aside to settle for 30 min. The supernatant was slowly transferred into a 15 mL falcon tube and centrifuged at ambient temperature (20 °C) for 15 minutes at 19,215 x g, in Heraeus Multifuge 3SR+ Centrifuge (Thermo Scientific, UK). The supernatant was removed and filtered through 0.22 µm PVDF filters (Cole Palmer, St. Neots, UK) and the filtered solution was kept at -20 °C for further analysis. A 100 µL of filtered solution was taken into an HPLC vial and 900 µL of HPLC grade water was added to it. A blank of 1000 µL containing HPLC grade water only was prepared. An external standard for sugars and organic acids was procured from Sigma Aldrich (Gillingham, UK). Standards were prepared in a concentration range from 0.0 to 1.0 mg. g^{-1}: fructose (> 99%; $r^2 = 0.999$), galactose (> 99%; $r^2 = 1$), glucose (> 99%; $r^2 = 0.999$), sucrose (> 99%; $r^2 = 0.998$), citric acid (> 99%; $r^2 = 1$), malic acid (> 99%; $r^2 = 0.999$), succinic acid (> 99%; $r^2 = 1$).

2.4.1.2. HPLC analysis

For this study, the HPLC method was used to quantify sugars and organic acids. The extracted samples were analysed on an Agilent 1260 Infinity II HPLC system (Agilent, Santa Clara, CA, USA), equipped with a quaternary pump, degasser, auto-sampler, thermostat-controlled column compartment, and diode array (DAD) and refractive index (RI) detectors. A Bio-Rad Aminex HPX-87H Column (300 x 7.8 mm, prepacked 9 µm particle size, pH range 1-3) (Watford, UK) with a micro-guard cation H guard column (30 x 4.6 mm) (Bio-Rad, Watford, UK) was used to achieve separation at 30 °C, with an isocratic elution where the mobile phase consisted of 10 mM sulphuric acid at a flow rate of 0.3 mL min^{-1}. Sugars and organic acids were separated on HPLC during a 40 min chromatographic run. Sugars were quantified using the Polymer Laboratories ERC- 7515 RI detector (Church Stretton, UK), with the flow cell purged after every 10 sample runs. Organic acids were quantified using a DAD detector at a wavelength of 190 nm. Compounds were quantified using authentic standards and analysed with Agilent ChemStation software (Santa Clara, CA, United States).

2.4.2. Sulphur analysis

2.4.2.1. Sulphur extraction

Sulphur extraction was performed and analysed as per the protocol presented by Bell *et al.* (2020a). All the glassware was acid washed with 10% nitric acid using a triple rinse. Lyophilized samples (0.05 g, n = 3) were weighed into acid-washed 15 mL glass boiling tubes. A 2.5 mL of 70% nitric acid was added, mixed, and left for 24 hours in a glass tube in the fume hood. After 24 hours, the pre-digested samples were heated for two hours at 90 °C in the fume hood. Once the samples were cooled, they were filtered through a 0.45 µM syringe filter in an Eppendorf tube to remove any

remaining fats and undissolved solids. One mL of filtrate was taken into a 15 mL falcon tube and 9 mL of 2% nitric acid containing Rhodium internal standard (10 ppb) was added to it to give an acid concentration of 3%.

2.4.2.2. ICP-OES method

Samples were analysed using Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES; manufactured by Perkin Elmer Optima 7300 Dual View). The sulphur content was determined using the radial signal at 181.975 nm. A suitable standard curve was obtained using sulphur standards (0.1 - 40 mg. g^{-1}) (Bell *et al.*, 2020a).

2.4.3. GSL analysis

2.4.3.1. GSL extraction

GSL extraction was performed and analysed by using LC-MS as per the protocol presented by Bell *et al.* (2015) and Jasper *et al.* (2020). Lyophilized leaf powder (0.04 g; n = 3) was weighed in an Eppendorf tube and was heated in a dry block at 80 °C for 10 min. 1 mL of preheated 70% (v/v) methanol (70 °C) was added to each sample and later placed in a water bath for 20 mins at 70 °C. Samples were cooled and centrifuged for 5 mins (16,050 x g, 20 °C) to collect loose material into a pellet. The supernatant was then filtered using 0.22 µm Arcrodisc syringe filters (VWR, Lutterworth, UK) into a fresh Eppendorf tube, it was further dried to completion in a speed vac and reconstituted with 1 mL distilled water. Crude extracts were frozen at -80 °C until LC-MS analysis began.

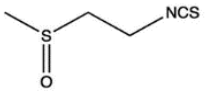
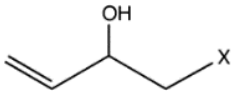
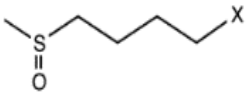
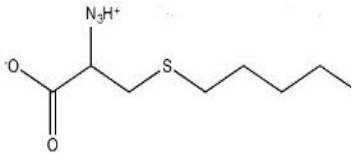
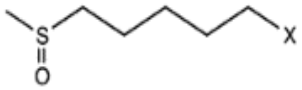
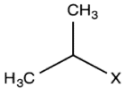
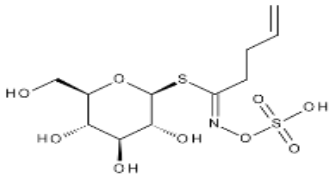
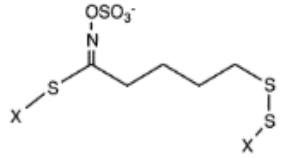
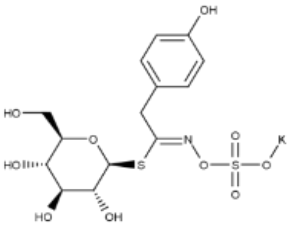
2.4.3.2. LC-MS analysis

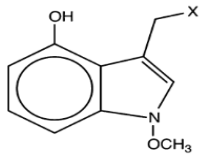
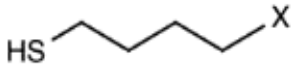
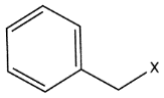
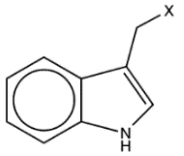
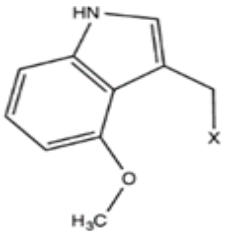
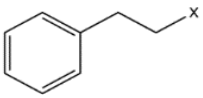
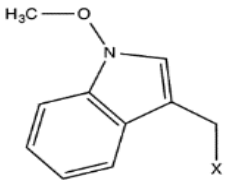
Immediately before LC-MS analysis, 200 µL of each sample was diluted with 800 µL of HPLC grade water in an amber HPLC vial. Samples and standards were run in random order with QC samples every 10 runs. A reference standard was purchased from PhytoPlan (Heidelberg, Germany) and was used to generate external calibration curves. External standards of glucoiberin (GIB; 99.61%, HPLC), progoitrin (PRO; 99.07 %, HPLC), sinigrin (SIN; 99%, HPLC), glucoraphanin (GRA; 99.86 %, HPLC), glucoalyssin (GAL; 98.8%, HPLC), gluconapin (GNP; 98.66%, HPLC), 4-hydroxyglucobrassicin (4-HGB; 96.19%, HPLC), glucotropaeolin (GTP; 99.61%, HPLC), glucoerucin (GER; 99.68 %, HPLC), glucobrassicin (GBC; 99.38 %, HPLC), and gluconasturtiin (GNT; 98.38 %, HPLC) were prepared for quantification of GSL compounds according to the method presented by Jin *et al.* (2009) and all compound purities were determined by HPLC. Pentyl GSL (GKR), glucorucolamine (GRM), glucoputranjivin (GPJ), diglucothiobeinin (DGTB), glucoberteroin (GBT), glucosativin (GSV), dimeric-4-mercaptobutyl GSL (DMB), 4-methylpentyl GSL (4MP), and hexyl GSL (HEX), were semi-quantified using SIN, as no standards are presently available for these compounds. Glucobrassicin (GBC) was used to semi-quantified the indole GSLs 4-methoxyglucobrassicin (4MGB) and neoglucobrassicin (NGB). An external reference standard of sinigrin hydrate for the quantification of GSL compounds was prepared and was as follows: A 12 mM solution was prepared in 70% methanol. A dilution series of concentrations was prepared as an external calibration curve with HPLC-grade water (224, 112, 56, 42, 28, 14 and 5.6 ng. μL^{-1}) (Jin *et al.*, 2009; Bell *et al.*, 2015; Jasper *et al.*, 2020) and a limit of detection (5.38 µmol L^{-1}) and limit of quantification (16.3 µmol L^{-1}) was established for the method by running serial dilutions of sinigrin.

LC-MS analysis was performed using the protocol by Bell *et al.* (2015) and Jasper *et al.* (2020). A negative ion mode on an Agilent 1260 Series LC system (Stockport, UK) equipped with a binary pump, degasser, autosampler, thermostat, column heater, photodiode array detector coupled to an Agilent 6120 Series single quadrupole mass spectrometry was used. Separation of samples was achieved on a Gemini 3 µm C18 110 A° column (150 x 4.6 mm; 1.8 µm) with a guard column, C18: 4mm x 3 mm; Phenomenex, Macclesfield, UK) (Jasper *et al.*, 2020). GSLs were separated during a 40 min chromatographic run, with a 5 min post-run sequence. Mobile phases consisted of ammonium formate (0.1 %; A) and acetonitrile (B) with the following gradient timetable: (i) 0 min (A–B, 95:5, v/v); (ii) 0–13 mins (A–B, 95:5, v/v); (iii) 13–22 mins (A–B, 40:60, v/v); (iv) 22−30 mins (A–B, 40:60, v/v); 30−35 mins (A–B, 95:5, v/v); (v) 35−40 mins (A–B, 95:5, v/v). The flow rate was optimized for the system at 0.4 mL min^{-1} with a column temperature of 30 °C, with 20 µL of the sample injected into the system.

Mass spectrometry analysis settings were as follows: Atmospheric pressure electrospray ionisation was carried out at atmospheric pressure in negative ion mode (scan range m/z 100–1500 Dalton) (Bell *et al.*, 2015; Jasper *et al.*, 2020). Nebulizer pressure was set at 50 psi, with the gas-drying temperature set at 350 °C and capillary voltage at 2,000 V. Compounds were identified using their primary ion mass, compared with authentic standards, and relative retention times in the literature (Cataldi *et al.*, 2007). All data were analysed using Agilent OpenLAB CDS ChemStation Edition for LC-MS (Agilent, version A.02.10). GSL concentrations were identified and quantified at a wavelength of 229 nm by matching ion spectra and fragmentation with standards and reported in Table 2.3.

Table 2. 3. Glucosinolate compounds identified in *E. sativa* leaves by LC-MS.

Common name	Abbreviation	R- group name	Identifying m/z $[M-H]^-$	Structure
Glucoiberin*	GIB	3-(methylsulfinyl) propyl	422	
-	GKR	pentyl	388	unknown
Progoitrin*	PRO	(R)-2-hydroxy-3-butenyl	388	
Sinigrin*	SIN	allyl	358	
Glucoraphanin*	GRA	4-(methylsulfinyl) butyl	436	
Glucorucolamine$a	GRM	4-(cystein-S-yl) butyl	494	
Glucoalyssin*	GAL	5-(methylsulfinyl) pentyl	450	
Glucoputranjivin$a	GPJ	1-methylethyl	360	
Gluconapin*	GNP	3-butenyl	372	
Diglucothiobeinin[a]	DGTB	4-(D-glucopyranosyldisulfanyl) butyl	600	
Glucoberteroin[a]	GBT	5-(methylthio) pentyl	434	

4-hydroxyglucobrassicin*	4HGB	4-hydroxy-3indolylmethyl	463	
Glucosativin[a]	GSV	4-mercaptobutyl	406	
-	DMB	Dimeric 4-mercaptobutyl	811	
Glucotropaeolin*	GTP	benzyl	408	
Glucoerucin*	GER	4-(methylthio) butyl	420	
Glucobrassicin*	GBC	indolyl-3-methyl	447	
4-methoxyglucobrassicin[b]	4MGB	4-methoxyindolyl-3methyl	477	
Gluconasturtiin*	GNT	2-phenethyl	422	
Neoglucobrassicin[b]	NGB	1-methoxy-3-indolymethyl	477	
-	4MP	4-methylpentyl	402	unknown
-	HEX	hexyl	402	unknown

* Authentic standard; [a] quantified using sinigrin; [b] quantified using glucobrassicin; [$] tentative identification.

2.4.4. Sensory analysis

2.4.4.1. Sensory panel selection and training

Sensory panels including trained (Panel 1) as well as untrained (Panel 2) individuals were recruited to assess the rocket leaf samples grown in Italy and the UK. In both cases, panellists were briefed about the purpose of the study and were asked to sign the consent form. Panel 1 members consisted of 11 trained individuals (n = 11) from the University of Reading Sensory Science Centre (Reading, UK) having a minimum of six months of experience in sensory evaluation, and a few having more than eight years of experience. All 11 individuals were present in both the trial sessions (Italy and the UK). They were trained in accordance with ISO 8586:2012 standards and subject to performance monitoring according to ISO 1132:2012 standards (Bell *et al.*, 2017a).

Panel 2 members were recruited from the Reading area who had previously participated in the sensory studies held at the University of Reading and had given consent to be contacted. These individuals were required to be over 18 years of age and be non-smokers. Panel 2 consisted of nine individuals (n = 9), and they were all presented in the Italian trial session, however, during the UK trial session, six of the same individuals (from the Italy trial) from panel 2 were present again, while a further three individuals agreed to take part in the study and were recruited to replace those who dropped out of the study (n = 9). Panel 2 individuals underwent an evaluation process consisting of 15 different sensory tasks to determine their sensitivity and discriminatory capabilities (Appendix 2.2). It took 11, one-hour sessions to train the individuals using supermarket-bought products (such as bagged rocket leaves, green peppers, peppercorns, condiment mustard, and dried garlic) and standard compounds (such as 1-octen-3-ol, quinine, and ally ITC) from Merk-Sigma, Gillingham, UK.

2.4.4.2. Sensory panel genotyping

Buccal swabs were taken in duplicates from all the individuals and were sent to Biosearch Technologies – LGC Groups (Hoddesdon, UK) for TAS2R38 genotyping. Both Panel 1 and Panel 2 individuals gave consent for their TAS2R38 genotyping data to be used in the studies conducted at the University. Ethical approval for all the sensory work and collection of panellists' tissue samples and genotype data was obtained from the University of Reading Research Ethics Committee with the study number UREC 18/23. Panellist genotypes are reported in Table 2.4.

Table 2. 4. Sensory panel genotypes of individuals assessing *E. sativa* leaves grown at two locations: Italy and the UK.

Panel (country)	PAV/PAV or PAV/AVI TAS2R38 diplotypes	AVI/AVI TAS2R38 diplotypes	Total
Panel 1 (Italy)	9	2	11
Panel 2 (Italy)	2	7	9
Panel 1 (UK)	9	2	11
Panel 2 (UK)	5	4	9

2.4.4.3. Vocabulary development

Independent vocabulary development sessions were conducted for both panels over three half-hour sessions by using the bagged rocket leaves from the supermarket and standard compounds before the actual samples. With the help of a facilitator, the panellist from both panels discussed the various sensory attributes associated such as aroma, mouthfeel, taste, flavour, and aftereffects of leaf samples which were presented to them. Each panel developed a consensus vocabulary, and the same terms were agreed upon by panellists from both panels. A list of sensory terms and their definitions is provided in Table 2.5. As a year passed between Italy and the UK trials, panellists from both panels were re-familiarised with the vocabulary by attending two additional half-hour

training sessions to ensure the consistency of responses. As we recruited additional three new members to Panel 2, they went through additional training and familiarisation sessions as above.

Table 2. 5. Definition for sensory attributes associated with the six lines of *E. sativa* leaves used during vocabulary development.

Attributes	Agreed definition
Aroma	
1) Pungent	A sharp aroma associated with perceived strength and elicited a tingling sensation in the nostrils
2) Mustard	Aroma associated with crushed mustard seeds or condiment mustard
3) Peppery	Aroma associated with ground peppercorns
4) Green	Aroma(s) associated with cut grass and freshness
5) Earthy	Resembling or suggestive of earth or soil
Mouthfeel	
6) Crisp	Brittle sensation on the teeth or tongue when chewing or biting leaves
7) Crunch	The audible sound heard when chewing the leaves
8) Firmness	Degree of the ease with which leaf can be broken and chewed by teeth
9) Moistness	Associated with the water content of the leaf when ingested
10) Warming	The sensation of increased temperature in the mouth while chewing leaves
11) Numbing	The sensation produced upon the tongue: associated with slight prickling
Taste	
12) Bitter	Sharp, unpleasant, or pungent taste on the tongue
13) Sweet	The pleasant taste associated with sugary foods
14) Umami	The taste associated with meaty or savoury foods
Flavour	
15) Peppery	The flavour associated with ground peppercorn
16) Green	The flavour associated with cut grass and freshness
17) Soapy	The flavour associated with soap and medicinal products
18) Mustard	The flavour associated with crushed mustard seeds
19) Burnt	The flavour associated with overcooked burnt foods; is reminiscent of burning rubber
Aftereffect	
20) Warming (mouthfeel)	Persistence of the sensation of heat in the mouth after swallowing
21) Tingling (mouthfeel)	A slight prickling sensation is produced in the throat after swallowing a leaf
22) Green (flavour)	Persistence of a grassy, fresh flavour

23) Drying (mouthfeel)	A sensation of dryness produced in the mouth after swallowing a leaf
24) Numbing (mouthfeel)	A sensation of numbness was produced after swallowing the leaf
25) Bitter (taste)	A persistence of bitter taste after swallowing leaf

2.4.4.4. Sensory analysis

The day 0 sensory evaluation on six lines of *E. sativa* took place on the following day after samples reached the University of Reading. Leaf samples were kept at refrigerated temperature (4 °C). These samples were allowed to come to room temperature before being served to the panellists. Day 5 samples were evaluated five days later under identical conditions. Samples were labelled with random three-digit codes and served in sterile Petri dishes and about 5-6 leaves were provided per panellist. For each line, two to three leaves were selected from each of the three bags at random for presentation. All the panellists were provided with water and frozen natural yoghurt for palate cleansing between samples. Sensory descriptors were entered into Compusense software (Guelph, ON, Canada) and the panellists were asked to score each attribute on anchored unstructured line scales (data scaled 0 - 100) with each anchor corresponding to the agreed extremes of each attribute mentioned in the definition (Table 2.5). Each sample was presented and assessed twice by each of the panellists (n = 40). Evaluation sessions were conducted in sensory booths (air-conditioned rooms, 23 °C) under artificial daylight within the Sensory Science Centre at the Department of Food & Nutritional Sciences, University of Reading, UK. They were asked to score the samples for aroma (pungency, mustard, peppery, green, and earthy), taste (bitter, sweet, and umami), flavour (peppery, green, soapy, mustard, and burnt), mouthfeel (crisp, crunch, firmness, moistness, warming, and numbing) and aftereffect attribute perceived on the tongue (warming, tingling, green, drying, numbing, and bitter). Aftereffect intensity was scored three times at the gap of 30 sec. Time

T0, T1, T2, and T3 were defined for aftereffect attributes by the panel assessors, where T0 is considered as the time just after consuming rocket leaves. T1 is the time after 30 s where again the assessor must score the aftereffect attribute. T2 is the time again after the next 30 sec when the panellist will score this aftereffect attribute and finally, for T3, the time after the 30 sec when they will again score. In total each assessor will score from T0 to T3 which is around 90 s for each trait. Once the assessment for day 0 (intake) was completed, the panellist was asked to revisit the University after five days for the day 5 postharvest shelf life study for the first cut trial for the Italian trial. This complete process was repeated for the second cut for day 0 (intake) and again for day 5 (postharvest shelf life) for the same Italian trial. After one year when samples were harvested in the UK trial, the entire process was again performed on the 1^{st} cut, day 0 (intake) and day 5 (postharvest shelf life) and on the 2^{nd} cut, day 0 (intake) and day 5 (postharvest shelf life).

CHAPTER 3

Evaluating phytochemical content in a recombinant inbred line mapping population of 'salad' rocket (*Eruca sativa*) grown in two locations: Italy and the UK.

3.1. Abstract

Rocket leaves are RTE salad that has many important phytochemicals that are thought to benefit human health. Due to its hot and pungent flavour, it is not widely accepted by many consumers, however, a few prefer their rocket hot. Sugars play a key role in determining taste and flavour as they can mask the bitterness and pungency of the rocket. This study quantifies the abundance of phytochemicals such as sugars, organic acids, and GSLs present in a mapping population of 'salad' rocket (*Eruca sativa*) grown at two different locations: Italy and the UK. Furthermore, this study presents a genetic linkage and QTL map for the phytochemical trait such as sugar and reveals the underlying genetic markers that contribute to the regulation of sugar accumulation.

Phytochemical analysis was conducted on a mapping population developed in collaboration with Elsoms Seeds Ltd. (Spalding, UK). To evaluate the effect of different environmental conditions and genetics on sugars, organic acids, and GSLs concentrations, field trials on 141 RILs of the F3 generation were conducted at two different locations, Italy, and the UK. The UK-grown trial showed approximately a two-fold higher average total sugar concentration as compared to the Italian-grown trial across the mapping population (UK average = 79.9 ± 1.10 mg. g^{-1} DW, Italy average = 40.05 ± 0.96 mg. g^{-1} DW). Among the four sugars, glucose was the most abundant sugar found in both trials, contributing 75% of the total sugars. These data show a significant influence

of growing location on an accumulation of sugars in rocket leaves. On average, no significant differences were observed for total acids and total GSLs between the trial locations, however, individual organic acids and GSLs varied significantly ($p < 0.0001$).

The phenotypic data obtained from the study were mapped onto the genotypic data to find QTL. A genetic linkage map was constructed using 285 high-quality markers having a map length of 889.2 cM, distributed onto 18 linkage groups covering all 11 chromosomes. This study identified a total of 20 QTL across the two trials, with 13 QTL identified from the UK trial and 7 QTL from the Italian trial. This is the first time a draft genetic linkage map and a QTL map have been developed for *Eruca sativa*. Once QTL were mapped, this identified regions of the genome that contribute to the regulation of sugar accumulation. The markers underlying these QTL are therefore potential candidates to assist breeders in selecting rocket plants with improved taste and flavour.

3.2. Introduction

Eruca sativa, commonly known as 'salad' rocket is a leafy RTE low-calorie food, which can be eaten raw with minimal preparation. Rocket species are grown commercially all over the world. Historically, they have grown in the countries and regions surrounding the Mediterranean Sea, as well as in India, Pakistan, Iran, and southern Europe, due to favourable growing conditions and climate (Martínez-Sánchez *et al.*, 2006b). The popularity and demand for rocket are growing with 40 million bags consumed annually in the UK. Throughout the year most of the rocket is imported from Italy and from different countries to fulfil the demand of the UK consumer population. This results in a difference in qualities such as appearance, taste, and flavour. Rocket is perceived as

being bitter by many consumers, due to the presence of a secondary metabolite GSL (Drewnowski and Gomez-Carneros, 2000) and their hydrolysis product, ITC.

It is known that a plant produces both primary metabolites (such as sugars, organic acids, amino acids etc.) involved in growth and metabolism as well as secondary metabolites (terpenes, phenolics, nitrogen-containing GSLs, etc.) involved in other metabolic pathways such as in plant defence mechanisms (Sudha and Ravishankar, 2002; Rolland *et al.*, 2006; Rosa *et al.*, 2009). Primary metabolites such as sugars (e.g., sucrose, glucose, fructose, galactose), along with imparting taste, also play an important role in maintaining the overall structure and growth of the plant. Organic acids such as citric, malic, and succinic are other important primary metabolites produced by plants that are involved in the (TCA cycle for the generation of metabolic energy where they not only supply energy in the form of adenosine triphosphate but also generate energy (Igamberdiev and Eprintsev, 2016; Zhang and Fernie, 2018). A limited number of studies have been conducted to evaluate the organic acid profile in *E. sativa*. Furthermore, the intermediate products of the TCA cycle produce precursors to amino acids (methionine) which are used in GSL biosynthesis (Cartea and Velasco, 2008; Hall *et al.*, 2012c).

The secondary metabolite GSL is a group of a bitter-tasting compound found in all the *Brassicaceae* crops including rocket (Steindal *et al.*, 2013; Helland *et al.*, 2016; Johansen *et al.*, 2017; Bell *et al.*, 2018). Although the sensory attribute of many GSLs are bitter, with a few having no taste at all, however, their presence is thought to benefit human health (Björkman *et al.*, 2011; Pasini *et al.*, 2011). A previous study on ‘salad’ rocket reported 11 GSLs (Chun *et al.*, 2013; Bell *et al.*, 2015), with the most prominent ones being glucoraphanin, glucoerucin, glucosativin, and its

dimer (dimeric-4-mercaptobutyl GSL) (Guijarro-Real *et al.*, 2020). Glucosativin and its dimer have been linked to bitterness (Jin *et al.*, 2009; Pasini *et al.*, 2011), but it is known that sugar can mask the bitterness of different foods (Sharafi *et al.*, 2013; Beck *et al.*, 2014; Chadwick *et al.*, 2016).

GSL and sugar are the two main components that determine the taste and flavour of the rocket. A study by Bell and Wagstaff (2017) on rocket leaves reported that the ratio between sugar and glucosinolate hydrolysis products was important in determining the taste and acceptance by the consumer. The sweet taste in the rocket is due to the presence of various sugars such as sucrose, glucose, fructose, and galactose and is preferred by many consumers as compared to the hot and pungent (Bell *et al.*, 2020b). So far, very little study has been performed to identify sugars in the rocket or the degree to which the effect of bitterness occurs in the RTE 'salad' rocket is poorly understood. Therefore, evaluating the sugar profile in the RTE 'salad' rocket is important for improved taste and flavour for consumer acceptance.

In the past, different approaches have been proposed to reduce the bitterness in *Brassicaceae* vegetables such as by lowering the GSLs contents for increased consumer acceptability. However, breeding for GSL is a complex issue as reduction or removal could result in adverse effects on the crop's survivability, or on the potential of the crop to impart health benefits to human consumers. Another approach could be increasing the concentration of sugars for more consumer acceptability, however, that would make the crop more attractive to pests and may be perceived as less healthy by the consumer. Therefore, identifying QTL is of particular importance in developing breeding lines to target these compounds (Chadwick *et al.*, 2016) as these can dissect the complex traits that

may be influenced by environmental factors (Jones *et al.*, 1997). A linkage map by using a QTL approach is the first step toward identifying the underlying gene(s) (Alarfaj *et al.*, 2021).

To elucidate the interaction between genetic and environmental components on different tastes and flavours, this study grew the mapping population of 141 RILs in replicated trials at two locations: Italy and the UK and measured the abundance of phytochemicals (sugar content, organic acid, and GSLs). Previous studies in the literature have reported that the phytochemical content was influenced by environmental factors, such as temperature, light, moisture, and soil quality (Jin *et al.*, 2009; Frezza *et al.*, 2010; Johansen *et al.*, 2016; Bonasia *et al.*, 2019). Climatic conditions which include light intensity and temperature does have a strong impact on the chemical composition of crops (Wagstaff, 2014) which may affect the nutritional quality (Bell *et al.*, 2020b). The above-mentioned phytochemicals are complex traits that are controlled by many genetic loci. For better tasting and healthier rocket, it is essential to breed rocket cultivars by developing genetic markers for phytochemicals by constructing linkage and QTL map. To the author's knowledge, no mapping population and therefore no linkage map has been constructed for *E. sativa*.

We hypothesised that there would be a significant difference in the accumulation of phytochemical content (such as sugars, organic acids, as well as higher GSLs) between the two locations. In this study, a draft genetic linkage map was constructed to identify QTL for phytochemicals such as sugar and assess the interaction between G x E by comparing the QTL at two locations. Identifying the underlying candidate gene/s could enable breeders to be used as markers in a future breeding programme for improved quality traits.

3.3. Material and Methods

3.3.1. Tissue preparation

Two elite inbred lines of 'salad' rocket for Parent B and Parent C (coded varieties by Elsoms Seeds Ltd.) were produced at Elsoms Seed Ltd. (Spalding, UK). See Chapter 2 for further details.

3.3.2. Reagents and chemicals

All the reagents and chemicals were procured from Sigma Aldrich (Poole, UK) unless otherwise stated. For further details, please refer to Chapter 2.

3.3.3. Sugar and organic acid analysis

Sugars and organic acids were analysed for the mapping population of 141 RILs of the 'salad' rocket grown at the two locations by using the HPLC instrument. See Chapter 2 for more details.

3.3.4. GSL analysis

The GSL profile of the mapping population of 141 RILs of 'salad' rocket grown at two locations was analysed using LC-MS according to the method presented by Bell *et al.* (2015) and Jasper *et al.* (2020). See Chapter 2 for further details.

3.3.5. Genome sequencing of Parent B and C

For genome sequencing, parent B and C lines were grown in a controlled environment growth chamber having a day temperature (22 °C/16 hours), night temperature (15 °C/8 hours), light

intensity (200 µmol m^{-2} s^{-1}) and water was provided as required. After 20 days, when plants matured, leaf tissues were sampled in triplicate (n = 3) and immediately frozen at -20 °C. DNA extraction was done using an E.Z.N.A.® isolation kit (Bell *et al.*, 2020a). Plant DNA DS Mini Kit (Omega Bio-Tek, Norcross, GA, United States) was used according to the manufacturer's protocol and samples were sent to the Earlham Institute (Norwich, UK) where QC analysis was done. At the University of Reading (UK), DNA samples for each parent were pooled and quantified using a Qubit fluorometer and dsDNA assay kit (Thermo Fisher Scientific, Loughborough, United Kingdom). The quality of DNA samples was assessed using a NanoDrop (Thermo Fisher Scientific) instrument.

3.3.6. Single Nucleotide Polymorphism (SNP) genotyping

The identification of SNPs was performed on the transcriptomes of parent B and parent C, which was done by Novogene (HK) Co. Ltd. (Hong Kong). Parent C, which is the reference genome for *E. sativa* (Bell *et al.*, 2020a) was used and the transcripts from parent B were compared to parent C. Illumina MiSeq and HiSeq2500 (Illumina Inc., San Diego, CA, United States) were used to generate the transcriptome sequencing data. 703 SNPs of high qualities were identified and selected based on their quality scores (Phred>60; $Q = -10 \log_{10} P$) which were used to generate a draft genetic linkage map. DNA was extracted from the 139 F3 mapping population samples using DNeasy Plant kits (Qiagen, Manchester, UK) and the protocol was followed according to the manufacturer's instructions. Extracted samples were sent to Bejo Zaden BV (Warmenhuizen, The Netherlands) to perform genotyping, according to the protocol by Haperen *et al.* (2020) where Kompetitive allele specific PCR primers were used.

3.3.7. Statistical analysis

Results from three biological replicates of each line (n = 3), for all the metabolites (sugar, acid, and GSL) were averaged and analyses were performed using XL Stat (Addinsoft, Paris, France). Normality tests were performed on each variable using the Shapiro-Wilk normality tests and were found to fit a normal distribution. Independently, a one-way analysis of variance (ANOVA) test was conducted to determine both within and between the trial variations of each rocket line. A *post hoc* Tukey's Honestly Significant Difference (HSD) test was applied to make multiple pairwise comparisons ($p<0.05$) between individual lines and environments (e.g., Italy vs. the UK). Principal Component Analysis (PCA) was performed using Spearman (n) correlation coefficient and significance analysis by using XL Stat (Addinsoft, Paris, France).

3.3.8. Linkage map construction

A total of 703 SNPs were selected based on their high-quality scores. After screening for, and removing, highly heterozygous SNPs and those which were heavily skewed (>90% of accessions expressing a single allele), out of 703 only 453 SNPs were chosen for JoinMap 4 software to build a linkage map. Of these 302 SNPs formed 18 linkage groups and 285 SNPs reached the final map. The remainder of 151 SNPs did not form enough statistically significant linages within the group to be placed in the map and thus remain unmapped. Of the 151 unmapped, 17 were removed because they collocated to other markers or lacked sufficient recombination for an algorithm to generate a reliable order. Linkage groups were formed using the 'recombinant frequency' parameter. Linkages with a threshold of 10 logarithms of odds (LOD) were considered strong linkages.

3.3.9. QTL analysis

For QTL mapping, the predicted means from the biological replicates (n = 3) were evaluated using ANOVA within XLStat (Addinsoft). MapQTL 6 (Van Ooijen, 2011) software was used to conduct QTL analysis. QTL were initially detected by interval mapping (IM) using a linear regression model with a maximum-likelihood principle. Significant markers were taken for automatic cofactor selection, and the best markers were used as cofactors in multiple QTL mapping (MQM) to confirm QTL. The LOD threshold was determined for each trait by permutation testing (5000 permutations) and QTL was deemed significant ($p<0.05$) if they exceeded this threshold.

3.4. Results and discussion

3.4.1. Phenotypic variation within the mapping population

Transgressive segregation was observed for compounds such as sugars, organic acids, and GSLs which were grown at two different locations, showing an influence of environmental conditions on phenotypes. The RIL mapping population contained lines with concentrations of the compound that were both higher and lower extremes, relative to the parental lines.

3.4.1.1. Sugar identification and concentration

The sugars (monosaccharides and disaccharides) identified in the present study were glucose, fructose, galactose, and sucrose. This study revealed that the average total sugar concentration was significantly higher in the UK-grown trial as compared to the Italian-grown ($p < 0.0001$). On average, a two-fold increase in total sugars was observed in the UK trial (Total sugars UK = 79.9 ± 1.10 mg. g^{-1} DW; Total sugars Italy = 40.05 ± 0.96 mg. g^{-1} DW) as compared to the Italian trial.

Amongst the four different sugars, glucose (UK average = 60.84 ± 0.9 mg. g^{-1} DW; Italy average = 25.8 ± 0.6 mg. g^{-1} DW) was the most abundant monosaccharide found in a mapping population contributing 75% of the total sugars. A previous study on rocket reported glucose as the primary photosynthetic product representing > 70% of the total soluble carbohydrates (Villatoro-Pulido *et al.*, 2013). The above result suggests accumulation of sugars within the leaves is influenced by the growing environment (Steindal *et al.*, 2015; Johansen *et al.*, 2016) which imparts taste attributes.

Table 3. 1. Summary of average sugar, organic acid, and GSL concentration (mg. g^{-1} DW) of a mapping population of 141 RILs of *E. sativa* grown at two locations: Italy and the UK (n = 3) with standard errors of mean values (±).

Compounds (mg. g^{-1} DW)	ITALY	UK	Significance (p-value)
Sugars			
Sucrose	5.6±0.2[a]	2.3±0.1[b]	<0.0001
Glucose	25.8±0.6[b]	60.8±0.9[a]	<0.0001
Galactose	3.0±0.1[b]	5.2±0.1[a]	<0.0001
Fructose	5.6±0.4[b]	11.5±0.2[a]	<0.0001
Total sugars	40.0±1.0[b]	80.0±1.1[a]	<0.0001
Organic acids			
Citric	102.8±2.1[a]	94.0±1.1[b]	<0.0001
Malic	47.1±1.1[b]	65.4±0.7[a]	<0.0001
Succinic	107.4±2.8	102.7±1.3	0.136
Total acids	257.3±5.6	262.0±2.3	0.458
Glucosinolates			
Glucoraphanin	1.4±0.1[b]	3.3±0.3[a]	<0.0001
Progoitrin	0.09±0.0[b]	0.15a±0.0[a]	<0.0001
Glucoalyssin	0.05±0.0[b]	0.19±0.1[a]	<0.0001
Diglucothiobeinin	0.1±0.0[b]	0.2±0.0[a]	<0.0001
Glucosativin	0.6±0.2[a]	0.3±0.1[b]	<0.0001
4-hydroxyglucobrassicin	0.02±0.0[a]	0.01±0.0[b]	<0.0001
Glucoerucin	2.8±0.2[a]	1.4±0.2[b]	<0.0001
Dimeric-4-mercaptobutyl	12.7±1.1[b]	13.9±1.1[a]	<0.0001
4-methoxyglucobrassicin	2.6±0.5[a]	0.5±0.1[b]	<0.0001
Neoglucobrassicin	0.4±0.0[a]	0.2±0.0[b]	<0.0001
Total GSLs	20.8±1.6	20.3±1.3	0.068

Significant differences (ANOVA Tukey's HSD test, $p \leq 0.05$). Differing small letters within each row denote a statistical difference. Values with no letters present no significant differences observed.

The present study reported a higher sugar concentration in the UK trial, which agrees with a previous study on a 'wild' rocket (*Diplotaxis tenuifolia*) which reported a higher sugar concentration in the UK-grown leaves as compared to those from Italy (Bell *et al.,* 2020b). Higher sugar concentration in the UK trial may be due to cold stress where plants are exposed to suboptimal climatic conditions [< 15 °C, 86.4 % RH (Relative Humidity)]. Soluble sugars assist in plant resistance to stress response and modify the cell reactive pathways by inducing stress response signals (Rosa *et al.,* 2009) affecting the accumulation of soluble sugar. A study on spinach reported increased sugar contents when subjected to low temperature (4 - 7 °C) or cold stress may be due to the up-regulation of the sucrose biosynthesis pathway, which generally occurs in plants during the development of freezing tolerance (Yoon *et al.*, 2017). Rocket species originated from warm and dry climates such as the Mediterranean region, the Middle East, and Pakistan (Bell and Wagstaff, 2019) which makes the crop more stressful when grown in wet and cool climatic conditions like in the UK.

On average, the concentration of glucose, galactose, and fructose in the UK-grown trial were 2.4-fold (60.8 mg. g^{-1} DW), 1.8-fold (5.2 mg. g^{-1} DW), and 2.1-fold (11.5 mg. g^{-1} DW) higher than the Italy trial (25.4 mg. g^{-1} DW, 2.9 mg. g^{-1} DW, and 5.6 mg. g^{-1} DW) (Table 3.1) respectively. The only exception to this pattern was sucrose, which showed a higher average concentration in the Italian trial (5.6 mg. g^{-1} DW) with 2.4-fold higher values than in the UK trial (2.3 mg. g^{-1} DW). Sucrose is the primary photosynthetic product of the Calvin cycle via triose phosphate, which gets transported from the source (leaves) to the sink (roots, developing organs such as young leaves, seeds, etc.) through the phloem (Rolland *et al.,* 2002). The source/sink relationships can be affected by many environmental factors such as temperature, drought, salinity etc., (Lemoine *et al.,* 2013).

The accumulation of sucrose in the Italian trial is due to plants' normal photosynthesis process. However, the accumulation of other hexoses in the UK trial could be due to environmental stress such as cold temperature altering the source/sink relationship. This results in a higher accumulation of glucose, fructose, and galactose as a hydrolytic product to maintain the metabolic activity in leaves. A study on four genotypes of strawberries reported a significant influence of growing location (Swiss environmental conditions) on the accumulation of sugars and acid content (Crespo *et al.*, 2010), however, fruits and leaves behave differently in terms of their accumulation.

On average, a six-fold higher variation in total sugar concentrations was observed across all the lines within the mapping population of the Italian trial (ranged from 11.5 to 67.3 mg. g^{-1} DW) (Figure 3.1a) as compared to the UK-grown trial which showed 2.5-fold variation (ranged from 53.0 to 132.0 mg. g^{-1} DW) (Figure 3.1b) (Appendix 3.1). Higher overall variation in the Italian population suggests more carbon from photosynthetic products has been used to produce other metabolites, such as volatiles, and left the plant with limited carbon to produce other metabolites such as sugars. A high temperature may lead to increased VOC production. Rocket leaves when stored at 10 °C for 14 days showed increased VOC resulting in off-odour production (Spadafora *et al.*, 2016). Therefore, it is recommended to store the leafy salads at low or refrigerated temperatures to avoid the formation of off odour during shelf life.

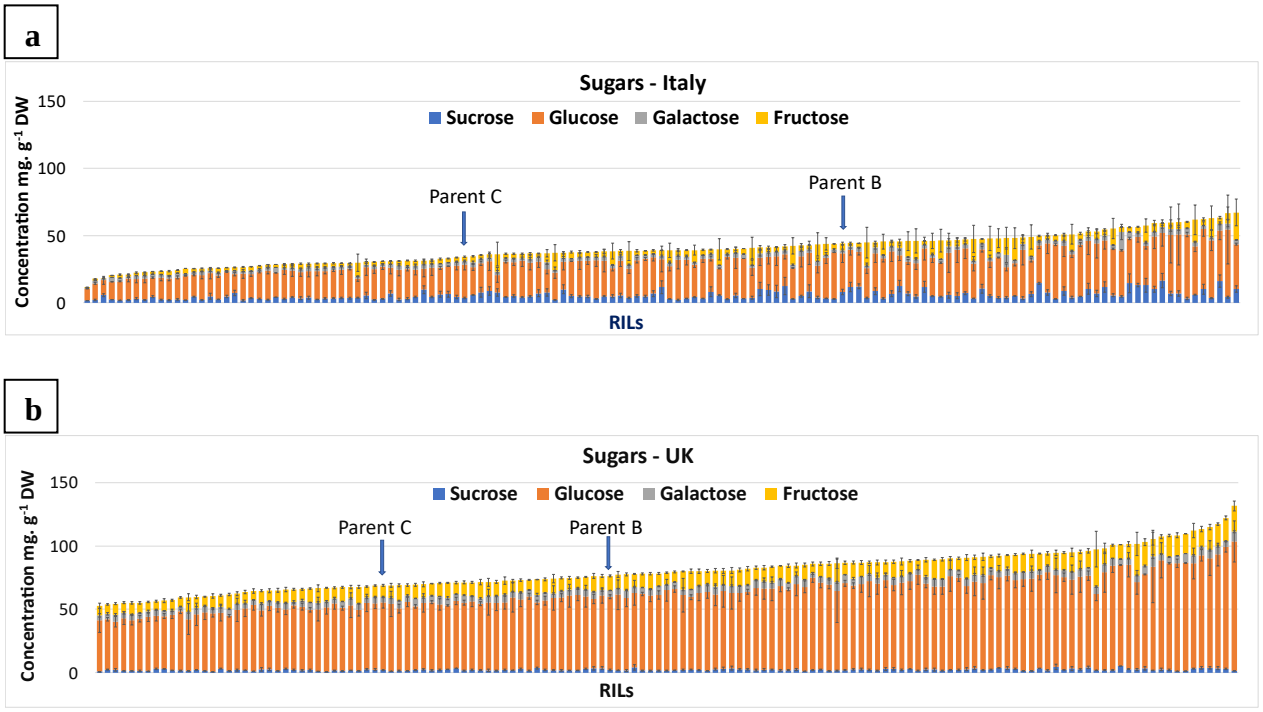


Figure 3. 1. Distribution of average sugar concentration in a mapping population of *E. sativa* grown at two locations (mg. g^{-1} DW). (a) 141 RILs in Italy; (b) 141 RILs in the UK. Parent B and Parent C were included in both trials indicated by blue arrows. Error bars signify the standard errors of the mean values of three biological replicates (n = 3). Colour code: see insight. Abbreviation: DW (Dry weight), RILs (Recombinant inbred lines).

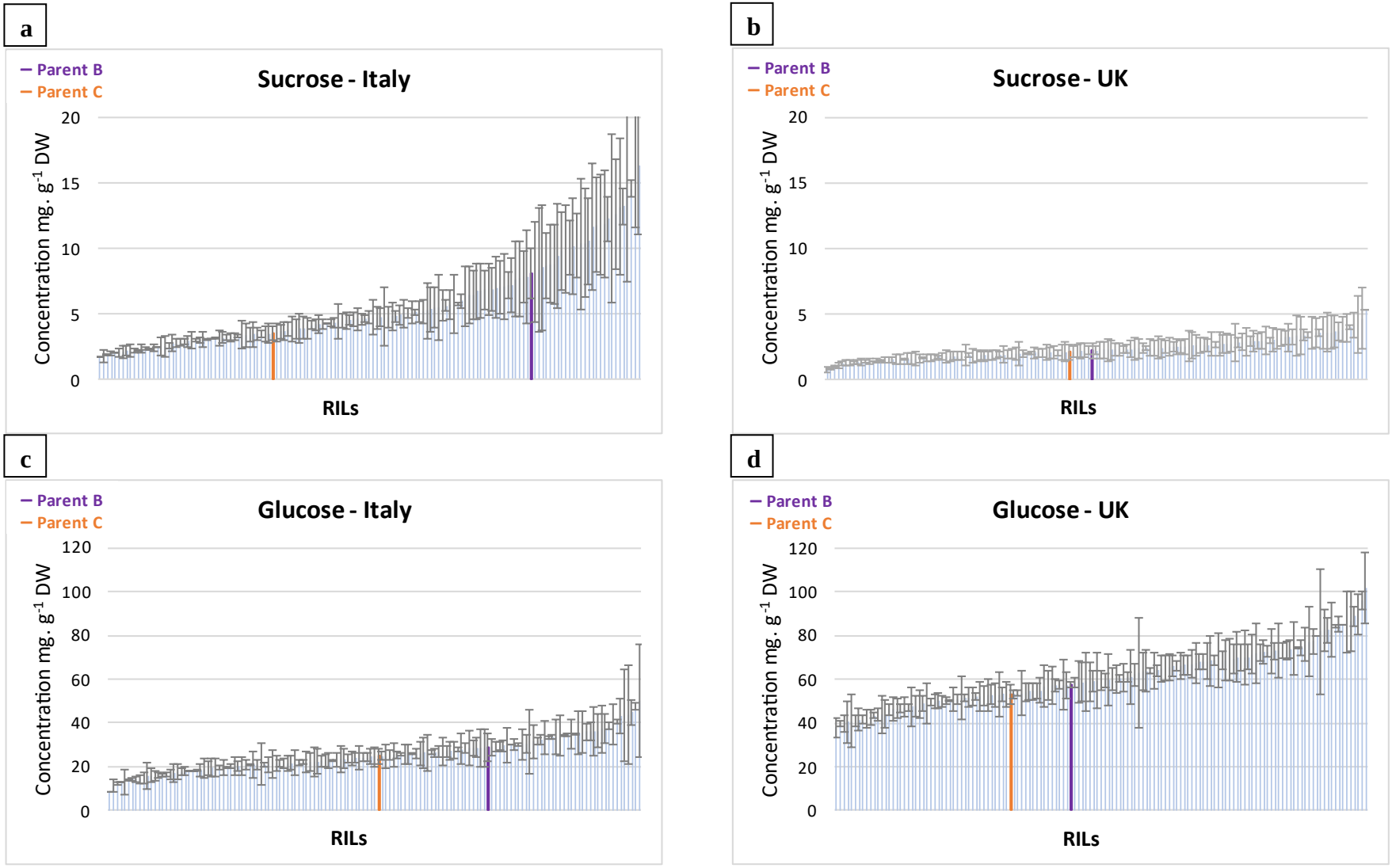
In each trial, Parent B showed a higher average sugar concentration (Total sugars Italy = 44.01 ± 8.8 mg. g^{-1} DW; Total sugars UK = 76.4 ± 7.2 mg. g^{-1} DW, p = 0.015) than Parent C (Total sugars Italy = 34.3 ± 7.2 mg. g^{-1} DW; Total sugars UK = 68.8 ± 7.2 mg. g^{-1} DW, p = 0.015) (Appendix 3.3). In the Italian trial, 34% of the RILs and in the UK trial 55% of the RILs showed a higher concentration of total sugars than Parent B, suggesting that there is a potential for breeding for higher sugars by using these RILs. Higher percentages of total sugar concentration in the extreme RILs than Parent B from each trial suggest the accumulation of favourable genes as the result of a recombination event. A study by Rieseberg *et al.* (1999) explained the concept of transgressive segregation as a beneficial tool for the development of new cultivars with improved traits in the breeding programme.

Within the respective trials, all the sugars were found to segregate significantly ($p<0.0001$) (Figure 3.2). Average sucrose concentration varied significantly in both trials ($p<0.0001$) (Table 3.1), where a higher concentration was observed in the Italy trial as compared to the UK trial. The Italian-grown trial showed a 10-fold variation in all the lines ranged from 1.7 to 16.4 mg. g^{-1} DW, whereas the UK-grown trial, showed a seven-fold variation (ranged from 0.8 to 5.3 mg. g^{-1} DW) (Figure 3.2a and 3.2b) (Appendix 3.2). Both parental lines showed different distribution patterns for sucrose. In the Italian trial, parental lines were located (Parent C towards lower; Parent B towards higher) at the end of the distribution range (Figure 3.2a), whereas in the UK-grown trial, both parents had converged towards the middle of the distribution (Figure 3.2b) showing less effect of environment on the genotype. A 50% of RILs showed higher sucrose content from the UK trial, whereas only 20% of the RILs showed higher sucrose in the Italy trial than Parent B.

On average glucose concentration varied significantly between both trials ($p<0.0001$) (Table 3.1). Average glucose concentration in the UK trial was higher as compared to the Italy trial and showed a 2.7-fold variation across the lines ranged from 37.9 to 101.9 mg. g^{-1} DW, whereas in the Italy trial, a 5.8 -fold variation was observed ranged from 8.6 to 50.3 mg. g^{-1} DW (Appendix 3.2). In both trials, Parent B showed a higher average glucose concentration than Parent C (Appendix 3.3), where Parent B showed a higher average glucose concentration in the UK trial than in the Italian trial. In the UK trial, 55% of the RILs showed more glucose concentration than Parent B. Both parents converged towards the middle of the distribution (Figure 3.2d) which suggested that more genes were unmasked in the RILs as compared to Parent B, and these extreme RILs can be useful in finding the molecular markers for higher glucose content. In the Italy trial, only 28% of the RILs showed a higher glucose concentration than Parent B (Figure 3.2c).

Average galactose concentrations vary significantly between each trial ($p < 0.0001$) with a higher concentration observed in the UK trial. A 20-fold variation was observed across all the lines within the Italian trial for galactose ranged from 0.7 to 13.8 mg. g^{-1} DW. Whereas, in the UK trial, a 2.8-fold variation was observed across all the lines ranged from 2.8 to 7.9 mg. g^{-1} DW (Appendix 3.2). For Galactose, Parent B showed a higher average concentration in the UK trial than Parent C, whereas in the Italy trial it was the opposite (Appendix 3.3). In the Italy trial, 57% of the RILs showed a higher concentration than parent C (Figure 3.2e), whereas, in the UK trial, 56% of RILs showed a higher galactose concentration (Figure 3.2f) than Parent B.

Fructose average concentration varied significantly between the two trials ($p < 0.0001$) (Table 3.1) whereas the UK trial showed a higher fructose concentration. In the UK trial, a 4.4-fold variation across the line was observed ranged from 6.7 mg. g^{-1} DW to 29.5 mg. g^{-1} DW, whereas in the Italian trial, a 40-fold variation was observed across the lines where the concentrations ranged from 0.5 mg. g^{-1} DW to 20.4 mg. g^{-1} DW (Appendix 3.2). In both trials, Parent B showed higher average values than Parent C (Appendix 3.3). In the Italian trial, 25% of RILs showed higher fructose concentration (Figure 3.2g) than Parent B, however, in the UK trial, 42% of the RILs showed higher fructose concentration than Parent B (Figure 3.2h).


a
Parent B
Parent C
Sucrose - Italy
Concentration mg. g-1 DW
0
5
10
15
20
RILs
b
Parent B
Parent C
Sucrose - UK
Concentration mg. g-1 DW
0
5
10
15
20
RILs
c
Parent B
Parent C
Glucose - Italy
Concentration mg. g-1 DW
0
20
40
60
80
100
120
RILs
d
Parent B
Parent C
Glucose - UK
Concentration mg. g-1 DW
0
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RILs

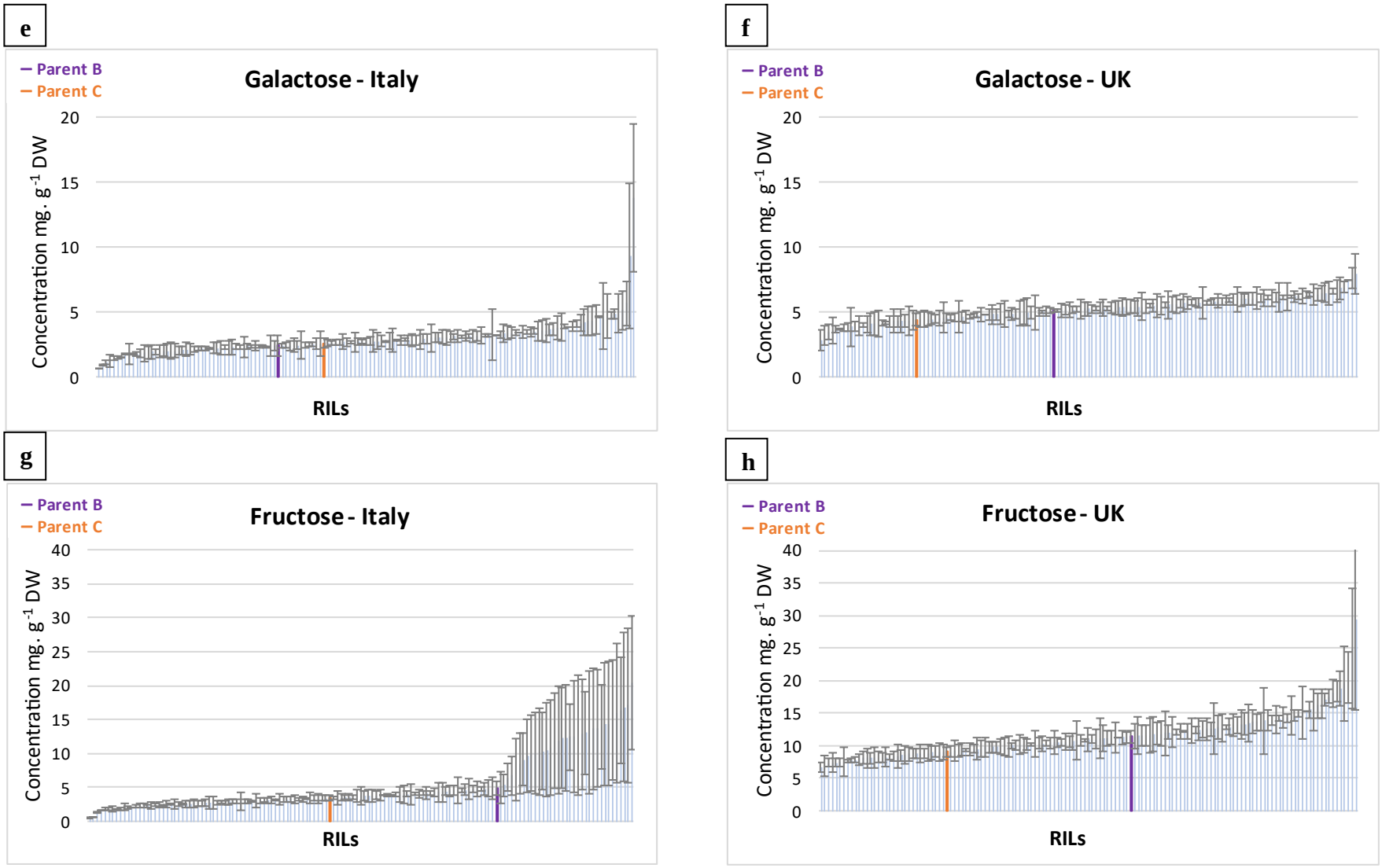


Figure 3. 2. Transgressive segregation of average individual sugars of 141 RILs of *E. sativa* grown at two locations (mg. g^{-1} DW). Sucrose (a) Italian trial; (b) UK trial, glucose (c) Italian trial; (d) UK trial, galactose (e) Italian trial; (f) UK trial, fructose (g) Italian trial; (h) UK trial. Parent B and Parent C were included in both trials indicated by purple and orange lines. Error bars signify the standard errors of the mean values of three biological replicates (n = 3). Abbreviation: DW (Dry weight), RILs (Recombinant inbred lines).

3.4.1.2. Organic acid identification and concentration

The three major organic acids identified in the present study were citric, malic, and succinic acid. The present study found no significant difference in average total acids and average succinic acid concentration between the two trial locations ($p = 0.456$, and 0.136), however, average citric acid and average malic acid concentrations vary significantly ($p < 0.0001$) (Table 3.1). Amongst the three different acids, succinic acid (Italy average = 107.4 ± 2.8 mg. g^{-1} DW; UK average = 102.7 ± 1.3 mg. g^{-1} DW) was the most abundant organic acid found in a mapping population followed by citric acid (Italy average = 102.8 ± 2.1 mg. g^{-1} DW; UK average = 93.9 ± 1.1 mg. g^{-1} DW) and malic acid (UK average = 65.4 ± 0.7 mg. g^{-1} DW; Italy average = 47.1 ± 1.1 mg. g^{-1} DW) (Table 3.1) respectively. The average malic acid concentration was 1.4-fold higher in the UK trial as compared to the Italian trial. Plants produce various organic acids as an intermediate product in the TCA cycle that are involved in various metabolic pathways such as biosynthesis of amino acids, energy production, regulation of osmotic pressure etc. (Ludwig, 2016). Accumulation of both citric and malic acids depends upon various factors such as genetics, degree of development and environmental conditions (cultural practices, irrigation, high and low temperature etc.) (Huang *et al.*, 2021). A study by Ayaz *et al.* (2006b) reported citric (22.1 mg. g^{-1} DW) and malic (15.1 mg. g^{-1} DW) acid content as the major organic acids in kale leaves. A previous study on seven accessions of *E. sativa* reported two major organic acids, namely citric acid (ranged between 10.4 ± 7.3 mg. g^{-1} DW and 30.9 ± 5.0 mg. g^{-1} DW) and malic acid (ranged between 46.8 ± 0.7 mg. g^{-1} DW and 83.5 ± 24.1 mg. g^{-1} DW) (Bell *et al.*, 2017a). The present study reported a 10-fold higher citric acid concentration as compared to the study by Bell *et al.* (2017a), while malic acid concentrations were within the same range. The higher citric acid concentration in the present study could be due to environmental stress (trials happening in the actual field conditions as compared to the previous study where plants were grown in the controlled environment) altering the metabolic pathway

(Rouphael *et al.*, 2012; Tahjib-Ul-Arif *et al.*, 2021), however, further study is needed to confirm the biosynthesis mechanism responsible for it.

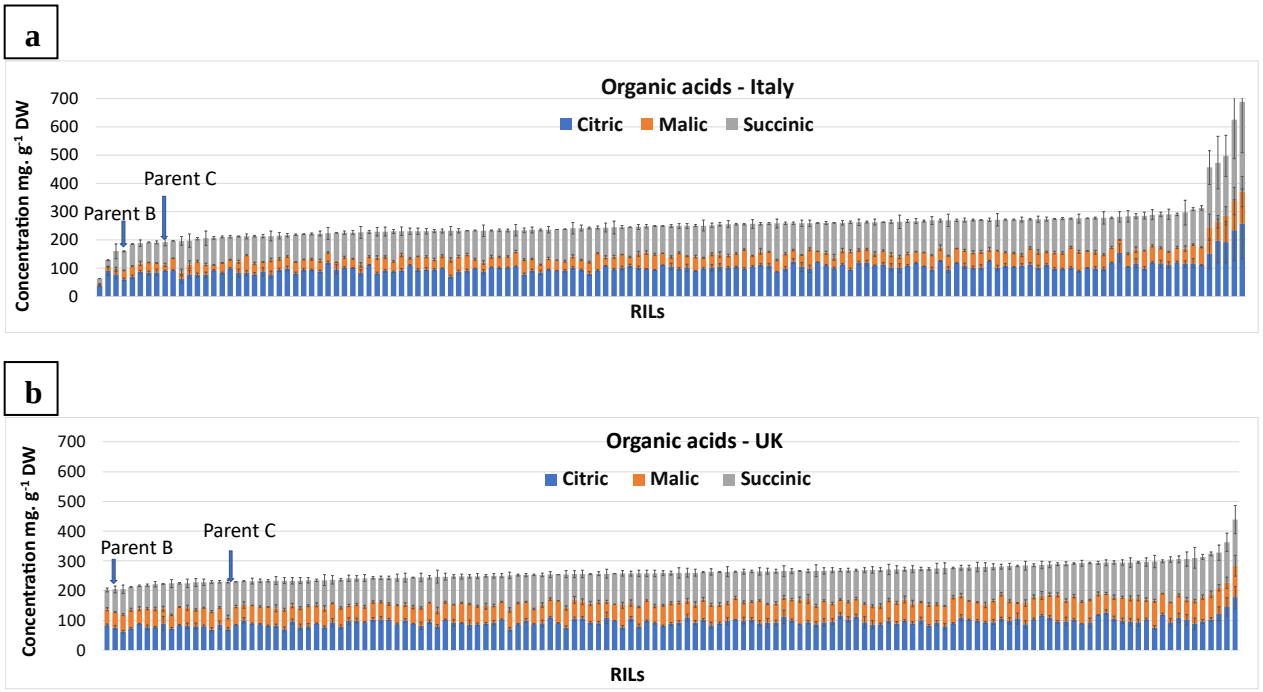


Figure 3. 3. Distribution of average organic acid concentration in a mapping population of *E. sativa* grown at two locations (mg. g^{-1} DW). (a) 141 RILs in Italy; (b) 141 RILs in the UK. Parent B and Parent C were included in both trials indicated by blue arrows. Error bars signify the standard errors of the mean values of three biological replicates (n = 3). Colour code: see insight. Abbreviation: DW (Dry weight), RILs (Recombinant inbred lines).

In the Italian trial, on average an 11-fold variation was observed across all the lines for total acids (ranged from 62.4 to 687.5 mg. g^{-1} DW) (Figure 3.3a), whereas the UK trial showed a two-fold variation ranged from 202.7 to 438.8 mg. g^{-1} DW (Figure 3.3b) across the lines within the mapping population (Appendix 3.1). Parent C contributed to higher average total acid in both trials (Appendix 3.3), with 94% of the RILs in the Italian trial and 89% of the RILS in the UK trial showing higher total acids than Parent C. In both trials, the relative position of parental lines (B and C) within the population for total acids were distributed towards the lower extreme of the

distribution as compared to the RILs, which could be due to inherited heterosis effects because of the initial cross (Figure 3.3a and 3.3b).

The average citric acid concentration varied significantly between the two trial locations ($p < 0.0001$) (Table 3.1), with a higher citric acid concentration observed in the Italy trial. A seven-fold variation was observed within the mapping population for citric acid concentration in the Italy trial ranged from 36.8 to 256.9 mg. g^{-1} DW (Figure 3.4a) as compared to the UK-grown trial where a 2.9-fold variation was observed which ranged from 61.8 to 177.7 mg. g^{-1} DW across the lines (Figure 3.4b). A higher average citric acid concentration was observed in Parent C as compared to Parent B (Appendix 3.3). In the Italy trial, 77% of the RILs showed a higher citric acid concentration than Parent C, whereas, in the UK trial, it was 94% of the RILs showed a higher citric acid concentration than Parent B. In both trials, the parental lines (B and C) showed lower citric acid concentration as compared to their RILs and showed a lot of transgressive segregation towards one direction (higher extreme) (Figure 3.4a and 3.4b) This suggests more genes were unmasked in RILs as compared to the parental lines which could be due to favourable environmental conditions resulting in higher accumulation of citric acid concentration.

Average malic acid concentration varied significantly between trial locations ($p < 0.0001$) (Table 3.1). The average malic acid concentration was higher in the UK-grown trial, whereas the average citric acid concentration was higher in the Italian trial. A 17-fold variation was observed across all the lines for malic acid concentration in the Italy trial ranged from 6.8 to 113.3 mg. g^{-1} DW (Figure 3.4c) as compared to the UK trial which showed a 2.6-fold variation ranged from 39.9 to 106 mg. g^{-1} DW (Figure 3.4d). A study by Ludwig (2016) argued that proteins present in the cytosol of the

plant cell are involved with organic acid metabolism, which is known to coordinate the concentration of malate in response to stress. Malic acid metabolism is regulated by a malic enzyme which plays a key role in plant stress resistance where a low temperature can induce an increase in malate content (Sun *et al.*, 2019). This may partly explain the higher malic acid concentration in the UK trial; however, more research is needed to confirm the underlying genetic mechanism responsible for this. In the Italy trial, 89% of the RILs and in the UK trial, 93% of the RILs showed higher malic acid contents than Parent B (Figure 3.4c and Figure 3.4d).

Higher accumulation of citric and malic acid within the leaves of 'salad' rocket as compared to other leafy salads could be an indicator of metabolic stress when grown under different environmental conditions (Bell *et al.*, 2017a; Huang *et al.*, 2021). Accumulation of citric and malic acid may mostly be due to their complicated metabolism (such as the TCA cycle in the mitochondrion, the glyoxylate cycle in glycoxysomes and citrate catabolism in the cytosol) and their vacuolar storage in the plant cell (Etienne *et al.*, 2013).

On average there were no significant differences observed between the trial locations for succinic acid concentrations ($p = 0.136$) (Table 3.1). In the Italian trial, average succinic acid concentrations ranged from 18.9 to 317.3 mg. g^{-1} DW (Figure 3.4e) showing a 17-fold variation across the lines as compared to the UK trial where a 2.4-fold variation was observed ranged from 65.7 to 155.1 mg. g^{-1} DW. Parent C showed a higher average concentration of succinic acid than Parent B in the UK trial (Appendix 3.3). In the UK trial, only 16% of the RILs showed higher succinic acid concentration than Parent C, which shows the distribution and segregation of 141 lines as transgressive. Whereas in the Italian trial both parental lines (B and C) showed lower succinic acid

concentrations than their RILs, with 89% of the RILs showing a higher succinic acid concentration than Parent C.

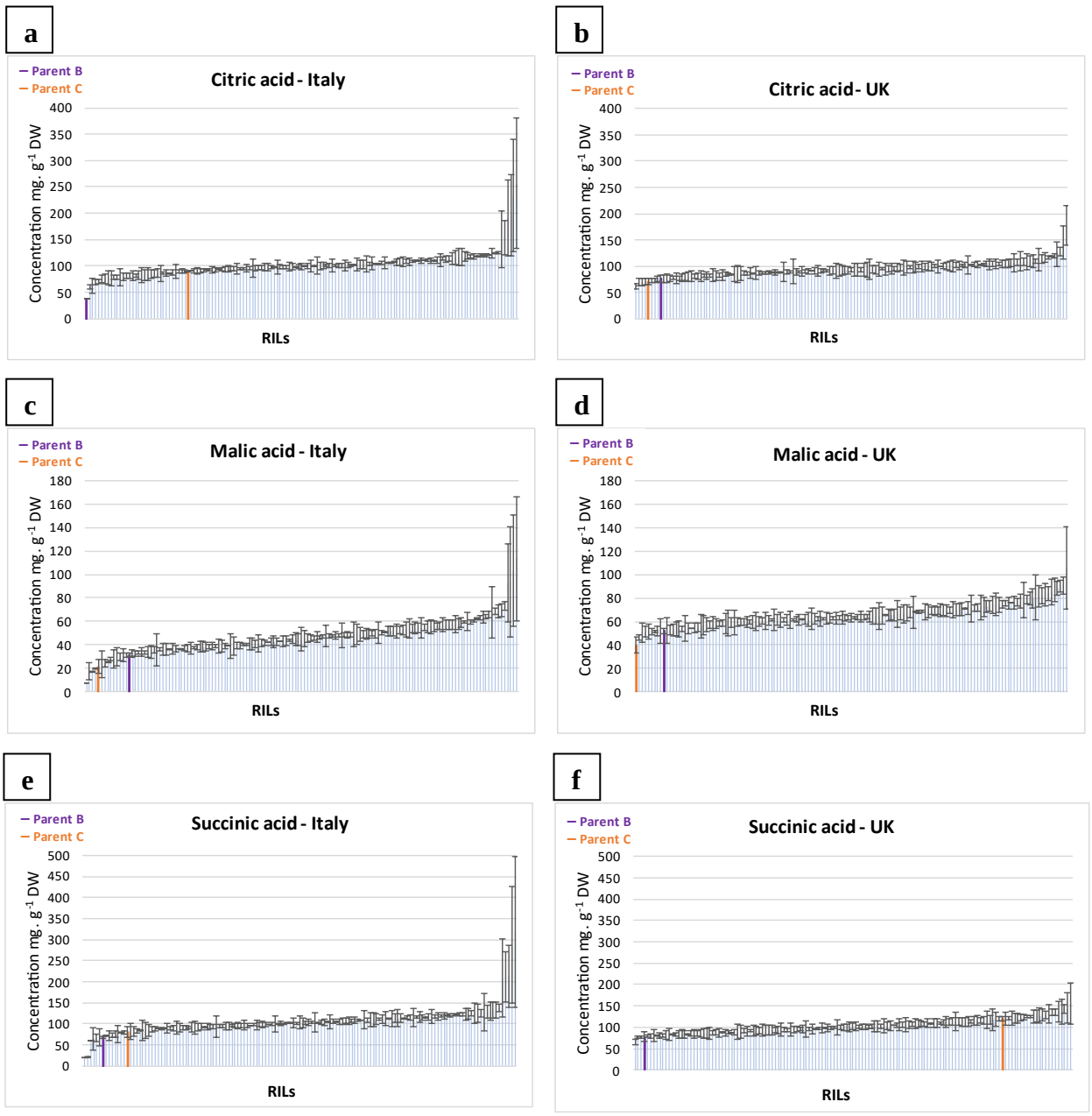


Figure 3. 4. Transgressive segregation of average individual organic acids of the 141 RILs of *E. sativa* grown at two locations (mg. g^{-1} DW). Citric acid (a) Italian trial; (b) UK trial, malic acid (c) Italian trial; (d) UK trial, succinic acid (e) Italian trial; (f) UK trial. Parent B and Parent C were included in both trials indicated by purple and orange lines. Error bars signify the standard errors of the mean values of three biological replicates (n = 3). Abbreviation: DW (Dry weight), RILs (Recombinant inbred lines).

3.4.1.3. Glucosinolate identification and concentration

The present study identified ten GSLs: glucoraphanin, glucoerucin, glucosativin, dimeric-4-mercaptobutyl GSL, glucorucolamine, glucoalyssin, diglucothiobeinin, glucobrassicin, 4-methoxyglucobrassicin, and neoglucobrassicin. On average there was not a significant difference between the Italian and UK-grown trials for total GSL concentrations ($p = 0.068$), but individual average GSL concentrations varied significantly (all $p < 0.0001$) between the trial locations (Table 3.1). The average total GSL concentration for the Italy trial was 20.8 ± 1.6 mg. g^{-1} DW, whereas the average total GSL concentration in the UK trial was found to be 20.3 ± 1.3 mg. g^{-1} DW (Table 3.1).

In the Italian trial, a two-fold variation was observed for total GSL concentrations ranged from 15.1 to 30.4 mg. g^{-1} DW (Figure 3.5a) across all the lines within the mapping population, whereas in the UK-grown trial, a 1.9-fold variation was observed ranged from 14.7 to 27.7 mg. g^{-1} DW (Figure 3.5b) across all the lines within the mapping population (Appendix 3.1). In both trials, Parent B contributed to a higher average total GSL concentration than Parent C (Appendix 3.3), which suggests the dominance of one genotype on the accumulation of secondary metabolites (Figure 3.5a and 3.5b). A previous study on rocket has shown that the accumulation of total GSL is genotype-dependent (Jin *et al.*, 2009; Jasper *et al.*, 2020). Furthermore, variation in the concentration of GSL and its hydrolysis products depends on both genetic and environmental factors (Cartea and Velasco, 2008). In the Italian trial, 8.5% of the RILs showed higher total GSL concentrations, whereas, in the UK trial, 7.8% of the RILs showed higher total GSL concentrations than Parent B (Figure 3.5b). Higher percentages of total GSL concentration in the extreme RILs than in Parent B were observed that suggest the formation of transgressive phenotypes due to the

combination/dispersion of favourable alleles from both parents (Rieseberg *et al.*, 1999; Mackay *et al.*, 2021).

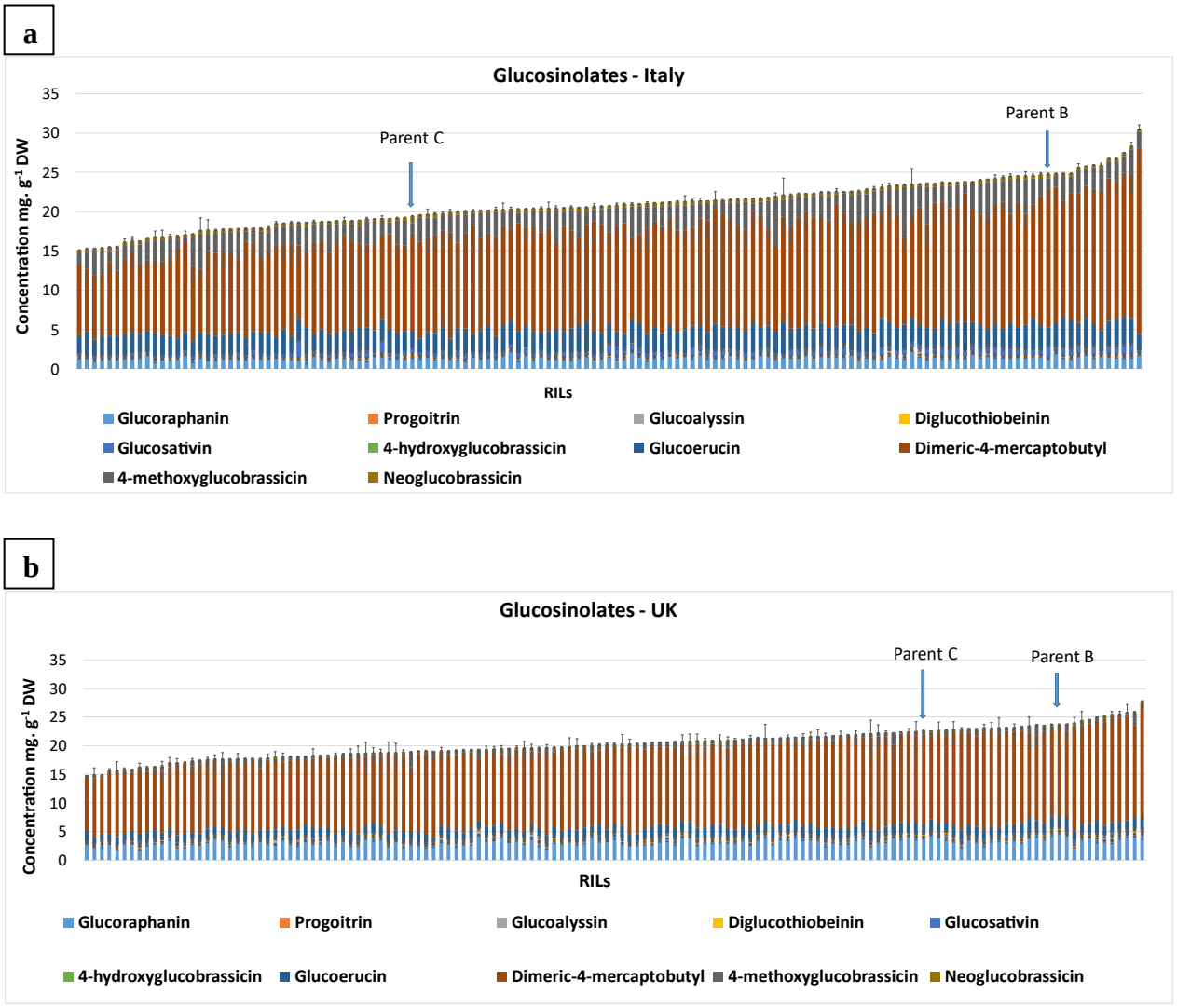


Figure 3. 5. Distribution of average GSL concentration in a mapping population of *E. sativa* grown at two locations (mg. g^{-1} DW). (a) 141 RILs in Italy; (b) 141 RILs in the UK. Parent B and Parent C were included in both trials indicated by blue arrows. Error bars signify the standard errors of the mean values of three biological replicates (n = 3). Colour code: see insight. Abbreviation: DW (Dry weight), RILs (Recombinant inbred lines).

Amongst the ten different GSLs, dimeric-4-mercaptobutyl GSL (UK average = 13.9 ± 1.1 mg. g^{-1} DW; Italy average = 12.7 ± 1.1 mg. g^{-1} DW) was the most abundant GSL found, contributing between 61-68% of the total GSLs. Similar results were reported by Pasini *et al.* (2011), where

their study reported around 60% of the total GSL in rocket was contributed by glucosativin and its dimer i.e., dimeric-4-mercaptobutyl GSL. However, a study by Bell *et al.* (2015) reported a much higher proportion of glucosativin/dimeric-4-mercaptobutyl GSL which was around 91.3% of total GSLs. A higher average concentrations of glucoraphanin (3.3 mg. g^{-1} DW, $p < 0.0001$), progoitrin (0.2 mg. g^{-1} DW, $p < 0.0001$), glucoalyssin (0.2 mg. g^{-1} DW, $p < 0.0001$), diglucothiobeinin (0.2 mg. g^{-1} DW, $p < 0.0001$) and dimeric-4-mercaptobutyl GSL (14 mg. g^{-1} DW, $p < 0.0001$) were observed in the UK trial, whereas, a higher average concentrations of glucosativin (0.6 mg. g^{-1} DW, $p < 0.0001$), 4-methoxyglucobrassicin (2.6 mg. g^{-1} DW, $p < 0.0001$), glucoerucin (0.6 mg. g^{-1} DW, $p < 0.0001$) and neoglucobrassicin (0.4 mg. g^{-1} DW, $p < 0.0001$) were observed in the Italy trial (Table 3.1). These results suggest that there is an influence of the growing environment on the accumulation of individual GSL concentrations. A previous study by Bell *et al.* (2020b) on the effect of temperature on *Eruca* and *Diplotaxis* species reported that glucoraphanin and dimeric-4-mercaptobutyl GSL were significantly higher in the cooler UK-grown trial while glucosativin concentration was higher in the warmer Italy trial and this trend was also found in the present study too.

The present study revealed that individual GSL varied significantly between the trial locations ($p<0.0001$). Average glucoraphanin concentrations in the Italian trial showed a 2.3-fold variation across all lines ranged from 1.0 mg. g^{-1} DW and 2.3 mg. g^{-1} DW (Figure 3.6a), whereas in the UK trial, a 2.4-fold variation ranged from 2.0 to 4.7 mg. g^{-1} DW (Figure 3.6b) was observed. A previous study on seven accessions of *E. sativa* reported glucoraphanin concentrations varied between 0.2 ± 0.1 mg. g^{-1} DW to 0.6 ± 0.4 mg. g^{-1} DW (Bell *et al.*, 2017a). Furthermore, their study reported glucoraphanin as a pleasant tasting compound. The present study reported higher average concentrations of glucoraphanin in the UK trial and agrees with a study by Bell *et al.* (2020b),

which reported that glucoraphanin concentrations increase with lower temperature which may be due to abiotic stress response and up-regulation of biosynthesis of secondary metabolites. Furthermore, a study on *Arabidopsis* has reported that low temperatures (9 °C) promote the synthesis of the aliphatic GSL (Kissen *et al.*, 2016) such as glucoraphanin. A similar trend was observed in the present study, where aliphatic GSLs such as glucoraphanin and glucoalyssin were significantly higher in the cooler UK-grown trial than in the warmer Italy trial, however, this effect was not observed for all aliphatic GSLs (Table 3.1). In the Italian trial, Parent C showed a higher average concentration of glucoraphanin as compared to Parent B, however, the opposite was true for the UK trial (Appendix 3.3). In the Italian trial, 50% of the RILs showed higher glucoraphanin concentrations than Parent C, whereas in the UK trial Parent B scored the highest concentration as compared to their RILs (Figure 3.6b).

The glucoerucin concentrations in the Italian-grown trial showed a 2.2-fold variation across the lines ranged from 1.8 to 4.0 mg. g^{-1} DW (Figure 3.6c), whereas in the UK trial, a 4.5-fold variation was found with concentrations ranged from 0.6 to 2.7 mg. g^{-1} DW (Figure 3.6d). In both trials, Parent B showed higher average glucoerucin concentrations as compared to Parent C (Appendix 3.3). In the Italy trial, 82% of the RILs showed a higher glucoerucin concentration than Parent B, whereas in the UK trial, only half of the RILs i.e., 43% of the RILs showed a higher concentration for glucoerucin than Parent B (Figure 3.6d).

Both glucoraphanin and glucoerucin do not impart any taste or flavour attribute (Bell *et al.*, 2020b), however, their respective hydrolysis products ITCs (sulforaphane and erucin) are known to be effective in lowering the risk of developing some forms of cancers (Cartea and Velasco, 2008;

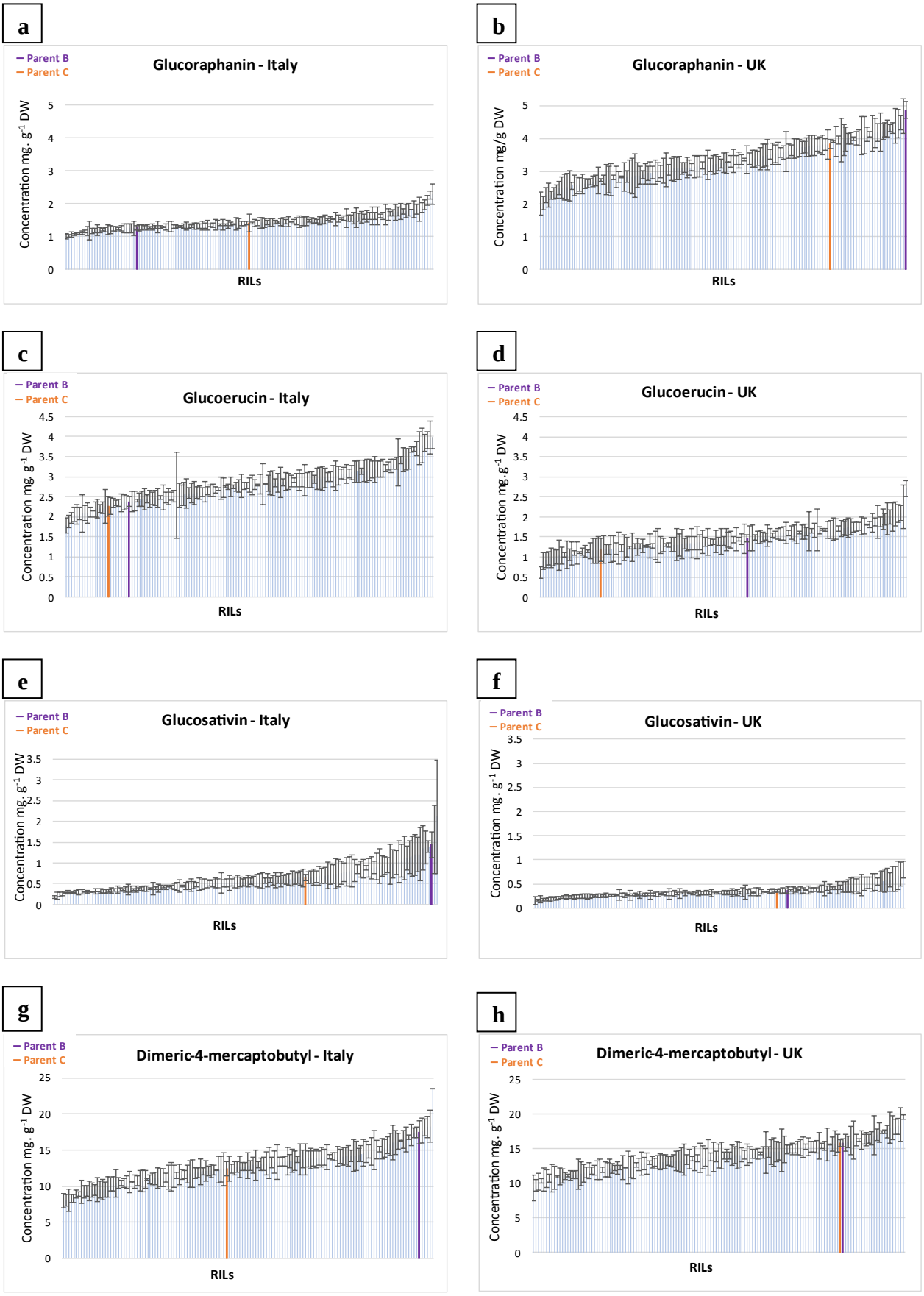
Traka *et al.*, 2013). Therefore, having a higher concentration of glucoraphanin and glucoerucin which is reported in the present study could be used as a beneficial tool in terms of health, however, more human trials are needed to confirm this.

Both species of rocket, i.e., *E. sativa* and *D. tenuifolia,* contain both the monomer (glucosativin) and dimer (dimeric-4-mercaptobutyl GSL) (Bell *et al.*, 2015). The present study revealed on average glucosativin concentrations in the Italian showed a 10-fold variation across the lines (ranged from 0.2 to 2 mg. g^{-1} DW) (Figure 3.6e), whereas, in the UK trial, an eight-fold variation was observed (ranged from 0.1 to 0.8 mg. g^{-1} DW). The previous study on *E. sativa* reported higher concentrations of glucosativin (ranged between 2.7 - 7.7 mg. g^{-1} DW) when grown under a controlled environment (Bell *et al.*, 2017a), however, the present study reported a lower concentration which could be due to different cultivation practices (controlled environment vs field-grown environment) influencing the accumulation. Studies in the literature reported the influence of different cultivation practices on the accumulation of phytochemicals (Bian *et al.*, 2015; Jasper *et al.*, 2020). In the Italian trial, 14% of the RILs showed a higher glucosativin concentration than Parent B, whereas in the UK trial 31% of RILs showed a higher glucosativin concentration than Parent B (Figure 3.6e). Regarding the dimer of glucosativin i.e., the average dimeric-4-mercapotobutyl GSL concentrations ranged from 8.0 to 23.5 mg. g^{-1} DW in the Italy trial where a 2.9-fold variation was observed across all the lines in a mapping population (Figure 3.6f). In the UK trial, a 2.1-fold variation was observed ranged from 9.2 to 19.6 mg. g^{-1} DW (Figure 3.6g). In the Italian trial, only 4% of the RILs showed a higher dimeric-4-mercaptobutyl GSL concentration than Parent B, whereas in the UK trial 16% of RILs showed a higher dimeric-4-mercaptobutyl GSL concentration than Parent B (Figure 3.6e). Parent B showed a higher average concentration of glucosativin as compared to Parent C (Appendix 3.3), which suggests the

dominance of one genotype over another, however, this trend was slightly different for its dimer. For dimeric-4-mercaptobutyl GSL, Parent B showed a higher average concentration than Parent C when grown in the Italy trial, however, both parents showed similar concentrations in the UK trial, which suggests either parent can be used as breeding stock.

One interesting finding that was observed in the present study is regarding the accumulation of respective monomer and dimer forms, where a significant difference ($p < 0.0001$) was observed showing that these could be genotype x environment (G X E) dependent. The exact mechanism of the biosynthesis pathway for this monomer and dimer is still unknown as the gene responsible for synthesis is unknown. Dimer concentrations showed higher concentration in the UK trial, whereas monomer concentration was higher in the Italy trial. A higher concentration of glucosativin (monomer) contributes to pungency, however, its dimer contributes to bitterness in rocket (Bell *et al.*, 2017a). Dimeric-4-mercaptobutyl GSL is a typical GSL to rocket genera that has a significant positive correlation with a bitter taste (Pasini *et al.*, 2011).

In the Italian trial, 4-methoxyglucobrassin showed a 10-fold variation ranged from 0.6 to 6.5 mg. g^{-1} DW (Figure 3.6i), whereas in the UK trial a 5-fold variation ranged from 0.5 mg. g^{-1} DW to 2.5 mg. g^{-1} DW was observed (Figure 3.6j). In the Italian trial, 90% of RILs showed a higher concentration than Parent B, whereas, in the UK trial, 66% of the RILs showed a higher concentration than Parent C (Figure 3.6J).


a
Parent B
Parent C
Glucoraphanin - Italy
Concentration mg. g-1 DW
RILs
b
Parent B
Parent C
Glucoraphanin - UK
Concentration mg/g DW
RILs
c
Parent B
Parent C
Glucoerucin - Italy
Concentration mg. g-1 DW
RILs
d
Parent B
Parent C
Glucoerucin - UK
Concentration mg. g-1 DW
RILs
e
Parent B
Parent C
Glucosativin - Italy
Concentration mg. g-1 DW
RILs
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Parent B
Parent C
Glucosativin - UK
Concentration mg. g-1 DW
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Parent B
Parent C
Dimeric-4-mercaptobutyl - Italy
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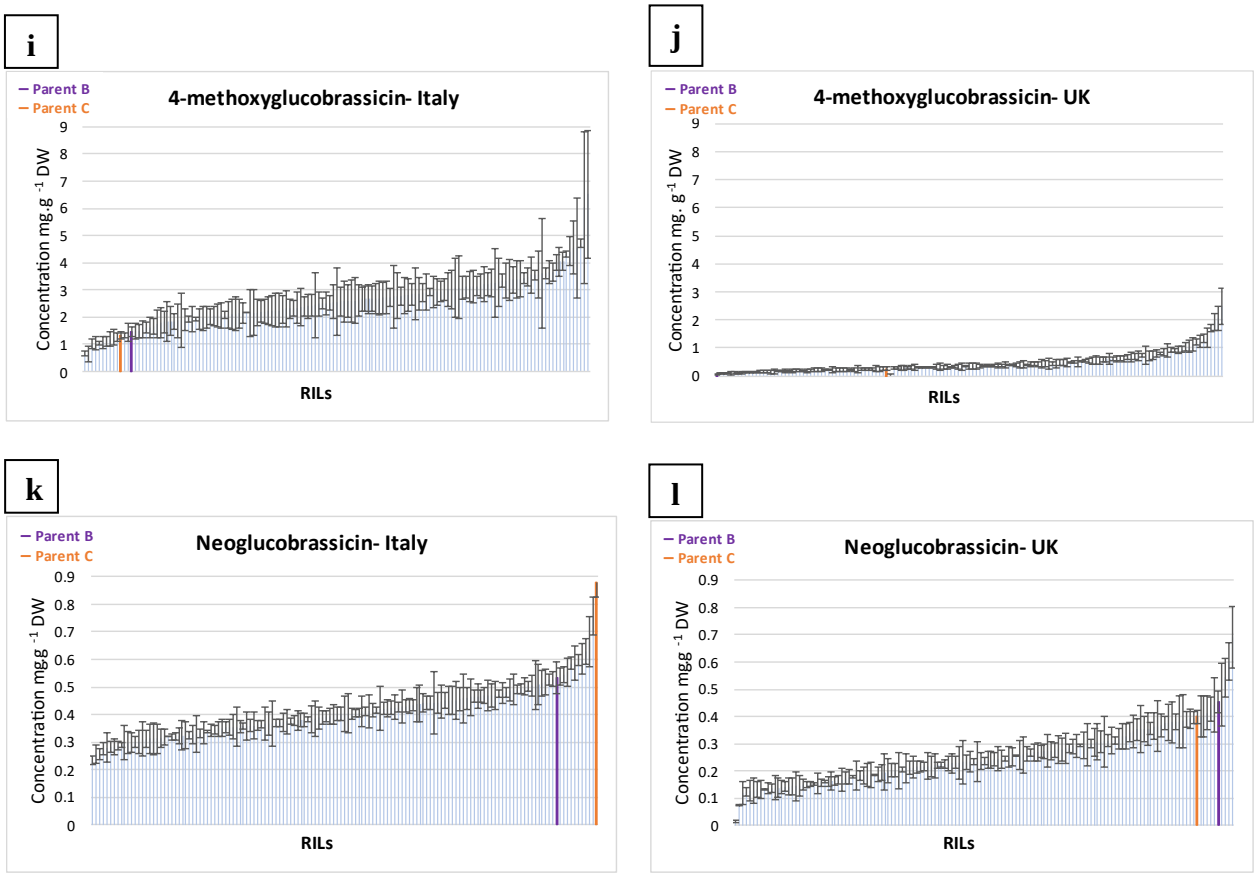


Figure 3. 6. Transgressive segregation of average individual GSLs of the 141 RILs of *E. sativa* grown at two locations (mg. g^{-1} DW). Glucoraphanin: (a) Italian trial; (b) the UK trial, glucoerucin: (c) Italian trial; (d) the UK trial, glucosativin: (e) Italian trial; (f) the UK trial, dimeric-4-mercaptobutyl GSL: (g) Italian trial; (h) the UK trial, 4-methoxyglucobrassin: (i) Italian trial; (j) the UK trial, neoglucobrassicin: (k) Italian trial; (l) the UK trial. Parent B and Parent C were included in both trials indicated by purple and orange lines. Error bars signify the standard errors of the mean values of three biological replicates (n = 3). Abbreviation: DW (Dry weight), RILs (Recombinant inbred lines).

In the Italian-grown trial, on average a four-fold variation was observed for neoglucobrassicin where the concentration ranged from 0.2 to 0.8 mg. g^{-1} DW (Figure 3.6k), whereas, in the UK trial, variation ranged from 0.01 to 0.7 mg. g^{-1} DW (figure 3.6l). The concentration of this GSL in both trials showed comparatively lower levels as compared to other individual GSLs. Transgressive segregation was only observed in the UK trial where 2.8% of RILs showed higher concentration than Parent B (Figure 3.6l) suggesting that fewer genes were expressed during recombination events which could be due to environmental stress. Both neoglucobrassicin and 4-methoxyglucobrassin are minor GSL, due to their low concentration, however, their presence plays

a significant role in the sensory perception of rocket where they contribute to bitter taste (Bell *et al.*, 2017a).

Within the mapping population of 141 RILs, individual lines showed an influence of the environment on the accumulation of sugars, organic acids, and GSLs. Of the 141 lines, line 34 (131.6 mg. g^{-1} DW) (Appendix 3.1) showed the highest total sugar concentration when grown in the UK trial, but only the 19th highest in the Italy trial. For the organic acids, line 86 (687.5 mg. g^{-1} DW) showed the highest total organic acids concentration in the Italy trial, but only the 95th highest in the UK trial. Line 85 (30.4 mg. g^{-1} DW) showed the highest total GSL concentration in the Italy trial but only 110th in the UK trial (Appendix 3.1). The present study found only line 61 for GSL compound, which showed similar values (Italy total GSL = 18.8 mg. g^{-1} DW; UK total GSL = 18.78 mg. g^{-1} DW) and was consistent over both the growing environments (Appendix 3.1). This line could be used as a potential candidate by breeders as it consists of a desirable amount of GSL; however, more replicated trials are needed to justify this.

3.4.2. Principal Component Analysis

PCA showed a visual comparison of the associations between sugars, organic acids, and GSLs content in a mapping population grown at two locations (Italy and the UK) (Figure 3.7). The PCA demonstrated a clear separation along principal components occurring between lines, location, and chemical compounds. Most of the information was contained in the first two principal components i.e., Principal components one (PC1), and two (PC2), which explained 54.44% of the total variation present in the data and were selected for the presentation. The majority of explained variation is found in PC1 accounting for 42.20 %, while PC2 accounts for 12.23% variation. No additional

information was captured from other principal components (PC3 and PC4) and hence not presented. A clear separation was observed between the Italian-grown and the UK-grown trial (Figure 3.7) with the Italian trial driving the traits such as individual GSLs and sucrose on the left side of the PC1 axis and the UK-grown trial driving mostly sugars with a few individual GSLs on the right side of the PC1 axis. The PC2 component separates most of the GSLs on the upper side with organic acids on the lower side of the PC2 component.

The correlation analysis revealed that sugars were associated with the UK-grown plants whereas GSLs vary between the trial locations. GSLs such as glucoraphanin, diglucothiobeinin, and glucoalyssin were positively associated with the UK-grown trial, whereas glucosativin, glucoerucin, 4-methoxyglucobrassicin, 4-hydroxyglucobrassicin, and neoglucobrassicin were closely associated with the Italy grown. These data suggest a clear impact of the growth environment on the accumulation of individual GSL rather than total GSL concentrations. Total sugars were positively correlated with glucose ($r = 0.981$, $p < 0.0001$), galactose ($r = 0.827$, $p < 0.0001$), fructose ($r = 0.779$, $p < 0.0001$), glucoraphanin ($r = 0.773$, $p < 0.0001$) and negatively correlated with GSLs such as 4-methoxyglucobrassicin ($r = -0.778$, $p < 0.0001$), glucoerucin ($r = -0.680$, $p < 0.0001$), neoglucobrassicin ($r = -0.534$, $p < 0.0001$), and 4-hydroxyglucobrassicin ($r = -0.410$, $p < 0.0001$) (Appendix 3.4). Minor GSLs such as 4-methoxyglucobrassicin, neoglucobrassicin, and 4-hydroxyglucobrassicin were negatively correlated with total sugars. A study by Bell *et al.* (2017a) on *E. sativa* leaves reported the role of minor GSLs in contribution to sensory attributes such as bitterness which contributes towards an increase in bitterness.

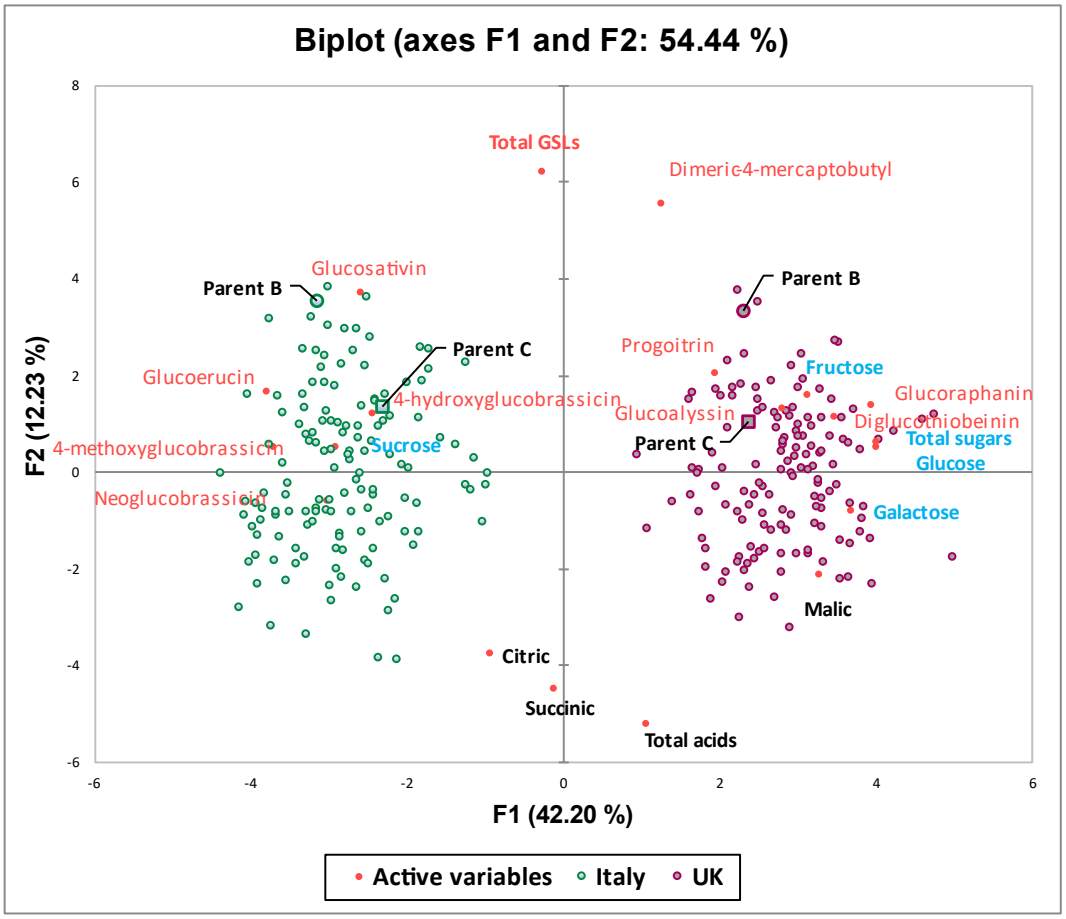


Figure 3. 7. Principal component analysis (PCA) biplots of phytochemical content of 141 RILs of *E. sativa* grown at two locations. Components PC1 vs PC2 (F1 and F2) account for 54.44% of the explained variation. Green dots represent the Italy trial; red dots represent the UK trial. A larger circle represents Parent B; a square represents Parent C.

GSL such as glucosativin was positively correlated with glucoerucin ($r = 0.594$, $p < 0.0001$), and 4-methoxyglucobrassicin ($r = 0.502$, $p < 0.0001$) and negatively correlated with glucoraphanin ($r = -0.520$, $p < 0.0001$), galactose ($r = -0.497$, $p < 0.0001$) (Appendix 3.4). A bitter taste in rocket is due to the presence of glucosativin (Pasini *et al.*, 2011). Glucoraphanin was positively correlated with glucose ($r = 0.780$, $p < 0.0001$), total sugars ($r = 0.773$, $p < 0.0001$) and diglucothiobeinin ($r = 0.743$, $p < 0.0001$) and negatively correlated with glucoerucin ($r = -0.672$, $p < 0.0001$), 4-methoxyglucobrassicin ($r = -0.688$, $p < 0.0001$), neoglucobrassicin ($r = -0.631$, $p < 0.0001$), glucosativin ($r = -0.520$, $p < 0.0001$) and sucrose ($r = -0.590$, $p < 0.0001$) (Appendix 3.4). Having a positive association of glucoraphanin within the UK-grown leaves would be beneficial as its

effect on health is well established in the literature (Traka *et al.*, 2013) and therefore identifying lines that produce glucoraphanin in the UK-grown conditions is useful for targeted nutrition.

In the Italian trial, Parent B was in close association with glucosativin, whereas Parent C was with sucrose as well as 4-hydroxyglucobrassicin. A positive correlation was observed between sucrose and 4-hydroxyglucobrassicin ($r = 0.356$, $p < 0.0001$) (Appendix 3.4). In the UK trial, Parent B was closely associated with dimeric-4-mercaptobutyl GSL, whereas Parent C with fructose as well as glucoalyssin. A positive correlation was observed between fructose and glucoalyssin ($r = 0.396$, $p < 0.0001$) (Appendix 3.4).

3.4.3. Genetic linkage map and QTL map

3.4.3.1. Genetic mapping

The identification of trait loci using SNPs as a marker in a bi-parental cross population in plants to construct a linkage map supplies a crucial strategy (Yu *et al.*, 2013). JoinMap 4 (Kyazma) was used to construct the map and MapQTL6 was used to map the traits. A genetic linkage map was generated using 285 markers mapping onto 18 linkage groups covering all 11 chromosomes (Table 3.2; Figure 3.8). It is known that *E. sativa* has 11 pairs of chromosomes ($2n = 22$) (Tripodi *et al.*, 2017), and having 18 linkage groups indicates that the distribution and the density of selected SNPs were not sufficient to match chromosomes and linkage groups.

The map length covered 889.2 cM, with the smallest and the largest linkage groups having 11.3cM (linkage group 11) and 96.9 cM (linkage group 16) map lengths, respectively. The average distance between markers over the map is 3.12 cM, with linkage group 3 having the most markers (37),

while linkage group 1a had the least (7) markers (Table 3.2). 17 gaps (defined as 10 cM or more without a marker) were observed on the consensus map, with the longest of 54.1 cM on linkage group 1a (Figure 3.8). Linkage group 8 was a more robust map as compared to linkage group 1a. Linkage group 8 was densely saturated with 28 markers having an average distance between the markers of 1.09 cM, whereas linkage group 1a was considered a less decent map as it had 7 markers sparsely distributed along the map length of 84.5 cM.

Table 3. 2. The consensus map illustrates several markers per linkage group, the length of each linkage group in cM and the average distance between the markers.

Linkage group	Map length (cM)	Number of markers	Average distance between markers
1a	84.5	7	12.07
2	21.6	9	2.40
3	47.3	37	1.28
4a	15.3	11	1.39
5	27.2	23	1.18
6	89	23	3.87
7	27.7	10	2.77
8	30.4	28	1.09
9	39.7	19	2.09
10	53.1	12	4.43
11	11.3	11	1.03
12	95	11	8.64
13	74.8	11	6.80
14	38	9	4.22
15	46.4	9	5.16
16	96.9	32	3.03
17	33.5	14	2.39
18	57.5	9	6.39
Total	889.2	285	3.12

Linkage group 4a had 11 markers with an average distance of 1.39 cM between markers and could be considered a slightly decent map. The letter suffix indicates that the chromosome is likely to split into several linkage groups, indicating that there is a region with no markers. More iterations of the mapping population will be needed to have tighter SNP density, which will assist in improving the map and will resolve the identities of linkage groups to corresponding chromosomes, as achieved in leafy vegetables such as lettuce and spinach (Atkinson *et al.*, 2013b; Cai *et al.*, 2018).

3.4.3.2. QTL mapping

A list of identified QTL for the metabolites was presented in Table 3.3 with LOD scores, LOD threshold, marker positions, confidence interval and explained variation percentages. Analysis revealed QTL for 20 compounds including sugar, organic acids, and a range of GSLs which were distributed on all the linkage groups (Table 3.3). The present study identified 13 QTL from the UK trial and 7 QTL from the Italy trial.

UK trial

In this trial, 13 QTL were identified, of which five were associated with sugars and eight with GSLs (Table 3.3). The five identified QTL for sugars were: fructose (two identified) and total sugars (three identified) and the eight identified QTL for GSLs were: 4-methoxyglucobrassicin (4-MGB) (five identified), neoglucobrassicin (one identified), glucosativin (one identified), and glucoerucin (one identified), however, none were identified in the Italy trial, despite producing higher concentrations of individual GSLs in the Italian trial.

Table 3. 3. Detection of QTL by multiple QTL mapping for all the traits accessed in the RIL mapping population of *E. sativa* grown in two locations: Italy and the UK. The linkage group represents the chromosome number to which QTL corresponds. All distances (marker position and QTL interval) are given in cM. QTL interval is the area in which the LOD score is 2 of the peak value and represents the extent to which a QTL is found. LOD is the log of odds score. Variance indicates the percentage of phenotypic variation within the population that can be explained by that QTL. The additive effect indicates which parental allele causes a positive change in the trait value. Positive values indicate that the Parent B allele increased the trait value, while negative values indicate that the Parent C allele increased the trait values.

Compounds	Linkage Group	Nearest Marker	Marker Position (cM)	QTL Interval (cM) ǂ	LOD	LOD Threshold	% Variance Explained	Additive Effect
UK Trial								
Fructose	6	296_63603_296.8	52.722	0.25	3.63	2.6	10.2	1.08307
Fructose	10	2418_17292_2418.2	53.112	17.114	2.93	2.6	8.1	0.951429
Total Sugars	2	567_85240_567.27	4.389	3.569	6.05	2.8	16.6	-8.49146
Total Sugars	2	327_144021_327.19	13.01	4.937	3.12	2.8	8.2	5.9644
Total Sugars	10	2418_17292_2418.2	53.112	14.603	2.91	2.8	7.6	4.33243
Glucosativin	3	967_40724_967.3	3.063	0.362	3.86	3.1	11.8	0.0403197
Glucoerucin	4a	198_1536_198.6	15.288	0.574	3.16	2.7	9.8	-0.0967997
4-methoxyglucobrassicin (4-MGB)	1a	27_784912_27.17	48	33.22	3.2	2.6	23.3	-0.289723
4-methoxyglucobrassicin (4-MGB)	1a	911_92462_911.8	71.1	2.31	4.9	2.6	38.7	-0.937912
4-methoxyglucobrassicin (4-MGB)	3	409_26377_409.5	3.553	0.3	4.63	2.6	13.1	-0.144341
4-methoxyglucobrassicin (4-MGB)	12	8_83778_8.22	0	21.604	3.24	2.6	8.1	0.117655
4-methoxyglucobrassicin (4-MGB)	14	73_1059485_73.200	31.519	6.041	2.94	2.6	10.3	-0.130833
Neoglucobrassicin	11	537_79980_5378	3.646	0.437	3.12	2.8	9.7	0.033543
Italian Trial								
Citric Acid	14	66_277019_66.68	34.787	4.268	4.44	2.7	13.9	-21.751
Malic Acid	16	134_275891_Novel00420	55.669	1.074	3.59	2.8	9.6	3.57446
Malic Acid	18	574_80050_574.2	23.114	28.509	4.75	2.8	15.8	-4.67481
Succinic Acid	10	157_209386_157.28	2.944	3.77	2.84	2.7	9.1	-5.77999
Total Acids	16	134_275891_Novel00420	55.669	1.074	2.97	2.6	9.4	10.4325
Progoitrin (putative)	14	73_1013087_73.188	25.746	20.703	3.52	2.9	20	-0.0357458
Progoitrin (putative)	16	73_1303249_73.252	40.522	1.208	2.9	2.9	16	0.0312223

QTL for sugars

In the UK trial, the QTL for total sugars were located on linkage groups 2 and 10 (Figure 3.8). On linkage group 2, the Parent C allele increases the trait value for this QTL, accounting for 16.6 % of the variation with a LOD score of 6.05 cM (Table 3.3; Figure 3.8). Furthermore, on the same linkage group 2, the trait values for total sugar were increased by the Parent B allele accounting for 8.2% of the variation. Parent B allele also increases the trait values for this QTL, which was associated with total sugars, located on the linkage group 10, accounting for 7.6% of the variation

with a LOD score of 2.91 cM. The two fructose-related QTL were identified on linkage groups 6 and 10 positioned at 52.7 and 53.1 cM, respectively (Figure 3.8). Parent B allele increases the trait values for fructose accounting for 10.2 and 8.1% variation, with a LOD score of 3.63 and 2.93 cM, respectively.

The QTL for fructose on linkage group 6 is much narrower, whereas the QTL for fructose on linkage group 10 is relatively broader suggesting their low marker density. The QTL for fructose is near the locus for total sugars on linkage group 10, which suggests there could be a strong underlying marker for these metabolites.

QTL for GSLs

In the UK trial, five QTL were identified for 4-MGB on linkage groups 1a, 3, 12, and 14, (Table 3.3; Figure 3.8) respectively. Parent B allele, as well as Parent C allele, increases the trait values for QTL associated with 4-MGB, however, on different linkage groups. On linkage groups 1a, 3, and 14, the Parent C allele increases the trait value for 4-MGB accounting for 38.7%, 13.1% and 10.3% of the variation, respectively. Furthermore, on linkage group 12, the Parent B allele increases the trait value of the above GSL accounting for 8.1% of the variation.

In this trial, the QTL for glucoerucin showed higher values by Parent C allele on linkage group 4a with a LOD score of 3.16 located at 15.28 cM (Table 3.3; Figure 3.8). The QTL for glucosativin was located at 3.06 cM and appeared on linkage group 3 where the Parent B allele increases the trait value accounting for 11.8% of the variation. The QTL for neoglucobrassicin is located on

linkage group 11, where the Parent B allele increased the concentration of this GSL with a LOD score of 3.12, accounting for 9.7% of the variation.

The QTL for 4-MGB on linkage groups 1a and 12, were broad, which suggested their low marker density, whereas the QTL for 4-MGB on linkage group 3 is much narrower. This study found a QTL for 4-MGB near the locus for glucosativin on linkage group 3, which suggests a strong correlation between them (Figure 3.8). The QTL for glucoerucin and neoglucobrassicin on linkage group 4a and linkage group 11 were much narrower.

Italy trial

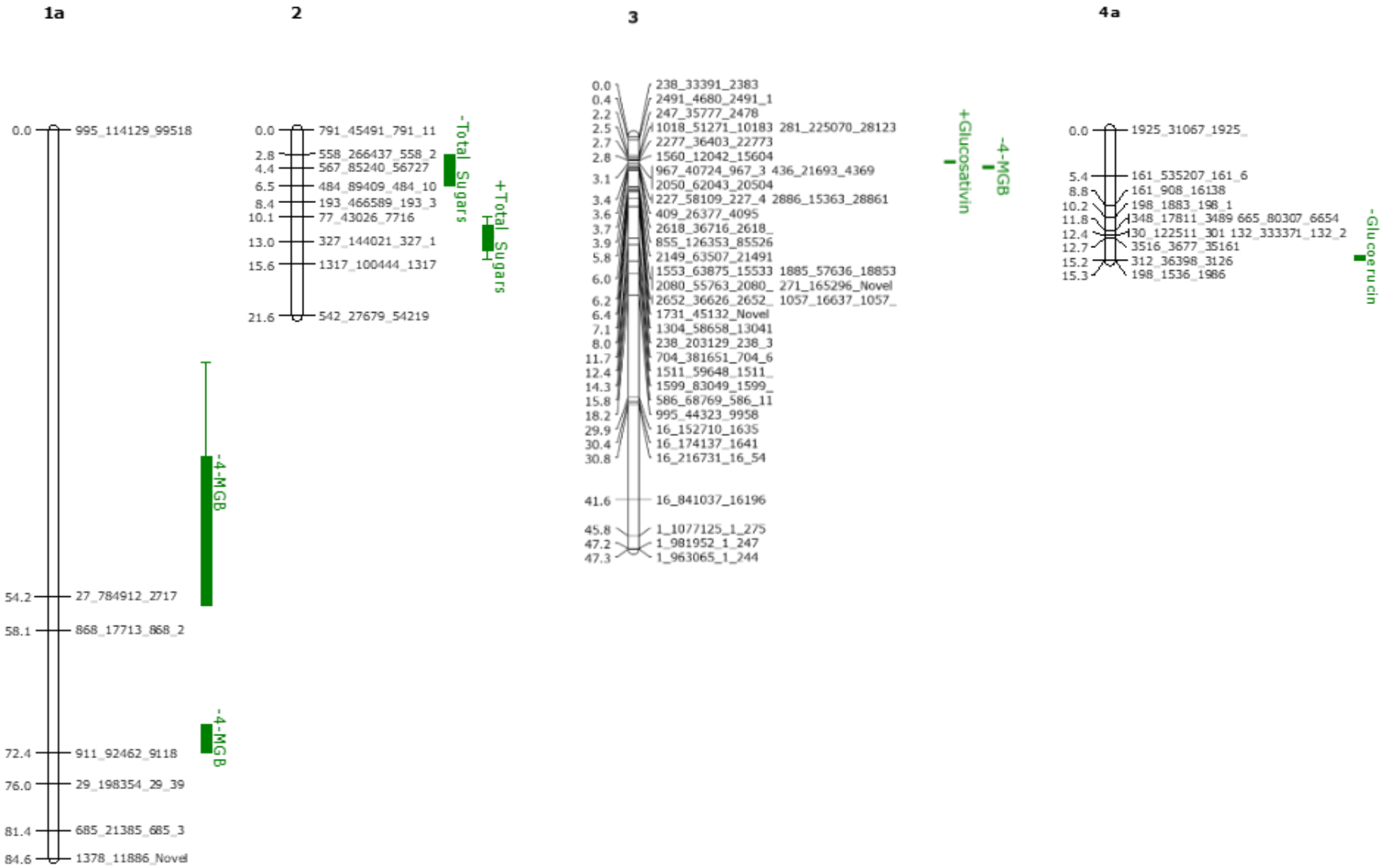
In the Italian trial, seven QTL were identified of which five QTL were identified for organic acid [one each for (citric, succinic, and total acids) and two identified for (malic acid)] and two QTL for GSL progoitrin (one each) (Table 3.3; Figure 3.8). No sugar-related QTL were observed for the Italy-grown trial, but several for organic acids and a couple for GSL were. Two putative QTL for progoitrin (Figure 3.8) were found in the Italy trial, on linkage groups 14 and 16, however, the QTL for progoitrin was much broader on linkage group 14 as compared to that on linkage group 16 (much narrower) (Figure 3.8).

The QTL for citric acid appeared on linkage 14, with a LOD score of 4.4, accounting for 13.9% of the variation while succinic acid appeared on linkage 10, with a LOD score of 2.84, accounting for 9.1% of the variation. The QTL for malic acid appeared on the linkage group 16 and 18, with LOD scores of 3.59 and 4.75, accounting for 9.6 and 15.8% variation respectively (Table 3.3; Figure

3.8). Of note, an additional co-locating locus for malic acid and total acids on linkage group 16 was observed, which suggests that there could be a strong underlying marker for these metabolites.

The putative QTL for progoitrin was driven by the Parent C allele showing the higher values for this trait, which appeared on linkage group 14 with a LOD score of 3.52, accounting for 20% of the variation. Furthermore, the Parent B allele also showed a higher concentration for this QTL and appeared on linkage group 16, with LOD scores of 2.9, accounting for 16% of the variation, respectively. Of all the QTL identified from the Italian and the UK mapping population trials, none have overlapped for any of the metabolites, which indicates that there is a strong genotype x environment interaction that determines the presence of both primary and secondary metabolites of *E. sativa.*

This is the first linkage map and QTL map constructed for the minor crop like a rocket (to the author's knowledge). Earlier, a linkage map and a QTL map were constructed for leafy crops such as spinach (Cai *et al.,* 2018) and for lettuce (Zhang *et al.*, 2007; Atkinson *et al.*, 2013b; Hunter *et al.*, 2022) where genes were identified for underlying leaf colour (spinach) and pinking and browning traits (lettuce).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3
4a
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76.0 29_198354_29_39
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84.6 1378_11886_Novel
-4-MGB
-4-MGB
0.0 791_45491_791_11
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4.4 567_85240_56727
6.5 484_89409_484_10
8.4 193_466589_193_3
10.1 77_43026_7716
13.0 327_144021_327_1
15.6 1317_100444_1317
21.6 542_27679_54219
-Total Sugars
+Total Sugars
0.0 238_33391_2383
0.4 2491_4680_2491_1
2.2 247_35777_2478
2.5 1018_51271_10183 281_225070_28123
2.7 2277_36403_22773
2.8 1560_12042_15604
3.1 967_40724_967_3 436_21693_4369
2050_62043_20504
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3.6 409_26377_4095
3.7 2618_36716_2618_
3.9 855_126353_85526
5.8 2149_63507_21491
6.0 1553_63875_15533 1885_57636_18853
2080_55763_2080_ 271_165296_Novel
6.2 2652_36626_2652_ 1057_16637_1057_
6.4 1731_45132_Novel
7.1 1304_58658_13041
8.0 238_203129_238_3
11.7 704_381651_704_6
12.4 1511_59648_1511_
14.3 1599_83049_1599_
15.8 586_68769_586_11
18.2 995_44323_9958
29.9 16_152710_1635
30.4 16_174137_1641
30.8 16_216731_16_54
41.6 16_841037_16196
45.8 1_1077125_1_275
47.2 1_981952_1_247
47.3 1_963065_1_244
+Glucosativin
-4-MGB
0.0 1925_31067_1925_
5.4 161_535207_161_6
8.8 161_908_16138
10.2 198_1883_198_1
11.8 348_17811_3489 665_80307_6654
12.4 30_122511_301 132_333371_132_2
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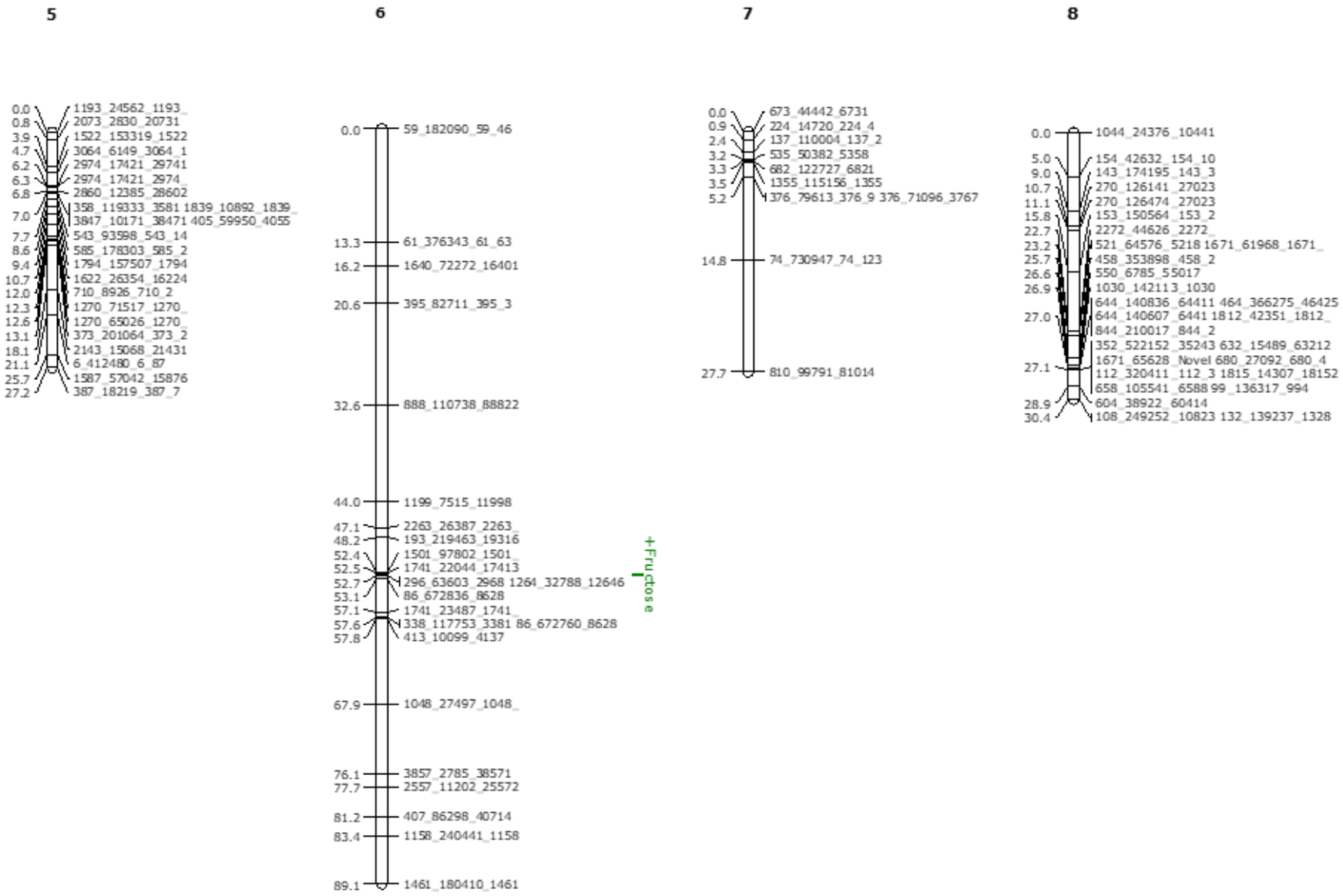
-Glucoe ru cin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9.4 1794_157507_1794
10.7 1622_26354_16224
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21.1 6_412480_6_87
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27.2 387_18219_387_7
6
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20.6 395_82711_395_3
32.6 888_110738_88822
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47.1 2263_26387_2263_
48.2 193_219463_19316
52.4 1501_97802_1501_
52.5 1741_22044_17413
52.7 296_63603_2968 1264_32788_12646
53.1 86_672836_8628
57.1 1741_23487_1741_
57.6 338_117753_3381 86_672760_8628
57.8 413_10099_4137
67.9 1048_27497_1048_
76.1 3857_2785_38571
77.7 2557_11202_25572
81.2 407_86298_40714
83.4 1158_240441_1158
89.1 1461_180410_1461
+Fructose
7
0.0 673_44442_6731
0.9 224_14720_224_4
2.4 137_110004_137_2
3.2 535_50382_5358
3.3 682_122727_6821
3.5 1355_115156_1355
5.2 376_79613_376_9 376_71096_3767
14.8 74_730947_74_123
27.7 810_99791_81014
8
0.0 1044_24376_10441
5.0 154_42632_154_10
9.0 143_174195_143_3
10.7 270_126141_27023
11.1 270_126474_27023
15.8 153_150564_153_2
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25.7 458_353898_458_2
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26.9 1030_142113_1030
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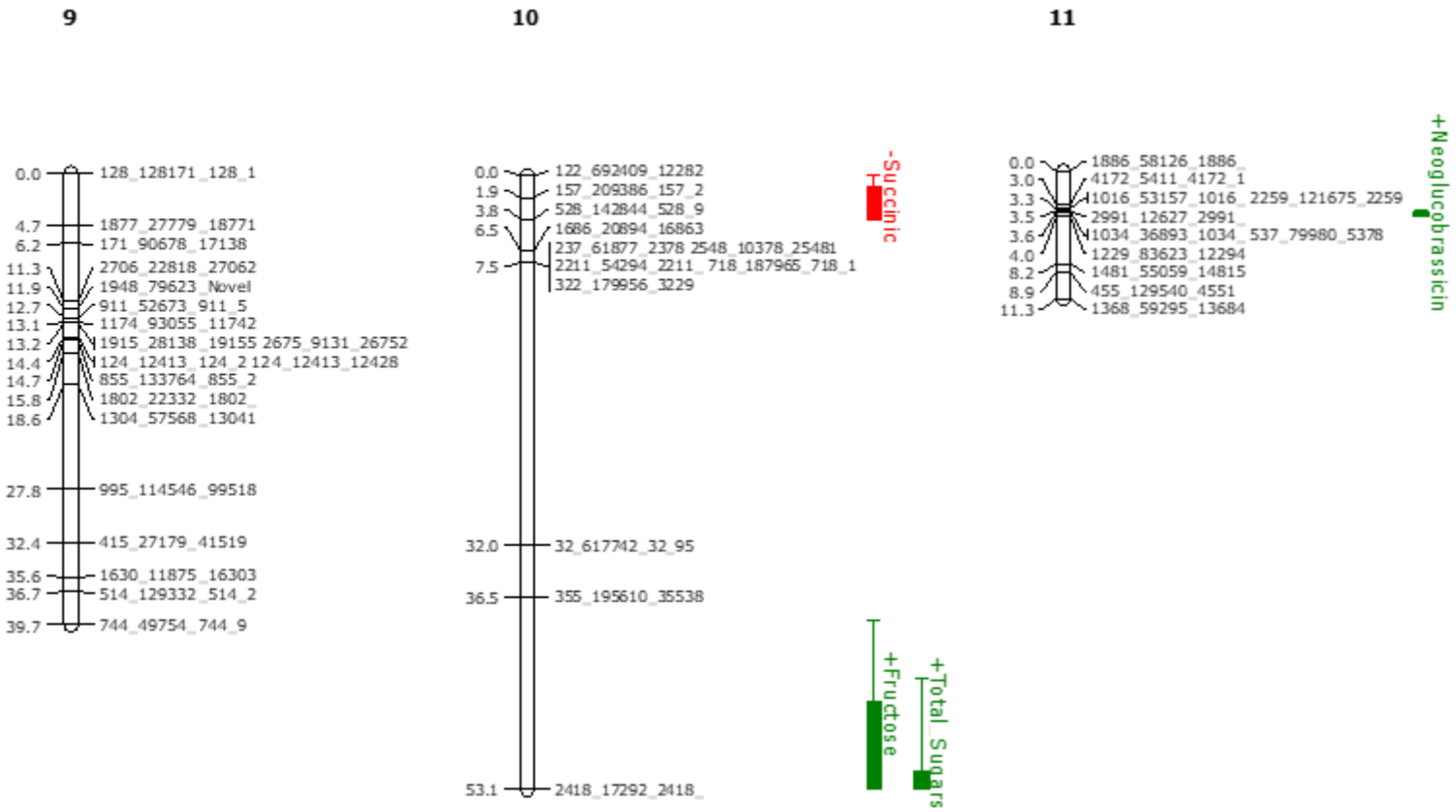
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32.0 32_617742_32_95
36.5 355_195610_35538
53.1 2418_17292_2418_
-Succinic
+Fructose
+Total Sugars
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3.0 4172_5411_4172_1
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3.5 2991_12627_2991_
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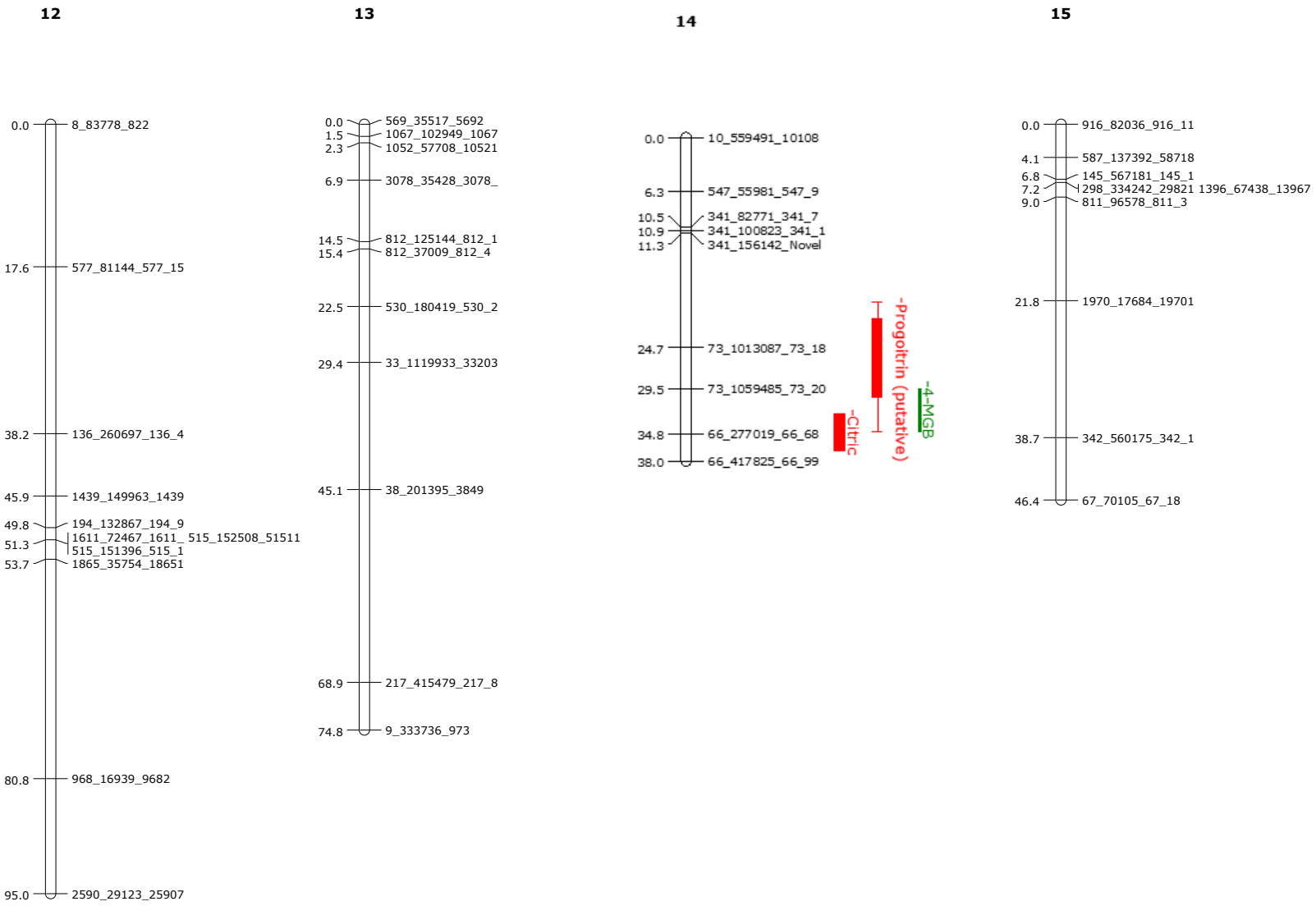
8.9 455_129540_4551
11.3 1368_59295_13684
+Neoglucobrassicin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515_151396_515_1
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95.0 2590_29123_25907
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-Citric
-Progoitrin (putative)
-4-MGB
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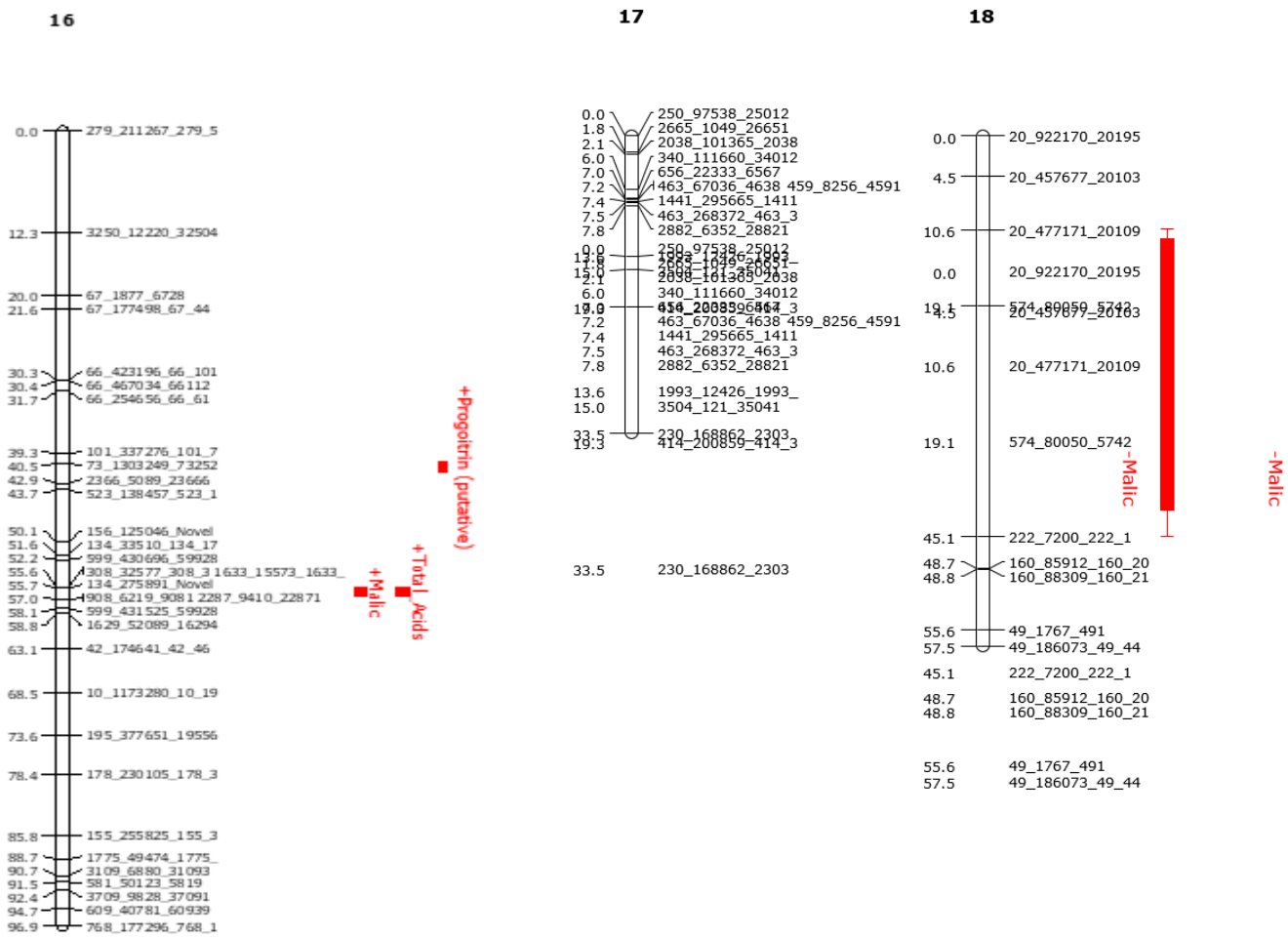


Figure 3. 8. Schematic of QTL localisation to marker map linkage groups. QTL distribution on the molecular linkage map of the RIL mapping population based on multiple QTL mapping. QTL driven by Parent B allele is shown as bar labelled with plus (+) symbols before trait and for Parent C allele with minus (-) symbol. Map positions are given in cM, listed on the right of each linkage group. The length of the bars indicates the LOD interval over the significant threshold for each QTL. Red bars represent the QTL detected in the Italian trial and the green bars represent the UK-grown trial. Bars represent one LOD interval, with the whiskers representing two LOD.

3.4.4. QTL x environment interaction

QTL for traits such as sugars, acids, and GSLs have been studied in two different environments. The use of two different cultivation environments provides a means of assessing gene x environment interactions. In the UK trial, 13 QTL were identified, whereas 7 QTL were identified in the Italian trial. The reason for having more QTL in the UK trial could be due to cold stress which could have affected the metabolic pathway which in turn produced more QTL. The trials conducted in Italy and the UK were designed for genetic analysis to provide robust phenotypes for QTL analysis so that it will determine whether there was any phenotypic plasticity over the environment (Zhang *et al.*, 2007; Atkinson *et al.*, 2013b). The present study was unable to find a single QTL that was independent of the environment. More markers will be needed by repeating the study with different environments and different seasons to make a tighter linkage map which will help construct a robust QTL analysis, which will provide QTL independent of the environment.

3.4.5. Study limitation

The main limitation of this study is having relatively low-density markers within the linkage map. This was not sufficient to fully resolve the linkage groups which were presented in Figure 3.8. The reason could be selecting higher quality SNPs for the study which in a way suggests that the data used for constructing the linkage and QTL map are robust. To overcome the issue of low-density markers, further iterations will be needed to improve density markers.

3.5. Conclusion

A mapping population of 139 F3 RILs were constructed by crossing two inbred lines (parent B and parent C); subsequently, phytochemicals were quantified by growing the population in two

different environments that are both commonly used for growing rocket commercially. Results from this study showed a higher sucrose concentration in the Italian population, whereas higher glucose, fructose, galactose, and total sugars concentration in the UK population. The UK-grown trial showed a two-fold higher average total sugar concentration as compared to the Italian-grown trial across the mapping population. These data show a significant influence of growing location on the accumulation of sugars in rocket leaves. More sugars in the UK trials may be due to cooler conditions enough to be stressful resulting in increased respiration rate and primary metabolism in plants thus accepting the proposed hypothesis regarding sugars. Total acids and total GSLs concentration showed no significant difference between trial locations, however, individual compounds vary significantly. Malic acid concentrations were higher in the UK trial, whereas accumulation of citric acid showed a higher concentration in the Italy trial. The more malic acid in the UK trial may be due to more oxidative stress from the cooler and humid environment in the UK as compared to Italy. Major GSL, glucosativin showed a higher concentration in the Italian trial, however, its dimer showed a higher concentration in the UK-grown trial suggesting that the accumulation of the respective monomer and dimer form is genotype x environment dependent. The present study thus accepts the hypothesis of significant differences in the accumulation of phytochemical content between two locations.

A linkage map was constructed using the genotypic data and later QTL analysis was performed on phenotypic and genotypic data. The data used for constructing the linkage and QTL map are robust and with further iterations, more density markers and resolution can be improved. As this is for the first time a draft linkage and QTL map was constructed for *E. sativa*, this study demonstrated progress has been made in using QTL mapping to understand the genetic basis of phytochemical

content. It also determined the impacts of the cultivation environment on the abundance of phytochemical contents in a segregating population of *E. sativa*.

Further study is needed to identify the genes underlying and regulating the QTL, which could be used in the breeding of a ‘salad’ rocket for improved quality traits. This information will be beneficial for growers and plant breeders as it demonstrates the influence of environmental conditions on the metabolic profile.

CHAPTER 4

Investigating the relationship between phytochemical content and sensory attributes from six lines of 'salad' rocket (*Eruca sativa*) grown at two different locations (Italy and the UK).

4.1. Abstract

Eruca sativa is RTE crop of *the Brassicaceae* family that contains nutritionally relevant compounds which provide a distinct peppery flavour and is gaining popularity due to its sensory and nutritional characteristics. Commercially, rocket leaves are harvested multiple times from the same plants. Previous work demonstrated that first-cut leaves of rocket were favoured more by consumers due to their mild hotness, however, multiple leaf cuts resulted in lower acceptance as the hotness and pungency increased. Sugars play an important role in determining the overall taste and flavour of fruit and vegetables as they can mask other tastes, such as bitterness. The present study investigates the relationship between the phytochemical content and sensory profile of *E. sativa* from the selected six lines, each grown at two locations (Italy and the UK), for the 1st and 2nd cut, and intake (day 0) and postharvest shelf life (day 5) respectively.

Instruments such as HPLC, LC-MS, and ICP-OES were used to measure sugars, GSLs, and sulphur content present in six lines of the *E. sativa*. Sensory analysis was carried out using two trained panels, differing in genotype for the TAS2R38 bitter taste receptors that were associated with the perception of a bitter taste for GSLs. A significant difference ($p<0.05$) in phytochemical content and sensory attributes were observed, which were influenced by both locations and lines. The present study found total sugar concentrations were significantly higher in the UK-grown crop as compared

to the Italian trial ($p<0.05$), with the 2nd cut of the UK-grown rocket leaves showing a higher sugar concentration compared to the 1st cut. Total GSL and sulphur contents were higher in the Italian trial and were positively correlated with sensory attributes such as bitterness and pepperiness, whereas sugars were higher in the UK-grown leaves and were positively correlated with a sweet taste. Individuals with PAV/PAV TAS2R38 diplotypes showed a reduced perception of the subtle flavour attributes of rocket leaves compared with AVI/AVI diplotypes. Sweetness reduces consumer perception of bitterness in foods, therefore having a sweeter rocket could attract more consumers to consume rocket while maintaining the maximum health benefits associated with the crop.

4.2. Introduction

Eruca sativa also known as 'salad' rocket or 'arugula' is an annual diploid herbaceous crop gaining popularity throughout the world (Jin *et al.*, 2009; Afsar *et al.*, 2020; Bell *et al.*, 2020b). The leaves of this *Brassicaceae* species are consumed all over the world either cooked or raw to garnish salads or as a snack or in a large variety of meals due to their spicy hot taste (Garg and Sharma, 2014). Leaves of the crop are usually sold in mixed salad bags (e.g., with watercress and spinach) or whole bags and in some niche markets as micro leaves. Eating fresh leaves is the best way to gain health benefits as cooking results in losses of health-promoting components (Bennett *et al.*, 2007; Palermo *et al.*, 2014; Giallourou *et al.*, 2016).

Rocket has many important phytochemicals such as GSLs, flavanols, vitamins, and minerals that are thought to benefit human health and consumption of *Brassicaceae* family crops is associated with a reduced risk of developing some types of cancers (Pasini *et al.*, 2011). It is known that several

factors such as genotypes, environmental conditions, and cultivation practices influence the abundance of phytochemicals present in *Brassicaceae* crop species (Biondi *et al.*, 2021). Rocket is a perishable crop with a short shelf life attributed to its high respiration rate during postharvest storage (Koukounaras *et al.*, 2007b). Stresses either due to harvest (cutting), processing, or storage temperature could alter the appearance, nutritional, and sensory quality of leaves. This is a major concern to growers/producers and supermarkets, as these leaves get accepted or rejected by the consumer based on these attributes (Bell *et al.*, 2016; Ansah *et al.*, 2018). Moreover, stress can increase the synthesis of secondary metabolites such as GSL.

Previous studies reported that climatic factors such as light intensity, temperature, water availability, and CO_2 enrichment influence the quality and nutritional content of fresh produce (Weston and Barth, 1997; Kader, 2002b). Of these climatic factors, light intensity and temperature play the most influential part (Rouphael *et al.*, 2012). Temperature, either high or low, affects the nutritional content of leafy produce. Higher temperature influences the uptake and metabolism of nutrients by crops as transpiration increases. Heat stress, which occurs in Mediterranean areas as well as in greenhouses or under polytunnels (in the present study) during the spring-summer season, can impact plant metabolism and slow down many physiological processes (Toscano *et al.*, 2019). Whereas, low temperature or cold stress could result in a higher accumulation of osmolytes such as soluble sugars (Browse and Xin, 2001).

Rocket is known for its distinct sensory characteristics, including trigeminal sensations, where the trigeminal nerve is responsible for sending warm and touch from face to brain. Trigeminal sensations such as ‘warmth’ and ‘intensity’ are found in rocket along with flavour characteristics

(such as pungency and pepperiness) and bitter taste (Bell and Wagstaff, 2019). A previous study by Bell *et al.* (2017b) reported that the first cut leaves of rocket were favoured more by consumers due to their mild hotness, whereas multiple leaf cuts resulted in lower acceptance as the hotness and pungency increased. Repeated harvesting is a common practice, in both genera of rocket (Hall *et al.*, 2012a). Most of the research is focused on determining the phytochemicals from the first harvest/1st cut and very few studies accessed the phytochemicals from the second harvest/2nd cut. It is further speculated that multiple cuts can increase the abundance of phytochemicals in rocket (Jasper *et al.*, 2020). Previous research on rocket leaves was focused on the visual and morphological traits and very few studies focused on the sensory attributes such as tastes, odours, and flavours (D'Antuono *et al.*, 2009; Pasini *et al.*, 2011; Løkke *et al.*, 2012; Bell *et al.*, 2017a). Limited studies have been performed focusing on how phytochemical content from the multiple harvests and postharvest shelf life affect the sensory attributes such as aroma, flavour, taste, mouthfeel, and aftereffect of rocket (Bell *et al.*, 2020b; Jasper *et al.*, 2020). The GSLs in rocket are the predominant cause of both bitter tastes as well as hotness, pungency, and peppery flavour. Furthermore, human perception of GSLs is influenced by differences in genotype for the bitter taste receptor TAS2R38. To date, only one study has investigated consumer taste perception and preferences for rocket by TAS2R38 genotype reporting that PAV/PAV individuals could perceive bitterness significantly (Bell *et al.*, 2017b). Their study further reported that hotness and liking were positively correlated with other with hotness and not bitterness as the main attribute for their liking. Therefore, to have a better understanding, the current study extends this type of investigation by accounting for both human taste differences alongside differences in rocket crops (the environmental growing conditions, the genotypes of rocket, and the phytochemical content; for both the 1st and the 2nd cut and shelf life).

We conducted a comprehensive study on environmental, phytochemical, and sensory analysis by choosing the six most diverse lines (genotypes) from a 'salad' rocket mapping population based on total GSL content. These six lines were grown at two different locations (Italy and the UK) to study the effect of multiple harvests and physiochemical changes during shelf life. These lines were further assessed by sensory panels having 'PAV/PAV' (supertaster), PAV/AVI (medium tasters), and 'AVI/AVI' (non-taster) individuals. Results from the present study will highlight the components important for determining taste and flavour, which will help breeders to select cultivars suitable for each environment.

The aim of this study was to measure the phytochemical content and investigate the relationship between sensory analysis and human taste receptor genotypes on these six lines of 'salad' rocket grown at two locations for two cuts and during postharvest shelf life. Sensory profiling included a comparison between the 1st and the 2nd cut of the 'salad' rocket at two shelf life points. We hypothesised that there would be a significant difference in phytochemical content such as sugars, GSLs, and sulphur between the locations, between the cuts (1st and 2nd) and between the changes during postharvest shelf life (day 0 and day 5). We further hypothesised that there would be significant differences in sensory profiling between six lines of 'salad' rocket when grown at two locations for the 1st and 2nd cut and change during postharvest shelf life. In addition, we also hypothesised that there would be a significant difference in sensory perception due to human taste receptor genotypes (TAS2R38) on six lines of 'salad' rocket grown at two locations.

4.3. Material and Methods

4.3.1. Tissue preparations

The selected six lines of ‘salad’ rocket will be referred to as lines 21 (low), 25 (low), 68 (high), 72 (low), 112 (high), and 130 (high), were chosen based on the abundance of high or low levels of GSL concentrations across the two growing locations. See Chapter 2 for further details.

4.3.2. Reagents and Chemicals

All the reagents and chemicals were procured from Sigma-Aldrich UK unless otherwise stated. Further details are mentioned in Chapter 2.

4.3.3. Sugar analysis

Sugars such as glucose, fructose, galactose, and sucrose were extracted and analysed using an HPLC instrument on six lines of ‘salad’ rocket grown at the two locations (Italy and the UK). See Chapter 2 for further details.

4.3.4. Glucosinolate analysis

The GSL profile for the six lines of the ‘salad’ rocket was extracted and analysed using LCMS as presented by Bell *et al.* (2015) and Jasper *et al.* (2020). See Chapter 2 for further details.

4.3.5. Sulphur analysis

The sulphur content of the six lines of ‘salad’ rocket grown at two locations was analysed using an ICP-OES machine. See Chapter 2 for further details on extraction and analysis.

4.3.6. Sensory analysis

The definition of sensory attributes for rocket was established using two trained panels at Sensory Science Centre (n = 20) (University of Reading, UK) using Quantitative Descriptive Analysis (QDA^{TM}). Further details on sensory selection, training, vocabulary development, and analysis were explained in Chapter 2.

4.3.7. Statistical analysis

ANOVA

The instrumental results presented are the average of three biological replicates (n = 3) for each sample. Outputs were analysed by (multiple ways) Analysis of variance (ANOVA) where multiple treatment effects were fitted (lines, location, cuts, and days) along with the interaction between (location x shelf life, location x cuts and location x days) using XL Stat version 2020.1.3 (Addinsoft, Paris, France). All multiple pairwise comparisons were conducted using Tukey's HSD test, with differences expressed at the 5% significance level ($p<0.05$). Sensory profile data were analysed using ANOVA where multiple treatment effects were fitted (lines, location, cuts, days, and TAS2R38 genotypes) with *post hoc* Tukey's HSD test for significance ($p<0.05$). Data were tested for the outliers using Grubb's test. Shapiro-Wilk and Jarque-Bera tests were conducted on the residuals to check for normality on all sensory variables. The residuals were tested for normal distribution, and for those that fit the normal distribution an ANOVA (a parametric test) was used otherwise Mann Whitney and Kruskal Wallis (a non-parametric test) was used.

Principal Component Analysis (PCA) and Multiple Factor Analysis (MFA)

Relationships between sensory attributes and non-volatile compounds (sugars, GSLs, and sulphur) were tested using Spearman correlation, with a significant correlation stated at $p<0.05$. This was succeeded by PCA on the averages of the sensory data with the average values for sugars, GSLs, and sulphur regressed onto it. For MFA, the average for the sensory data was taken over by assessors and correlated with the average values from the instrumental data via multiple factor analysis (MFA). MFA is a multivariate data analysis method for visualising complex data. All the above analysis was done using XL Stat version 2020.1.3 (Addinsoft, Paris, France).

4.4. Results and discussion

4.4.1. Phytochemical analyses

4.4.1.1. Sugar profile

The present study revealed that the average total sugar concentration was significantly ($p<0.05$) higher in the UK-grown trial as compared to the Italian-grown. The UK-grown trial showed approximately three-fold higher average total sugars as compared to the Italy-grown trial (UK total sugars = 144.5 mg. g^{-1} DW, Italy total sugars = 45.66 mg. g^{-1} DW) (Figure. 4.1.i) (Table 4.1).

Amongst the four different sugars quantified, glucose concentration (UK average = 98.3 mg. g^{-1} DW, Italy average = 29.17 mg. g^{-1} DW) (Table 4.1) was the most abundant monosaccharide found in rocket leaves grown at both trials, followed by fructose (UK average = 28.38 mg. g^{-1} DW, Italy average = 6.72 mg. g^{-1} DW), sucrose (UK average = 9.8 mg. g^{-1} DW, Italy average = 5.73 mg. g^{-1} DW), and galactose (UK average = 8.0 mg. g^{-1} DW, Italy average = 4.03 mg. g^{-1} DW), respectively with glucose contributing around 64-68% of the total soluble sugars. Previous studies confirmed glucose to be the dominant monosaccharide in rocket leaves ranging from 21.1 ± 0.9 to 93.9 ±3.0 mg. g^{-1} DW representing > 70% of the total soluble sugars (Villatoro-Pulido *et al.*, 2013; Bell *et al.*,

2017a; Bell *et al.*, 2020b). A study by Steindal *et al.* (2015) reported the total sugar content for curly kale brassicale ranged between 60 – 120 mg. g^{-1} DW, the present study reported a similar range for total sugars in *E. sativa* leaves. A study on spinach by Yoon *et al.* (2017) reported a higher total sugar concentration when grown in an open field (263.4 mg. g^{-1} DW) as compared to the greenhouse-grown (73.0 mg. g^{-1} DW). A similar trend was observed in the present study when *E. sativa* leaves were grown in two different cultivation environments i.e., field grown (the UK) and polytunnel grown (Italy) trial, suggesting the role of climatic conditions in regulating the concentration of sugars in brassicaceous leafy vegetables.

Table 4. 1. ANOVA pairwise comparisons ($p<0.05$) of six lines of *E. sativa* for sugars, GSLs, and sulphur between the Italy and UK trials as representative averages (n = 3). Values are expressed as mg. g^{-1} dry weight.

Compounds	Italy	UK	Significance (p-value)
Sucrose	5.734 b	9.801 a	<0.0001
Glucose	29.169 b	98.321 a	<0.0001
Galactose	4.032 b	8.007 a	<0.0001
Fructose	6.725 b	28.382 a	<0.0001
Total Sugars	45.660 b	144.510 a	<0.0001
GIB	0.003 b	0.004 a	<0.0001
GKR	1.484 a	0.179 b	<0.0001
PRO	0.007 a	0.000 a	0.206
SIN	0.000 a	0.000 b	<0.0001
GRA	0.567 a	0.720 a	0.115
GRM	2.850 a	0.330 b	<0.0001
GAL	0.092 a	0.010 b	<0.0001
GPJ	0.037 a	0.000 b	<0.0001
GNP	0.000 b	0.002 a	<0.0001
DGTB	2.019 a	0.745 b	<0.0001
GBT	0.118 a	0.005 b	<0.0001
4HGB	0.005 b	0.021 a	<0.0001
GSV	1.910 a	2.572 a	0.085
DMB	29.272 a	4.886 b	<0.0001
GTP	0.011 a	0.006 b	<0.0001

GER	0.350 a	0.163 b	<0.0001
GBC	0.008 a	0.007 a	0.679
4MGB	0.117 a	0.038 b	<0.0001
GNT	0.000 b	0.000 a	<0.0001
NGB	1.136 a	0.411 b	<0.0001
4MP	0.467 a	0.052 b	0.000
HEX	0.064 a	0.040 a	0.090
BTL	0.150 a	0.002 b	<0.0001
Total GSL	40.67 a	10.19 b	<0.0001
Sulphur	14.78 a	10.26 b	<0.0001

Letters within columns denote statistical significance; values with the same letters present are not statistically significant from one another.

Within the specific six lines of 'salad' rocket grown at two locations, lines 112 (171.22 mg. g^{-1} DW), followed by line 130 (167.85 mg. g^{-1} DW), and 68 (166.33 mg. g^{-1} DW), showed significantly ($p \leq 0.05$) higher average total sugar concentration in the UK-grown leaves as compared to the Italian-grown leaves (Appendix 4.1) (Figure. 4.1.ii). The above results indicate that the growing conditions (temperature and light intensity) influence the accumulation of total sugar. Similar results on accumulation for total sugars were found in Chapter 3 of this thesis, where the UK-grown leaves accumulate more sugars than Italian grown (Table 3.1). Under low growth temperatures, crops generally experienced abiotic cold stress where the water absorption by roots gets suppressed. To protect the plant from dehydration, the osmotic adjustment occurs resulting in the accumulation of soluble solutes (Ito *et al.*, 2014). Yoon *et al.* (2017) reported cold stress may up-regulate the sucrose biosynthesis pathway resulting in increased sugar contents in spinach. This might be one of the reasons the present study reported a higher concentration of sugars in the UK-grown leaves. Previous studies on *Brassicaceae* vegetables such as swede roots and kale have reported a higher accumulation of total sugars when grown at low temperatures (9 °C) (Steindal *et al.*, 2015; Johansen *et al.*, 2016).

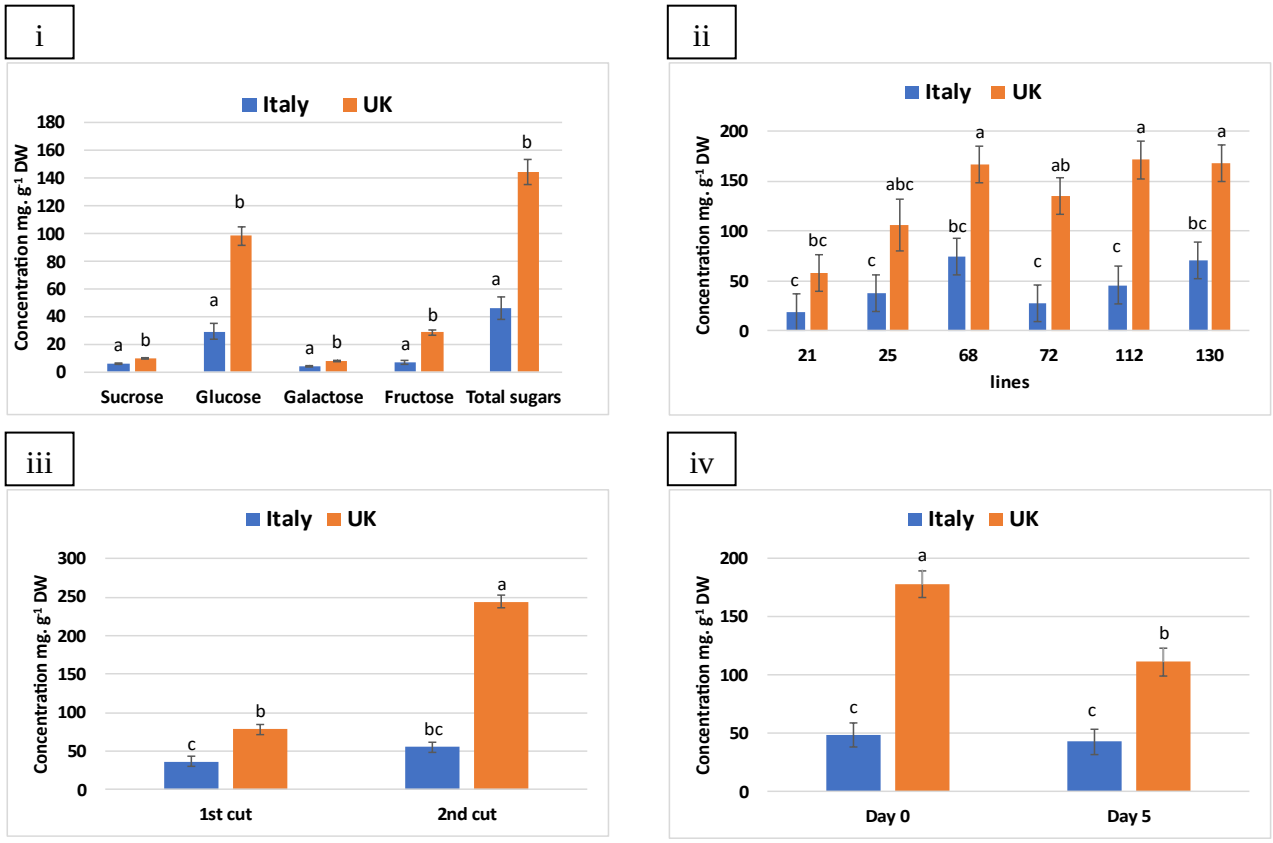


Figure 4. 1. Average total sugar concentration (mg. g^{-1} DW) of six lines of *E. sativa* leaves grown in Italy and the UK (n = 3). (i) for different sugars, (ii) for individual six lines, (iii) for the 1st and 2nd cut, and (iv) for day 0 and day 5. Error bars signify the standard error of the mean values of three biological replicates. Where there is no common letter above the bars within a chart, it represents significant differences between the bars (Tukey's HSD test, $p<0.05$). Abbreviations: DW, Dry weight. The present study found a significant difference in total sugar concentrations between the 1st and the 2nd cut ($p<0.05$). A 1.5-fold higher average concentration of total sugars was observed in the 2nd cut for the Italian-grown trial, as compared to the 1st cut (Italy 1st cut = 36.33 mg. g^{-1} DW, Italy 2nd cut = 54.99 mg. g^{-1} DW) (Appendix 4.2). On average, a 3.1-fold higher concentration of total sugars was observed in the 2nd cut for the UK-grown trial, as compared to the 1st cut (UK 1st cut = 78.4 mg. g^{-1} DW, UK 2nd cut = 243.68 mg. g^{-1} DW) (Figure, 4.1.iii). Higher sugars in the 2nd cut as compared to the 1st cut could be due to cutting or wounding injury on leaves. In general, mechanical injury or wounding leads to changes in metabolism, especially for sugars where it increases the activities of sucrose hydrolysing enzymes, such as sucrose synthase and

invertase in plants. A recent study on mutant leaves of *Arabidopsis thaliana* reported a higher accumulation of sugars when leaves were stressed due to wounding (Lukaszuk *et al.*, 2016).

The Italian trial did not show any statistically significant difference ($p<0.05$) for average total sugar concentrations across the change in shelf life (day 0 and day 5) (Italy day 0 = 48.53 mg. g^{-1} DW, Italy day 5 = 42.79 mg. g^{-1} DW) (Figure 4.1.iv). However, on average, a 1.6-fold higher total sugar concentration was observed on day 0 (intake) of the UK-grown trial as compared to day 5 (postharvest shelf life) (UK day 0 = 177.83 mg. g^{-1} DW, UK day 5 = 111.19 mg. g^{-1} DW) ($p<0.05$) (Appendix 4.3). Sugars were thought to be lost between day 0 (intake) and day 5 (postharvest shelf life) in the UK-grown leaves due to higher levels of respiration compared to the Italian trial, however, the present study did not measure the gas exchange. A higher respiration rate caused due to cold stress may indicate a more active metabolism which can result in a more rapid loss of acids, sugars, and other components. Furthermore, these compounds determine the flavour and nutritive value of fresh produce (Martínez-Sánchez *et al.*, 2012; Ansah *et al.*, 2018). Therefore, to maintain the nutritional quality of the fresh produce, it is necessary to keep the fresh produce at a low temperature to reduce the respiration rate. During storage, sucrose gets hydrolysed to the corresponding hexose such as glucose and fructose, however, both glucose and fructose are used as a substrate for sugar metabolism in plants, which explains a decrease in sugar concentration (Halford *et al.*, 2011). A study by Helland *et al.* (2016) reported a reduction in total sugar content with an increase in storage time for brassica vegetables swede root and turnip due to an increase in respiration rate. This was further supported by Nei *et al.* (2006) who reported a decrease in total sugars due to respiration in shredded cabbage when stored at 5 °C for four days, suggesting a relationship between respiration and sugar consumption.

We thought a similar trend would be observed for the Italian-grown leaves, with a lower concentration of total sugars on day 5 (postharvest shelf life) due to higher levels of respiration and further, due to the time, it arrives in the UK causing additional stress on leaves (due of transportation). However, the present study found no significant differences in total sugars between day 0 and day 5, suggesting that perhaps rocket is a weed crop and can withstand stressful conditions. Studies in the literature reported that mechanical vibration levels during transportation can accelerate physiological and biochemical reactions affecting the nutritional quality of fresh produce which was observed in tomatoes (Al-Dairi *et al.*, 2021) and mushrooms (Tao *et al.*, 2021).

4.4.1.2. Glucosinolate profile

The total average concentration of GSLs was significantly higher ($p<0.05$) in the Italian-grown leaves as compared to the UK-grown leaves. The Italian-grown trial showed 4-fold higher average concentrations of total GSLs as compared to the UK-grown trial (Italy total GSLs = 40.66 mg. g^{-1} DW, UK total GSLs = 10.2 mg. g^{-1} DW) (Table 4.1). The results from the present study on total GSL concentrations were in agreement with the previous study by Tripodi *et al.* (2017) reporting the total GSLs content ranged between 2.10 - 40.96 mg. g^{-1} DW for *Eruca* accessions. Furthermore, the results from the present study suggest locations and different environmental growth conditions such as high temperature was causing stress on rocket leaves to produce more secondary metabolites such as GSLs (Francisco *et al.*, 2011; Jasper *et al.*, 2020). An increased accumulation of GSL may be related to metabolic changes associated with natural and/or stress-induced senescence, where amino acid methionine, phenylalanine, tyrosine, and tryptophan the precursors for GSL

biosynthesis, allocate these amino acids during senescence and thus favour GSL production (Hodges *et al.*, 2006).

Amongst the 23 GSLs identified in the present study, average dimeric 4-mercaptobutyl GSL (DMB) concentration (29.3 mg. g^{-1} DW) was the most abundant GSL found in the Italian trial making 72% of the total GSL, which is in agreement with the study by Pasini *et al.* (2011) on *E. sativa* leaves for DMB concentration. The next abundant GSL reported was glucorucolamine (GRM) (2.85 mg. g^{-1} DW), followed by glucosativin (GSV) (1.9 mg. g^{-1} DW), glucoraphanin (GRA) (0.57 mg. g^{-1} DW) and glucoerucin (GER) (0.35 mg. g^{-1} DW) (Table 4.1) respectively on average. In the UK trial, on average DMB (4.9 mg. g^{-1} DW) was again the most abundant GSL accounting for around 48% of the total GSLs followed by GSV (2.6 mg. g^{-1} DW), GRA (0.72 mg. g^{-1} DW), GRM (0.33 mg. g^{-1} DW), and GER (0.16 mg. g^{-1} DW) respectively. The previous study in the literature showed GSV, DMB, GRA, and GER being the most widely accepted main GSLs found in the rocket genus (Bell and Wagstaff, 2019).

For the six lines of 'salad' rocket grown at two locations, the highest average total GSLs concentration was found in lines 68 (54.87 mg. g^{-1} DW) and 130 (52.45 mg. g^{-1} DW) followed by line 112 (41.95 mg. g^{-1} DW) in the Italian trial ($p<0.05$). However, no significant differences between lines were observed in the UK-grown trial (Appendix 4.1) (Figure 4.2.i). These results suggest that the growing environmental conditions had an impact on lines (genotypes), where a high degree of interaction leads to biosynthesis and accumulation of individual GSLs (Cartea and Velasco, 2008; Bell and Wagstaff, 2019).

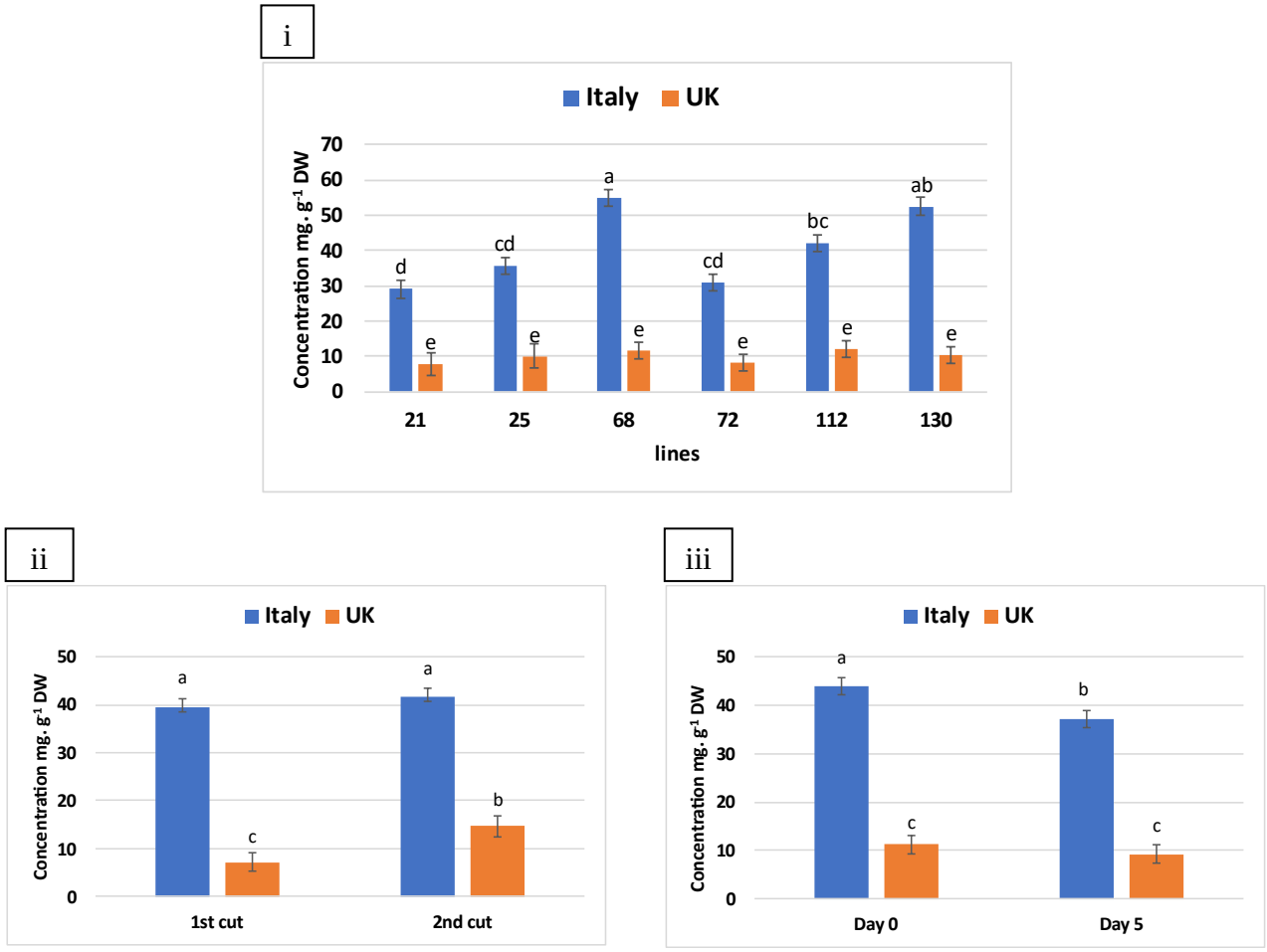


Figure 4. 2. Average total GSL concentration (mg. g^{-1} DW) of six lines of *E. sativa* leaves grown in Italy and the UK (n = 3).Blue colour: Italian-grown; orange colour: UK-grown. (i) for individual six lines, (ii) for the 1st and 2nd cut, and (iii) for day 0 and day 5. Error bars signify the standard error of the mean values of three biological replicates. Where there is no common letter above the bars within a chart, it represents significant differences between the bars (Tukey's HSD test, $p<0.05$). Abbreviations: DW, Dry weight.

When compared between the 1st and 2nd cut for total GSL concentrations, the Italian- grown trial showed no significant differences, whereas a two-fold higher average total GSL concentration was observed in the 2nd cut (14.74 mg. g^{-1} DW) leaves as compared to the 1st cut (7.21 mg. g^{-1} DW) for the UK-grown trial ($p<0.05$) (Appendix 4.2) (Figure 4.2.ii). For individual GSL, such as for GRM, a two-fold higher average GSL concentration was observed in the 2nd cut in the Italian trial as compared to the 1st cut, whereas, in the UK trial, a six-fold higher average GSL concentration was observed in the 2nd cut leaves as compared to the 1st cut for GSV, however, the rest of the GSLs did not vary significantly between cuts (Appendix 4.2). This suggests the accumulation of individual

GSL is location-dependent, however, the exact mechanism of its biosynthesis pathway is still unknown.

Previous studies in the literature suggested that a crop like a rocket undergoes multiple harvests and accumulates more phytochemicals (GSLs) due to the initiation of wound response (Martínez-Sánchez *et al.*, 2008; Bell *et al.*, 2020b). This was further supported by Pimpini and Enzo (1996), reporting that some markets in Italy, prefer multiple harvests to the 1st cut for intense aroma and improved quality in terms of leaf consistency. Furthermore, it is a most common practice and an effective way to produce higher levels of secondary metabolites (Jacobo-Velázquez *et al.*, 2015; Toscano *et al.*, 2019). For instance, wounding response when applied to vegetables such as carrots, produced a higher level of phenolic compounds (secondary metabolites) that have potential applications in the treatment and prevention of chronic diseases (Jacobo-Velázquez *et al.*, 2011). Another study on cabbage reported a four-fold increase in some GSL especially glucobrassicin (GBC) after chopping, with a possible explanation that cutting triggers a defence mechanism which can also occur due to wound response by an insect (Verkerk *et al.*, 1997). In Italy, it is a common practice to harvest a single crop for more than two harvests at an interval of 20-60 days depending on the season, the production systems, and the market destination (Siomos and Koukounaras, 2007).

When compared between day 0 (intake) and day 5 (postharvest shelf life) for total GSL concentrations, the Italian-grown trial showed a higher average total GSL concentration for day 0 (44.07 mg. g^{-1} DW) as compared to day 5 (37.17 mg. g^{-1} DW), whereas no significant differences were observed for the UK-grown leaves ($p<0.05$) (Appendix 4.3) (Figure 4.2.iii). A previous study on the 'commercial supply chain' on *E. sativa* leaves reported a higher concentration of GSL and its hydrolysis product (ITC) during postharvest shelf life over varying timeframes (Bell *et al.*,

2017c). However, the present study reported a decrease in total GSL concentrations with the postharvest shelf life with a possible explanation that only two-time frames were considered as compared to the earlier study on the 'commercial supply chain' which considered five-time frames. Rodrigues and Rosa (1999) reported a decrease in total GSL content with an increase in postharvest shelf life for broccoli when stored for five days. A decrease in total GSLs could be due to the onset of senescence. Moreover, plant tissue may also get damaged with an absence of oxygen during storage resulting decrease in total GSL content where endogenous myrosinase enzymes could hydrolyse GSLs (Kim and Ishii, 2007). However, the myrosinase activity was not measured in the present study. A study by Helland *et al.* (2016) reported changes in GSL and sugar concentrations during storage influencing sensory attributes such as appearance, taste, and flavour of fresh-cut swede and turnip which belongs to the *Brassicaceae* family. Therefore, to maintain the nutritional and sensorial attributes, it is recommended to store the *Brassicaceae* vegetables at a low temperature (5 °C).

4.4.1.3. Sulphur content

The sulphur content was significantly higher ($p<0.05$) in the Italian-grown trial as compared to the UK-grown trial. The Italian-grown trial showed a 1.4-fold higher average sulphur content as compared to the UK-grown trial (Italy total = 14.78 mg. g^{-1} DW, UK total = 10.26 mg. g^{-1} DW) (Table 4.1). A higher sulphur content (24.18 mg. g^{-1} DW) in *E. sativa* leaves was reported when grown in a conventional soil system having Mediterranean soil property called 'Terra Rossa' clay soil (classified as Alfisols according to the USDA soil taxonomy) (Di Gioia *et al.*, 2018). For six lines of *E. sativa* grown at both locations, lines 68 (17.56 mg. g^{-1} DW), followed by line 112 (16.13

mg. g^{-1} DW) and 130 (15.46 mg. g^{-1} DW) showed significantly ($p<0.05$) higher average sulphur content in the Italian-grown leaves as compared to the UK-grown (Appendix 4.1) (Figure 4.3.i).

The 2nd cut leaves in the Italian trial showed a significantly higher average sulphur content as compared to the 1st cut (Italy 1st cut = 14.01 mg. g^{-1} DW, Italy 2nd cut = 15.54 mg. g^{-1} DW). A similar pattern was observed in the UK-grown trial, which showed a significantly higher average sulphur content in the 2nd cut (UK 2nd cut = 12.15 mg. g^{-1} DW), as compared to the 1st cut (UK 1st cut = 9.00 mg. g^{-1} DW) (Appendix 4.2) (Figure 4.3.ii) although the UK sulphur concentrations were both significantly lower than the Italian concentrations. Since the Italian trial experienced considerably higher temperatures than the UK trial our results are consistent with the hypothesis that heat stress induces sulphur synthesis, potentially via up-regulation of the sulphur-related gene (Bell et al., 2020a), where the *SULTR* gene facilitates the transport of sulphate from vacuoles into the cytoplasm of the plant cell.

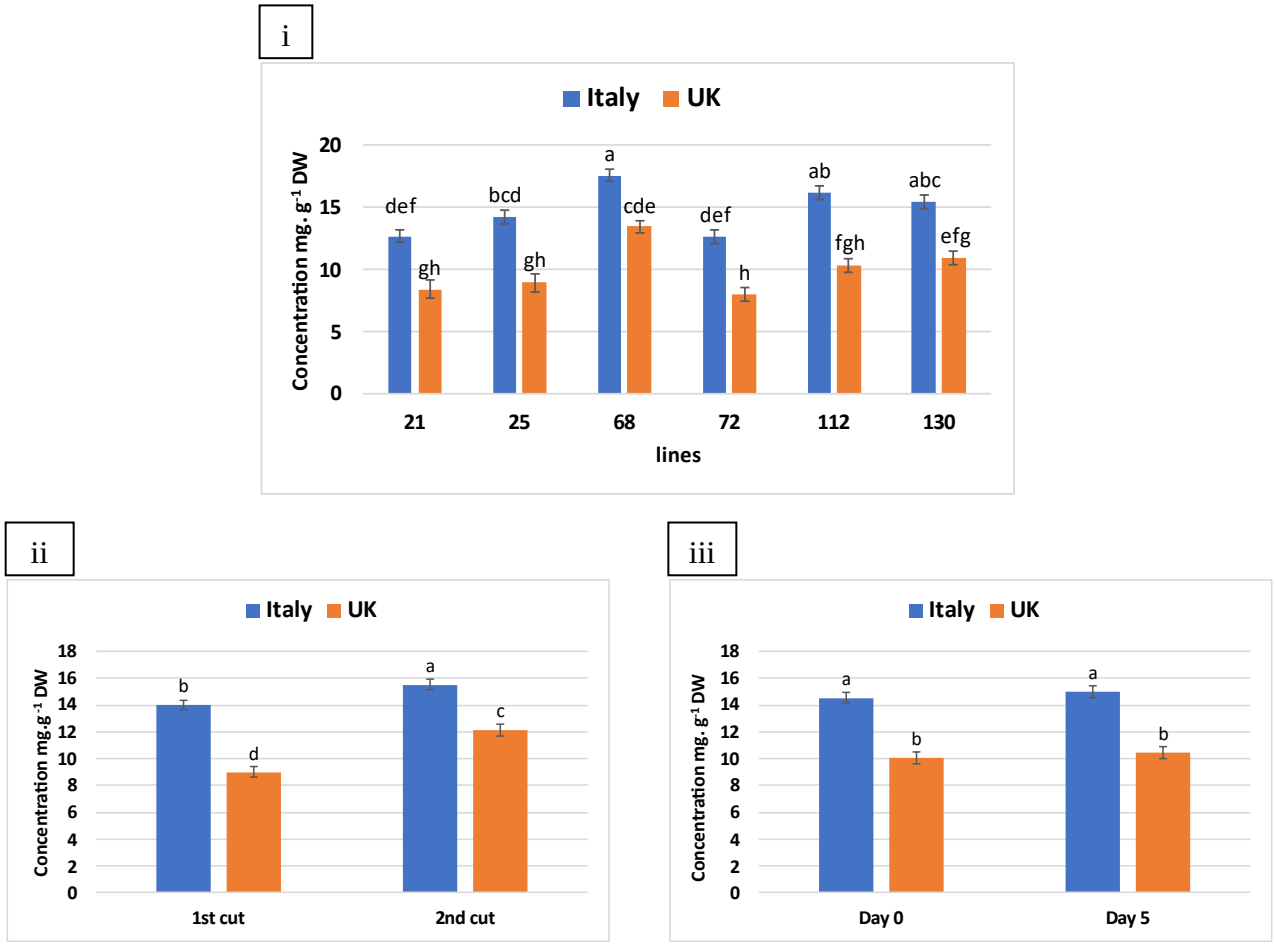


Figure 4. 3. Average sulphur content (mg. g^{-1} DW) of six lines of *E. sativa* leaves grown in Italy and the UK (n = 3).Blue colour: Italian-grown; orange colour: UK-grown. (i) for individual six lines, (ii) for the 1st and 2nd cut, and (iii) for day 0 and day 5. Error bars signify the standard error of the mean values of three biological replicates. Where there is no common letter above the bars within a chart, it represents significant differences between the bars (Tukey's HSD test, $p<0.05$). Abbreviations: DW, Dry weight.

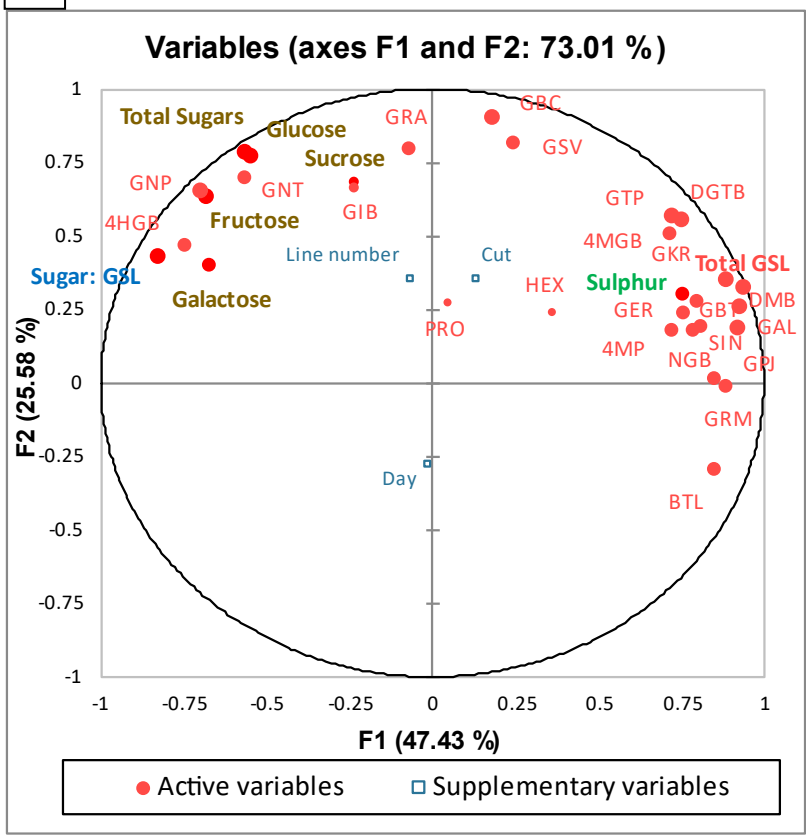
When comparing day 0 (intake) and day 5 (postharvest shelf life) for average sulphur content, both the Italian trial and the UK trial showed no statistically significant difference ($p<0.05$). However, the sulphur content in the UK-grown leaves remained significantly lower than the Italian-grown (Italy day 0 = 14.55 mg. g^{-1} DW, Italy day 5 = 15.00 mg. g^{-1} DW); (UK day 0 = 10.06 mg. g^{-1} DW, UK day 5 = 10.45 mg. g^{-1} DW) (Figure 4.3.iii) (Appendix 4.3). The results for sulphur content have been influenced by locations, cuts, and days and were in line with changes in total GSL content.

Sulphur is necessary for the synthesis of GSLs, and sulphur-containing amino acids participate in the formation of proteins. Sulphur present in the soil is absorbed by the roots in the form of sulphate ions and gets transported to leaves via the xylem. Sulphate ions get mainly reduced to cysteine and or transformed into methionine in the leaves. A low level of GSL content was reported in rape seeds which was due to the result of low sulphate content present in the soil (Josefsson and Appelqvist, 1968), however, the accumulation of GSL differs with the environment, varieties, and within organelles.

4.4.1.4. PCA on non-volatile compounds

PCA provides a visual comparison of the associations between sugars, GSLs, and sulphur content in six lines of *E. sativa* grown at two locations (Italy and the UK) (Figure 4.4). The PCA demonstrated clear associations between lines, locations, cuts, and days based on their non-volatile compounds. Most of the information was contained in the first two principal components i.e., Principal components one (PC1) and two (PC2), which explained 73.01% of the total variance in the data. The majority of explained variation is found in the PC1 component (x-axis) accounting for 47.43%, while PC2 (y-axis) accounts for 25.58%. In this study, only PC1 and PC2 components were selected for presentation as the rest of the other components (PC3 and PC4) did not provide any additional information. A clear separation was observed between the Italian-grown and the UK-grown trial (Figure 4.4.B). The PC1 component separated the six lines of 'salad' rocket grown by location (Italy and the UK), while the PC2 component separated the six lines mostly by the 1st and 2nd cut for the UK trial and by postharvest day 0 (intake) and day 5 (postharvest shelf life) for the Italian trial, respectively.

A


Variables (axes F1 and F2: 73.01 %)
Total Sugars
Glucose
Sucrose
Fructose
Galactose
Sugar: GSL
Sulphur
Line number
Cut
Day
GRA
GBC
GSV
GNP
GNT
GIB
4HGB
GTP
DGTB
4MGB
GKR
Total GSL
HEX
GER
GBT
DMB
GAL
PRO
4MP
SIN
NGB
GPJ
GRM
BTL
F1 (47.43 %)
F2 (25.58 %)
Active variables
Supplementary variables

B

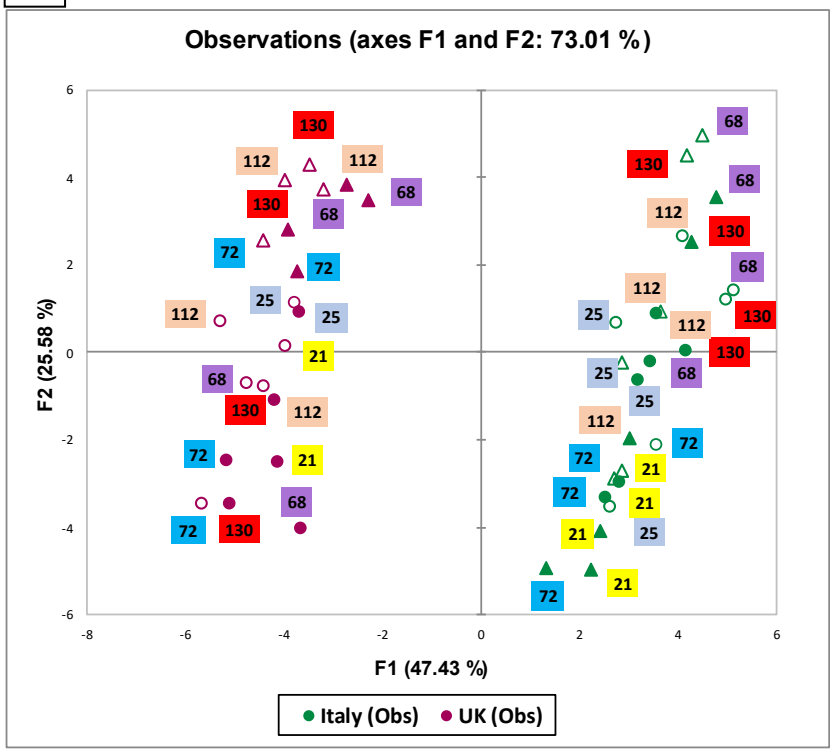

Observations (axes F1 and F2: 73.01 %)
F1 (47.43 %)
F2 (25.58 %)
Italy (Obs)
UK (Obs)

Figure 4. 4. Principal component analysis (PCA) shows the separation of an average of sugars, GSLs, and sulphur data of six lines of *E. sativa* leaves grown at two locations and regressed with supplementary data from lines, cuts, and days. (A) Distribution of variables; (B) Projection of six lines of *E. sativa*. Circles and triangles represent individual lines. Green colour: Italian trial; Red colour: the UK trial. Open circle: 1st cut, day 0; Closed circle: 1st cut day 5. Open triangle: 2nd cut, day 0; Closed triangle: 2nd cut, day 5.Abbreviations; DW: dry weight; GIB: glucoiberin; GKR: pentyl GSL; PRO: progoitrin; SIN: sinigrin; GRA: glucoraphanin; GRM: glucorucolamine; GAL: glucoalyssin; GPJ: glucoputranjivin; GNP: gluconapin; DGTB: diglucothiobeinin; GBT: glucoberteroin; 4HGB: 4-hydroxyglucobrassicin; GSV: glucosativin; DMB: dimeric 4-mercaptobutyl GSL; GTP: glucotropaeolin; GER: glucoerucin; GBC: glucobrassicin; 4MGB: 4-methoxyglucobrassicin; GNT: gluconasturtiin; NGB: neoglucobrassicin; 4MP; 4-methylpentyl; HEX: hexyl GSL; BTL: butyl GSL. Colour code: see highlight.

The correlation analysis revealed that GSLs and sulphur were the predominant compounds associated with the Italian trial, whereas sugars dominated the associations with the UK-grown trial. Total GSLs concentrations were highly correlated with both major GSL such as DMB ($r = 0.975$, $p < 0.0001$), GER ($r = 0.772$, $p < 0.0001$), SIN ($r = 0.825$, $p < 0.0001$), and minor GSL such as such as GKR ($r = 0.947$, $p < 0.0001$), GAL ($r = 0.922$, $p < 0.0001$), DGTB ($r = 0.897$, $p < 0.0001$), 4MGB ($r = 0.831$, $p < 0.0001$), as well as with sulphur ($r = 0.780$, $p < 0.0001$) (Appendix 4.4). Studies in the literature have reported that hydrolysis of GER results in the production of erucin which is known to be effective against some forms of cancer (Cartea and Velasco, 2008; Bell *et al.*, 2020b). Therefore, identifying lines that produce GER in high abundance under Italian growing conditions is useful in a breeding programme when targeting nutrition. The present study is in agreement with the study by Di Gioia *et al.* (2018) which reported a positive linear relationship between total GSL content and sulphur content. Total GSLs were negatively correlated with overall ratio of sugar and GSL ($r = -0.623$, $p < 0.0001$), 4HGB ($r = -0.546$, $p <0.0001$), and galactose ($r = -0.475$, $p = 0.001$) (Figure 4.4.A) (Appendix 4.4). Studies in the literature on *Brassicaceae* vegetables have reported a positive correlation between bitter taste and GSLs such as GSV, PRO, SIN, GAL, 4-MGB, GBC, etc., (Fenwick *et al.*, 1983; D'Antuono *et al.*, 2009) and a negative correlation between GSLs and sweet taste (Nor *et al.*, 2020). The present study agrees with the previous statement on the negative correlation between total GSLs and sugar such as galactose which imparts a sweetness.

Total sugars were highly and positively correlated with all three individual sugar [glucose ($r = 0.993$, $p < 0.0001$), fructose ($r = 0.886$, $p < 0.0001$), and galactose ($r = 0.754$, $p < 0.0001$)], and with a few individual GSL such as GNP ($r = 0.876$, $p < 0.0001$), GNT ($r = 0.877$, $p < 0.0001$), and 4HGB ($r = 0.748$, $p < 0.0001$), as well as with the ratio of sugar and GSL ($r = 0.877$, $p < 0.0001$) and were negatively correlated with BTL ($r = -0.684$, $p < 0.0001$) (Appendix 4.4). The present study found total sugars to be positively correlated with GRA ($r = 0.631$, $p < 0.0001$). Previous studies reported that GSL such as GRA is a precursor to sulforaphane a health-beneficial compound, which is pleasant and not bitter (D'Antuono *et al.*, 2008; Bell *et al.*, 2017a). Various studies in the literature have documented the health benefits associated with the consumption of GRA and its hydrolysis product sulforaphane (Sarikamis *et al.*, 2006; Ghawi *et al.*, 2013). Less bitterness of GRA is perhaps due to its abundance which is correlated with higher levels of sugar to make the overall impact of the rocket leaves less bitter in taste. This is consistent with research on other crops such as broccoli as well in cauliflower (Schonhof *et al.*, 2004), Brussels sprouts (Van Doorn *et al.*, 1998), and lettuce (Chadwick *et al.*, 2016), where prevalent bitter compounds had their taste-masked if sugars were present in high abundance. The PCA also revealed that lines such as 68, 112, and 130 were closely associated with GSLs and sulphur content in the Italian trial (Figure 4.4.B) and lines 68, 112, and 130 were closely associated with sugars in the UK trial.

4.4.2. Sensory analysis

4.4.2.1. Aroma traits

The aroma attributes of 'salad' rocket leaves were defined as pungent, mustard, peppery, green, and earthy. Amongst the five aroma traits, peppery, green, and earthy were found to vary significantly

(p <0.0001, 0.013, and <0.0001) between the Italian and the UK trial (Table 4. 2) and were found to be more strongly detected in the Italian trial. The most abundant aroma was green, which was scored slightly higher in the Italian trial. The study from Lytou *et al.* (2019) reported that volatile profiles are known to be influenced by variation in climate and so the difference between the UK and the Italian climate could play a role in determining the intensity of these aromas (Bell *et al.*, 2020b).

Amongst the five aroma traits, no significant difference was observed between the 1st and 2nd cut, however, there were differences in shelf life. Pungent and mustard traits were significantly reduced between day 0 (intake) and day 5 (postharvest shelf life) (p = 0.003, and 0.001) in both trials. A study by Helland *et al.* (2016) found a decreased odour intensity in *Brassicaceae* swede roots during storage (10 days) with a possible explanation for the evaporation of odour compounds from the surface, however, the present study did not measure the aroma intensity.

Genetic differences in the bitter taste receptor TAS2R38 showed no significant difference between PAV/PAV and AVI/AVI human genotype (Table 4. 2). This suggests genetic differences in the bitter taste receptors (TAS2R38) did not affect the perception of these aromas.

Peppery, green, and earthy traits did not vary between six lines of *E. sativa*, however, pungent, and mustard traits varied significantly (p = 0.021, and 0.007) (Table 4. 3) which agrees with the study from Bell *et al.* (2015), who reported that GSLs and their hydrolysis products, such as ITCs, are responsible for pungent aroma in ‘salad’ rocket. The perception of pungent and mustard aroma is linked with the hydrolysis product of GSV called sativin which is predominantly present in both

genera of rocket, however, a recent study reported that it is rather a combination of GSLs, ITCs, and several other volatile compounds (Bell *et al.*, 2021). Amongst the six lines of 'salad' rocket samples, line 68, followed by line 130, scored the highest values for pungent and mustard traits (Figure 4. 3), which suggests that these lines can be a potential candidate that can be used in a breeding programme for those consumers who like pungent and mustard flavour in their rocket.

4.4.2.2. Mouthfeel traits

Mouthfeel traits were defined as crispy, crunchy, firm, moist, warming, and numbing by sensory panels. Amongst these six attributes, only crispy, crunchy, and firmness, showed significant ($p < 0.0001$, 0.001, and 0.021) differences between the UK and the Italian-grown leaves. The UK-grown leaves were, overall, significantly crisper, crunchier, and firmer than those from the Italian trial (Table 4. 2). Warming and numbing traits did not vary significantly between trial locations. According to Sami *et al.* (2016), soluble sugars are known to help maintain the turgidity of leaves when experiencing abiotic stress resulting in crunchier, firmer, and crisper attributes and that may be the reason that lines which are grown in the UK trial, scored higher values for firmness, crunchiness, and crispness in their leaves.

Amongst the six mouthfeel attributes, crunchiness, firmness, warming, and firmness traits showed a significant difference between the 1st and 2nd cut (Table 4. 2). Leaves from the 2nd cut score higher values for crunchiness, firmness, and warming attributes ($p = 0.025$, <0.0001, and <0.0001) than those from the 1st cut. A previous study on 'salad' rocket reported that when crops are harvested for the 2nd cut, it causes more stress resulting in a higher accumulation of ITC derivate which is associated with the perception of warming mouthfeel (Bell *et al.*, 2020b). Regarding the postharvest

quality characteristics, it has been reported that some markets in Italy prefer the 2nd cut over the 1st for leaves consistency (uniformity), better preserves and a more intense flavour (Pimpini and Enzo, 1996; Bell *et al.*, 2020b). When compared between day 0 (intake) and day 5 (postharvest shelf life), only warming and numbing were found to be significantly reduced over the shelf life ($p < 0.0001$, and < 0.0001). The taste genotype (TAS2R38) of individuals showed no significant effect on the mouthfeel perception (Table 4. 2).

Crispiness, crunchiness, firmness, warming, and numbing traits vary significantly between the lines ($p < 0.0001$, 0.003, < 0.0001, < 0.0001, and < 0.0001) with lines 68 and 130 scorings higher values than lines 112 for most of these traits (Table 4. 3). Other lines such as lines 21, 25, and 72 scored comparatively lower values for the above traits. Similar results were found by Bell *et al.* (2017a), who reported that warming and initial heat in the mouthfeel were found to be significantly different between accessions.

4.4.2.3. Taste traits

Taste attributes such as bitterness, sweetness, and umami varied significantly between the two locations (Table 4. 2). Bitter taste was significantly higher in the Italian trial ($p < 0.0001$) whereas sweet and umami tastes were significantly higher in the UK trial ($p < 0.0001$ and < 0.0001). Bitter taste in rocket as well as in other *Brassicaceae* families is due to the presence of secondary metabolites such as GSLs, however, their accumulation depends on both genetics and environmental conditions (Cartea and Velasco, 2008). Higher temperature (Italian trial in the present study) accumulated more GSL in leaves (Jahangir *et al.*, 2009a; Jasper *et al.*, 2020) resulting in a bitter-tasting compound such as GSV, SIN, PRO, 4MGB etc. Low growing temperature can accumulate

more sugars in leaves (also observed in the present thesis reported in Chapter 3) which may result in sweetness perception (Steindal *et al.*, 2015) and can mask the bitter taste in food (Sharafi *et al.*, 2013; Beck *et al.*, 2014; Chadwick *et al.*, 2016; Nor *et al.*, 2020). Umami taste in rocket could be due to the presence of amino acids e.g. glutamic or aspartic acid, as a previous study on seven accession of *E. sativa* reported a higher abundance of both glutamic and aspartic acid (Bell *et al.*, 2017a), however, more study is needed to justify this.

No significant difference was observed between the 1st and the 2nd cut leaves for bitter, sweet, and umami taste (Table 4. 2). However, sweet taste varied significantly ($p = 0.008$) between the two shelf life points and scored higher values for day 0 (intake). Sugars are synthesised during photosynthesis and will be highest during day 0 (Koch, 1996; Rolland *et al.*, 2002) and as the shelf life progresses, respiration will utilise sugars for survival and thus shows a decline in sugars (Able *et al.*, 2005; Nei *et al.*, 2006).

The panellist genotype for the bitter taste receptor TAS2R38 showed a significant difference in the perception of sweet taste ($p < 0.0001$). The less bitter-sensitive 'AVI' panellists scored the 'salad' rocket samples to be significantly sweeter overall. Although the mean rating for bitter taste was lower, this was not significant. It is likely that for the more bitter-sensitive 'PAV' panellists, the differences in sweet taste were suppressed by the overriding presence of bitter-tasting compounds in these individuals (Table 4. 2).

The only taste attribute that differs significantly ($p < 0.0001$) between six lines of *E. sativa* was bitterness, with line 68 scoring significantly more bitterness in the leaves than lines 21 and 72 (Table

4. 3). This could either be because of differences in the abundance of bitter-tasting compounds or because of a lower concentration of sugars to mask them.

4.4.2.4. Flavour traits

The flavour attributes of 'salad' rocket leaves were described as peppery, green, soapy, mustard, and burnt by the sensory panellists and varied significantly between the two locations for peppery and soapy flavour ($p < 0.0001$ and 0.020). The peppery flavour scored higher values in the Italian trial, whereas the soapy flavour in the UK trial (Table 4. 2). A recent study on rocket has reported that peppery flavour is linked with GSL such as GER (Bell *et al.*, 2020b) and the present study reported a higher concentration of GER in the Italian trial (Table 4.1). Green, mustard, and burnt flavour did not vary significantly between trial locations.

The only flavour attribute affected by the cuts was mustard, which was significantly higher in the 2^{nd} cut ($p = 0.038$). The rest of the flavours did not vary significantly between cuts. It is speculated that multiple cuts in rocket species can increase the accumulation of GSLs and their hydrolysis products (ITCs) (Jasper *et al.*, 2020) which is responsible for peppery and mustard flavour. The presence of ITC is linked with mustard flavour in rocket as well as in *Brassicaceae* which explains the perception of this attribute (Bell *et al.*, 2018).

When the flavour data was assessed for shelf life between day 0 (intake) and day 5 (postharvest shelf life), peppery, green, and mustard flavours all significantly decreased over the shelf life ($p = 0.001$, 0.038, and <0.0001) (Table 4. 2). The decrease in the above flavours during the storage period could be due to enzymatic breakdown as a result of tissue damage (cutting) or due to plant

senescence (Spadafora *et al.*, 2016; Bell *et al.*, 2018) resulting in the formation of ketones which are volatile organic chemical (VOCs) responsible for the formation of green flavour. This is usually at this point consumers at home consume rocket and perceive this type of flavour profile. Thus, keeping RTE salads at a low temperature and handling RTE salads carefully could retain the flavour profile.

The genotype for bitter taste receptor TAS2R38 was related to a significantly ($p < 0.0001$ and < 0.0001) higher perception of soapy and burnt flavours in the less bitter sensitive AVI panellists. In terms of the six lines of *E. sativa*, the peppery and mustard flavour varied significantly for line 68, scoring a higher value than for most of the other lines ($p < 0.0001$) (Table 4. 3). This suggests that line 68 could be used as a breeding candidate for those customers who like their rocket peppery. Similar results were reported by Bell *et al.* (2017a) suggesting that peppery and mustard flavour was found to be significantly different between accessions.

4.4.2.5. Aftereffect traits

Warming, tingling, green, drying, numbing, and bitter traits were scored for aftereffects attributes at an interval of 30 seconds once swallowed. The aftereffect attributes varied significantly between the two locations for green [(T0, T1, and T2) ($p = 0.047$, 0.013, and 0.019)], drying [(T0, T1, and T2) ($p < 0.0001$, 0.001, and 0.041)], numbing [(T0, T1, T2, T3) ($p = 0.006$, 0.004, 0.004, and 0.041)], and bitter [(T0, T1, T2, and T3) ($p < 0.0001$, < 0.0001, 0.004, and < 0.0001)] traits (Table 4. 2). Green flavour post-consumption was higher in the Italian trial at (T0, T1, and T2) whereas drying sensation was higher in the UK trial at (T0, T1, and T2). The numbing sensation was higher in UK-grown

leaves at all time points (T0, T1, T2, and T3) whereas the bitter taste was higher in the Italian trial at all time points (T0, T1, T2, and T3).

The aftereffect attributes were significantly higher for warming sensation [(T0, T1, T2, and T3) ($p < 0.0001$, < 0.0001, < 0.0001, and 0.0001)] as well as bitter taste [(T0, T1, T2, and T3) ($p = 0.043$, 0.002, 0.002, and 0.0001)] at all time points for the 2nd cut. It was also significantly higher for tingling sensation at [(T1 and T2) ($p = < 0.0001$ and 0.008)]. Bell and Wagstaff (2019) reported similar findings for the 2nd cut leaves reporting that these were primarily favoured by growers and processors for their perceived increased trigeminal sensations.

When compared between day 0 (intake) and day 5 (postharvest shelf life), the aftereffect traits tended to reduce significantly over shelf life for sensations such as warming ($p = 0.003$ and < 0.0001), tingling ($p < 0.0001$, 0.021), and numbing ($p < 0.0001$, < 0.0001), as well as for bitter taste ($p < 0.0001$ and 0.015) (Table 4. 2).

For the six lines of the 'salad' rocket, the aftereffect attributes varied significantly for sensations such as warming, tingling, and numbing as well as for the bitter taste at all time points. Line 68 generally scores a higher aftereffect attribute than most of the other lines (Table 4. 3). This line scored higher values for aftereffect attributes such as warming [(T0, T1, T2, T3) ($p < 0.0001$, < 0.0001, < 0.0001, 0.014)], tingling [(T0, T1, T2, T3) ($p < 0.0001$, < 0.0001, 0.001, 0.009)], numbing [(T0, T1, and T2) ($p < 0.0001$, < 0.0001, and 0.002)] as well as for bitter taste [(T0, T1, T2, T3) ($p < 0.0001$, < 0.0001, 0.003, and 0.02)]. This suggests that line 68 could be a potential candidate to breed a rocket line for those consumers who like their rocket to have trigeminal attributes. It is not

always that major GSLs and their hydrolysis products are associated with sensory attributes, sometimes minor GSLs also play an important role in sensory perception (D'Antuono et al., 2009; Bell et al., 2017a). Although minor GSLs are found in low concentrations, their contribution plays a significant role in sensory attributes such as tingling, warming, numbing, and bitter aftereffects (Bell et al., 2017a) suggesting the fact that both major and minor GSLs and their hydrolysis products are important compounds in the characterising the sensory attributes of rocket.

Table 4. 2. Sensory attributes ratings averaged across six lines of *E. sativa* samples to demonstrate the overall effects of locations, 1st versus 2nd cut, day 0 (intake) and day 5 (postharvest shelf life) and as perceived by 20 panellists varying in human taste genotype TAS2R38.PAV genotypes include both the homozygous and heterozygous genotypes whereas AVI genotypes include only the homozygous genotype.

	Location		**Significance**	**Cut**		**Significance**	**Day**		**Significance**	**TAS2R38**		**Significance**
	Italy	**UK**	tested by ANOVA/MW	**1st**	**2nd**	tested by ANOVA/MW	**0**	**5**	tested by ANOVA/MW	**PAV**	**AVI**	tested by ANOVA/MW
Aroma												
Pungent	36.6	37.1	ANOVA	36.4	37.2	ANOVA	37.4[b]	33.7[a]	ANOVA	34.8	37.1	ANOVA
Mustard	32.7	33.9	ANOVA	32.6	33.9	ANOVA	35.5[b]	30.9[a]	MW	30.3	37.7	ANOVA
Peppery	32.9[b]	26.9[a]	MW	29.9	30.7	ANOVA	31.7	28.8	MW	27.2	34.8	ANOVA
Green	39.1[b]	34.6[a]	ANOVA	37.6	36.7	ANOVA	38.0	36.3	ANOVA	34.7	40.8	ANOVA
Earthy	25.1[b]	19.1[a]	MW	22.7	22.3	ANOVA	23.0	21.9	ANOVA	17.2	30.3	ANOVA
Mouthfeel												
Crisp	36.2[a]	42.0[b]	MW	37.7	39.9	ANOVA	39.4	38.0	ANOVA	39.1	38.1	ANOVA
Crunchy	31.1[a]	36.0[b]	ANOVA	31.5[a]	35.1[b]	ANOVA	34.2	32.2	ANOVA	30.7	36.9	ANOVA
Firmness	32.4[a]	35.3[b]	ANOVA	31.0[a]	36.6[b]	ANOVA	33.9	33.5	ANOVA	33.5	33.9	ANOVA
Moistness	39.7	38.7	ANOVA	40	38.4	ANOVA	39.8	38.8	ANOVA	36.7	43.1	ANOVA
Warming	28.6	26.6	ANOVA	24.6[a]	31.1[b]	ANOVA	30.1[b]	25.3[a]	ANOVA	26.3	29.7	ANOVA
Numbing	13.4	15.0	MW	12.8	15.5	MW	16.1[b]	12.0[a]	MW	11.5	17.8	ANOVA
Taste												
Bitter	40.8[b]	38.8[a]	ANOVA	39.9	40.0	ANOVA	40.4	39.4	ANOVA	41.4	37.7	ANOVA
Sweet	16.3[a]	22.1[b]	MW	19.0	18.6	ANOVA	20.0[b]	17.6[a]	MW	16.6[a]	22.1[b]	MW
Umami	18.7[a]	24.3[b]	MW	21.4	20.8	ANOVA	21.8	20.4	ANOVA	17.8	26.1	ANOVA
Flavour												
Peppery	35.1[b]	31.4[a]	ANOVA	32.6	34.5	ANOVA	34.9[b]	32.1[a]	ANOVA	31.6	36.3	ANOVA
Green	40.0	36.9	ANOVA	39.5	37.7	ANOVA	39.9[b]	37.4[a]	ANOVA	35.8	42.9	ANOVA
Soapy	13.04[a]	19.3[b]	MW	16.2	15.2	ANOVA	16.3	15.3	ANOVA	10.4[a]	23.8[b]	MW
Mustard	32.3	33.3	ANOVA	31.7[a]	33.9[b]	ANOVA	34.7[b]	30.8[a]	ANOVA	30.5	36.0	ANOVA
Burnt	12.1	11.3	ANOVA	10.8	12.8	MW	12.4	11.1	ANOVA	6.8[a]	19.0[b]	MW

Aftereffect												
Warming__T0	17.8	18.3	ANOVA	15.1[a]	21.1[b]	MW	19.0[b]	16.9[a]	MW	18.6	17.0	ANOVA
Warming__T1	11.9	13.3	ANOVA	10.2[a]	15.1[b]	MW	13.1[b]	11.9[a]	MW	14.4	9.7	ANOVA
Warming__T2	8.7	10.2	ANOVA	7.6[a]	11.2[b]	MW	9.4	9.2	ANOVA	11.6	5.9	ANOVA
Warming__T3	6.8	8.5	ANOVA	6.1[a]	9.2[b]	MW	7.5	7.7	ANOVA	9.9	4.1	ANOVA
Tingling__T0	12.1	10.4	ANOVA	10.1	12.8	MW	12.5[b]	10.3[a]	MW	10.7	12.3	ANOVA
Tingling__T1	8.7	7.8	ANOVA	7.1[a]	9.7[b]	ANOVA	9.0[b]	7.7[a]	ANOVA	8.4	8.3	ANOVA
Tingling__T2	6.6	6.0	ANOVA	5.3[a]	7.5[b]	MW	6.6	6.0	ANOVA	7.0	5.4	ANOVA
Tingling__T3	5.1	4.7	ANOVA	4.1	5.8	MW	5.0	4.9	ANOVA	5.7	3.8	ANOVA
Green__T0	25.9[b]	23.8[a]	ANOVA	25.5	24.4	ANOVA	25.8	24.1	ANOVA	25.4	24.3	ANOVA
Green__T1	21.0[b]	19.3[a]	ANOVA	20.2	20.2	ANOVA	20.6	19.8	ANOVA	22.3	17.1	ANOVA
Green__T2	17.3[b]	16.5[a]	MW	16.5	17.4	ANOVA	16.8	17.0	ANOVA	19.8	12.7	ANOVA
Green__T3	14.5	14.2	ANOVA	13.7	15.2	ANOVA	14.2	14.6	ANOVA	17.5	9.8	ANOVA
Drying__T0	20.3[a]	25.3[b]	ANOVA	22.1	23.0	ANOVA	23.0[b]	22.0[a]	ANOVA	24.2	20.0	ANOVA
Drying__T1	17.9[a]	22.0[b]	MW	19.2	20.2	MW	20.1	19.3	MW	22.2	15.9	ANOVA
Drying__T2	15.8[a]	19.0[b]	MW	16.3	18.0	MW	16.6	17.7	ANOVA	20.3	12.4	ANOVA
Drying__T3	14.1	17.0	ANOVA	14.7	16.1	MW	14.8	15.9	ANOVA	18.7	10.3	ANOVA
Numbing__T0	10.6[a]	13.1[b]	MW	10.6	12.9	MW	12.8[b]	10.5[a]	MW	11.6	11.8	ANOVA
Numbing__T1	8.4[a]	11.1[b]	MW	8.7	10.6	MW	10.5[b]	8.6[a]	MW	10.3	8.5	ANOVA
Numbing__T2	6.6[a]	9.1[b]	MW	6.7	8.7	MW	8.0	7.3	ANOVA	8.7	6.1	ANOVA
Numbing__T3	5.5[a]	7.7[b]	MW	5.9	7.2	MW	6.6	6.3	ANOVA	7.5	5.0	ANOVA
Bitter__T0	24.8[b]	23.7[a]	ANOVA	23.2[a]	25.6[b]	ANOVA	25.3[b]	23.3[a]	ANOVA	27.3	19.9	ANOVA
Bitter__T1	19.9[b]	19.1[a]	ANOVA	18.3[a]	21.0[b]	ANOVA	20.0[b]	19.2[a]	ANOVA	23.0	14.5	ANOVA
Bitter__T2	16.2[b]	15.9[a]	MW	14.9[a]	17.3[b]	ANOVA	15.9	16.2	ANOVA	19.5	10.9	ANOVA
Bitter__T3	13.9[b]	13.6[a]	ANOVA	12.8[a]	14.8[b]	ANOVA	13.3	14.1	ANOVA	17.0	8.9	ANOVA

The table represents the results of ANOVA type II sum of squares significance values for parametric and Mann-Whitney (MW) test for a non-parametric test for residuals to satisfy the normal distribution. Different small letters (a and b) in each row confirm whether the differences were significant with letter b showing for higher values. Abbreviations: T0, T1, T2, and T3 represent ratings taken on initial tasting (T0) and post swallowing as aftereffects at 30 s (T1), 60 s (T2), and 90 s (T3).

Table 4. 3. Average panel scores for sensory attribute ratings of six lines of *E. sativa* samples averaged across location, cut, shelf life and all the 20 panellists (regardless of taste genotype).

	line no.						Significance
	21	**25**	**68**	**72**	**112**	**130**	**tested by ANOVA/KW**
Aroma							
Pungent	32.2^{a}	34.4^{ab}	40.2^{b}	32.7^{a}	36.3^{ab}	37.0^{ab}	ANOVA
Mustard	29.2^{a}	31.8^{ab}	37.8^{b}	31^{ab}	34.5^{ab}	33.5^{ab}	KW
Peppery	27.8	29.2	33.1	28.6	30.8	31.1	ANOVA
Green	36.6	37.7	37.6	37.2	36.8	37.1	ANOVA
Earthy	21.8	22.9	23.2	21.3	22.3	23.1	ANOVA
Mouthfeel							
Crisp	34.6^{ab}	33.3^{a}	40.8^{bc}	38^{abc}	39.8^{bc}	43.3^{c}	KW
Crunchy	28.1^{a}	28.5^{a}	35.9^{b}	32.8^{ab}	34.4^{ab}	36.8^{b}	ANOVA
Firmness	30.9^{ab}	29.0^{a}	35.3^{bc}	32.7^{ab}	33.4^{ab}	38.7^{c}	ANOVA
Moistness	39.8	39.8	38.9	40	40.6	36.8	ANOVA
Warming	23.3^{ab}	22.9^{a}	36.4^{d}	21.7^{a}	31.6^{c}	27.6^{bc}	ANOVA
Numbing	11.7^{a}	10.5^{a}	19.0^{c}	11.4^{a}	16.9^{bc}	13.2^{ab}	KW
Taste							
Bitter	38.1^{ab}	40.1^{bc}	43.1^{c}	35.5^{a}	40.5^{bc}	41.7^{bc}	ANOVA
Sweet	16.2	17.3	19.6	20.1	20.1	18.5	ANOVA
Umami	20.6	18.9	22	20.4	23	21.3	ANOVA
Flavour							
Peppery	30.5^{ab}	30.4^{ab}	38.9^{d}	27.7^{a}	37.2^{cd}	34.5^{bc}	ANOVA
Green	38	39.7	37.9	38.3	38.1	39.8	ANOVA
Soapy	15.7	17.2	15	15.6	15.1	16.4	ANOVA
Mustard	27.5^{a}	28.3^{ab}	39.6^{d}	27.3^{a}	37.4^{cd}	33.1^{bc}	ANOVA
Burnt	11.2	11.7	13.2	8.7	13.1	12.3	ANOVA
Aftereffect							
Warming__T0	14.9^{ab}	13.4^{a}	23.6^{c}	13.9^{a}	22.0^{c}	17.8^{b}	KW
Warming__T1	10.2^{ab}	9.3^{a}	15.8^{c}	10.0^{a}	15.4^{c}	12.8^{b}	KW
Warming__T2	7.7^{ab}	7.1^{a}	11.5^{c}	7.9^{ab}	11.2^{c}	9.5^{bc}	KW
Warming__T3	6.1^{ab}	5.7^{a}	8.9^{c}	6.7^{ab}	9.1^{c}	7.9^{bc}	KW
Tingling__T0	10.0^{a}	9.3^{a}	14.5^{b}	8.4^{a}	14.4^{b}	10.6^{a}	KW
Tingling__T1	7.7^{ab}	7.0^{a}	10.2^{b}	6.2^{a}	10.3^{b}	8.0^{ab}	ANOVA
Tingling__T2	5.9^{ab}	5.1^{a}	7.8^{b}	5.0^{a}	7.7^{b}	6.1^{ab}	KW
Tingling__T3	4.3^{ab}	4.3^{ab}	5.8^{ab}	4.1^{a}	6.2^{b}	4.6^{ab}	KW
Green__T0	24.8	25.5	24.7	24	25	25.8	ANOVA

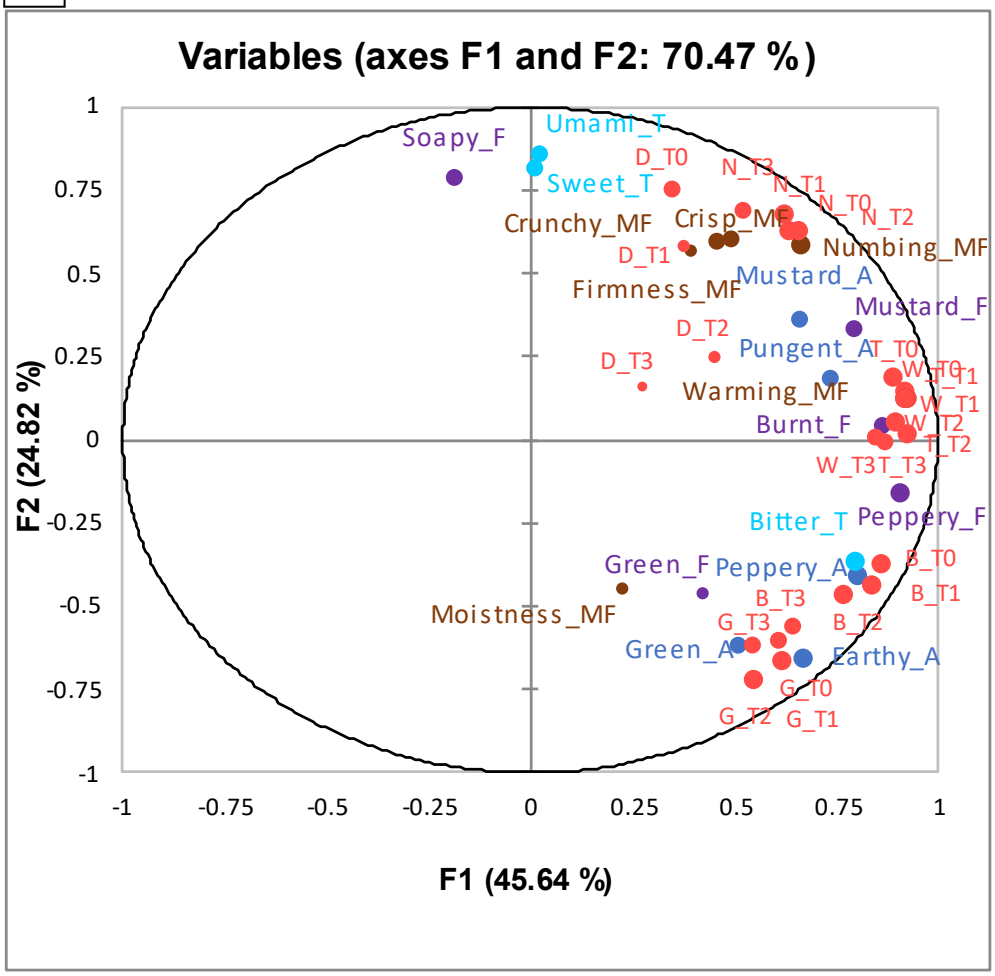
Green__T1	19.9	20.5	20.3	19.3	20.4	20.8	ANOVA
Green__T2	16.8	16.9	17	16.1	16.7	18	KW
Green__T3	13.8	14.2	14.5	13.9	14.5	15.3	ANOVA
Drying__T0	21.5	21.9	23.6	21	23.7	22.8	ANOVA
Drying__T1	19	18.5	20	18.5	20.8	20.8	KW
Drying__T2	17	16.5	17.4	16.3	17.8	17.7	ANOVA
Drying__T3	15.2	14.7	15.5	15.1	16	15.5	ANOVA
Numbing__T0	9.5^{a}	9.6^{a}	15.0^{b}	9.3^{a}	14.4^{b}	11.1^{a}	KW
Numbing__T1	7.6^{a}	8.0^{a}	11.7^{b}	8.1^{a}	11.4^{b}	9.6^{ab}	KW
Numbing__T2	5.8^{a}	6.6^{a}	9.4^{b}	6.7^{a}	9.3^{b}	7.4^{ab}	KW
Numbing__T3	5.1	5.9	7.7	5.5	7.7	6.4	KW
Bitter__T0	22.3^{ab}	24.6^{abc}	27.5^{c}	20.9^{a}	24.3^{abc}	25.7^{bc}	ANOVA
Bitter__T1	18.0^{ab}	19.2^{abc}	21.8^{c}	17.1^{a}	19.2^{abc}	21.0^{bc}	ANOVA
Bitter__T2	15.4^{ab}	16.2^{ab}	17.7^{b}	14.1^{a}	16.0^{ab}	16.9^{b}	ANOVA
Bitter__T3	12.9^{ab}	13.9^{ab}	14.5^{ab}	12.4^{a}	13.7^{ab}	14.9^{b}	ANOVA

The table represents the results of ANOVA type II sum of squares significance values for parametric and Kruskal Wallis (KW) test for a non-parametric test for residuals to satisfy the normal distribution. Different small letters (a, b, and c) in each row confirms whether the differences were significant or not with letter c showing the highest value. Abbreviations: T0, T1, T2, and T3 represent ratings taken on initial tasting (T0) and post swallowing as aftereffects at 30 s (T1), 60 s (T2), and 90 s (T3).

4.4.2.6. PCA on sensory attributes

Principal component analysis (PCA) provides a visual comparison of differences in sensory attributes (aroma, mouthfeel, taste, flavour, and aftereffect) between the six lines of 'salad' rocket grown at two locations (Italy and the UK) (Figure 4.5). The PCA demonstrated a clear difference between locations, lines, cuts, and shelf life based on their sensory attributes. Most of the information was contained in the first two principal components i.e., Principal components one (PC1) and two (PC2) which explained 70.47% of the total variation present in the data. The majority of explained variation is found in PC1 (x-axis) accounting for 45.64%, while PC2 (y-axis) accounts for 24.82%. A clear separation was observed between the Italian-grown and the UK-grown trial on the y- axis (Figure 4.5 B).

A


Variables (axes F1 and F2: 70.47 %)
F2 (24.82 %)
F1 (45.64 %)
1
0.75
0.5
0.25
0
-0.25
-0.5
-0.75
-1
-1
-0.75
-0.5
-0.25
0
0.25
0.5
0.75
1
Soapy_F
Umami_T
D_T0
N_T3
Sweet_T
N_T1
N_T0
N_T2
Crunchy_MF
Crisp_MF
Numbing_MF
D_T1
Mustard_A
Firmness_MF
Mustard_F
D_T2
Pungent_A
T_T0
D_T3
W_T0
W_T1
Warming_MF
W_T1
Burnt_F
W_T2
T_T2
W_T3
T_T3
Bitter_T
Peppery_F
Green_F
Peppery_A
B_T0
B_T3
B_T1
Moistness_MF
G_T3
B_T2
Green_A
Earthy_A
G_T0
G_T2
G_T1

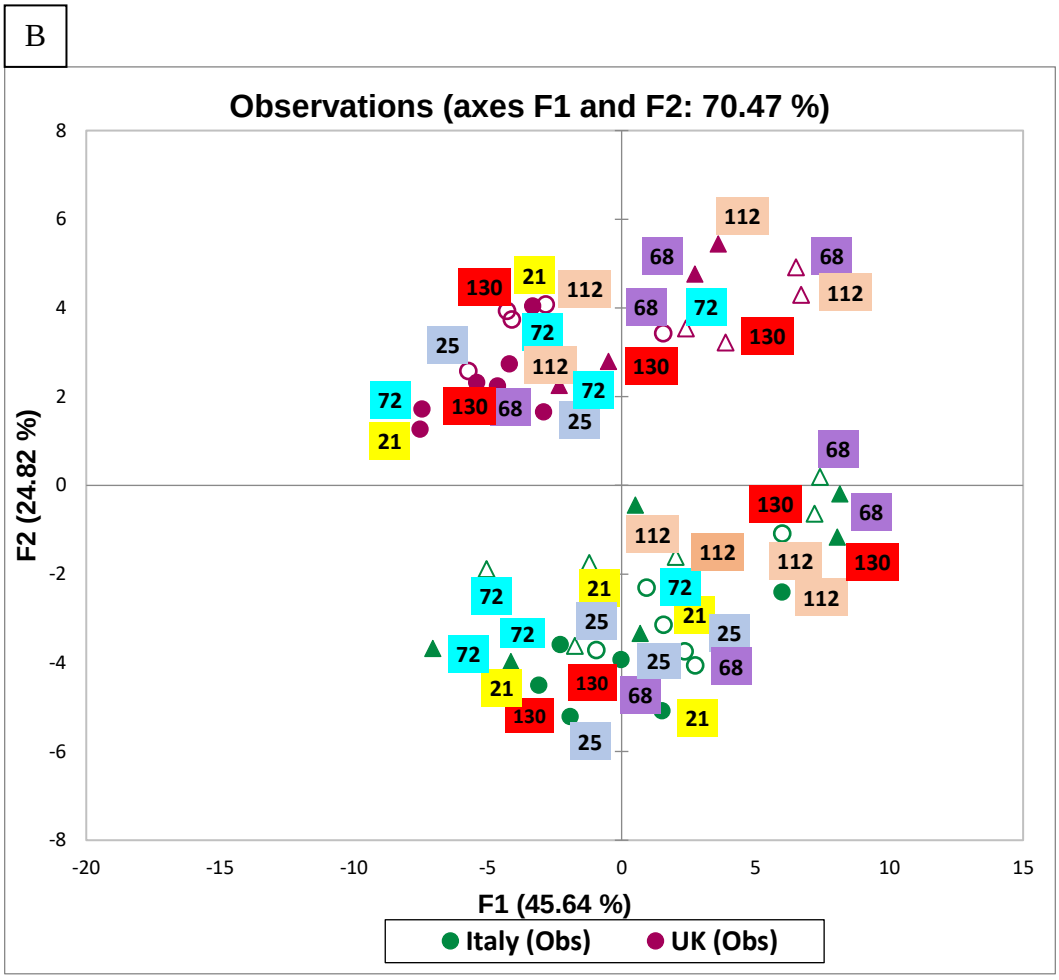


Figure 4. 5. Principal component analysis (PCA) shows the separation of sensory attributes of the six lines of *E. sativa* grown at two locations. (A) Distribution of variables; (B) Projection of the six lines of *E. sativa*, with circles and triangles representing an individual line. Green colour: Italian trial; Red colour: the UK trial. Open circle: 1st cut, day 0; Closed circle: 1st cut day 5. Open triangle: 2nd cut, day 0; Closed triangle: 2nd cut, day.Abbreviations: A: aroma; MF: mouthfeel; T: taste; F: flavour; W: warming; T: tingling; G: green; D: drying; N: numbing; B: bitter. T0, T1, T2, and T3 represent ratings taken on initial tasting (T0) and post swallowing as aftereffects at 30 s (T1), 60 s (T2), and 90 s (T3).Colour code: see highlight.

The correlation analysis revealed bitter taste was highly and positively correlated with peppery aroma (r = 0.873, p <0.0001), warming mouthfeel (r = 0.728, p <0.0001), peppery flavour (r = 0.874, p <0.0001) and to less extent with burnt flavour (r = 0.679, p <0.0001) (Appendix 4.5). Furthermore, bitter taste was also positively correlated with aftereffects traits such as warming (T0, T1, T2) (r = 0.668, 0.632, 0.623) (p <0.0001, <0.0001, <0.0001), tingling (T0, T1, T2) (r = 0.654, 0.604, 0.628) (p <0.0001, <0.0001, <0.0001), and bitter taste (T0, T1, T2, T3) (r = 0.897,

0.844, 0.742, 0.699) ($p < 0.0001$, < 0.0001, < 0.0001, < 0.0001) and negatively correlated with sweet taste ($r = -0.315$, $p = 0.038$) and soapy flavour ($r = -0.434$, $p = 0.004$) (Appendix 4.5). The negative correlation between bitter and sweet taste has been reported by previous studies on a variety of horticultural crops (Beck *et al.*, 2014; Chadwick *et al.*, 2016; Helland *et al.*, 2016; Bell *et al.*, 2017a).

Furthermore, the sweet taste was significantly correlated with sensory traits such as with umami ($r = 0.798$, $p < 0.0001$), soapy ($r = 0.755$, $p < 0.0001$), and crispy mouthfeel ($r = 0.587$, $p < 0.0001$) and negatively correlated with green aroma ($r = -0.375$, $p = 0.013$), green aftereffect (T2, T3) ($r = -0.653$, -0.611) ($p < 0.0001$, < 0.0001) and bitter T3 ($r = -0.585$, $p < 0.0001$) attribute (Appendix 4.5). A sensory study on brassica swede root vegetables perceived the sweetness, juiciness, and crispness when grown at a 'low' growth temperature (9 °C) due to higher levels of sugars (Johansen *et al.*, 2016).

The six lines of the 'salad' rocket were separated on the PC1 axis with lines 68, 130, and 112 positioned on the right side of the PC1 component whereas lines 72, 21, and 25 were on the left side (Figure 4.5.B). Lines 68 and 130 grown in Italy were driven by sensory attributes such as peppery flavour, peppery aroma, bitter taste, and bitter aftereffect, whereas lines 68 and 112 from the UK trial were driven by sweet and umami taste. This implies that lines 68, 130, and 112 could be the potential lines that breeders could select to breed rocket for consumers who prefer their rocket peppery and sweet. Whereas lines such as 72, 21, and 25 could be selected as pre-breeding lines for those who prefer their rocket mild.

4.4.3. Bitter taste TAS2R38 genotype

Selected six lines of *E. sativa* leaves were accessed by sensory panellists having PAV/PAV (supertaster) and AVI/AVI (non-taster) diplotypes and their scorings were reported in appendix 4.6. Individuals with AVI allele could perceive the attributes such as aroma (Figure 4.6a), mouthfeel (Figure 4.6b), taste (Figure 4.6c) and flavour (Figure 4.6d), significantly ($p<0.05$), whereas individuals with PAV allele could perceive the aftereffect attributes, more significantly ($p \leq 0.05$) (Figure 4.6 e).

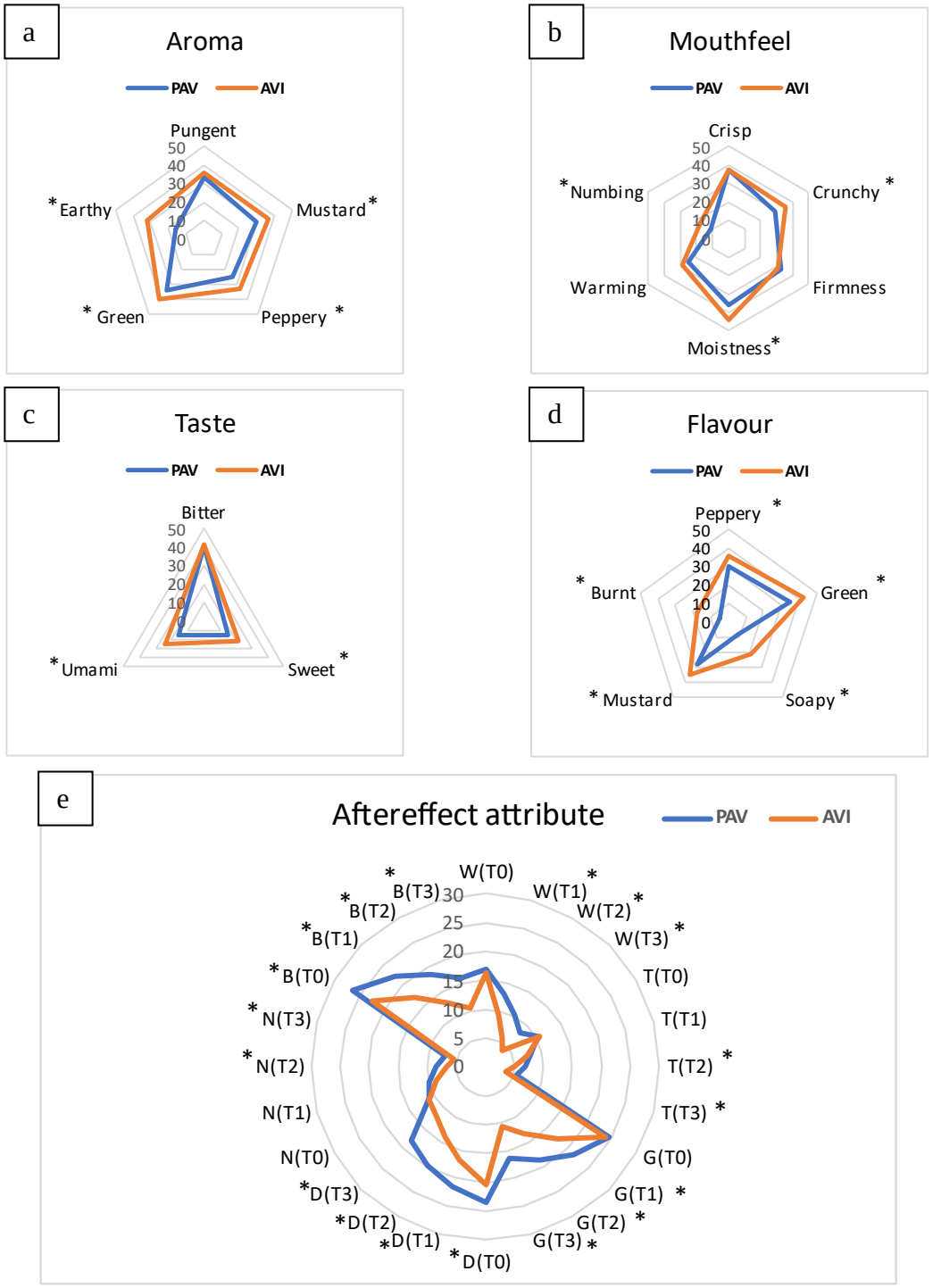


Figure 4. 6. Panellist scored for sensory attributes: (a) aroma, (b) mouthfeel, (c) taste, (d) flavour, and (e) aftereffect attribute for six lines of *E. sativa* according to TAS2R38 taste receptor diplotypes using a spider web diagram. * represents significant differences between TAS2R38 taste receptor diplotypes (Tukey's HSD test, $p<0.05$). Blue colour: PAV/PAV diplotype; Orange colour: AVI/AVI diplotype. Abbreviations: W; warming, T; tingling, G; green, D; drying, N; numbing, B; bitter. T0, T1, T2, and T3 represent ratings taken on initial tasting (T0) and post swallowing as aftereffects at 30 s (T1), 60 s (T2), and 90 s (T3).

4.4.3.1. PCA on TAS2R38 diplotypes

PCA showed the visual comparison of sensory attributes scores by the panellists for the six lines of 'salad' rocket for human genotype TAS2R38 (Figure 4.7). Most of the information was contained in the first two principal components (65.12%) i.e., Principal components one (PC1), and two (PC2). The PC1 component explains 42.93% of the total variations present in the data, while PC2 explains 22.19%.

A clear separation between the human taste receptor TAS2R38 genotype PAV/PAV and AVI/AVI was observed (Figure 4.7). The correlation analysis showed that the burnt flavour was positively correlated with soapy flavour ($r = 0.685$, $p < 0.0001$), mustard aroma ($r = 0.626$, $p < 0.0001$), earthy aroma ($r = 0.609$, $p < 0.0001$), warming mouthfeel ($r = 0.613$, $p < 0.0001$), numbing mouthfeel ($r = 0.714$, $p < 0.0001$), umami taste ($r = 0.603$, $p < 0.0001$), mustard flavour ($r = 0.609$, $p < 0.0001$) as well as with sweet taste ($r = 0.455$, $p = 0.002$) and negatively correlated with green T3 ($r = -0.467$, $p = 0.002$), and drying T3 ($r = -0.552$, $p = 0.001$) aftereffect attributes (Appendix 4.7).

The aftereffect warming_T1 was dominant on the PC1 component and were positively correlated with most of the aftereffect traits such as warming_T0 ($r = 0.911$, $p < 0.0001$), warming_T2 ($r = 0.952$, $p < 0.0001$), warming_T3 ($r = 0.918$, $p < 0.0001$), tingling_T0 ($r = 0.809$, $p < 0.0001$), tingling_T1 ($r = 0.906$, $p < 0.0001$), tingling_T2 ($r = 0.916$, $p < 0.0001$), tingling_T3 ($r = 0.895$, $p < 0.0001$), drying_T0 ($r = 0.734$, $p < 0.0001$), drying_T1 ($r = 0.811$, $p < 0.0001$), drying_T2 ($r = 0.741$, $p < 0.0001$), numbing_T0 ($r = 0.745$, $p < 0.0001$), numbing_T1 ($r = 0.789$, $p < 0.0001$), numbing_T2 ($r = 0.805$, $p < 0.0001$), numbing_T3 ($r = 0.743$, $p < 0.0001$), bitter_T2 ($r = 0.779$, $p < 0.0001$), bitter_T3 ($r = 0.765$, $p < 0.0001$) and negatively correlated with moistness mouthfeel (r

= -0.585, p <0.0001), and green flavour (r = -0.504, p = 0.001) (Appendix 4.7). The present study agrees with the previous study on seven accessions of on 'salad' rocket that bitter aftereffects were negatively correlated with moistness mouthfeel (Bell *et al.*, 2017a).

Lines 68, 112, and 130 from the Italian-grown trial were closely associated with aftereffect traits (such as bitterness, numbing, warming, tingling, drying, and green) with panellists having PAV alleles could distinguish these attributes significantly. Whereas panellists with the AVI allele could perceive the rest of the attributes such as mouthfeel, aroma, taste, and flavour significantly (Figure 4.7). This suggests that individuals with PAV/PAV 'super-tasters' have reduced perception of subtle sensory attributes of rocket leaves when compared to the AVI/AVI 'non-tasters'. When breeding the rocket for different groups of consumers, it is necessary to keep individuals having PAV allele in mind as this group perceives bitterness strongly and thus avoids consuming rocket which in turn will not get the health benefits associated with the crop.

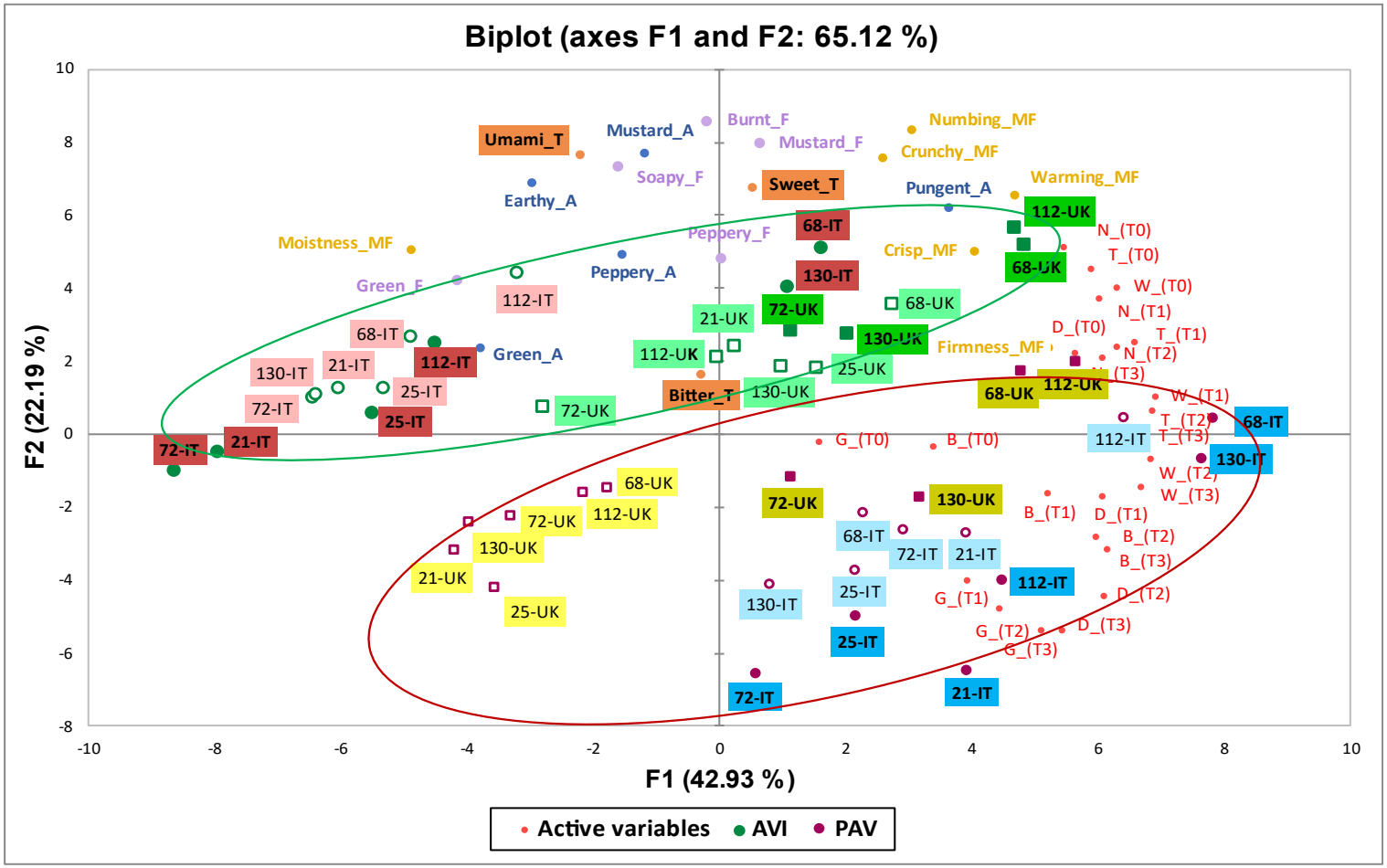


Figure 4. 7. Principal Component Analysis (PCA) of panels scoring sensory attributes of six lines of *E. sativa* grown at two locations for TAS2R38 human genotype for PAV and AVI allele. Biplot displays PC1 and PC2 components, which represent 65.12% of the variation within the data. PAV/PAV diplotype: red ellipse; AVI/AVI diplotype: a green ellipse. Circles and squares represent individual lines with an open circle: 1st cut for Italy; closed circle: 2nd cut for Italy. Open square: 1st cut for the UK; closed square: 2nd cut for the UK. Abbreviations: IT, Italy; A: aroma; MF: mouthfeel; T: taste; F: flavour; W: warming; T: tingling; G: green; D: drying; N: numbing; B: bitter. T0, T1, T2, and T3 represent ratings taken on initial tasting (T0) and post swallowing as aftereffects at 30 s (T1), 60 s (T2), and 90 s (T3).Colour code: see highlight.

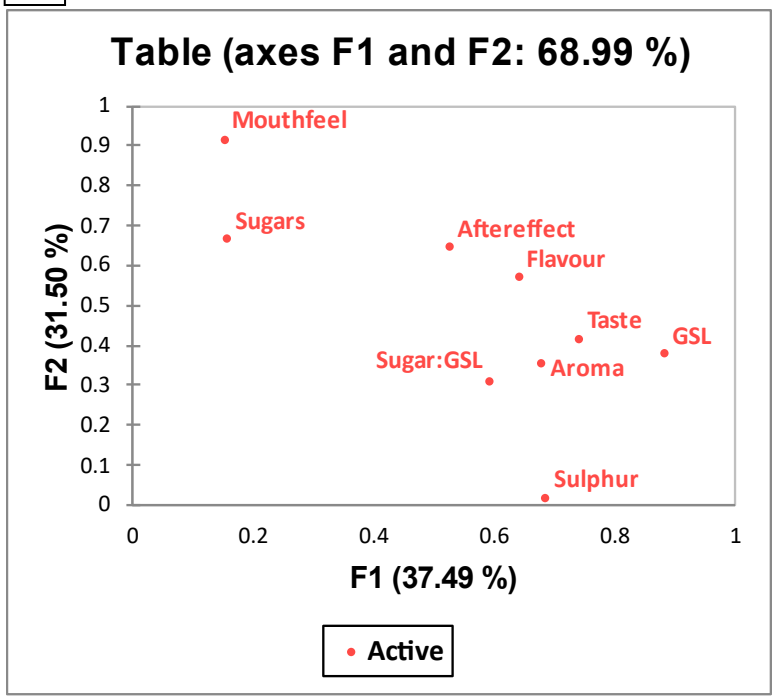
Studies have reported that while TAS2R38 genotype and taster phenotype contribute to food preference and choices of dietary intake, age, food adventurousness, gender, fungiform papillae density and social and cultural influences also play a significant role (Beckett *et al.*, 2014; Hoppu *et al.*, 2020). This was further supported by Bell *et al.* (2017b) in their study revealing that the liking of the rocket was not solely based on their ability to perceive bitterness but also on consumers' exposure and familiarity with rocket leaves. If we consider all the above factors, it will help plant breeders to understand consumer needs to develop a new cultivar with desired quality traits (taste and flavour).

4.4.4. Multiple factor analysis (MFA)

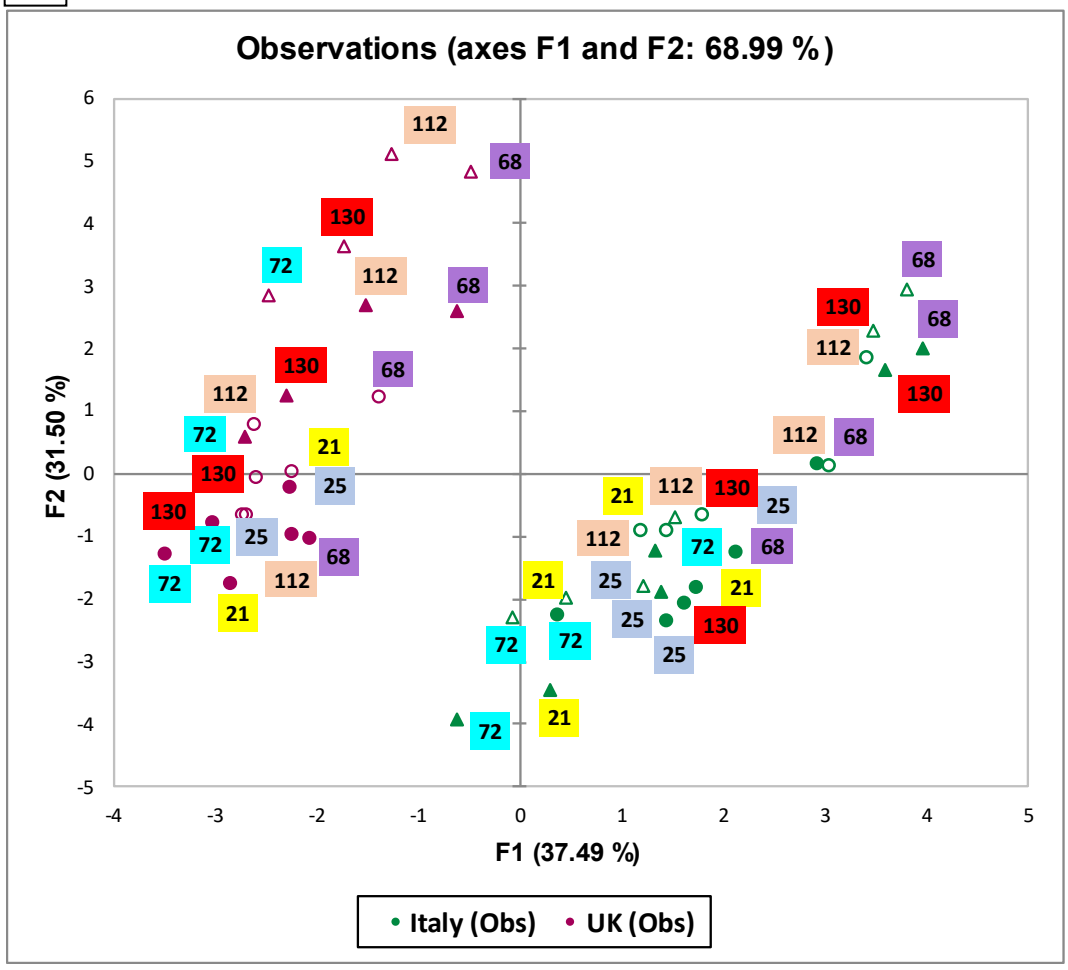
To visualise the overall picture, an MFA was conducted on the entire data (instrumental and sensory). It was used to simultaneously provides a visual comparison of the association between sensory and phytochemical content of six lines of *E. sativa* grown at two locations (Italy and the UK) (Figure 4.8.A). The correlation maps of observations and variables are shown in (Figures 4.8, B and C) respectively. The variable map (Figure 4.8 C) showed that sensory analysis was linked with instrumental data such as sugars, sulphur, and GSLs and was superimposed. The correlation analysis showed bitter taste to be positively correlated with many of the major and minor GSLs such as DMB ($r = 0.762$, $p < 0.0001$), GSV ($r = 0.563$, $p < 0.0001$), GER ($r = 0.660$, $p < 0.0001$), GKR ($r = 0.783$, $p < 0.0001$), GAL ($r = 0.742$, $p < 0.0001$), DGBT ($r = 0.825$, $p < 0.0001$), 4MGB ($r = 0.661$, $p < 0.0001$), and total GSL ($r = 0.769$, $p < 0.0001$) and was negatively correlated with galactose ($r = -0.344$, $p = 0.023$) as well as with the sugar to GSL ratio ($r = -0.361$, $p = 0.016$) (Figure 4.8.C) (Appendix 4.8). This suggests the association between bitter taste and many of the major GSLs such as DMB, GSV, and GER. This was further supported by various studies in the

literature (Van Doorn *et al.*, 1998; Schonhof *et al.*, 2004; Pasini *et al.*, 2011). A study from Pasini *et al.* (2011) reported DMB as a typical GSL in both genera of rocket that was significantly correlated with a bitter taste. Minor GSLs such as GKR, GAL, GRM, and 4MGB, although they do not occur in higher concentrations, however, were the major contributor to sensory attributes (Bell *et al.*, 2015) compared to just using total GSLs as a comparator for sensory traits (D'Antuono *et al.*, 2009).

A



B


Observations (axes F1 and F2: 68.99 %)
F2 (31.50 %)
F1 (37.49 %)
Italy (Obs)
UK (Obs)

C

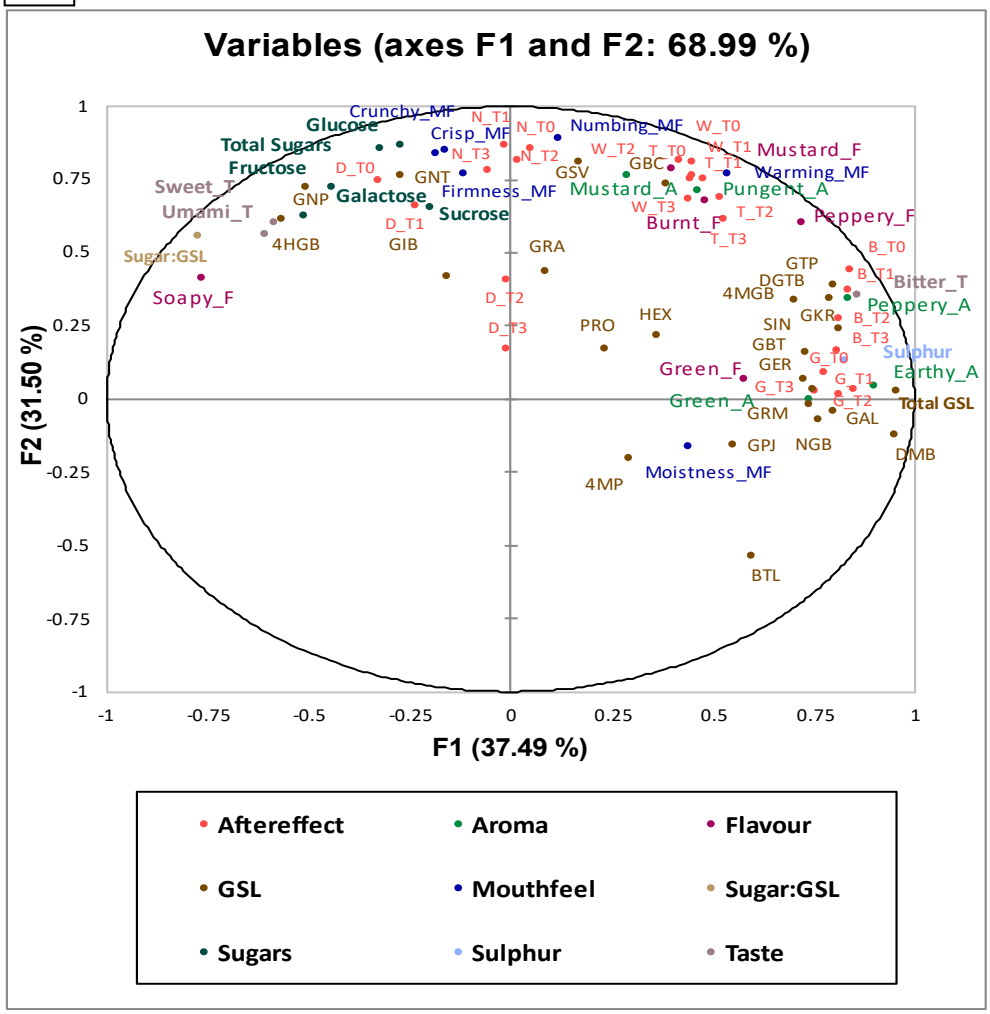


Figure 4. 8. Multiple Factor Analysis (MFA): (A) Representation of groups of variables: (B) Representation of six lines of *E. sativa* grown at two locations: (C) Distribution of variables. Circles and triangles represent an individual line. Open circle: 1st cut, day 0; Closed circle: 1st cut day 5. Open triangle: 2nd cut, day 0; Closed triangle: 2nd cut, day 5. Abbreviations; GIB: glucoiberin; GKR: pentyl GSL; PRO: progoitrin; SIN: sinigrin; GRA: glucoraphanin; GRM: glucorucolamine; GAL: glucoalyssin; GPJ: glucoputranjivin; GNP: gluconapin; DGTB: diglucothiobeinin; GBT: glucoberteroin; 4HGB: 4-hydroxyglucobrassicin; GSV: glucosativin; DMB: dimeric 4-mercaptobutyl GSL; GTP: glucotropaeolin; GER: glucoerucin; GBC: glucobrassicin; 4MGB: 4-methoxyglucobrassicin; GNT: gluconasturtiin; NGB: neoglucobrassicin; 4MP; 4-methylpentyl; HEX: hexyl GSL; BTL: butyl GSL; A: aroma; MF: mouthfeel; T: taste; F: flavour; W: warming; T: tingling; G: green; D: drying; N: numbing; B: bitter. T0, T1, T2, and T3 represent ratings taken on initial tasting (T0) and post swallowing as aftereffects at 30 s (T1), 60 s (T2), and 90 s (T3).Colour code: see highlight.

Total GSLs were positively correlated with DMB ($r = 0.975$, $p < 0.0001$), GKR ($r = 0.947$, $p < 0.0001$), GAL ($r = 0.922$, $p < 0.0001$), GTP ($r = 0.877$, $p < 0.0001$), bitter aftereffects (T0, T1, T2, T3) ($r = 0.797, 0.808, 0.767, 0.794$) with (all $p < 0.0001$) and negatively correlated with the sugar to GSL ratio ($r = -0.623$, $p < 0.0001$) (Appendix 4.8). The present study agrees with the previous

study on seven accessions of the 'salad' rocket suggesting that total GSL were positively correlated with bitter aftereffect attributes (Bell *et al.*, 2017a). The present study further revealed that major GSL, such as GER, was positively correlated with sensory attributes such as earthy aroma ($r = 0.743$, $p < 0.0001$), GTP ($r = 0.739$, $p < 0.0001$), sulphur ($r = 0.820$, $p < 0.0001$), total GSL ($r = 0.772$, $p < 0.0001$), DMB ($r = 0.732$, $p < 0.0001$), and aftereffect attributes such as green_T2 ($r = 0.748$, $p < 0.0001$) and negatively correlated with sweet taste ($r = -0.532$, $p < 0.0001$), umami taste ($r = -0.543$, $p < 0.0001$) and soapy flavour (r -0.567, $p < 0.0001$) (Appendix 4.8). Previous studies reported that GSLs and sugars can influence flavour and taste (Beaulieu and Baldwin, 2002) and changes in the content of these phytochemical compounds could influence sensory attributes of fresh-cut swede and turnip *Brassicaceae* (Helland *et al.*, 2016).

The present study reported the sweet taste was positively correlated with fructose ($r = 0.817$, $p < 0.0001$), total sugars ($r = 0.685$, $p < 0.0001$), glucose ($r = 0.648$, $p < 0.0001$) and the sugar to GSL ratio ($r = 0.747$, $p < 0.0001$) and 4HGB ($r = 0.737$, $p < 0.0001$) and were in close association. Moreover, sweet taste was positively correlated with few of the GSLs such as GNP ($r = 0.782$, $p < 0.0001$), 4HGB ($r = 0.737$, $p < 0.0001$), GNT ($r = 0.656$, $p < 0.0001$) and negatively correlated with GRM ($r = -0.776$, $p < 0.0001$), GAL ($r = -0.640$, $p < 0.0001$), DMB ($r = -0.589$, $p < 0.0001$), GER ($r = -0.532$, $p < 0.0001$), BTL ($r = -0.777$, $p < 0.0001$) and total GSLs ($r = -0.590$, $p < 0.0001$) suggesting that bitter-tasting compounds such as GSLs can suppress sweet taste (Nor *et al.*, 2020) (Appendix 4.8). The sweet taste was correlated, but not significantly with GRA ($r = 0.385$, $p = 0.001$) which according to previous studies, is a non-bitter compound (Bell *et al.*, 2018). The present study agrees with other studies in the literature on sweet taste being negatively correlated with bitter taste (Helland *et al.*, 2016; Nor *et al.*, 2020).

A clear separation for six lines of 'salad' rocket between the Italian-grown and the UK-grown trial was observed (Figure 4.8.B). The Italian trial was split between genotypes/lines with lines 68, 112, and 130 on the upper side of the PC2 component whereas lines 21, 25, and 72 on the lower side of the PC2 component. Line 68, 112, and 130 in the Italian trial were closely associated with a higher concentration of GSLs such as GKR, GAL, DMB, DGBT, GBT, GTP, total GSLs and sulphur content, which were responsible for sensory attributes such as peppery aroma, earthy aroma, warming mouthfeel, peppery flavour, and bitter taste. Whereas lines 21, 25 and 72 are closely associated with other GSLs such as GAL, 4MP, and GPJ which were responsible for attributes such as moistness mouthfeel, and green aroma.

In the UK trial, lines such as 68, 72, and 112 were closely associated with glucose, total sugars, fructose, and a few GSL such as GNP, 4HGB, GNT and ratio of sugars and GSL and were responsible for sensory attributes such as sweet taste, umami taste, and soapy flavour. The present study further revealed that aroma, flavour, mouthfeel, and aftereffect attributes were as important for discriminating between lines as the taste attribute focused on by other studies (Pasini *et al.*, 2011; Bell *et al.*, 2017a).

The above results suggest that these lines could be a potential candidate for consumers who like their rocket peppery, pungent with a sweet taste. Similar thoughts were shared by Groenbaek *et al.* (2019) who reported that growers should take into account the growing season and the life cycle of cultivars to meet consumers' sensory preferences. These findings revealed that sensory attributes such as aroma, flavour, mouthfeel, and aftereffects traits are as important as the taste attribute and

should be taken into consideration while breeding a new cultivar (Pasini *et al.*, 2011; Bell *et al.*, 2017a).

4.5. Conclusion

There were significant differences in non-volatile compounds (sugars, GSLs, and sulphur) and sensory attributes between the six lines of 'salad' rocket samples grown at two locations: Italy, and the UK. The present study reported a higher concentration of sugars in the UK-grown trial as compared to the Italian-grown plants. The most abundant sugar was glucose, followed by fructose, sucrose, and galactose respectively found in both trials. When compared between six lines of the 'salad' rocket, the most abundant sugars were found in line 68, followed by lines 130 and 112. The present study reported that the 2nd cut UK-grown rocket showed a higher sugar concentration compared to the 1st cut, this could indicate an appropriate agronomic strategy to employ to encourage consumer acceptability towards rocket, except that postharvest shelf life suffers. Sugars were lost during postharvest shelf life from the UK-grown trial, perhaps due to respiration that occurred postharvest. Therefore, slowing down respiration will noticeably improve the flavour during shelf life; however, more study needs to be conducted to justify this. Total GSLs and sulphur contents were higher in the Italian trial as compared to the UK. This study thus accepts the proposed hypothesis of a significant difference in phytochemical content (such as sugars, GSLs, and sulphur) between location, between the 1st cut and 2nd cut and between day 0 (intake) and day 5 (postharvest shelf life). Significant variability in total sugars, total GSLs and sulphur content was observed between location, cuts, and lines and high accumulators of these compounds may be a valuable resource for growers/breeders (Bell *et al.*, 2015).

After analysing the chemical and sensory data, human genotype analysis was conducted on the perception of six lines of 'salad' rocket grown at two locations. Sensory results showed that a sweet taste was negatively correlated with a bitter taste. It showed that the Italian trial was mostly associated with a bitter taste, whereas the UK trial was associated with a sweet and umami taste. It was further revealed that total GSLs and sulphur, which were higher in the Italian trial, were associated with a bitter taste whereas sugars, which were higher in the UK trial, were associated with a sweet taste by the panel assessors. Furthermore, this study accepts the hypothesis of significant differences in sensory profiling between six lines of 'salad' rocket when grown at two locations for the 1st and 2nd cut and change during postharvest shelf life. Lines 68, 112, and 130 showed some interesting sensory characteristics; when grown in the UK growing conditions, leaves of 'salad' rocket perceived a sweeter taste whereas when grown in Italian growing conditions these leaves perceived more pepperiness and pungency attributes. This suggests that depending on consumers' sensory preferences, growers could take location and season into account by growing these lines accordingly.

The present study also showed that panellists with PAV/PAV genotype could distinguish most of the aftereffect attributes such as warming (T0, T1, T2, T3), tingling (T0, T1, T2, T3), numbing (T1, T2, T3), drying (T0, T1, T2) and bitter (T1, T2, T3) traits significantly which were likely to be related to GSL and their hydrolysis product. Whereas the panellists with AVI/AVI genotype could distinguish most of the subtle sensory attributes such as aroma (mustard and earthy), mouthfeel (crunchy, warming, and numbing), taste (sweet and umami), and flavour (soapy, mustard, and burnt) significantly accepting the hypothesis for significant difference between PAV/PAV and AVI/AVI panellists for phytochemical and sensory attributes. Results from the present study have highlighted the components important for determining the taste/flavour of 'salad' rocket leaves by

assessors differing in TAS2R38 genotypes. This will allow the breeding and selection of cultivars for specific environments as well as consumer groups that have a known sensory profile which will result in more acceptability for the ‘salad’ rocket.

CHAPTER 5

General Discussion

5.1. Discussion

The concluding chapter of this thesis highlights the key findings and the impact of this Doctoral project along with contribution to the industry, study limitations, and suggestions for future work. Through the work presented in this thesis, we have undertaken a comprehensive genetic, environmental, phytochemical, and sensory analysis of *Eruca sativa* genotypes derived from a mapping population and conducted robust triplicated experiments in different countries, regions, climates, and cultivation practices to find molecular markers that will help to develop cultivars with improved nutritional 'quality'.

The first phase of the research focused on evaluating and measuring the abundance of phytochemicals (sugars, organic acids, and GSLs) content present in a mapping population of *E. sativa* grown at two separate locations (Italy and the UK) and constructing a linkage and QTL map for metabolites, such as sugar. The second phase of the research was focused on investigating the relationship between sensory attributes and human taste receptors and the accumulation of the phytochemical content on selected six lines of *E. sativa* grown at two separate locations (Italy and the UK). Sensory profiling was conducted for a comparison between the 1st cut and the 2nd cut on the crops and changes during postharvest shelf life. Furthermore, it investigated whether the human taste receptor genotypes perceived any differences in the sensory attributes.

5.1.1. Why was this study conducted?

E. sativa (also known as 'salad' rocket) has been recognised as a rich source of phytonutrient's is a RTE leafy vegetable and has been cultivated worldwide and is well known in medicinal and various cuisines. It has many vital phytochemicals such as GSLs and ITCs that are considered as imparting taste and flavour. Numerous factors such as genetics (crop and human), stresses (abiotic and biotic), season, and cultivation practices influence the attributes such as taste and flavour of 'salad' rocket (Bell and Wagstaff, 2017). So far, little research has been conducted to improve the consistency and perceived nutritional 'quality' of 'salad' rocket leaves, which are contributing factors to the 40% of food waste from the salad industry. One reason has been not meeting consumer expectations of consistency as the crop lacks morphological as well as nutritional uniformity (uniform taste, flavour, and texture). One of the biggest complaints from industrial processors and retailers is regarding inconsistency in quality throughout the year and between the growing locations (Ansah *et al.*, 2018). To improve the crop quality, genome sequencing of the salad rocket was needed to underpin the search for molecular markers for attributes such as bitterness, sweetness, or hotness. The mapping population was grown at separate locations (Italy and the UK) in the hope of finding markers that were stable for a particular trait across different growing environments. This research will help the breeder to select plants containing these attributes to develop a new variety for commercial use.

The main aims of this thesis were to:

(A) to understand the influence of crop genetics and the environment on phytochemical content in a mapping population of 'salad' rocket (*Eruca sativa*) grown at two separate locations in

replicated trials: Italy and the UK. To identify the QTL responsible for the accumulation of sugars that will be used in the future breeding programme of *E. sativa* for nutritional quality.

(B) to understand the influence of crop genetics and environment on the relationship between plant phytochemical content and sensory perception on the selected six lines of 'salad' rocket (*E. sativa*) grown at two separate locations: Italy and the UK. Furthermore, to investigate the relationship between the difference in the genotype of individuals for the bitter taste receptors TAS2R38 and sensory attributes.

5.1.2. Study Findings

To achieve the above aims, in Chapter 3, replicated field trials were conducted on the 141 F3 RILs of the *E. sativa* mapping population which includes Parent B and Parent C. The various phytochemicals such as sugars, GSLs, and organic acids were evaluated and quantified. A previous study reported a higher accumulation of GSLs when plants were grown under raised temperatures (40 °C as compared to 20 °C) (Jasper *et al.*, 2020). The present study reported a higher accumulation of individual phytochemicals to be temperature dependent. A few individual phytochemicals (such as glucose, fructose, galactose, malic acid, GRA, and DMB) were higher in the UK-grown cooler growing conditions, whereas, sucrose, citric acid, GER and GSV were higher in the Italian hot-grown environment. Chapter 3 showed a positive association between sugar accumulation and the UK-growing conditions (Figure 3.7). Total sugars and malic acid accumulated more in *E. sativa* leaves grown in the UK trial than in Italy, possibly due to environmental stress responses arising from the cooler UK temperatures, or because sugars are not turned over so quickly via respiration in the UK crop and therefore accumulate in the leaf, remaining there when the crop is harvested in

the morning. For example, a higher accumulation of the beneficial GSL glucoraphanin was observed when the crop was grown in the UK, whereas a higher accumulation of glucoerucin was found in the Italian-grown leaves (Table 3.1). Glucoraphanin, which is the precursor of ITC, sulforaphane and glucoerucin, a precursor of ITC, erucin, are beneficial health compounds in human health where their effects are well established in the literature (Ying-juan, 2012; Traka *et al.*, 2013). Furthermore, it was revealed in Chapter 4 that glucoraphanin is not bitter and was positively correlated with sugars (Figure 4.8), which will allow consumers to consume the salad rocket leaves without experiencing any bitterness. The research in this thesis demonstrates that crop grown in the cooler UK environment is likely to contain more sugars and a higher abundance of beneficial GSL glucoraphanin than the equivalent grown in the hotter Italian environment. Therefore, if improved crop nutrition is a target, it may prove beneficial for breeders/growers to breed and grow those lines in the UK which will result in a crop that is both nutritious and good acceptability for consumers.

Chapter 3 also reported that the accumulation of phytochemicals is genotype dependent. In a mapping population of 139 RIL, parent B and parent C showed a different accumulation pattern. Parent B showed higher concentrations for most of the compounds as compared to Parent C when grown in an Italian environment (Appendix 3.3). Whereas, in the UK-grown trial, a mixed pattern for sugars, organic acids, and GSLs was observed between parents. Environmental conditions play a key role in influencing the phytochemical content between the two parental lines as well as across the trial locations. In this thesis, we aimed to select a subset of lines from the mapping population that represented the extremes of phytochemical diversity. Whilst these still showed variation in the absolute amount of each phytochemical grown in different locations, they did remain at these extremes compared to the rest of the population and thereby demonstrate the need to combine the

selection of an appropriate genotype with optimal growing conditions if a grower or retailer is aiming to promote a crop that has a particular flavour or nutritional attributes.

Sensory attributes such as the taste and flavour of 'salad rocket' are two important components that are influenced by season and environmental growing conditions (Bell *et al.*, 2020b) which are determined by both GSLs and sugars. Studies have reported GSLs as bitter-tasting compounds and sweetness can mask the bitterness in food. Sugars, other than being used as a substrate for various metabolic pathways, also impart sweetness to food. A positive correlation between total sugars and sweet taste was found in Chapter 4 (Figure 4.8 C). GSL is another metabolite that is the most researched compound in both brassica and rocket because of their potential role as an antimicrobial and their association with human health benefits. GSLs are the plant defence compounds (Jahangir *et al.*, 2009b) produced by all the members of brassica vegetables as well as rocket, that imparts bitterness and pepperiness to leaves. A positive correlation between total GSL and bitterness was reported in Chapter 4 (Figure 4.8 C). Not all GSLs are bitter and some, such as glucoraphanin and glucoerucin, do not have a taste at all. It is not clear what the role of these non-tasting compounds is *in planta*, but they do present an opportunity to develop a crop that is enriched for these compounds that have health benefits (such as lowering the risk of developing certain types of cancer) that remain organoleptically acceptable to the consumer.

Further investigation of the impact of preharvest temperatures and environmental conditions on sensory quality and phytochemical accumulation on rocket was undertaken as studies in the literature do not document these aspects in a single experiment. This was achieved by growing six phytochemically 'extreme' lines (genotypes) of *E. sativa* from the mapping population in two

different environments and investigating the relationship between phytochemicals and sensory analysis by panellists who differ in TAS2R38 bitter taste receptors. A previous study on the consumers by Bell *et al.* (2017b) on *E. sativa* revealed that the ability of individuals to detect and perceived different sensory attributes are related to their TAS2R38 bitter taste receptor genotype. PAV allele individuals could distinguish the aftereffects traits significantly (Figure 4.6 e) whereas individuals with AVI alleles could perceive the rest of the sensory attributes such as aroma (Figure 4.6 a), flavour (Figure 4.6 b), taste (Figure 4.6 c), and mouthfeel (Figure 4.6 d) significantly. The present study further suggested that future breeding programmes should consider such interacting factors between human genotypes and target the development of lines with different organoleptic profiles.

Commercially, rocket leaves are harvested multiple times for leaf consistency and to promote the development of a hot and peppery taste. However, studies on multiple cuts on the same plant and their influence on taste and flavour are missing from the literature. Chapter 4 evaluated the effect of multiple cuts on the same plants on the accumulation of phytochemical composition and its relationship with sensory attributes. This study further found that the accumulation of phytochemicals is dependent on cultivation practices such as polytunnel grown, open field grown, number of harvests and postharvest shelf life. Higher accumulation of sugars was reported when plants were grown in the field as compared to a polytunnel; however, these plants are compromised in terms of postharvest survival which was reported in Chapter 4. The second cut accumulates more sugars (two-fold) in the UK-grown environment compared to Italy, which could be due to environmental stress and the cutting/wounding effect on the leaves during harvest. Furthermore, sugars were lost during shelf life in the UK trial which could be due to cold stress triggering respiration during storage. A clear separation between the two trials, with lines 68, 130, and 112

were positively associated with sensory attributes such as pepperiness, pungency, and sweetness and lines 21, 25, and 72 with attributes such as moistness, green flavour, and green aroma (Figure 4.5 B). The result from the present study suggests that lines 68, 130, and 112 could be used as potential breeding lines to select rocket for consumers who prefer their rocket 'peppery' and 'sweet'. Furthermore, lines 21, 25, and 72 could be selected as pre-breeding lines for those who prefer their rocket 'mild'.

From this study, it was revealed that the sweet taste in rocket was due to the sugars, although other compounds, such as sweet-tasting amino acids (e.g., alanine, glutamine, glycine, serine, threonine, and proline) may also contribute (Bell *et al.*, 2017a). The next step was to identify molecular markers for breeders so that they can breed rocket with consistent quality. To find molecular markers we conducted genotyping of the mapping population and constructed the first linkage map for *Eruca sativa*. We mapped QTL for the phytochemical traits, such as sugars, and further determined if any QTL were co-located across the two environments. We identified 13 QTL from the UK trial for compounds such as fructose, total sugars, glucosativin, glucoerucin, 4-methoxyglucobrassicin, and neoglucobrassicin and seven QTL such as citric acid, malic acid, succinic acid, total acids and progoitrin (putative) from the Italian trial. This study could not identify a single QTL that can be overlapped between two locations for the same trait, which highlights the challenge of developing a crop that performs consistently in terms of organoleptic properties when it is grown in different locations.

5.1.3. Study limitations and further recommendations

The present study did not measure the other phytochemicals that are present in *E. sativa* leaves such as amino acids, flavanols, and volatile organic compounds (VOC) such as alcohols, aldehydes, ketones, and indoles which would have provided us with more detailed knowledge of sensory attributes such as taste and flavour. The present study assumed that sweetness in rocket leaves was due to the presence of sugars, however, studies in the literature provided evidence that other phytochemicals such as amino acids (alanine, threonine, proline, and glutamine) and VOC (1-penten-3 one, 2-hexenal, 2-pentenal, 2-penten-1-ol) have potential to impart sweetness to foods (Bell *et al.*, 2016; Bell *et al.*, 2017a). A previous study on *E. sativa* accessions suggested that the flavour attribute of rocket is not entirely due to the abundance of GSL compounds, but also due to the presence of flavanols such as kaempferol-3 (2-sinapoylglucoside)-4′-glucoside (Bell and Wagstaff, 2014).

The present study did not measure the yield for second-cut rockets grown in the UK when only four of the six lines survived. Furthermore, no microbiological work was conducted on *E. sativa* leaves to evaluate the effect of mesophilic bacteria and total yeast and moulds present during the shelf life. A previous study on rocket reported the development of an off flavour during the shelf life was due to the presence of microorganisms (Yahya *et al.*, 2019).

In Chapter 3, while constructing the linkage map, the present study identified relatively low SNP density markers within the linkage map. The present study could not fully match the chromosome numbers with linkage groups, which may be due to the higher quality SNPs that were selected in the present study for clustering. With further iterations on the mapping population, marker density

and resolution can be improved. Furthermore, this study used the F3 mapping population which means a degree of heterozygosity remains in the population.

Further recommendations

In the present study, we mapped QTL, to identify regions of the genome in *E. sativa* that regulate sugar accumulation, however, further study is needed to identify the underlying genes which would be used in the breeding of 'salad' rocket for improved quality traits. The next step would be to confirm the QTL mapping and use fine mapping to identify more makers on the chromosomal region to find the underlying gene. However, identifying QTL may or may not lead to the underlying gene of interest. Fine mapping or high-resolution mapping will provide a greater number of additional molecular markers linked to QTL (< 5 cM but ideally <1 cM away from the gene) (Collard *et al.*, 2005). Another proposed method which could be used is bulked-segregant analysis to identify additional markers linked to targeting a specific region on a chromosome. Furthermore, identifying a QTL suitable for all the growing conditions would allow breeders to develop a cultivar for commercial use, which will be more consistent with the quality that will provide the consumer with a better-quality product.

Through this study, we found that leaves when subjected to abiotic stress, accumulate various phytochemicals. It is predicted that climate change will have a major impact on food production and thus on food security. Extreme weather conditions such as higher than average temperature, more flooding or drought or salinity or higher percentage of carbon dioxide in the atmosphere etc., have already been experienced. Further research is suggested to evaluate the impact of these stresses on the same 141 lines of mapping population of the 'salad' rocket. This will provide a

better understanding of the particular stress on an accumulation of phytochemicals which might confirm the molecular markers identified in the present study. This strategy of combining genetic and chemical information will make it possible to breed rocket for increased consumer acceptance while maintaining the maximum health benefits associated with the crop.

It is further recommended to conduct clinical trials to test any beneficial health effects of rocket on humans. Much work is needed to be done to understand how GSLs present in rocket leaves interacts with the human gut microbiota to know how different individuals assimilate, metabolite, and excrete ITCs. Few studies are conducted on broccoli vegetables where individuals were asked to consume broccoli and their then biological samples were analysed to see the effect on health (Conaway *et al.*, 2000; Sivapalan *et al.*, 2018; Kellingray *et al.*, 2020). However, the abundance of individual GSL differs with different organs in the plant and within different brassica families. Furthermore, to properly assess the health-promoting effects of rocket leaves, nutritional intervention studies are needed to be conducted to know how much rocket leaves are needed to be consumed in the daily diet to get the benefits on health. It is also recommended to find the sensory thresholds for GSLs which are related to bitterness in the present study

Use of new plant breeding technologies

It is predicted that by 2050, the world needs to produce up to 70% more food to feed the growing population despite climate change, biodiversity loss and pressure on finite natural resources such as land, water, and energy. Moreover, the war in Ukraine has imbalanced the global food supply and demand. To fulfil the demand of the growing population, plant breeding with various techniques such as crosses between two species, hybridisation, mutagenesis etc., could be the

solution and was successful to a certain extent, however, it can lead to random outcomes (Qaim, 2020). Therefore, to overcome the above issues, new plant breeding technologies (NPBT) such as gene editing (GE) (may or may not introduce foreign DNA) and genetically modified (GM) (often referred to as transgenic, where foreign DNA is introduced) crops could be an alternative. NPBT has opened a new horizon as these could contribute to higher yields, lower use of fertilisers and pesticides, reduce postharvest losses, better crop resilience to climatic stress and most importantly more nutritious foods (Qaim, 2020). Having said so, GM crops are not widely used and accepted, even after 30 years of research and commercial applications due to continuous widespread concerns over possible negative health consequences on the environment and hesitancy and fear of losing export markets with Europe to developing countries.

The GE technology is fairly new and is developed a decade ago by scientists. CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats) has emerged as one of the foremost techniques to accurately insert and alter DNA from the same species (i.e., cis-genic) or the different species (i.e., trans-genic) with the target specificity in the crop genome (Zaidi *et al.*, 2019). The use of CRISPR/Cas9 could be a potential solution to manipulate the genome sequence of *E. sativa* by altering a site-specific locus by finding the gene that is responsible for sugar accumulation. However, developing a design specifics guide RNA (sgRNA) with a complementary sequence would be time-consuming and challenging as this has not previously been developed for *E. sativa*. Furthermore, how much it will be used specifically in *E. sativa* to be able to resolve the issue with product consistency and nutritional quality, will be interesting to know. Regulatory approaches for GE crops are still evolving. Therefore, urgent attention is needed for an appropriate regulatory response toward the social acceptance of GE crops when released to consumers (Araki and Ishii, 2015).

Recently a study on the consumers conducted by Shew *et al.* (2018) on 'willingness-to-consume' and 'willingness-to-pay' for CRISPR-produced food compared to GM foods, reported that consumers would consume both foods. Their study further added that if efforts are made to increase consumer biotechnology knowledge and awareness, it may increase consumer acceptance of CRISPR-produced foods and biotechnology. Therefore, it is necessary for researchers, academics, and regulatory bodies to proactively discuss and educate the consumer on the socially acceptable integration of genome editing crops to improve global food security (Araki and Ishii, 2015). Things are changing slowly post Brexit in the UK. Recently, a new bill was introduced in the parliament and the UK government has given a green signal to the GE tomatoes to be grown commercially and sold in England (Li *et al.*, 2022). So, there is hope that GE crops will soon be a reality.

5.1.4. Contribution to the industry

The information generated from this study could be useful to growers, manufacturers, retailers, and consumers as the information could be used to guide them to extend the quality of the fresh produce. This will avoid food loss that leads to economic losses to the growers and all parties involved along the supply chain. In terms of agronomic practices, *Eruca* does not need any additional fertilizers such as nitrogen or sulphur as compared to similar crops such as lettuce (Hall *et al.*, 2012a). It can withstand drought as it has the potential to grow and survive in an arid region (Garg and Sharma, 2014). Furthermore, the *Eruca* crop can be grown with high plant density with efficient use of land (Frezza *et al.*, 2010). The above-mentioned agronomic factors thus satisfy the objective of sustainable farming/agriculture which is the utmost priority in current and future farming practices (using resources efficiently). This information will be useful to growers/farmers

who can use fewer resources and thus save money as well as resources. Moreover, during processing, *Eruca* leaves do not need chlorinated water (sanitiser) or MAP to extend shelf life, this will be a useful tool for manufacturers as the installation of MAP machinery needs capital investments. The use of chlorinated water during washing may leave a chlorine odour on leaves, which could sometimes not be widely accepted by consumers. Moreover, at the European level, there is still a discussion about its use (Gil *et al.*, 2014). However, the present study was not affected by either the use of MAP or chlorinated water to extend the shelf life of rocket leaves, which could be a win-win situation for both manufacturers and consumer.

The results from the present study will benefit the plant breeding companies such as Elsoms Seeds (one of the sponsors of this project) in terms of breeding for improved and consistent taste and flavour. Elsoms Seeds will be able to use the results from Chapter 3 where a potential marker for sugars was identified. These could be applied in the breeding programme using marker-assisted selection. The use of the marker-assisted selection method will provide better insight which may greatly increase the efficiency and effectiveness of the breeders to develop a novel cultivar which will have a consistent nutritional quality.

The other sponsor, Bakkavor Holdings Ltd (which owns 36 smaller companies and has relationships with several other growers) will be benefited where the postharvest data generated in Chapter 4 will inform the small growers with preharvest decision-making. This information will help the growers to have consistent ‘quality’ in terms of flavour and taste at the point of harvest. The postharvest data from Chapter 4 on phytochemical content (such as sugars and GSLs) on

selected six lines, will help growers to select those lines for the consumers with various preferences regarding taste.

In organic farming as well as in the agroecosystems, extract of GSL could be used as a botanical fungicide as this is emerging as an alternative tool. A study in the literature proposed the use of *E. sativa* as an intercropping herb with mustard to reduce the effect of mustard aphids (Garg and Sharma, 2014). Furthermore, *E. sativa* oil and leaf extract could be used in the pharmaceutical industry as they had shown good potential as insect repellents. Previous studies on *E. sativa* have reported the possible role of individual GLS as an antimicrobial agent (Yahya *et al.*, 2019).

5.2. Conclusion

E. sativa is a promising crop for future improvement through selective breeding as it has numerous nutritional and sensory quality traits that are beneficial to human health. 'Quality' is subjective and needs to be understood and accounted for when improving crops. While selecting the breeding programme for the development of a new cultivar, attention and priority must be given to the phytochemical content and sensory characteristics and must not be solely focused on the morphological traits, and this message has been disseminated throughout the present thesis. The present thesis also revealed that abiotic stresses can be used as a tool to enhance the phytochemical content in *E. sativa* crops (growing salad crops in a glasshouse condition by lowering the growing temperature), however, the effects of practical applications can vary depending upon crop genetics, agronomical practices, environmental conditions, and the combination of the above factors.

Any improvement of cultivars of any crop species should therefore account for diverse taste preferences to ensure high acceptance and consumption, resulting in better consumer health. Care should be taken to account to cater for the diverse preferences of consumers, such as for those who prefer their rocket ‘mild’, and others who prefer ‘peppery’ and ‘sweet’ must also be marketed appropriately to get the health benefits of ‘salad’ rocket. It should further be subdivided according to sensory properties and their intended consumer demographic, just the same way apples are subdivided according to their differing sweet and sour tastes. Finally, with the available genetic resources and the falling costs of sequencing and bioinformatics, and the use of the sensor-based platform to measure the phenotypic traits such as near-infrared spectroscopy, it will soon be possible to produce nutritively superior varieties of ‘salad’ rocket.

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APPENDICES

Appendix 1. 1. Paper as a co-author.

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ORIGINAL RESEARCH
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The *Eruca sativa* Genome and Transcriptome: A Targeted Analysis of Sulfur Metabolism and Glucosinolate Biosynthesis Pre and Postharvest

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Rocket (*Eruca sativa*) is a source of health-related metabolites called glucosinolates (GSLs) and isothiocyanates (ITCs) but little is known of the genetic and transcriptomic mechanisms responsible for regulating pre and postharvest accumulations. We present the first *de novo* reference genome assembly and annotation, with ontogenic and postharvest transcriptome data relating to sulfur assimilation, transport, and utilization. Diverse gene expression patterns related to sulfur metabolism, GSL biosynthesis, and glutathione biosynthesis are present between inbred lines of rocket. A clear pattern of differential expression determines GSL abundance and the formation of hydrolysis products. One breeding line sustained GSL accumulation and hydrolysis product formation throughout storage. Multiple copies of MYB28, SLIM1, SDI1, and ESM1 have increased and differential expression postharvest, and are associated with GSLs and hydrolysis product formation. Two glucosinolate transporter gene (GTR2) copies were found to be associated with increased GSL accumulations in leaves. Monosaccharides (which are essential for primary metabolism and GSL biosynthesis, and contribute to the taste of rocket) were also quantified in leaves, with glucose concentrations significantly correlated with the expression of numerous GSL-related genes. Significant negative correlations were observed between the expression of glutathione synthetase (GSH) genes and those involved in GSL metabolism. Breeding line **"B"** showed increased GSH gene expression and low GSL content compared to two other lines where the opposite was observed. Co-expression analysis revealed senescence (*SEN1*) and oxidative stress-related (*OXS3*) genes have higher expression in line **B**, suggesting that postharvest deterioration is associated with low GSL concentrations.

Keywords: rocket (*Eruca sativa* and *Diplotaxis tenuifolia*), isothiocyanate, postharvest, sulfur assimilation, glucosinolate transport, transcription factor, MYB28, SLIM1

INTRODUCTION

Sulfur (S) is a critical macronutrient that plants require for growth and development (Kopriva et al., 2016). Sulfate (SO_4^{2-}) is utilized as a primary means of synthesizing numerous S-containing metabolites, such as amino acids (cysteine and methionine), alkyl-cysteine-sulfoxides, glutathione (GSH), and glucosinolates (GSLs; Frerigmann and Gigolashvili, 2014). GSL compounds are present in species of the order Brassicales, and are abundant in many vegetables and condiments worldwide, such as rapeseed (*Brassica napus*), Chinese cabbage (*Brassica rapa*), cabbage (*Brassica oleracea* var. *capitata*), and broccoli (*B. oleracea* var. *italica*; Yan and Chen, 2007). GSLs are also found in the leafy vegetable *Eruca sativa* ("salad" rocket), which has gained significant popularity amongst consumers over the last 10 years (Bell and Wagstaff, 2014). Rocket is known for its distinctive flavor, aroma, and pungency, and can be eaten raw without the need for cooking (Bell et al., 2017a), which can lead to a loss of nutritional benefits.

Sulfur assimilated by Brassicales plants is thought to be a strong determining factor in the biosynthesis of GSLs (Pandey et al., 2017). GSLs themselves are not bioactive, and are hydrolyzed by myrosinase enzymes (β-thioglucoside glucohydrolase; TGG) when tissue damage takes place. They form numerous breakdown products including isothiocyanates (ITCs; Wittstock and Burow, 2010), which are of foremost interest for their anticarcinogenic effects in humans (Satyan et al., 2006). The retention of GSLs in the postharvest storage period of rocket is therefore of critical importance for maximizing the potential health benefits for consumers (Martinez-Sanchez et al., 2006).

Salad rocket produces the ITC sulforaphane (SF; a breakdown product of 4-methylsulfinylbutyl GSL; glucoraphanin, GRA), which has been well documented for its potent anticarcinogenic properties (Herr et al., 2010). SF is abundant in broccoli, however its hydrolysis from GRA is often inhibited or prevented due to high cooking temperatures employed by consumers, which denatures myrosinase at temperatures >65°C (Rungapamestry et al., 2007).

A previous study by Bell et al. (2017b) observed that both GSL and ITC concentrations increased significantly in rocket salad post-processing, but that this varied according to cultivar. The study also highlighted that abundances at the point of harvest were not reflective of those found after 1 week of cold storage. The authors proposed that in response to the harvesting and washing process, stress responses within leaf tissues were initiated, leading to the increase in synthesis of GSLs and subsequent hydrolysis into ITCs. Sugar content, by comparison, showed little dynamic change and little reduction in the same samples, which could have implications for sensory perception and consumer acceptance (Bell et al., 2017a). For these reasons, GSLs and their breakdown products are of importance and interest to plant breeders and the scientific community.

Glucosinolates are synthesized as part of plant defense mechanisms against pests and diseases (Winde and Wittstock, 2011), and can also act as important S storage molecules (Kopriva et al., 2016). Compounds such as glucosativin (4-mercaptobutyl GSL; GSV) and glucorucolamine (4-cystein-S-yl-butyl GSL; GRL) are unique to the genera *Eruca* and *Diplotaxis* ("wild" rocket; Kim et al., 2007). GSV can exist in a dimer form (dimeric 4-mercaptobutyl GSL; DMB), and diglucothiobeinin [4-(β-D-glucopyranosyldisulfanyl)butyl GSL; DGTB] is a unique GSL dimer of these species (Bell et al., 2015). Despite the advances made in elucidating the *Arabidopsis thaliana* and *B. oleracea* GSL pathways, very little novel gene discovery has taken place outside of these species. The reason for this is the lack of genome sequence available for niche Brassicales species like *E. sativa*, and reliance upon knowledge about common compounds in related species, which is not able to account for the large differences observed in the GSL profile of rocket. Much is now known about the "core" GSL biosynthesis pathway in Arabidopsis and the regulatory mechanisms that respond to different biotic and abiotic stimuli (Francisco et al., 2016). Six main R2R3 MYB transcription factors (TFs) have been identified as regulators of GSL synthesis.

Aliphatic GSLs are regulated by MYB28, MYB29, and MYB76 TFs, and indolic GSLs by MYB34, MYB51, and MYB122 (Frerigmann and Gigolashvili, 2014). These MYBs are in turn regulated by basic helix-loop-helix (bHLH) TFs such as MYC2, which are involved in plant defense response (Kazan and Manners, 2013). Other transcriptional regulators, such as *SLIM1* (*SULFUR LIMITATION 1*) and *SDI1* (*SULFUR DEFICIENCY INDUCED 1*) also interact with MYB TFs to regulate the use and efficiency of sulfur within the plant. As GSLs are a major sulfur sink (up to 30% of total plant S-content) the synthesis and catalysis of these compounds is crucial in times of stress (**Figure 1**; Chan et al., 2013; Aarabi et al., 2016).

Individual downstream GSL biosynthesis genes are regulated in response to a wide range of stress stimuli in response to changes in both MYB and MYC activity. Some of the most studied are genes encoding methylthioalkylmalate synthase (MAM) enzymes (regulators of GSL side chain lengths), genes encoding CYP79 enzymes (catalysts of the conversion of chain elongated amino acids into their respective aldoximes; Bell, 2019), and genes encoding CYP83 enzymes (that convert indolic aldoximes into corresponding thiohydroxymates; Bak and Feyereisen, 2001). Studies relating regulation to common horticultural practice, or transcriptomic regulation and response, are lacking. The effects of stresses imposed by harvesting, washing, processing, and storage differs between cultivars is not understood. *E. sativa* is a crop with great potential for enhancement of nutritional value, and it is therefore essential to understand how GSL biosynthesis and sulfur metabolism are regulated in order to direct breeding programs.

We present a *de novo E. sativa* reference genome sequence, and report on the specific effects harvest, wash treatment, and postharvest storage have on GSL biosynthesis and sulfur metabolism gene expression through RNA sequencing (RNAseq) in three elite inbred lines. We also present evidence of transcriptomic changes between first and second cuts of rocket plants, and how this in turn leads to elevated concentrations of both GSLs and ITCs. We hypothesized that each rocket line would vary in its ability to retain and synthesize GSLs post

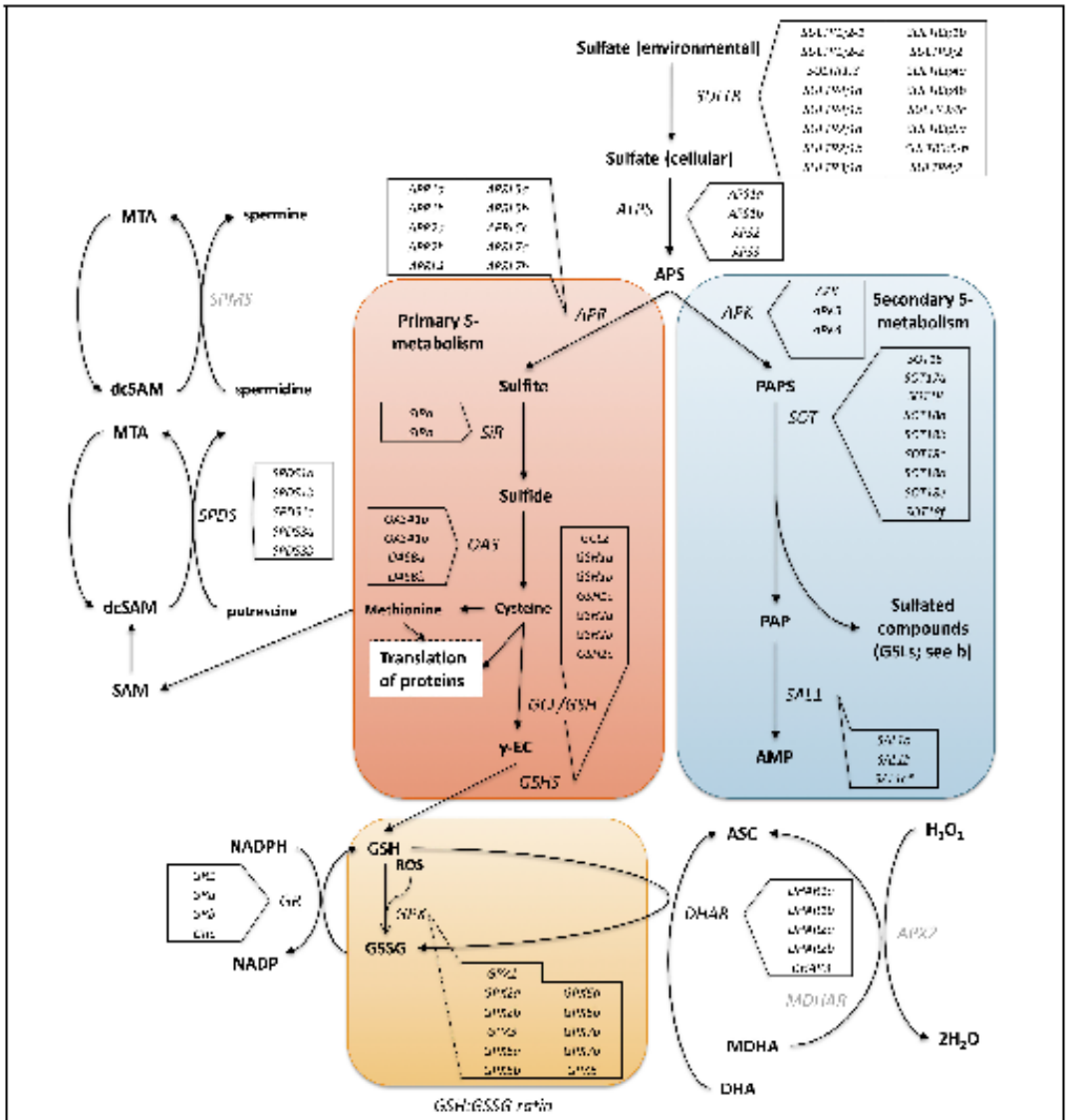

FIGURE 1 | The primary (red box) and secondary (blue box) sulfur metabolism pathways of *A. thaliana* with identified homologous genes within the *Eruca sativa* genome annotation (see boxed insets) adapted from Chan et al. (2013). Environmental sulfur is assimilated and integrated into key amino acids (cysteine and methionine) and enzymes. Sulfur metabolism is also intrinsically linked with oxidative stress via glutathione synthesis. Under stress conditions 5′-phosphoadenosine-3′-phosphate (PAP), glutathione disulfide (GSSG), and reduced glutathione (GSH) direct sulfate toward GSH production. The GSH:GSSG redox state ratio is also known to influence sulfur assimilation rates (orange box). *SOT* (sulfotransferase) genes link secondary sulfur metabolism with the final sulfation step of GSL biosynthesis, and it is thought that *SAL1* plays an important role in regulating the activity of these genes through interaction with PAP. Genes with identified orthologs in the *E. sativa* genome annotation are written in black; those with no identified homologous sequence are written in gray. *SULTR*, sulfate transporter; ATP, adenosine triphosphate; *ATPS*, *ATP sulfurylase*; *APR*, *APS reductase*; *APK*, *APS kinase*; SIR, sulfite reductase; *OASTL*, *O-acetylserine lyase*; *GCL*, *glutamate cysteine ligase*; γ-EC, γ-glutamyl-cysteine; *GSHS*, *GSH synthetase*; *GR*, *glutathione reductase*; *GPX*, *glutathione peroxidase*; ASC, ascorbate; DHA, dehydroascorbate; *DHAR*, *DHA reductase*; MDHA, monodehydroascorbate; *MDHAR*, *MDHA reductase*; *APX2*, *ascorbate peroxidase 2*; H_2O_2, hydrogen peroxide; PAPS, 5′-phosphoadenosine-3′-phosphosulfate; SAM, S-adenosyl methionine; dcSAM, decarboxylated SAM; MTA, methylthioadenosine; *SPDS*, *spermidine synthase*; *SPMS*, *spermine synthase*. *Indicates a novel gene identification within the genome annotation.

washing and during shelf life cold storage, as well as vary in their relative abundances between first and second cuts.

MATERIALS AND METHODS

Plant Material for Genome Sequencing

Three elite inbred lines of salad rocket were produced through self-pollination for five generations at Elsoms Seeds Ltd. (Spalding, United Kingdom) from 2010 to 2016, giving an estimated inbreeding coefficient of 0.969 (Falconer and Mackay, 1996). Each line was derived from germplasm accessions obtained from the Leibniz-Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK Gatersleben, Germany). For reasons of commercial sensitivity these lines (**A**, **B**, and **C**) and their lineage will not be identified.

For genome sequencing, plants of each line were grown under controlled growth room long-day cycle light conditions (200 μmol m^{-2} $s^{-}1$; 22°C day, 15°C night) and watered as required. Leaf tissues were sampled and immediately frozen at −20°C. DNA was extracted using an E.Z.N.A. Plant DNA DS Mini Kit (Omega Bio-Tek, Norcross, GA, United States) in triplicate according to the manufacturer protocol, and sent to the Earlham Institute (Norwich, United Kingdom) for QC analysis. DNA samples for each line were pooled and quantified using a Qubit fluorometer and dsDNA assay kit (Thermo Fisher Scientific, Loughborough, United Kingdom) and assessed for quality using NanoDrop (Thermo Fisher Scientific). QC data for the sequenced DNA samples are provided in **Supplementary Table 1**.

Genome Sequence Library Preparation and Assembly

De novo reference genome sequence was produced by interleaving Illumina MiSeq and HiSeq2500 sequence data (Illumina Inc., San Diego, CA, United States). DNA sequencing and assembly was performed as a service by the Earlham Institute. *De novo* genome sequencing and assembly was performed using PCR free paired-end (PE) and long mate pair (LMP) sequencing. After DNA sample QC, line **C** was selected for sequencing and reference genome assembly. One PCR free PE library was constructed from gDNA, and sequenced on one lane of an Illumina HiSeq2500 in rapid run-mode (v2) using 250 bp PE reads. LMP sequencing was also conducted using one set of Nextera libraries (Illumina) from gDNA, and sequenced on one lane of an Illumina MiSeq with 250 bp PE reads. After data QC and assembly of the high coverage PE library, LMP libraries were mapped to determine their suitability for assembly improvement. Three additional libraries were selected and re-sequenced to a higher depth of coverage on a single lane of an Illumina HiSeq2500 in rapid run-mode, to again yield 250 bp PE reads.

Genome Sequencing Bioinformatics

FASTQ files were converted to BAM format using PicardTools (v1.84[1]; FastqToSam option) and then assembled using DISCOVAR *de novo* sequence assembler (build revision 52488; Weisenfeld et al., 2014). All LMP libraries were processed using NextClip (Leggett et al., 2014) to analyze and create a high quality read subset for scaffolding the DISCOVAR-assembled sequences. SOAP (Li et al., 2008) and SSPACE (Boetzer et al., 2011) were used to scaffold the DISCOVAR assembly using data from three of the NextClip-processed LMP read libraries.

Genome Annotation

Annotation was performed by Novogene Co., Ltd. (Hong Kong). A homology and *de novo*-based approach was taken in order to identify TEs. The homology-based approach used known repetitive sequence databases: RepBase (Jurka et al., 2005), RepeatProteinMask, and RepeatMasker.[2] *De novo* repeat libraries were created using LTR_FINDER (Xu and Wang, 2007), RepeatScout (see text footnote 2), and RepeatModeler.[3]

An integrated approach was taken to compute consensus gene structures, such as cDNA, proteins in related species, and *de novo* predictions (**Figure 2A**). The homology-based approach used the related genomes of *Arabidopsis lyrata*, *A. thaliana*, *B. napus*, *Boechera stricta*, *Capsella rubella*, and *Raphanus sativus* to compare against *E. sativa* to find homologous sequences, and predict gene structures (using BLAST and genewise; Kent, 2002; Birney et al., 2004). *Ab initio* statistical models were also used to predict genes and their intron-exon structures; e.g., Augustus (Stanke et al., 2006), GlimmerHMM (Majoros et al., 2004), and SNAP.[4] EVidenceModeler (EVM; Haas et al., 2008) software was then used to combine *ab initio* predictions, protein, and transcript alignments, and RNAseq data into weighted consensus gene structures. Lastly, PASA was used to update the consensus predictions by adding UTR annotations and models for alternative splicing isoforms. All predicted proteins were functionally annotated using alignments to SwissProt, TrEMBL (Bairoch and Apweiler, 2000), KEGG (Kanehisa and Goto, 2000), and InterPro (Zdobnov and Apweiler, 2001; **Figure 2B**).

The full reference genome sequence and annotation can be found in the European Nucleotide Archive (Assembly accession no: GCA_902460325; Study ID: PRJEB34051; Sample ID: ERS3673677; Annotation accession number ERZ1066251).

Plant Material Growth and Collection for RNA, Elemental, and Phytochemical Analyses

Seeds were sown in a random order in seedling compost, and raised under controlled environment conditions in plastic trays inside a Weiss-Technik Fitotron cabinet (Weiss-Technik UK Ltd., Loughborough, United Kingdom). Daytime temperature was set to 20°C, and nighttime temperatures to 14°C (long day cycle; 16 h light, 8 h dark). Light intensity was set at 200 μmol m^{-2} s^{-1}. During a 1-h period of "dawn" and "dusk," light and temperature changes were ramped on a gradient. Humidity was ambient. After 10 days of growth, seedlings were transplanted to 1-L pots in standard peat-based compost.

[1]http://broadinstitute.github.io/picard/

[2]http://www.repeatmasker.org/

[3]http://www.repeatmasker.org/RepeatModeler.html

[4]http://homepage.mac.com/iankorf/

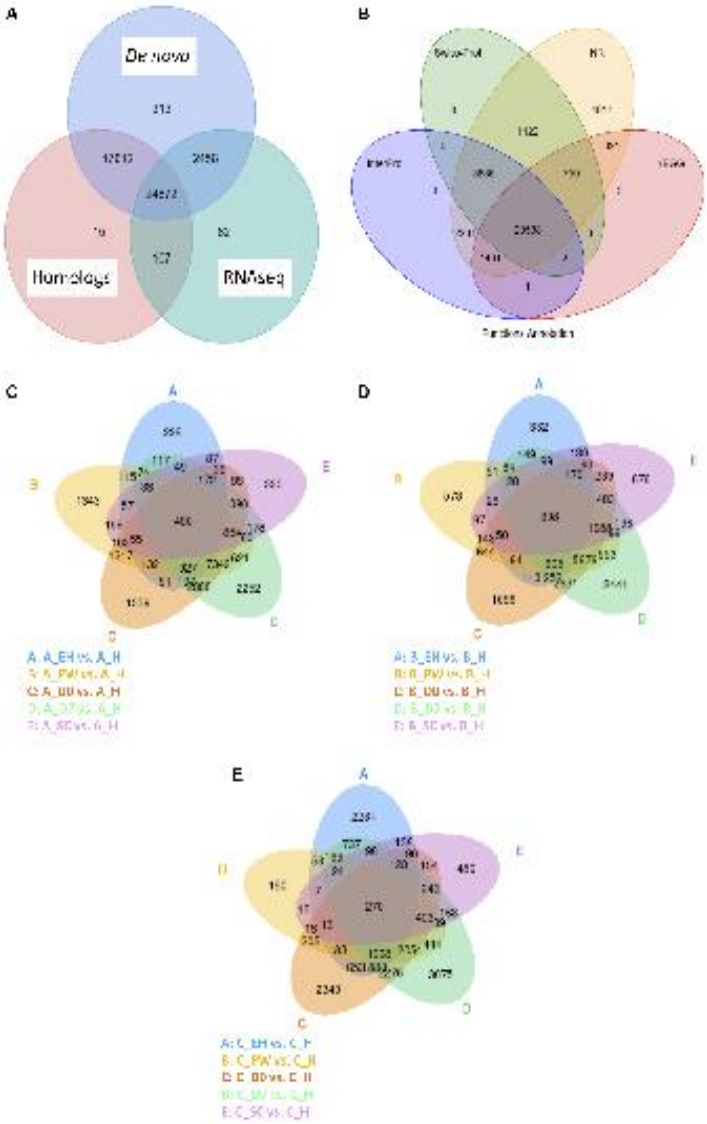

FIGURE 2 | Venn diagrams of the *Eruca sativa* reference genome annotation gene identification sources **(A)** and functional annotation databases used to assign putative gene identities **(B)**. Also shown are Venn diagrams of global differentially expressed genes (DEGs) at an early harvest **(EH)**, second harvest **(SC)**, pre-wash **(PW)**, post-wash **(D0)**, and 7-day shelf life **(D7)** time points relative to a first harvest **(H)** time point of three elite breeding lines: **A (C)**, **B (D)**, and **C (E)**. The numbers of DEGs identified under each condition are contained within the ellipses and their overlaps.

Postharvest (post sample H), leaves were stored for 2 days in a cold store (4°C; Bell et al., 2017b). Samples for D0 and D7 were washed individually in mildly chlorinated water (sodium hypochlorite, 30 ppm; Suslow, 2000) for 2 min, then rinsed for 1 min with distilled water (all at >14°C to avoid cold-shock). Leaves were dried of excess moisture for 1 min using a kitchen salad spinner, then placed in fresh bags, sealed, and stored overnight at 4°C. Shelf life leaves were stored in the cold and dark (4°C) for 7 days (D7) – typical of the use-by date of commercially bagged leaves.

All samples were taken between the hours of 1–3 pm to mitigate diurnal fluctuations in phytochemical content and gene

expression (Huseby et al., 2013). Immediately after each of the aforementioned samples was taken, leaves were frozen using liquid nitrogen and ground into a fine powder using a pestle and mortar. Samples were stored at −80°C in tubes and lyophilized prior to chemical analysis. A subset of non-lyophilized sample was kept aside for RNA extractions.

RNA Extraction and Quality Control

RNA sequencing and bioinformatics was conducted on 18 plants from three elite inbred lines designated **A**, **B**, and **C**; giving a total of 54 plant samples. Time points corresponded to three harvest times ("early harvest" at 22 days after sowing, **EH**; "harvest" at 30 days after sowing, **H**; "second cut," **SC**; leaves harvested from the same **H** plants 43 days after sowing), and three consecutive postharvest time points (harvested at 30 days after sowing and designated: "pre-wash," **PW**; "day 0" of shelf life, 1 day post wash, **D0**; and "day 7" of shelf life, **D7**). See **Supplementary Figure 1** for a schematic of the experimental design.

RNA for RNAseq and qRT-PCR analyses was extracted using RNeasy Plant Mini Kits (Qiagen, Manchester, United Kingdom) according to the manufacturer "Plants and Fungi" procedure. As part of the protocol, an on-column DNase digestion was incorporated according to the manufacturer RNase-Free DNase Set (Qiagen) protocol. Samples were checked for degradation and contamination prior to sequencing using agarose gel electrophoresis (1%, TAE buffer), Qubit, and NanoPhotometer (Implen, CA, United States) methods. Briefly, $\geq$2 μg of total RNA was obtained for each sample at a minimum concentration of $\geq$50 ng μL^{-1}. RNA integrity was determined and evaluated using an Agilent 2100 Bioanalyzer (Fleige and Pfaffl, 2006). QC data for all RNA samples is provided in **Supplementary Table 2**.

RNAseq Library Preparation and Sequencing

After QC procedures, sequencing libraries of three replicates were prepared using NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, MA, United States) following the manufacturer's instructions, and index codes were added to attribute sequences to each sample. mRNA was purified from total RNA by using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5×). First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H-). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3′ ends of DNA fragments, NEBNext Adaptor with hairpin loop structure were ligated to prepare for hybridization. In order to select cDNA fragments of 150–200 bp in length preferentially, the library fragments were purified with an AMPure XP system (Beckman Coulter, MA, United States). Three microliters USER Enzyme (NEB) was used with size-selected, adaptor-ligated cDNA at 37°C for 15 min, followed by 5 min at 95°C before PCR. PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. Finally, PCR products were purified (AMPure XP system) and library quality was assessed using an Agilent 2100 Bioanalyzer system.

The clustering of the index-coded samples was performed on a cBot Cluster Generation System using HiSeq PE Cluster Kit cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina Hiseq platform and 125/150 bp PE reads were generated.

RNAseq Bioinformatics

Raw data (raw reads) of FASTQ format were firstly processed through Novogene Co., Ltd., in-house perl scripts. Clean reads were obtained by removing reads containing adapter, reads containing ploy-N, and low quality reads from the raw data. Q20, Q30, and GC content of the clean data were calculated.

An index of the reference genome was built using Bowtie (v2.2.3), and PE clean reads were aligned to the reference genome using TopHat (v2.0.12; Langmead et al., 2009; Anders et al., 2010; Langmead and Salzberg, 2012). TopHat was selected as the mapping tool as it can generate a database of splice junctions based on the gene model annotation file, and thus a better mapping result is achieved than other non-splice mapping tools.

HTSeq (v0.6.1) was used to count the read numbers mapped to each gene (Trapnell et al., 2010). FPKM (Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced) of each gene was calculated based on the length of the gene and reads count mapped to each gene. Differential expression analysis of each sample point/inbred line (three biological replicates) was performed using the DESeq R package as described by Anders et al. (2010; 1.18.0). After normalization, the resulting *p*-values were adjusted using Benjamini and Hochberg's approach for controlling the false discovery rate (*q*-value). Genes with a *q*-value < 0.05 were assigned as being significantly differentially expressed.

RNAseq Validation by qRT-PCR

Independent RNA extractions were conducted for qRT-PCR validation, and quality checked according to the same protocols and instrumentation as for RNAseq. cDNA synthesis was conducted using qPCRBIO cDNA Synthesis Kit (PCR Biosystems Ltd., London, United Kingdom) according to the manufacturer instructions. cDNA was then diluted 10× prior to analysis. All 54 biological samples were tested in triplicate.

PCR primers were designed using PRIMER3[5] using default settings. Ten genes related to GSL biosynthesis and transcription were selected at random for the validation analysis (*BCAT4, CYP83B1, MYB122-1a, MYB51a, SOT16, SUR1, TGG1b, TGG1d, TGG1j*, and *UGT74B1*), with *ACT11* used as a reference gene (Hu et al., 2009). Gene sequences of *E. sativa* were obtained using NovoFinder (Novogene Co., Ltd.), and primer annealing sites were designed to span intron–intron boundaries where possible (see **Supplementary Table 3**).

Analysis was performed using the $2^{-\Delta\Delta}$ method (Livak and Schmittgen, 2001) on a Roche LightCycler 480 Instrument and the manufacturer Advanced Relative Quantification protocol

[5]http://bioinfo.ut.ee/primer3/

(v1.5.1). Primer efficiencies were determined by analyzing each primer set with log-fold dilutions of cDNA (**Supplementary Table 3**). 2× qPCRBIO SyGreen Blue Mix Lo-ROX (PCR Biosystems Ltd.) was used to prepare a master mix for all reactions. Reaction volumes totaled 10 µL, and the PCR method used was as per the manufacturer recommendations.

Data were normalized and expressed as the log2-fold change relative to *ACT11*. RNAseq data for each of the tested genes were similarly converted for direct comparison of the two methodologies (**Supplementary Figure 3**). An ANOVA test found no significant difference between the two data sets.

Co-expression Module Identification and Gene Set Enrichment Analysis

Full gene expression data of lines **A**, **B**, and **C** were analyzed using the webCEMiTool (Co-Expression Module Identification Tool) pipeline (Russo et al., 2018; Cardozo et al., 2019). A variance filter value of 0.01 was used to ensure the highest level of statistical stringency. RNAseq normalization mean variance dependencies were corrected using the Variance Stabilizing Transformation (VST) option. Pearson's correlation method was selected for identification of the gene modules. As part of the pipeline, a Gene Set Enrichment Analysis (GSEA) was performed using each module as a gene set. A Normalized Enrichment Score (NES) was generated for each phenotype, as well as a Benjamini-Hochberg *q*-value.

Intact Glucosinolate Extraction and Analysis by LC-MS

Intact GSLs were extracted according to the protocol used by Bell et al. (2015). Immediately before LC-MS analysis, samples were diluted with 4 mL of HPLC-grade water. Samples were analyzed in a random sequence with standards and QC samples. External standards of sinigrin (SIN; >99%, TLC), GRA (99.86%, HPLC), glucoalyssin (GAL; 98.8%, HPLC), 4OHB (96.19%, HPLC), and GER (99.68%, HPLC) were prepared for quantification of GSL compounds. SIN was used to quantify DGTB, GSV, and DMB, as no standards are available for these compounds. 4OHB was used to quantify the indole GSLs 4MOB and neoglucobrassicin (NGB). All standards with the exception of SIN (Sigma Merck, Gillingham, United Kingdom) were purchased from PhytoPlan (Heidelberg, Germany). Limits of detection (LOD) and quantification (LOQ) were established for the method by running serial dilutions of SIN (LOD = 2.14 $mg.kg^{-1}$; LOQ = 6.48 $mg.kg^{-1}$).

LC-MS analysis was performed in the negative ion mode on an Agilent 1260 Infinity Series LC system (Agilent, Stockport, United Kingdom) equipped with a binary pump, degasser, auto-sampler, column heater, and diode array detector, coupled to an Agilent 6120 Series single quadrupole mass spectrometer. Separation of samples was achieved on a Gemini 3 µm C_{18} 110Å (150 × 4.6 mm) column (with Security Guard column, C_{18}; 4 mm × 3 mm; Phenomenex, Macclesfield, United Kingdom). GSLs were separated during a 40 min chromatographic run, with a 5 min post-run sequence. Mobile phases consisted of ammonium formate (0.1%; A) and acetonitrile (B) with the following gradient timetable: (i) 0 min (A–B, 95:5, v/v); (ii) 0–13 min (A–B, 95:5, v/v); (iii) 13–22 min (A–B, 40:60, v/v); (iv) 22–30 min (A–B, 40:60, v/v); 30–35 min (A–B, 95:5, v/v); (v) 35–40 min (A–B, 95:5, v/v). The flow rate was optimized for the system at 0.4 mL min^{-1}, with a column temperature of 30°C, and 20 µl of sample injected. Quantification was conducted using a diode array detector at a wavelength of 229 nm.

MS settings were as follows: Atmospheric pressure electrospray ionization was carried out in negative ion mode (scan range m/z 100–1500 Da). Nebulizer pressure was set at 50 psi, gas-drying temperature at 350°C, and capillary voltage at 2,000 V. Compounds were identified using their primary ion mass $(M\text{-}H)^-$, and comparison to authentic standards (Cataldi et al., 2007; Lelario et al., 2012). Data were analyzed using Agilent OpenLAB CDS ChemStation Edition for LC-MS (vA.02.10). GSL concentrations from each time point were averaged over three biological replicates with two technical replicates of each ($n = 6$). This approach was also conducted for glucosinolate hydrolysis product (GHP) and monosaccharide content.

Glucosinolate Hydrolysis Product Extraction and Analysis by GC-MS

GHPs were extracted according to the protocol presented by Ku et al. (2016) with the following modification: samples were hydrolyzed in $d.H_2O$ for 3 hours at 30°C before extraction with dichloromethane (DCM) for 21 h. This duration was optimized for maximum yields of GHPs by comparison of extraction times: 3 h incubation in $d.H_2O$ at 30°C with immediate DCM extraction; 3 h incubation in $d.H_2O$ at 30°C, with three, nine, and 21 h post incubation with DCM.

GC-MS analysis and GHP identification was conducted according to the method presented by Bell et al. (2017b). Concentrations of all GHPs were calculated as equivalents of SF standard (Sigma).

Monosaccharide Extraction and Analysis by HPLC

Free monosaccharides were extracted according to the method presented by Bell et al. (2017a), with the exception that 0.2 g of lyophilized leaf powder was extracted. Extracts were analyzed on an Agilent 1100 series HPLC system equipped with a binary pump, degasser, and auto-sampler, with an external column heater (50°C). A Bio-Rad Aminex HPX-87H (300 × 7.8 mm, 9 µm particle size) column with a Micro-Guard Cation H guard column (Bio-Rad, Watford, United Kingdom) was used to achieve separation with an isocratic gradient of 5 mM sulfuric acid, and a flow rate of 0.6 mL per min. A Polymer Laboratories ERC-7515 refractive index detector (Church Stretton, United Kingdom) was used to detect monosaccharides. Compounds were quantified using authentic standards and analyzed with Agilent ChemStation software (Santa Clara, CA, United States).

Sulfur Content Analysis by ICP-OES

Lyophilized samples were weighed into acid washed glass boiling tubes, and pre-digested in 70% nitric acid for 24 h, before

being heated to 90°C for 2 h using a heat block. Once cooled, these were filtered through a 0.45 μM syringe filter, and diluted to give an acid concentration of 3%. These samples were analyzed using Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES; Perkin Elmer Optima 7300 DV). Sulfur content was determined using the radial signal at 181.975 nm. Due to the small plant size and limited amounts of dried leaf powder, EH samples were not included in sulfur content analysis.

Statistical Analyses

All statistical analyses (not included in bioinformatics sections) were performed using XL Stat (Addinsoft, Paris, France). Shapiro–Wilk normality tests were conducted for all variables, all of which were concluded to fit a normal distribution. ANOVA with *post hoc* Tukey's Honest Significant Difference (HSD) tests were performed to generate multiple pairwise comparisons between sampling points for each cultivar (i.e., **H** vs. **D7** for cultivar **B**) and between cultivars at each respective time point (i.e., **A** vs. **B** for time point **H**) for phytochemical and elemental data (**Supplementary Data File 1**). Principal Component Analysis (PCA) was performed using Pearson correlation coefficient analysis, $n-1$ standardization, Varimax rotation, and Kaiser Normalization. Phytochemical data were regressed onto the gene expression data as supplementary variables for the targeted analysis.

RESULTS

Eruca sativa Genome Assembly and Annotation

Elite breeding line **C** was assembled into 49,933 contigs (≥500 bp). This line was chosen as the reference sequence because of its higher DNA concentration and optimal 260/280 ratio (**Supplementary Table 1**). The resulting assembly was ~851 Mb in size (**Table 1**). Transposable elements (TEs) within the *E. sativa* genome comprise 66.3% of its content. The majority of TEs are long terminal repeat (LTR) retrotransposons (37.3%), with long interspersed nuclear elements (LINEs; 3.3%) and short interspersed nuclear elements (SINEs; 0.3%) having lower relative abundance. A total of 18.2% of all TEs identified were of unknown classification (**Table 2**).

A combined method of *de novo* prediction, RNAseq (of leaf, stem, and root tissue), and homology with related species' genomes was used to predict gene numbers in *E. sativa*. A total of 45,438 protein-coding genes were identified within the assembly, with an average length of 1,889.6 bp, and an average of 4.8 exons per gene. This genome size is smaller than that predicted for radish (*R. sativus*), and larger than *A. lyrata* (**Table 3**), and is consistent with what is known of Brassicales phylogeny (Arias and Pires, 2012). A total of 98.3% of predicted genes were found to have homology with other plant species (**Figure 2B** and **Supplementary Table 4**). The average coding sequence (CDS) length of genes identified in rocket was 1,069.4 bp, which is most similar to that found in *A. lyrata*. Average intron and exon lengths were 224.8 and 218.3 bp, respectively; which is most similar to the *A. lyrata* genome.

RNAseq Analysis of *E. sativa* Plants

Global Differential Gene Expression

After sample QC and clean-up over 2.6 billion clean PE reads were produced, averaging ~49 million reads per sample. Q20 (<1% error rate) averaged 96.3%, Q30 (<0.1% error rate) averaged 90.8%, and GC content ranged from 44.5 to 47.4%.

The total numbers of differentially expressed genes (DEGs) for line **A**, **B**, and **C** are presented in **Figures 2C–E**, respectively. Few significant DEGs were observed between **EH** and **H** samples for lines **A** and **B** (<333; **Figures 2C,D**), whereas they were observed at a higher rate in **C** (2,234; **Figure 2E**). This indicates a high degree of plasticity of **C** across growth stages.

This trend was reversed at **PW**, where 180 DEGs were observed compared to **H** in **C**, and 1,343 were observed in **A**. During shelf life (**D0** and **D7**) **C** expressed a greater number of DEGs compared to **H**, than **A** or **B** (2,340 at **D0** and 3,075 at **D7**, respectively). By contrast, DEGs at **SC** were much less variable between the three lines (330–676) indicating a greater degree of uniformity of expression in the second cut.

Up and down regulation of global DEGs relative to **H** are presented in **Figure 3**. Several differences between the lines are illustrative of the complex and varied responses genotypes have when grown under the same environmental conditions. Lines **A** and **B** have very similar up/down expression patterns of genes at ontogenic (**EH** and **SC**) and postharvest (**D0**, and **D7**) time points. The exception to this was at **PW**, where line **A** showed higher numbers of down (7,023) and up (6,564) regulated genes than **B** (5,800 down; 4,868 up) relative to their respective numbers at **H**. The largest differences in up/down expression patterns can be seen for line **C**. At **EH**, more than double the numbers of genes were up/down regulated relative to **H**, compared to lines **A** and **B**. At **SC**, the opposite was observed, suggesting that

TABLE 1 | Summary of genome assembly and annotation of *Eruca sativa*.

Genome assembly	≥0 bp	≥1,000 bp	Largest contig	Total (≥500 bp)
Contig number	1,041,818	12,352	1,477,633	49,933
Total length	850,956,505	562,271,846		586,731,295

Assembly related statistics

GC%	N50	NG50	N75	NG75	L50	LG50	L75	LG75
36.25	196,831	136,378	87,576	2,634	789	1,256	1,889	7,243

TABLE 2 | Transposable elements content in the reference genome.

Type	*De novo* + Repbase*		TE proteins$		Combined TEs#	
	Length (bp)	% in genome	Length (bp)	% in genome	Length (bp)	% in genome
DNA	69,251,054	8.14	21,607,510	2.54	76,517,426	8.99
LINE	20,290,781	2.38	17,153,783	2.02	28,200,567	3.31
SINE	2,134,305	0.25	0	0	2,134,305	0.25
LTR	311,377,915	36.59	91,001,347	10.69	317,124,290	37.27
Other^	106,176	0.01	0	0	106,176	0.01
Unknown^^	155,033,031	18.22	0	0	155,033,031	18.22
Total	547,675,259	64.36	129,571,414	15.23	563,873,839	66.26

*RepeatMasker based on the ncbi algorithm combined with the known Repbase and de novo repeat library created by RepeatModeler/Repeat Scout/LTR_finder.
$RepeatProteinMask based on Repbase.
#The non-redundant set of results combining De novo + Repbase TEs and TE proteins.
^Repeats that can be classified by RepeatMasker, but not included by classes above.
^^Repeats that could not be classified by RepeatMasker.

TABLE 3 | Predicted protein-coding genes within the *E. sativa* reference genome.

Gene set		Number	Average gene length (bp)	Average CDS length (bp)	Average exons per gene	Average exon length (bp)	Average intron length (bp)
*De novo**	Augustus	50,179	1,701.56	1,024.89	4.48	228.88	194.57
	Glimmer HMM	73,989	1,335.36	725.53	3.03	239.16	299.86
	SNAP	80,264	1,231.13	728.3	4.02	180.98	166.27
	Genemid	100,165	2,127.25	585.82	3.07	191.03	745.87
	Genscan	71,813	3,942.04	786.32	3.92	200.11	1,079.4
Homolog^	*Arabidopsis lyrata*	32,667	1,867.18	1,084.09	4.86	223.12	202.93
	Arabidopsis thaliana	27,416	1,870.34	1,218.4	5.13	237.58	157.91
	Brassica napus	101,040	1,764.75	1,001.16	4.91	204.06	195.48
	Boechera stricta	27,416	2,006.68	1,181.2	5.09	231.86	201.61
	Capsella rubella	26,521	1,958.82	1,248.6	5.19	240.53	169.46
	Raphanus sativus	49,733	2,064.75	1,194.41	4.94	241.57	220.66
RNAseq^^	Cufflinks	43,200	2,848.16	1,723.58	6.03	285.65	223.41
	PASA	37,870	1,744.08	1,034.72	4.77	216.9	188.13
EVM		59,643	1,665.12	926.2	4.17	222.2	233.23
PASA-update		59,491	1,656.25	929.33	4.17	222.9	229.36
Final set		45,438	1,889.6	1,069.44	4.76	224.81	218.3

*The combined results by EVM of 5-ab initio gene predictions.
^The combined results by EVM of homology-based gene prediction.
^^The combined results by EVM of transcriptome data sets.

second cuts of line **C** are more similar to first cuts (**H**) than **A** and **B**, in terms of their gene expression. This is of relevance to growers and breeders, as it indicates that greater transcriptional and metabolic consistency may be achievable between successive cuts of rocket if an appropriate cultivar is selected. This trend was also observed postharvest, where line **C** had several thousand fewer genes up/down regulated than **A** and **B**. As will be discussed in following sections, the apparently reduced transcriptional response to stimuli, such as harvesting, processing, and cold storage, may be indicative of greater resilience to stress in line **C**.

Co-expression and Gene Set Enrichment Analysis

Co-expression analysis of RNAseq data produced eight gene modules (**Figure 4**). These contained 583 (**M1**), 184 (**M2**), 112 (**M3**), 107 (**M4**), 72 (**M5**), 65 (**M6**), 58 (**M7**), and 38 (**M8**) genes, respectively (**Figure 4A**). **Figure 4B** shows that there are distinct and significant module expression patterns for each of the three genotypes (**A**, **B**, and **C**). Line **A** had significant positive expression of modules **M1**, **M2**, and **M7** in comparison to the other two lines. Line **C** had significantly greater expression in modules **M5** and **M6**, and line **B** in module **M8**. These patterns are most distinct in the **Figure 4A** plots for **M3**, **M4**, and **M5**; but there are also subtle differences within genotypes where there are distinct increases/decreases for the postharvest time points (**PW**, **D0**, and **D7**). This can be most clearly seen in **M2**, **M6**, **M7**, and **M8**.

The genes contained within each respective module are listed in **Supplementary Data File 2**. Several of these modules contain genes related to the GSL biosynthesis pathway, as well as myrosinases. Module **M1** is the by far the largest and contains

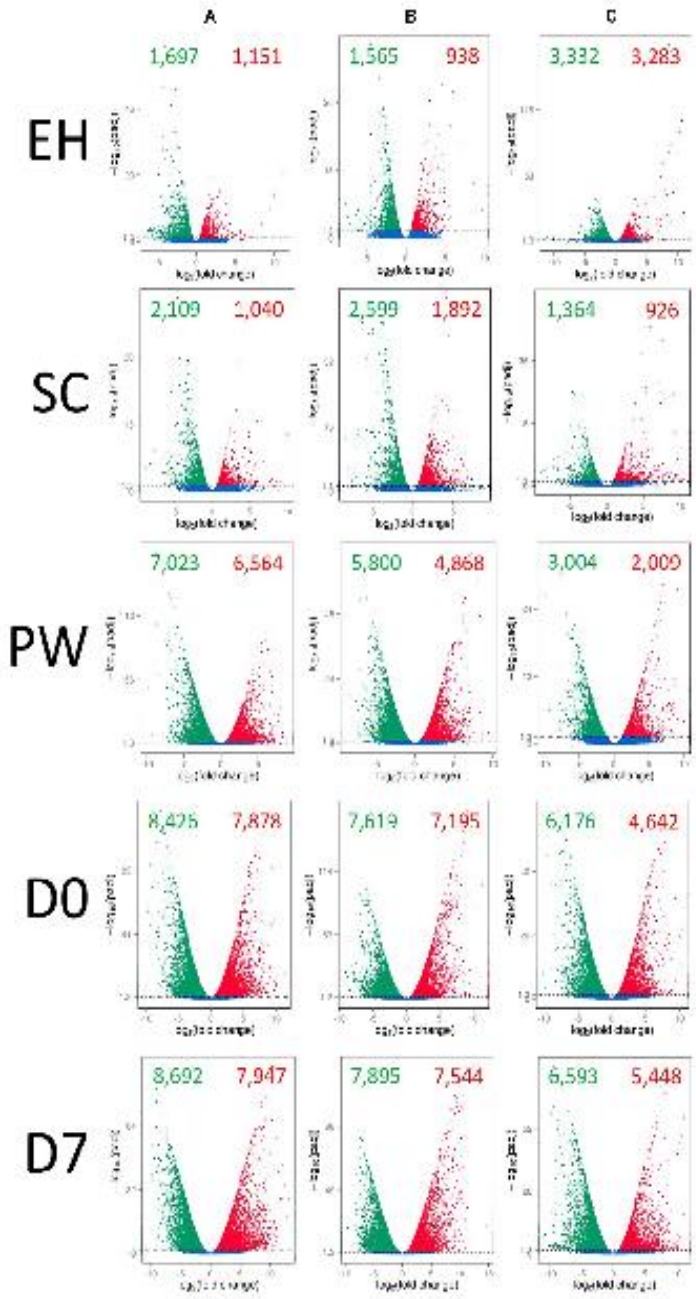

FIGURE 3 | Global differential gene expression and distribution in three elite inbred lines of *Eruca sativa* (**A–C**) across five time points, relative to harvest (**H**) samples for each line. Individual volcano plots display the log2-fold change of gene expression on the *x*-axis, and the degree of statistical significance on the *y*-axis (*q*-value < 0.05, with removal of variations between biological replicates). Red dots, significantly up-regulated genes (relative to H); green dots, significantly down-regulated genes (relative to H); blue dots, no significant change in expression. **EH**, early harvest; **SC**, second harvest; **PW**, pre-wash; **D0**, post-wash; and **D7**, 7-day shelf life.

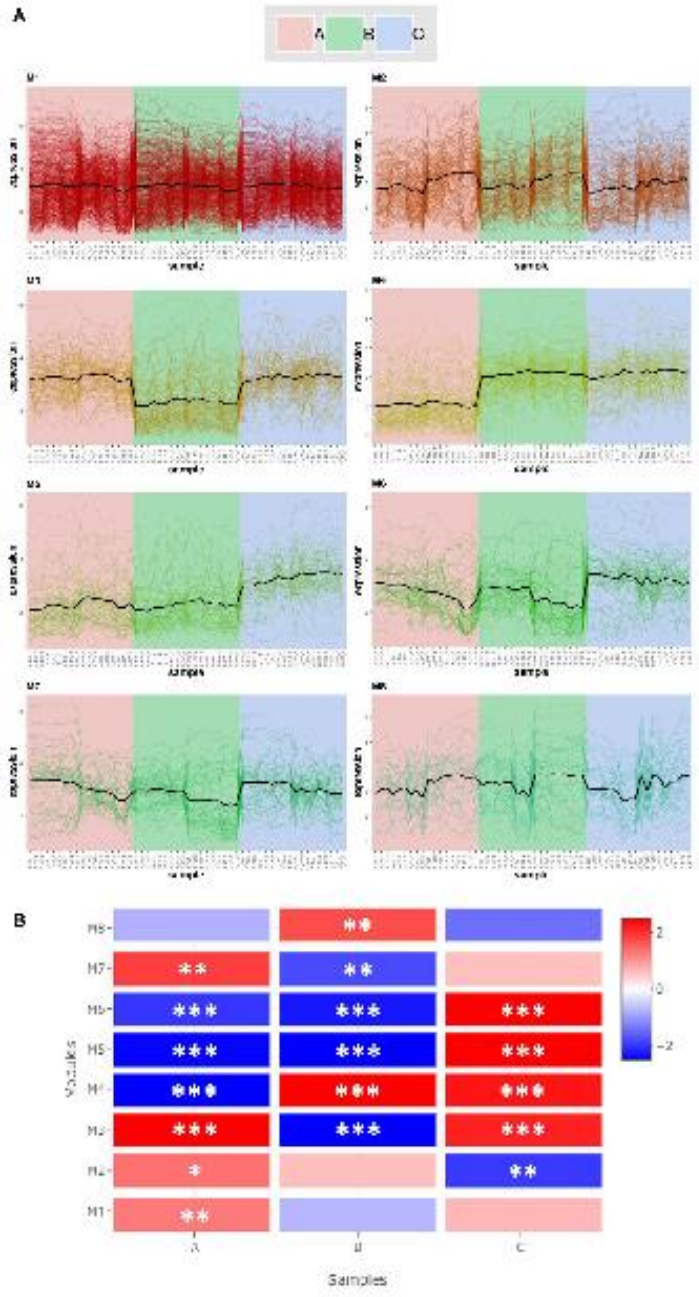

FIGURE 4 | Co-expression module analysis **(A)** and Gene Set Enrichment Analysis (GSEA; **(B)** of three elite inbred lines of *Eruca sativa*. Eight gene modules (M1–M8) were identified within the transcriptomes of lines **A**, **B**, and **C** (see inset for color coding). Modules contain 583 **(M1)**, 184 **(M2)**, 112 **(M3)**, 107 **(M4)**, 72 **(M5)**, 65 **(M6)**, 58 **(M7)**, and 38 **(M8)** genes, respectively **(A)**. The heat-map **(B)** illustrates differences in module activity (red, high; blue, low) between each genotype, and asterisks indicate the level of Normalized Enrichment Score (NES) significance: $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$. Complete gene lists for each module and the corresponding enrichment analysis statistics are presented in **Supplementary Data File 2**.

"hub" genes related to photosynthesis (*LHCA4* and *PSAH2*). It contains a number of indole GSL biosynthesis genes, such as *CYP79B2a*, *CYP83B1*, *SOT16*, *CYP81F2b*, *IGMT1a*, and *IGMT4a*. Two TGG1 myrosinase genes are also present (*TGG1b* and *TGG1e*) as well as two putative *TGG6* genes. This module had significantly higher expression in line **A** than the two others (**Figure 4B**). Of note in **M2** is the presence of the TF *HY5*, which as previously been associated with the diurnal regulation of GSL biosynthesis in *A. thaliana* (Huseby et al., 2013).

Contained within **M6** are two genes pertinent to sulfur assimilation and aliphatic GSL biosynthesis; *SiRa* (sulfite reductase) and *MYB28b*. MYB28 has been shown to upregulate expression of SiR in Arabidopsis (Sønderby et al., 2007) as well as other genes in the sulfur metabolism pathway.

M8 is the smallest of the gene modules identified, but has a notable hub gene related to senescence: *STR15*, also known as *SENESCENCE1* (*SEN1*). This gene has been previously linked with expression of defense-related signaling pathways (Schenk et al., 2005) and increases in senescence-induced oxidative stress (Hye et al., 2004). Also of note within this module is *OXS3* (*OXIDATIVE STRESS 3*), which is part of cellular oxidative stress response (Blanvillain et al., 2009). This module had significantly higher expression in line **B** relative to **A** and **C** (**Figure 4**), and as will be discussed, may contribute to differences in observed shelf life GSL phenotypes.

Sulfur Assimilation and Glucosinolate Biosynthesis Pathway Gene Expression

Sulfate Assimilation Gene Expression

Figure 5 presents differential gene expression within the sulfate assimilation pathway of *E. sativa*. All significances quoted hereafter were at the $q < 0.05$ significance level. In the primary stages of sulfur metabolism, sulfate is activated via adenylation to adenosine-5′-phosphosulfate (APS), catalyzed by ATP sulfurylase (ATPS; Anjum et al., 2015). In *E. sativa* four ATPS-encoding genes were identified: *APS1a*, *APS1b*, *APS2*, and *APS3* (**Figure 1**). Very few significant DEGs were observed between sample points for each respective rocket line (see **Supplementary Data File 3** for full values and statistics of each sample comparison). However, between **H**, **SC**, and **PW**, each respective line did show significant differential expression of ATPS genes.

In the second stage of the pathway, APS is reduced to sulfite by adenosine-5′-phosphosulfate reductase (APR; Capaldi et al., 2015). Four APRs were identified (*APR1a*, *APR1b*, *APR2a*, and *APR2b*) as well as six APR-like genes (*APRL4*, *APRL5a*, *APRL5b*, *APRL5c*, *APRL7a*, and *APRL7b*). *APR1a* and *APR2a* showed significant differential expression across multiple samples and time points (**Figure 6**). Line **B** displayed low relative expression of these genes compared to **A** and **C**. Line **C** exhibits significantly higher expression postharvest compared to **H**; 2.2 log2-fold (**D0**) and 2.7 log2-fold (**D7**) increases of *APR1a*, and 0.9 log2-fold (**D0**) and 1.1 log2-fold (**D7**) increases of *APR2a* were observed. We hypothesize that this may be indicative of a greater ability to assimilate sulfate via APR enzymes to facilitate and maintain secondary metabolite biosynthesis for longer into shelf life.

Two copies of genes encoding sulfite reductase (SiR; *SiRa* and *SiRb*) were identified. *SiRa* showed significantly higher levels of expression in line **C** (**Figure 6**). Line **C** had no significant change in activity of this gene relative to time point **H**, however both lines **A** and **B** had significantly lower expression postharvest (**Figure 6**).

Sulfur Metabolism Transcription, Regulation, and Transport Gene Expression

Three copies of SDI1 (*SDI1a*, *SDI1b*, and *SDI1c*) and three copies of SLIM1 (SULFUR LIMITATION 1, aka ETHYLENE INSENSITIVE-like 3; *SLIM1a*, *SLIM1b*, and *SLIM1c*) were identified within the genome annotation. These genes are thought to play critical roles in the management and use-efficiency of sulfur in plants, and have been linked with optimization of GSL biosynthesis under S-limited conditions in *A. thaliana* (Aarabi et al., 2016).

SDI1a and *SDI1c* were differentially expressed between each line (**Figure 6** and **Supplementary Data File 3**), with **C** having the highest levels of expression postharvest. It might be expected that each line would see a similar trend of expression over the course of shelf life, as additional sulfur is not obtainable; however only line **C** displayed this (**Figure 6**).

SLIM1b was significantly higher at time points **D0** and **D7** relative to **H** (a 1.4 and 1.1 log2-fold significant increase, respectively) in line **C**. Expression of *SLIM1c* by comparison was not significantly different for each respective plant line between time points, but there were clear and significant differences in expression between lines (**Supplementary Data File 3**). Line **C** had highest expression of this gene, followed by **A**; with **B** having significantly lower expression overall (**Figure 6**). Previous studies have shown that SLIM1 down regulates APK gene expression and GSL biosynthesis as a way of conserving sulfur for primary metabolism (Chan et al., 2019). Our data suggest that this is only the case between *SLIM1a* and *APK3* ($r = -0.597$, $q < 0.001$; **Supplementary Data File 1**). *SLIM1a* expression was positively (and significantly) correlated with *APK* expression ($r = 0.521$), and *SLIM1b* and *SLIM1c* with *APK4* ($r = 0.575$ and 0.698, respectively; **Supplementary Data File 1**). This suggests *E. sativa* has a complex and interacting network of sulfur metabolism genes, where functions may not necessarily be analogous to those found in *A. thaliana*.

Sixteen sulfur transport (*SULTR*) genes were identified within the annotation; of note were *SULTR1;2a*, *SULTR2;1a*, *SULTR2;1b*, *SULTR4;1a*, and *SULTR4;2*. *SULTR1;2a* has been associated with the uptake of environmental sulfate in root tissues (**Supplementary Data File 3**), but we detected low levels of expression in leaf tissues. Postharvest, line **C** had differential expression of this gene compared to **A** and **B** in **D7** samples (**Figure 6**). This was more pronounced for *SULTR2;1a* and *SULTR2;1b*, and both **A** and **B** had significant reductions in expression at **D0** and **D7** relative to **H**. **SC** samples showed significant increases relative to **H**, with the exception of *SULTR2;1a* in **B**.

SULTR4;1 and *SULTR4;2* genes also had distinct patterns of expression between lines. *SULTR4;1a* saw significant increases in expression in postharvest samples relative to **H** (**Figure 6**).

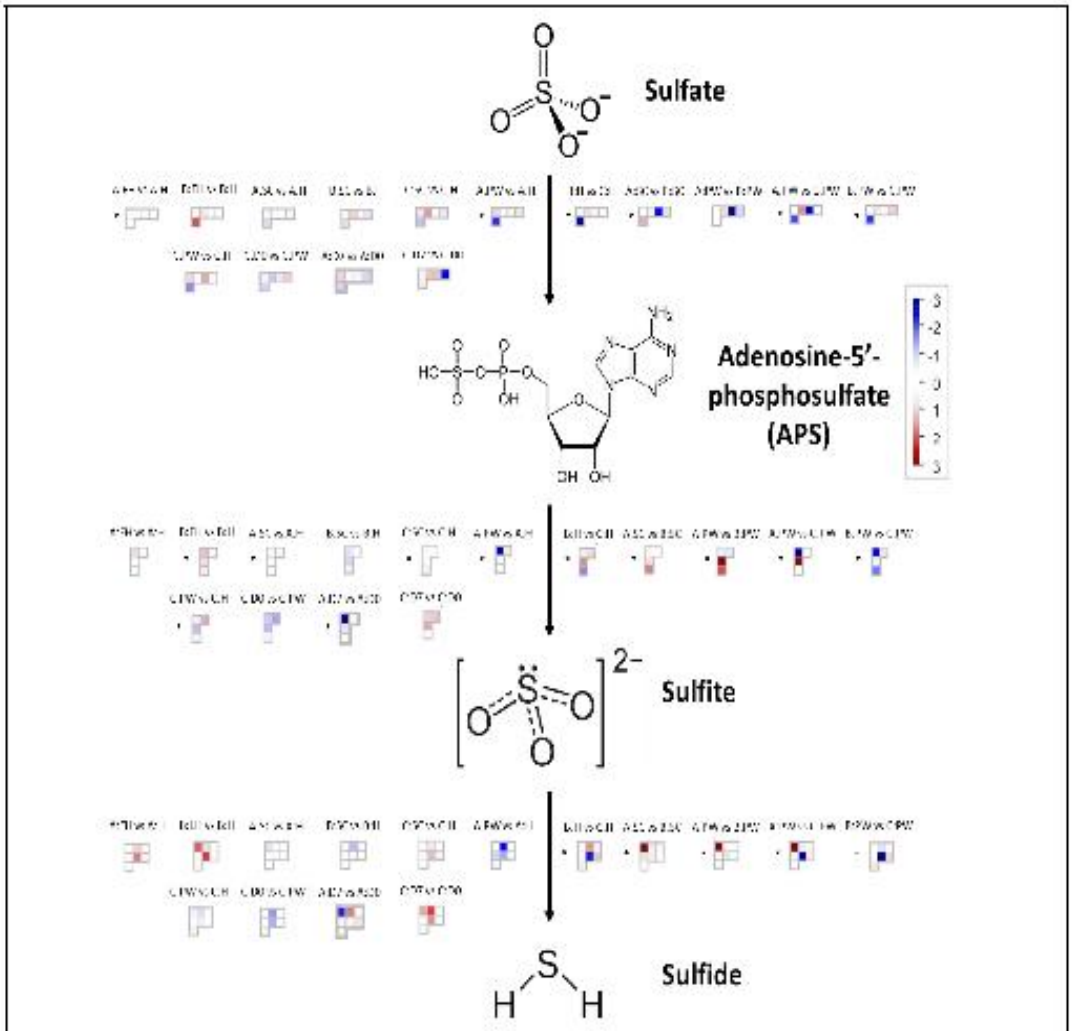

FIGURE 5 | RNAseq expression data (FPKM) for genes involved with sulfate assimilation in three elite inbred lines of *Eruca sativa*. A custom MapMan (version 3.6.0RC1) annotation file of the *E. sativa* reference genome was created using Mercator4 (version 1.0, plaBI dataBase, Institute of Biology, Aachen, Germany), and used to visualize differential expression of genes within the sulfate assimilation pathway. Asterisks denote significance of up/down regulation. **EH**, early harvest; **H**, harvest; **SC**, second harvest; **PW**, pre-wash; **D0**, post-wash; and **D7**, 7-day shelf life.

Line **A** had higher expression of *SULTR4;2* during growth before declining significantly post-wash (**D0**). The opposite trend was seen in **C**, where gene expression peaked at **D7**. These data are suggestive of more active intra-leaf sulfur transport in line **C** postharvest, and may be associated with the higher expression of APR, SiR, SDI1, and SLIM1 genes to facilitate more efficient S utilization during this period.

Glutathione Synthesis

With the exception of *GSH2b*, glutathione synthetase genes were most highly expressed in rocket line **B**, with significant increases observed postharvest (**Figure 6**). Lines **A** and **C** were unchanged between sample points for these genes, but had a marked difference in expression for *GSH2b* relative to each other. **B** had negligible levels of *GSH2b* expression.

As both glutathione and secondary S-containing metabolites, such as GSLs, have been associated with antioxidant responses (Chan et al., 2019) the differences observed between each of the lines in terms of both GSL concentrations and glutathione-related gene expression, may be indicative of different adaptive metabolic strategies for dealing with oxidative stress postharvest. Lines **A** and **C** favor secondary sulfur metabolism and the

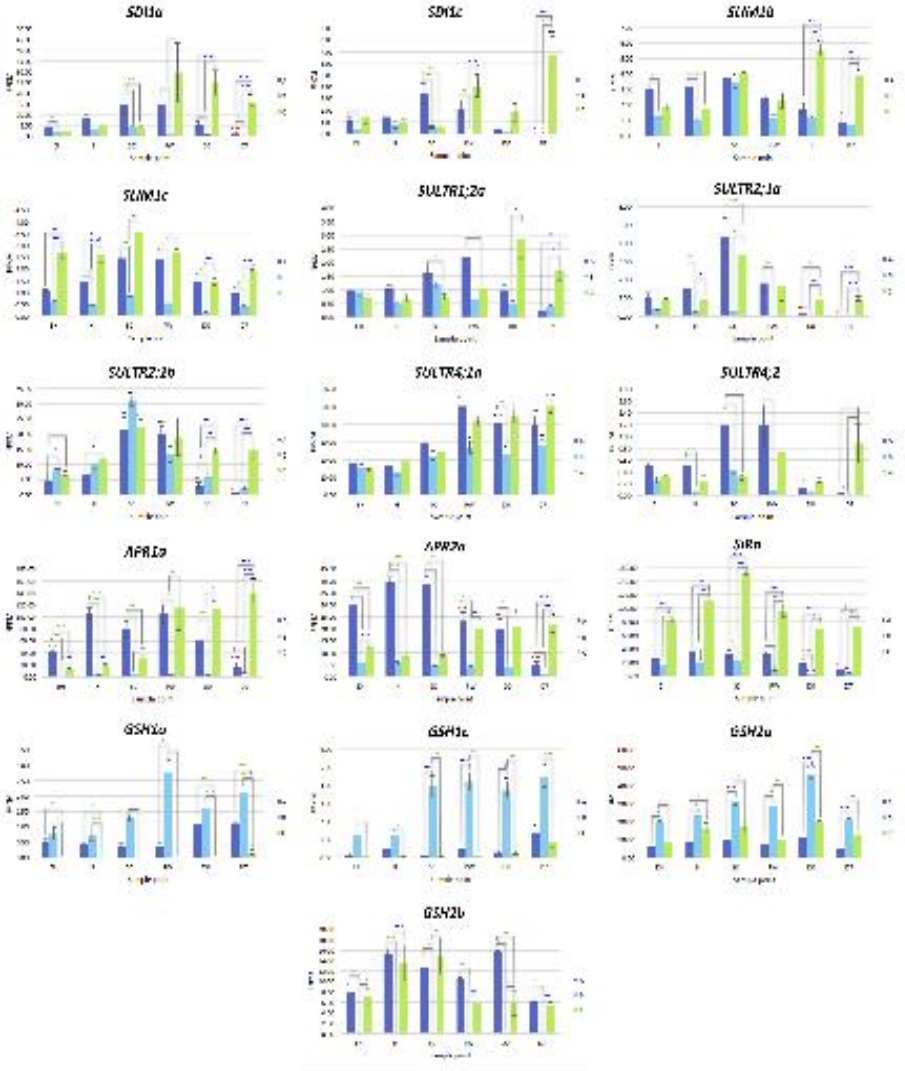

FIGURE 6 | RNAseq expression data (FPKM) for genes involved with sulfate transport and redox response in three elite inbred lines of *Eruca sativa* (**A**, dark blue; **B**, light blue; **C**, green). Standard errors of the mean expression values are represented by error bars. Asterisks denote levels of significance of up and down regulation within sample points (between each inbred line) and relative to the point of harvest for each respective sample point: $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; green, significant up regulation between lines **A**, **B**, and **C**; blue, significant down regulation between lines **A**, **B**, and **C**; black, significant up regulation relative to **H**; red, significant down regulation relative to **H**. **EH**, early harvest; **H**, harvest; **SC**, second harvest; **PW**, pre-wash; **D0**, post-wash; and **D7**, 7-day shelf life.

synthesis of GSLs, and B favors primary sulfur metabolism and glutathione synthesis.

Glucosinolate-Related Transcription Factors

MYC2a and *MYC2c* were highly expressed in line **A**, and had uniform patterns of relative expression. **SC** had the highest expression values for this line, suggesting a general response to mechanical wounding and stress, however this was not significantly different from **H**. The only significant difference for *MYC2c* between **H** and **SC** was in line **B** (a 0.7 log2-fold increase; **Figure 7**).

MYB28a and *MYB28b* display high degrees of differential expression between each rocket line. While **A** has high expression of *MYB28a* in samples **EH**, **H**, **SC**, and **PW**, it is has by

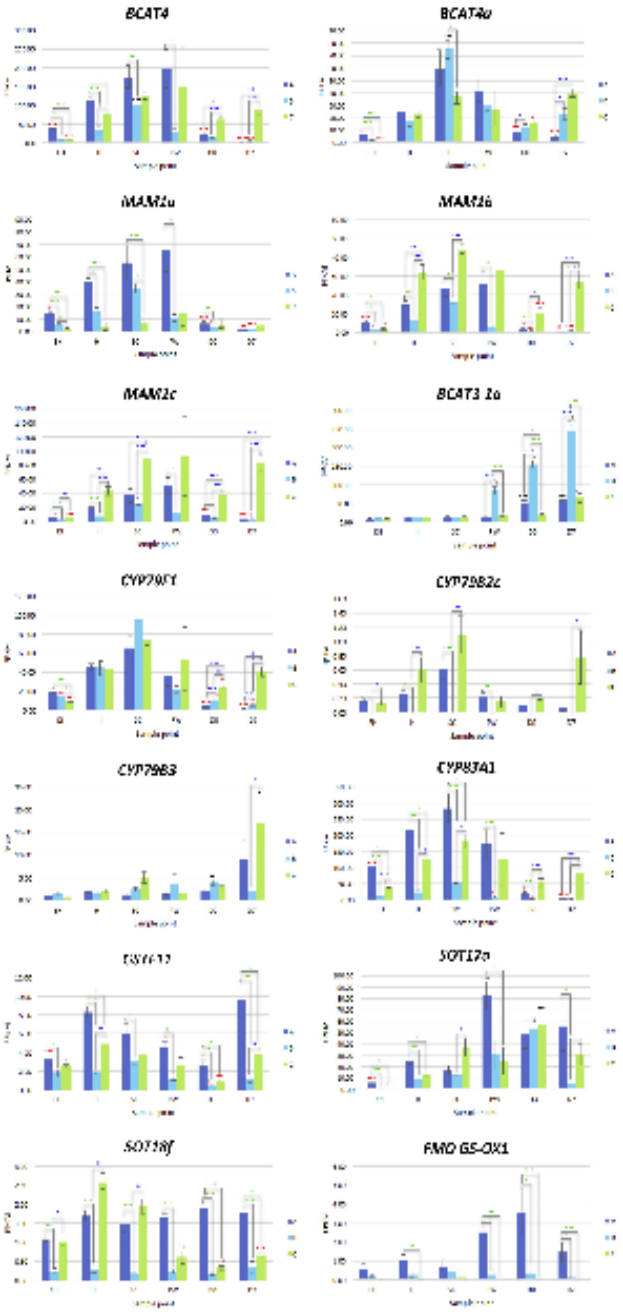

FIGURE 7 | RNAseq expression data (FPKM) for genes involved with glucosinolate biosynthesis in three elite inbred lines of *Eruca sativa* (**A**, dark blue; **B**, light blue; **C**, green). Standard errors of the mean expression values are represented by error bars. Asterisks denote levels of significance of up and down regulation within sample points (between each inbred line) and relative to the point of harvest for each respective sample point: $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; green, significant up regulation between lines **A**, **B**, and **C**; blue, significant down regulation between lines **A**, **B**, and **C**; black, significant up regulation relative to **H**; red, significant down regulation relative to **H**. **EH**, early harvest; **H**, harvest; **SC**, second harvest; **PW**, pre-wash; **D0**, post-wash; and **D7**, 7-day shelf life.

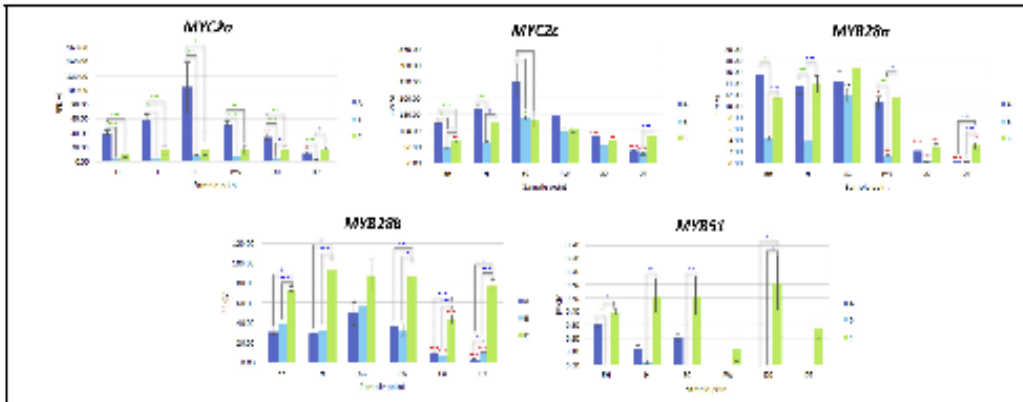

FIGURE 8 | RNAseq expression data (FPKM) for glucosinolate transcription factors in three elite inbred lines of *Eruca sativa* (**A**, dark blue; **B**, light blue; **C**, green). Standard errors of the mean expression values are represented by error bars. Asterisks denote levels of significance of up and down regulation within sample points (between each inbred line) and relative to the point of harvest for each respective sample point: *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; green, significant up regulation between lines **A**, **B**, and **C**; blue, significant down regulation between lines **A**, **B**, and **C**; black, significant up regulation relative to **H**; red, significant down regulation relative to **H**. **EH**, early harvest; **H**, harvest; **SC**, second harvest; **PW**, pre-wash; **D0**, post-wash; and **D7**, 7-day shelf life.

comparison lower expression of *MYB28b* compared to **C** (**Figure 9**). **C** on the other hand has relatively high expression for both of these TFs, and displays significantly higher expression postharvest, up to and including **D7**. Combined with what is known about these TFs in other Brassicaceae species, it is likely that the differences in GSL concentrations observed postharvest are linked to the differential expression of *MYB28a* and *MYB28b* between the respective lines.

Also of note is that expression of MYB28 genes were positively associated with expression of SDI1 gene copies. Previous research has shown that the SDI1 protein binds to MYB28, inactivating expression and reducing GSL biosynthesis (Chan et al., 2019). In *E. sativa* the opposite appears to be true, with significant positive correlations between respective expression of two MYB28 copies and *MYB29* with SDI1 copies (*SDI1a* and *MYB29*, $r = 0.72$; *SDI1b* and *MYB28a*, $r = 0.507$; *SDI1c* and *MYB28c*, $r = 0.459$; **Supplementary Data File 1**). At **D7**, both **A** and **B** had significantly lower expression levels compared with **H** (a 2.2 and 3.7 log2-fold reduction of *SDI1a*, respectively; and a 3.9 and 3.2 log2-fold significant reduction of *SDI1c*, respectively).

Glucosinolate Biosynthesis

Rocket contains two genes encoding BCAT4, and two genes encoding BCAT3; converting 2-oxo acids to homomethionine and dihomomethionine. *BCAT3-1a* displayed no significant variation between lines during growth, but saw significant increases for all (compared to **H**) at **D0** and **D7** (**Supplementary Data File 3**). The most marked and statistically significant increase was in **B**. It is unclear how this "preference" for BCAT3 activity over BCAT4 is regulated or affects the synthesis pathway, but the relative and respective activity of these genes is associated with GSL content.

Only orthologs of MAM1 were identified, with no corresponding MAM2 or MAM3 genes present within the annotation. Each of the three MAM1 copies had differing expression patterns (**Figure 8**). Line **A** displayed higher relative expression of *MAM1a*, whereas **C** had greater expression for *MAM1b* and *MAM1c*. **B** however maintained low expression for all three of these genes. **A** had reduced expression activity during shelf life, whereas in **C**, levels were significantly higher compared to **H** (**Figure 8**).

One *CYP79F1* homolog was found in rocket, with no expression found for a corresponding *CYP79F2* gene. The lack of a *CYP79F2* homolog in rocket may be suggestive of a loss of function, and/or redundancy with other enzymes. Of note for *CYP79F1* expression was the significant differences observed between **EH** and **H**, indicating that earlier harvests of rocket leaves may have a reduced ability for GSL biosynthesis compared with later ones and second cuts (**SC**). Expression was significantly greater in **C** during shelf life. In the conversion of aldoximes to nitrile oxides, *CYP83A1* expression was higher in **A** and **C** than **B**, with line **C** having significantly higher expression in shelf life samples (**Figure 8**).

Glucosinolate Hydrolysis

Eleven TGG1 (myrosinase) and three TGG2 copies (**Supplementary Data File 3**) were identified within the annotation. Some of these genes appear to have differential expression according to ontogeny and shelf life time point, with some copies expressed at **EH** with none during postharvest (e.g., *TGG1h*, *TGG2a*, and *TGG2c*; **Figure 9**). Others however display the inverse of this, with increased relative expression postharvest (*TGG1a*; **Supplementary Data File 3**). It is known that myrosinases TGG1 and TGG2 are functionally redundant in Arabidopsis, however it has also been noted that their activity and specificity is linked with developmental processes, and may explain some of the high levels of expression observed at **EH**.

An explanation for the lack of nitrile GHPs in rocket may be that the high expression of *NSP5* is inhibited by the five EPITHIOSPECIFIER MODIFIER 1 (ESM1) orthologs found in

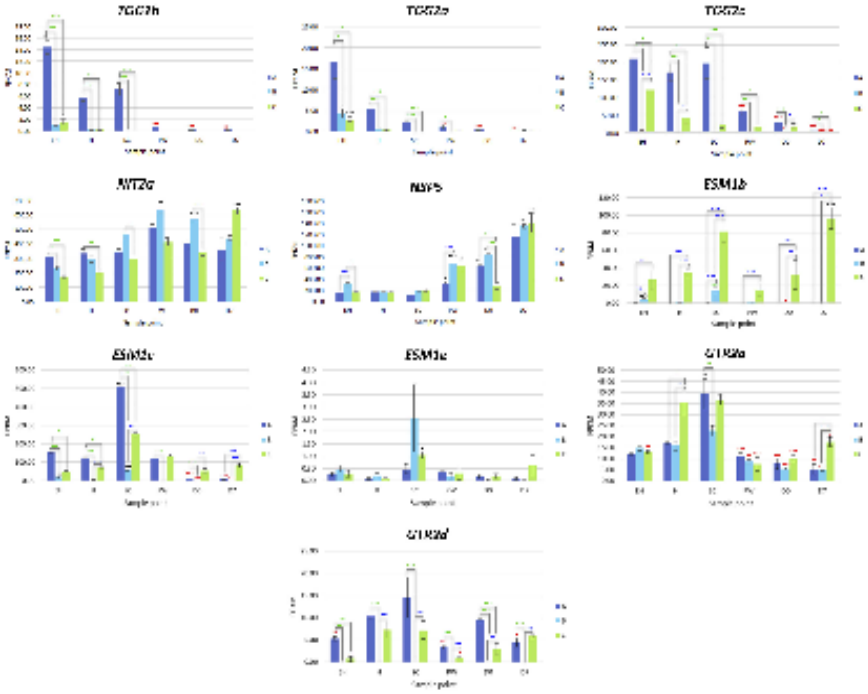

FIGURE 9 | RNAseq expression data (FPKM) for genes involved with glucosinolate hydrolysis and transport in three elite inbred lines of *Eruca sativa* (**A**, dark blue; **B**, light blue; **C**, green). Standard errors of the mean expression values are represented by error bars. Asterisks denote levels of significance of up and down regulation within sample points (between each inbred line) and relative to the point of harvest for each respective sample point: $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; green, significant up regulation between lines **A**, **B**, and **C**; blue, significant down regulation between lines **A**, **B**, and **C**; black, significant up regulation relative to **H**; red, significant down regulation relative to **H**. **EH**, early harvest; **H**, harvest; **SC**, second harvest; **PW**, pre-wash; **D0**, post-wash; and **D7**, 7-day shelf life.

the rocket genome. These proteins are known to inhibit the action of NSPs and promote ITC formation. Expression was significantly greater in line **C** for *ESM1b* (**Figure 11**) at all sample points, and fits with the observed pattern of sustained GHP formation postharvest. The lower activities in **A** and **B** did not however correspond to a reciprocal decrease in the relative concentrations of GHPs, and neither were nitrile concentrations at anything above trace levels. Much further work is needed to explain the genetic regulation of GHP formation in rocket and the high expression of *NSP5*.

Glucosinolate Transporters

Eight GSL transporter genes were identified in the rocket annotation; four GTR1 and four GTR2 genes. These genes are involved in leaf distribution and long-range phloem GSL distribution, respectively. Expression of *GTR2a* and *GTR2d* were significantly correlated with GSL abundance and GHP formation in the analyzed leaf tissues. Of particular note is that **B** had no expression of *GTR2d* at any of the sample points, indicating that the gene may be non-functional. If this transport system is impaired in **B**, it would explain the significantly lower abundance of GSLs observed in leaves (**Figure 10**). Coupled with the high expression of glutathione-related genes and similar sulfur content of **B** compared to lines **A** and **C**, the inactivity of this gene copy may have significant effects on leaf sulfur transport, metabolism, and antioxidant response. The lower GSL content in leaves may therefore be compensated by increased glutathione synthesis.

Sulfur and Phytochemical Composition of *E. sativa*

Sulfur Content of *E. sativa*

Total sulfur content for each of the breeding lines is presented in **Figure 10A**. No significant differences were observed between lines and sample time points ($p = 0.434$). A lack of statistical difference between lines and between time points demonstrates that observed GSL profiles and abundance cannot be inferred from sulfur content of the leaves. As gene expression analysis of sulfur metabolism-related genes has shown, there is a distinct difference between lines **A** and **C** compared with line **B** in the utilization of available sulfur for GSL biosynthesis.

Glucosinolate Profiles and Contents of *E. sativa*

For each of the cultivars between the first (**H**) and **SC**, an increase in total GSL concentrations was observed due to elevations of

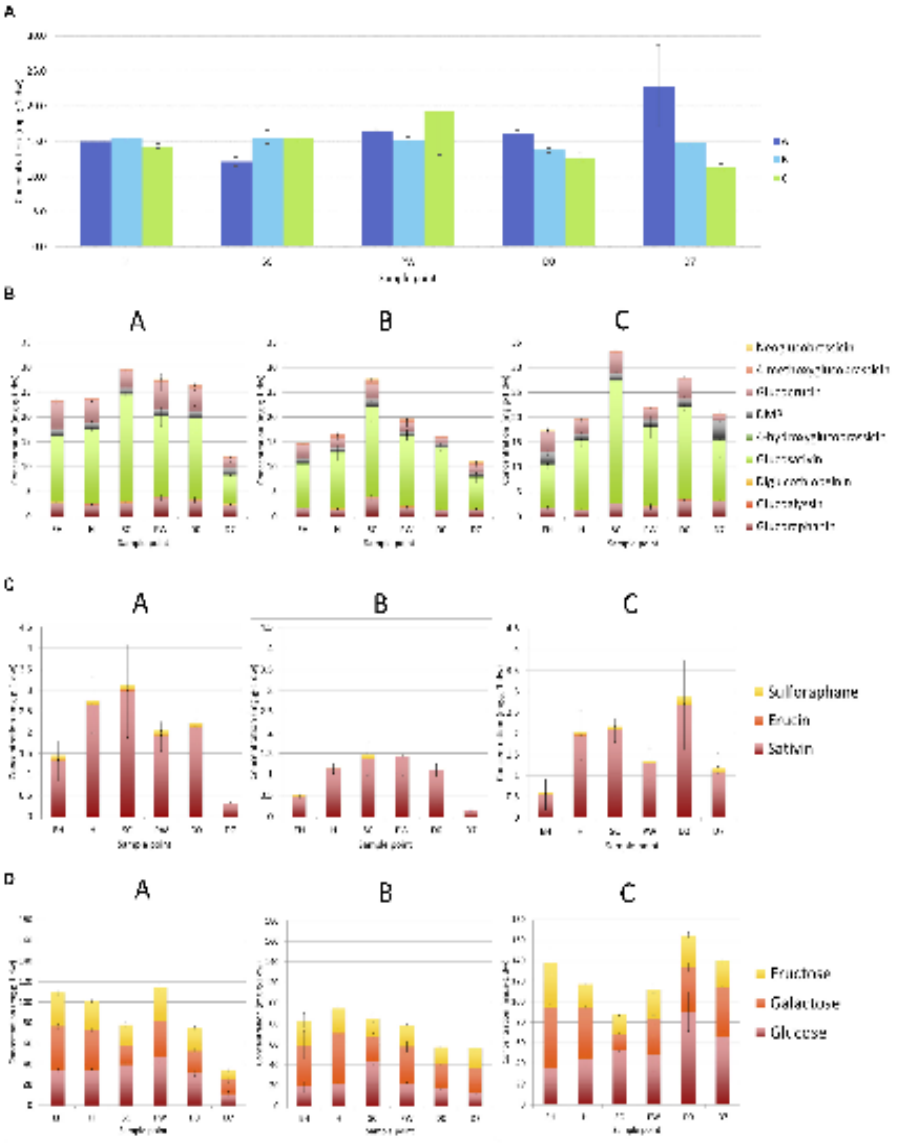

FIGURE 10 | Elemental sulfur **(A)**, glucosinolate **(B)**, glucosinolate hydrolysis product **(C)**, and monosaccharide **(D)** concentrations observed in elite inbred lines of *Eruca sativa* (**A**, **B**, and **C**). Concentrations are expressed as mg.g^{-1} of dry weight. Error bars represent standard error of the mean of each analyte detected. See insets for compound color coding. For ANOVA and Tukey's HSD pairwise significance values see **Supplementary Data File 1**. **EH**, early harvest; **H**, harvest; **SC**, second harvest; **PW**, pre-wash; **D0**, post-wash; and **D7**, 7-day shelf life.

GSV (**A**, a 1.4-fold increase, $p < 0.0001$; **B**, a 1.6-fold increase, $p < 0.0001$; **C**, a 1.8-fold increase, $p < 0.0001$; **Figure 10B**) and GRA (**B**, a 2.6-fold increase, $p < 0.0001$; **C**, a 1.8-fold increase, $p < 0.0001$; **Supplementary Data File 1**). Line **C** produced the highest total concentrations of GSLs in **SC** (a 1.7-fold increase; $p < 0.0001$), and line **B** also saw significant elevations compared to **H** (a 1.6-fold increase; $p < 0.0001$).

Line **A** contained the greatest GSL concentrations compared to **B** and **C**, until **D7** where content declined significantly (a 0.6-fold decrease compared to **D0**, $p < 0.0001$; **Supplementary**

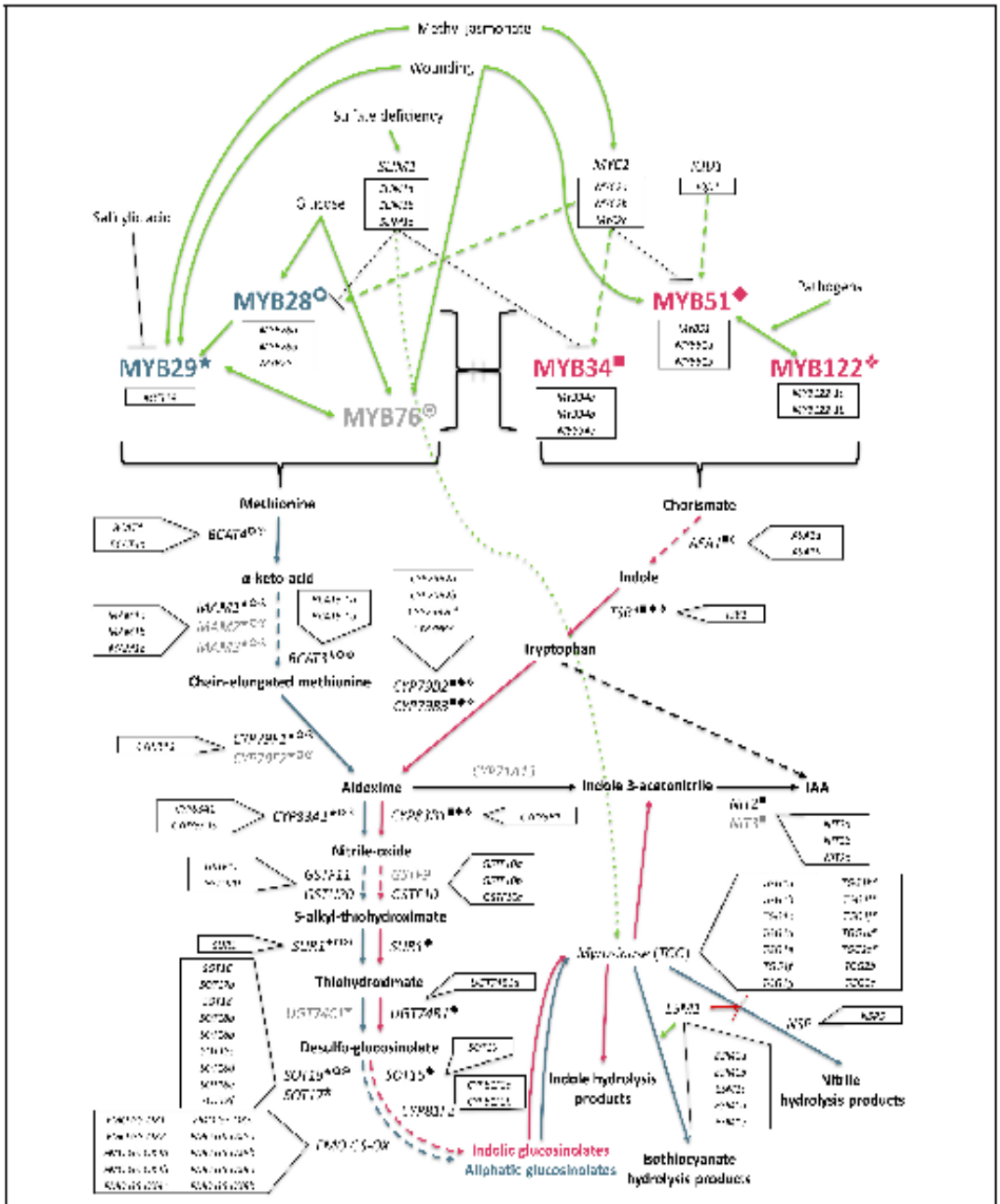

FIGURE 11 | The GSL biosynthesis pathway (adapted from Gigolashvili et al., 2009) is initiated by a complex and interacting network of abiotic and biotic factors. Aliphatic synthesis pathway shown in teal, and the indolic pathway shown in pink, is regulated by R2R3-MYB transcription factors. Known interactions between MYBs and specific genes within each respective pathway are highlighted as follows: ○ MYB28; ★ = MYB29; ⊙ = MYB76; ■ = MYB34; ♦ = MYB51; ✱ = MYB122. Genes with identified orthologs in the *E. sativa* genome annotation are written in black; those with no identified homologous sequence are written in gray. *SUM1, sulfur limitation 1*; *IQD1, protein IQ domain 1*; *BCAT, methionine aminotransferase*; *MAM, methylthioalkylmalate synthase*; *CYP, cytochrome P450*; *GST, glutathione-S-transferase*; *SUR1, C-S lyase 1*; *UGT, UDP-glycosyltransferase*; *FMOGS–OX, flavin-containing monooxygenase*; *ASA1, anthranilate synthase alpha subunit 1*; *TSB1, tryptophan synthase beta chain 1*; IAA, Indole-3-acetic acid; *NIT, nitrilase*; *ESM1, epithiospecifier modifier protein 1*; *NSP, nitrile specifier protein*.

Data File 1). C by comparison contained high concentrations of GSLs during shelf life, peaking at **D0**, with a non-significant decrease at **D7** (0.3-fold reduction). This line did not demonstrate the same decline in GSLs toward the end of shelf life as in the other two, and displays a propensity for maintaining GSLs for longer into the shelf life period.

Glucosinolate Hydrolysis Product Profiles and Contents of *E. sativa*

Glucosinolate hydrolysis product concentrations are presented in **Figure 10C** (see **Supplementary Data File 1** for ANOVA and Tukey's HSD significances). As with previous studies of rocket (Fechner et al., 2018), three main GHPs were detected: sativin (1,3-thiazepane-2-thione; hydrolysis product of GSV; SAT), erucin (ITC of glucoerucin; GER), and SF. The fluctuations in total GHP concentration mirror those observed for GSLs, however the increases between **H** and **SC** are much less pronounced, with no significant differences between cuts.

As with GSLs, line **B** displayed the lowest concentrations of GHPs, whereas the differences between lines **A** and **C** are less apparent. The trend of reduction of GHPs over shelf life is also visible for lines **A** and **B**, though only significant in **B** (a 0.9-fold reduction, $p < 0.0001$). Concentrations remained higher in line **C** (1.2 mg g^{-1} dw, a 0.6-fold reduction from **D0**).

Monosaccharide Profiles and Contents of *E. sativa*

Monosaccharides are important in terms of sensory attributes and the masking of bitter and pungent sensory attributes in rocket (Bell et al., 2017a) altering consumer perception and preference. Glucose is also known to influence stress responses and interact with MYB TFs (Gigolashvili et al., 2009; **Figure 11**). The concentrations of sugars observed in *E. sativa* lines are presented in **Figure 10D** (see **Supplementary Data File 1** for ANOVA and Tukey's HSD significances).

Unlike previous reports (Bell et al., 2017b) the changes in sugar concentrations in this study were dynamic across each of the respective time points. Both lines **A** and **B** contained low total concentrations compared to line **C**. Line **B** displayed consistent concentrations, with no significant differences observed. **A** showed a similar trend to GSL and GHP concentrations by declining at the end of shelf life (**D7**; a 0.5-fold decrease from **D0**, $p < 0.0001$).

Line **C** is distinct from the others in terms of its sugar profile and the relative differences between sample points. Concentrations increased postharvest (**D0** and **D7**; a 1.4 and 1.2-fold increase relative to **H**, respectively), perhaps owing to a breakdown of stored carbohydrate to facilitate respiration. Line **C** sugar content consists primarily of glucose, whereas **B** tended to have greater concentrations of galactose, and **A** was composed of similar amounts of each monosaccharide.

Principal Component Analysis of Sulfur and Glucosinolate Metabolism Genes

Hereafter, only correlations significant at the $p < 0.001$ level are presented and discussed in relation to the PCA. *SULTR4;1a* was significantly correlated with GRA concentrations ($r = 0.577$), which is associated with shelf life samples for lines **A** and **C** (**Figure 12B**, cluster II). **Figures 12A,B** show a distinct separation between ontogenic and shelf life samples along PC1. The increased expression of sulfur transport genes such as this postharvest may provide some explanation as to why GSL concentrations increase in the initial stages shelf life (**PW**), as S may be re-mobilized to facilitate biosynthesis. Efficient transport and storage of sulfur pre-harvest may also facilitate better retention and decreased degradation of GSLs postharvest. This can be seen in **Figure 12B** (**V**) where *SULTR3;1a* and *SULTR3;2* are associated with pre-harvest expression.

Sulforaphane and SAT concentrations were significantly correlated with *APR2a* gene expression (**Figure 12C I and II**; $r = 0.58$, SF; $r = 0.464$, SAT) and associated in particular with **A** ontogenic samples and **PW**. *APR2* is known to contribute to sulfur accumulation and homeostasis, as well as facilitating cysteine synthesis, and is associated with increased myrosinase activity and GSL recycling. Line **A** (on average) contained the highest ontogenic concentrations of GRA, SF, GSV, and SAT (**Figures 10B,C**); this is supported by a significant correlation and association with GSL-related TFs *MYB28a* ($r = 0.486$, SF; $r = 0.53$, SAT), *MYC2a* ($r = 0.596$, SF; $r = 0.626$, SAT) and *MYC2c* ($r = 0.584$, SF; $r = 0.583$, GSV; $r = 0.634$, SAT; **Figure 12C I and II**), as well as a drought tolerance-related gene *SAL1b* ($r = 0.595$, GRA; $r = 0.547$, SF; $r = 0.499$, SAT; **Figures 12A II, III and IV, 12C II**). **A** was also associated with increased activity of *MAM1a* (**Figure 12C II**), facilitating greater GRA biosynthesis through chain elongation. It may be that lines **A** and **C** have increased relative GRA concentrations at **EH** and **H**, but preferentially express *MYB28c* and *MYB28b*, respectively. It is unknown if the function of each MYB28 TF is redundant in rocket, but these data would suggest that there is some clear overlap of function, though the expression of *MYB28b* in particular is associated with increased GSL biosynthesis postharvest (**Figure 8**).

The lower relative expression in line **B** for many of these genes is consistent with its lower GSL and hydrolysis product concentrations, irrespective of sample point. GRA/SF, GSV/SAT, and GER concentrations were significantly and negatively correlated with *SPERMIDINE SYNTHASE 1c* (*SPDS1c*; $r = -0.622$, GRA; $r = -0.614$, SF; $r = -0.6$, GSV; $r = -0.454$, SAT; $r = -0.604$, GER), which had a high degree of co-separation in all **B** samples (**Figure 12C III**). This association may be related to increased primary S metabolism and reduced partitioning of methionine for secondary S metabolites (**Figure 1**).

DISCUSSION

The Complexity of the *E. sativa* Genome and Future Novel Gene Discovery

The presented reference genome and annotation for *E. sativa* shows a huge amount of complexity. The transcriptomic evidence presented here also illustrates just how variable traits and expression can be between breeding lines under controlled environmental conditions. In the three inbred lines tested, global differential expression of genes was highly variable, and suggests mechanisms present in commercial rocket that

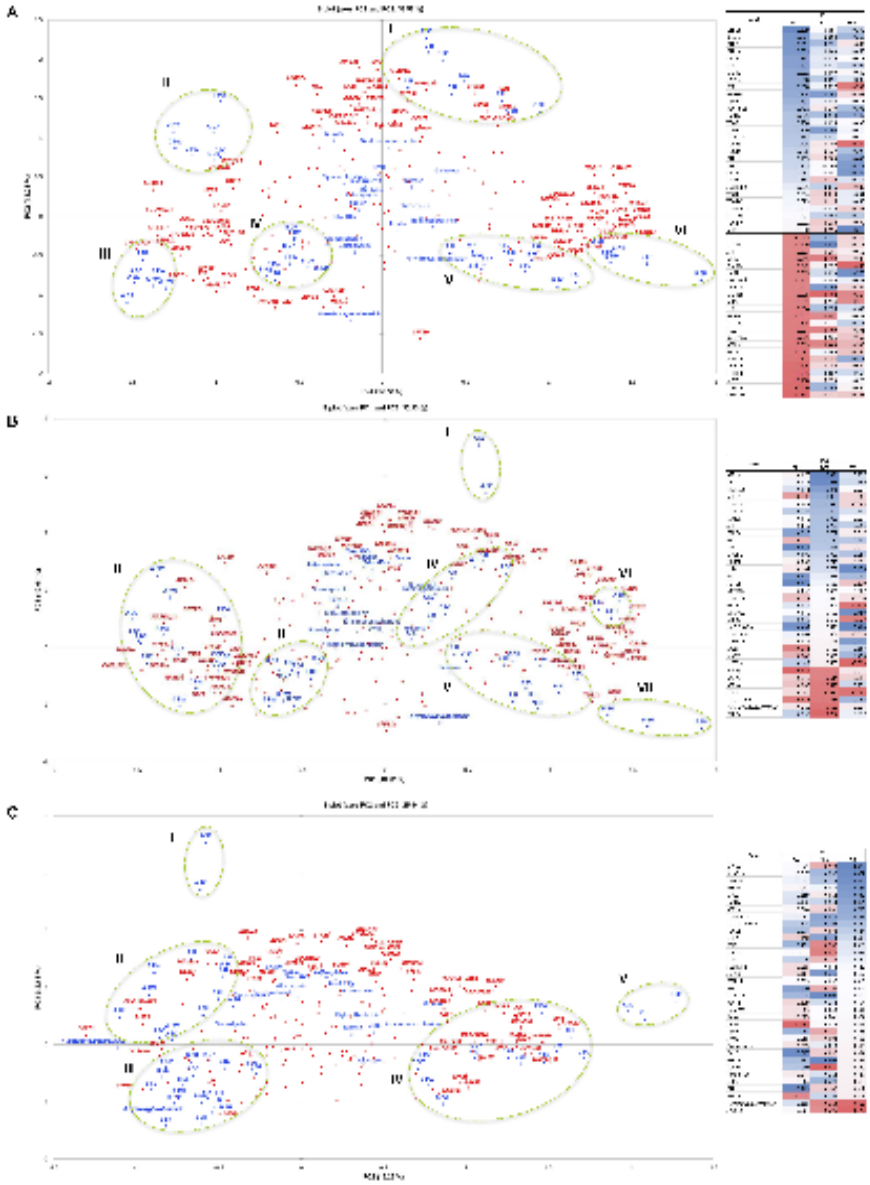

FIGURE 12 | Principal Component Analysis of sulfur assimilation pathway, glucosinolate biosynthesis, and glucosinolate hydrolysis gene expression data (FPKM) for three *Eruca sativa* elite inbred lines (**A**, **B**, and **C**) across ontogenic and postharvest sample points. Biplot **(A)** displays Principal Components (PCs) 1 and 2, which represent 33% of variation within the data. Biplot **(B)** displays PC1 and PC3, explaining 32.3% of variation within the data; and **(C)** displays PC2 and PC3, explaining 25.8% of the variability. The PCA plots presented are the results of Varimax rotation. Each biplot is accompanied by a factor loadings table sorted according to PC1 **(A)**, PC2 **(B)**, and PC3 **(C)**; Italics denotes a supplementary variable, and asterisks denotes a putative novel gene within the reference annotation. Blue coloration denotes high factor loading scores, red denotes low. Only genes with loading values >0.5 were included, and each is represented within the biplots in red (bold italics). Red circles represent individual genes included in the analysis (*n* = 177). Blue circles represent sample point variables and have accompanying labels (blue bold). Blue squares denote phytochemical data regressed onto the PCA as supplementary variables. Bold data labels indicate phytochemical components with >0.5 factor loadings scores. Green dotted ellipses denote clusters of variables and are numbered using Roman numerals, which are quoted within the text. **EH**, early harvest; **H**, harvest; **SC**, second harvest; **PW**, pre-wash; **D0**, post-wash; and **D7**, 7-day shelf life.

underlie differences in postharvest quality and shelf life performance. This is exemplified in line **C**, which displayed differential patterns of expression, both during growth stages and postharvest (**Supplementary Data File 3**). A literature search suggests some studies treat cultivars of *E. sativa* the same, without regard for potential differences in phytochemistry or postharvest quality (Jin et al., 2009; Selma et al., 2010), and that produce is genetically uniform. This study demonstrates wide variation between genotypes, and there is significant potential for further crop improvement for enhanced shelf life nutritional quality. Development of these data in *Eruca* is a major step forward for a crop once considered to be niche, and which now joins a growing list of minor crops in the genomic era. While this study has highlighted the orthologous genes that are likely to be involved with sulfur assimilation, GSL accumulation, and postharvest stress response, much further research will be needed to unpick novel gene functions and interactions.

Eruca sativa Has a Distinct and Complex Glucosinolate Pathway

Evolutionary divergence between *E. sativa* and other Brassicales species has led to a unique GSL synthesis pathway, displaying extensive gene duplication. Aside from the duplications of MYB28 found in salad rocket, one TF prominent in GSL biosynthesis has no orthologous sequence or expression in the tested rocket lines: MYB76. Similarly, other genes, such as MAM2, MAM3, CYP79F2, CYP71A13, GSTF9, UGT74C1, and NIT3, are all absent from the reported rocket genome annotation. While this may be amended with future annotation iterations and sequence improvements, it is conceivable that these genes may have been lost over the course of evolutionary time and divergence with *A. thaliana*.

It is not clear what the function(s) of gene copies and paralogues may be in *Eruca*. It may be the result of segmental duplications within the genome, such as those observed in the *Brassica* A genome (Jiang et al., 2011), and future, more detailed studies of the *Eruca* genome structure may reveal the nature and number of any such events. For example, *B. rapa* and *B. oleracea* contain two copies of SOT18 (Liu et al., 2014), whereas in *E. sativa* we report seven. *B. rapa* has two copies of FMO_{GS-OX} genes, and salad rocket has at least ten. The related *Diplotaxis tenuifolia* ("wild" rocket) transcriptome has been reported to contain three copies of MYB28 (Cavaiuolo et al., 2017), and is consistent with the hypothesis that duplication occurred after *Eruca* and *Diplotaxis* diverged with a common ancestor in the *B. oleracea* lineage.

One example of recent novel gene discovery outside of *Arabidopsis* and *Brassica* species GSL synthesis pathways is *GLUCORAPHASATIN SYNTHASE 1* (*GRS1*) in radish; which is thought to have evolved from a mutation in a 2-oxoglutarate-dependent dioxygenase (2OGD; Kakizaki et al., 2017). Similar mutations and modification of genes have likely occurred in *E. sativa* and led to the evolution of GSV, GRL, DGTB, and DMB. Future work will elucidate the genes responsible for synthesis of these GSLs. The development of the genomic and transcriptomic resources in this study are an important first step in achieving this.

Genes in Sulfate and Glucosinolate Pathways Are Strongly Correlated With Glucosinolate Biosynthesis

Principal Component Analysis highlighted several genes that are significantly correlated with the abundance of GSLs in *E. sativa*. In terms of sulfur assimilation, the expression of genes *SULTR4;1b* and *APR2a* appear to be strongly associated with both aliphatic and indolic GSL biosynthesis. *SULTR4;1* facilitates transport of sulfate from cell vacuoles into the cytoplasm, and has been previously linked with the activity of MYB28 and MYB29 (Sønderby et al., 2010), which is supported by this study. Likewise, co-expression analysis found that *MYB28b* and *SiRa* belong to the same gene module (**M6**); suggesting a transcriptional relationship between aliphatic GSL biosynthesis and primary sulfur metabolism. It may be that expression of *MYB28b* expedites the synthesis of GSLs by facilitating greater availability of sulfate. This is in turn linked with the activity of *APR2*, which is known to be responsible for regulating sulfur homeostasis (Kopriva et al., 2015). This gene has also been associated with increased GSL recycling and myrosinase activity (Maruyama-Nakashita et al., 2003). It is likely that the transport of sulfate and its management in terms of recycling is pivotal for GSL abundance and flux in rocket at any given time.

Postharvest Maintenance of Glucosinolate Content Is Related to Senescence and Oxidative Stress

As shown in **Figure 10A**, the content of sulfur between the three tested breeding lines was not significantly different. In light of the observed differences in gene expression and GSL accumulations, we theorize that primary and secondary sulfur metabolism pathways "compete" for assimilated environmental sulfur. As content was not significantly different postharvest (**PW**, **D0**, and **D7**) compared to pre-harvest first cut (**H**) in any of the breeding lines, the degree of remobilization and ability to synthesize/recycle GSLs is under strict genetic control. The evolutionary advantages of this are unclear, but as shown in **Figure 10B**, the amount of total sulfur assimilated during growth is not reflected in the postharvest concentrations of GSLs. Line **B** exemplifies this: it contains statistically no more or less sulfur than lines **A** or **C**, yet synthesizes far fewer GSLs and any given time point.

The natural strategy of the leaf is to remobilize sulfur around parts of the plant as required, such as in times of deficiency. The transcriptome of severed leaves in the postharvest context is an evolutionary dead end, and not subject to natural selection. As such, the differences we have observed can be attributed to different strategies for dealing with unexpected physiological stress, nutrient deficiencies, or as part of senescence responses. This is exemplified by the high relative expression of **M8** (**Figure 6**) in line **B**, where *SEN1* and *OXS3* are present; suggesting a deficiency in its ability to cope with oxidative stress compared to lines **A** and **C**.

Co-expression Patterns Reveal Possible Links Between Indole Glucosinolate Biosynthesis, Hydrolysis and Catabolism

The identified modules of expression contained genes involved with or linked to GSL biosynthesis and hydrolysis. Notably, module **M1** contains a number of indole GSL biosynthesis genes and myrosinases. In other Brassicales, indolic GSL biosynthesis is being increasingly linked with auxin and camalexin biosynthesis in related species, via shared reactions with indole-3-acetaldoxime (IAOx; Malka and Cheng, 2017). The high activity of such indole GSL-related genes in rocket suggests that indole GSLs have a high turnover *in planta*, as concentrations are typically low in rocket (**Figure 10B**). This is also supported by the high expression of genes such as *NIT2a* (**Figure 9**), which codes for nitrilase involved in the metabolism of indole-3-acetic acid (IAA). The co-expression of several myrosinases (TGG1s and TGG6s') within **M1** suggests that the hydrolysis of indole GSLs is intrinsically tied to these catabolic processes, and may therefore explain why indolic GSLs are found in such relatively low concentrations in rocket compared to other species (such as *B. oleracea*). The diversity of myrosinase genes identified within the rocket genome and the association of specific copies in **M1** also implies that these may have evolved specific paralogous functions within the indole-GSL pathway. These data therefore provide new insights into the role of indolic GSLs in non-model species, and numerous avenues for future study.

The Number of Identified Myrosinase Gene Copies Is Indicative of Specialized Functionalities

Perhaps of most interest and significance in this study is the high copy numbers of myrosinase genes (TGGs) present in the *Eruca* annotation. Both *Arabidopsis* and *B. rapa* have four myrosinase gene copies of TGG1 and TGG2, and *B. oleracea* has six (Liu et al., 2014). Our data indicate that *Eruca* has at least 14 copies, as well as two copies encoding PEN2 myrosinase. There has evidently been a massive diversification and duplication of these enzymes in rocket, but it has yet to be established if this is reflected in functionality and spatial expression. The high number of identified TGG1, TGG2, and (poorly characterized) TGG6 genes in *Eruca* also suggests diversified function, as paralogous gene duplications relieve the evolutionary pressure upon orthologous genes; thereby allowing for redundancy with the original function, and subsequently diversification of function over the course of evolutionary time (Selzer et al., 2018). The presented annotation therefore provides new information regarding myrosinase and PEN2 variability.

Such duplications demonstrate the importance of the pathway, offering resilience against random mutations and/or loss of function. Divergence indicates the roles of GSLs (and their associated downstream and upstream metabolites) are under evolutionary pressure to adapt to environmental conditions; perhaps as a means of deterring feeding insects or protecting against infections when cells are damaged. The duplication of myrosinase genes may also be linked to the unusual GSL profile of salad rocket, which contains several compounds not found outside of the *Eruca* and *Diplotaxis* genera. The mechanisms behind hydrolysis of compounds such as GSV and DMB are presently unknown, and the hydrolysis products of GRL and DGTB have not yet been identified. Similarly, the co-expression of TGG1 and TGG6 myrosinases with indolic GSL biosynthesis genes (**Supplementary Data File 2**) suggests specific functionality and involvement with catabolic processes therein. Through co-expression analysis we have identified four candidate myrosinases in gene module **M1** for future investigation.

CONCLUSION

Eruca sativa is a promising crop for future improvement, having numerous nutritional and sensory quality traits of benefit and interest to the consumer. We have produced the first reference genome sequence and annotation for the species that will aid in these efforts. The transcriptomic information associated with different harvest and shelf life time points indicates that there are complex mechanisms governing the nutritional quality of rocket leaves, that links sulfur metabolism, GSL biosynthesis, senescence, and oxidative stress responses. Through co-expression analysis we have identified multiple genes for future studies to target. These data will also assist in understanding how the unique GSL compounds found in *E. sativa* are synthesized, and what functions they have within the plant.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the European Nucleotide Archive: Assembly accession no GCA_902460325, Study ID: PRJEB34051, Sample ID: ERS3673677, Annotation accession number ERZ1066251.

AUTHOR CONTRIBUTIONS

LB and CW conceived and designed the experiment and analyses. RT and SK produced the breeding line seed material for genome sequencing and the RNAseq experiment. LB grew plants in controlled environment, performed RNA extractions and quality control, qRT-PCR validation, glucosinolate analysis by LC-MS, hydrolysis product analysis by GC-MS, performed ANOVAs, Pearson's correlation analyses, co-expression module analysis, and Principal Component Analyses of phytochemical and gene expression data. MP performed sugar analysis by HPLC. MC performed sulfur content analysis by ICP-OES. LB wrote the manuscript, with contributions from MC, RT, SK, LM, and CW. LB, LM, and CW obtained the funding. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.525102/full#supplementary-material

Supplementary Figure 1 | RNAseq experimental design and sampling diagram. Three elite inbred lines of *Eruca sativa* were grown under controlled environment conditions and sampled at each of the six time points indicated (in triplicate). **EH**, early harvest; **H**, harvest; **SC**, second harvest; **PW**, pre-wash; **D0**, post-wash; and **D7**, 7-day shelf life.

Supplementary Figure 2 | Pearson correlation matrix of RNAseq biological sample replicate gene expression values. Replicates of each sample showed a high degree of correlation (r^2 = >0.884) indicating robust reproducibility of gene expression between the individual plants tested at each respective sample point.

Supplementary Figure 3 | qRT-PCR (green) vs. RNAseq (orange) gene expression of ten randomly selected glucosinolate biosynthesis and hydrolysis-related genes. Data are expressed as the normalized log2-fold change in expression relative to the reference gene *ACT11*. ANOVA revealed no significant difference between the two data sets. **EH**, early harvest; **H**, harvest; **SC**, second harvest; **PW**, pre-wash; **D0**, post-wash; and **D7**, 7-day shelf life.

Supplementary Table 1 | Genome sequencing pooled DNA sample quality control data for three *Eruca sativa* elite inbred lines.

Supplementary Table 2 | RNAseq sample quality control data.

Supplementary Table 3 | qRT-PCR primers and efficiencies.

Supplementary Table 4 | Numbers of genes with homology or functional assignment.

Supplementary Data File 1 | Analysis of Variance outputs with Tukey's HSD pairwise comparisons between sample points and each respective rocket breeding line: Tab 1 – glucosinolate analysis; Tab 2 – GHP analysis; Tab 3 – sugar analysis. Highlighted values are significant at the following levels: $p = < 0.05$ (yellow), $p = < 0.01$ (orange), and $p = < 0.001$ (green). Tab 4 contains a Pearson's correlation analysis matrix for sulfur and glucosinolate-related gene expression values and phytochemical observations. Values in bold are significant correlations at the $p = < 0.001$ threshold.

Supplementary Data File 2 | List of genes identified by CEMiTool (Co-Expression Module Analysis) and the associated enrichment analysis statistics for each *Eruca sativa* genotype. Contains RNAseq read counts, log2-fold changes, *p*-values, and *q*-values (padj) for all genes within modules M1 to M8 for each of the three rocket lines and the respective sample points. Significant up/down regulation is denoted by green/red highlighting, respectively. KEGG annotation numbers and UniProt gene descriptions for orthologous genes in *A. thaliana* are provided.

Supplementary Data File 3 | RNAseq read counts, log2-fold changes, *p*-values, and *q*-values (padj) for sulfur metabolism, glucosinolate biosynthesis, hydrolysis, and transport genes for each of the three rocket lines and the respective sample points. Significant up/down regulation is denoted by green/red highlighting, respectively. KEGG annotation numbers and UniProt gene descriptions for orthologous genes in *A. thaliana* are provided.

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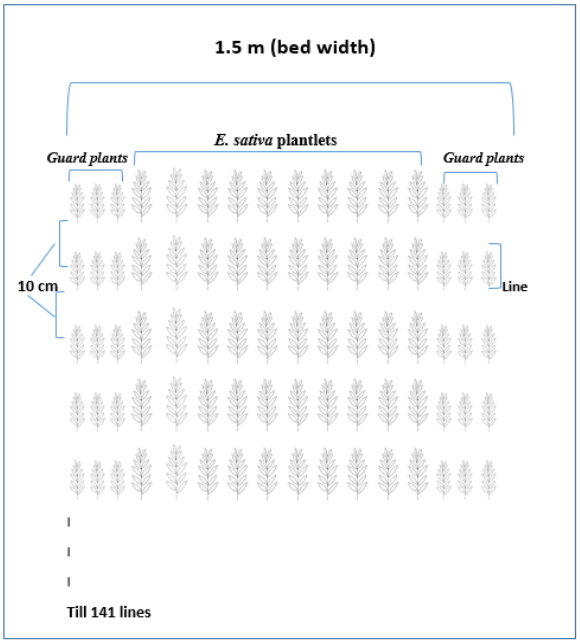
Conflict of Interest: RT and SK are employed by the company Elsoms Seeds Ltd.

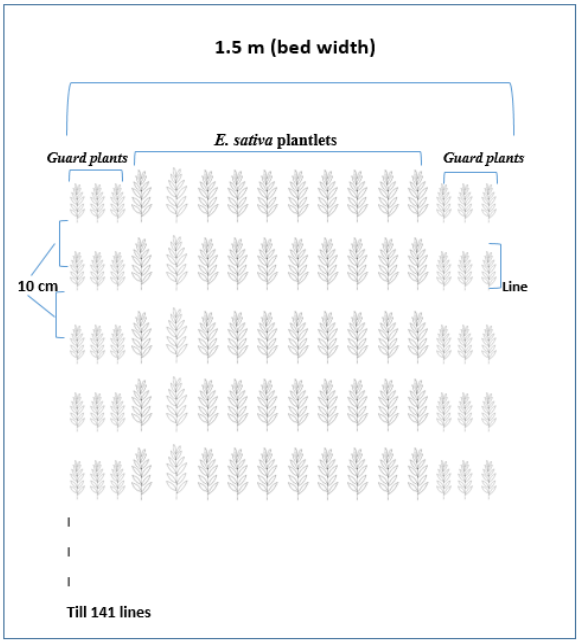
The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix 2. 1. Schematic representation of field trial.

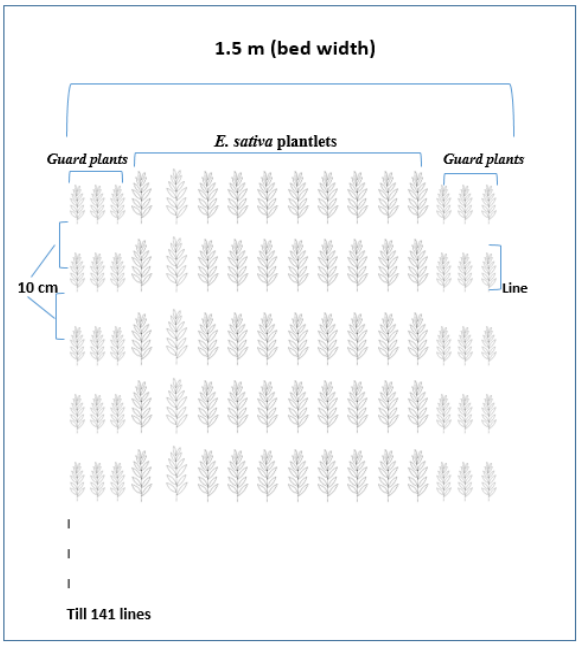
Block-1 (139 RILs and mapping parents)



Block- 2 (139 RILs and mapping parents)



Block-3 (139 RILs and mapping parents)



Appendix 2. 2. Screening Questionnaire – Solution Requirements.

Table 1			**Marking /50**
Task one: Please taste each of the solutions in the order given below and describe what you can taste. Please ensure that you cleanse your palate between samples using the water and biscuits provided.			**Office only, please**
040	5.8g sucrose/litre (stock)		Out of 6
279	1g salt/litre		
512	0.013g quinine/litre		
811	0.3g citric acid/litre		
795	0.1 g ferrous/litre		
365	0.3g MSG/litre		
Task Two - Please taste all the solutions and try to identify what it tastes. The taste will either be bitter, salty, sour, or sweet.			Out of 4:4 points for 4 correct. 2 for top 3 correct. 1 point for identifying the strongest.
Describe taste	SOUR		
Now rank the solutions in order from weakest to strongest			
Strongest -1	401 = 1.5g citric acid/litre (stock solution)		
2	120 = 1g/Litre (100mL stock plus 50mL water, i.e., total of 150 mL)		
3	516 = 0.5g/Litre (100mL stock plus 200mL water, i.e., total of 300 mL))		
Weakest - 4	309 = 0.25g/Litre (100mL stock plus 500mL water, i.e., total of 600 mL) *(50mL plus 250mL)*		
Task Three – Please sniff each of the tubes, one at a time, in the order given below, and describe what you can smell			Out of 3
1	Chocolate extract		
2	Vanilla essence		
3	Cloves		
Table 2			
Task Four - Please taste all the solutions and try to identify what it tastes. The taste will either be bitter, salty, sour, or sweet.			Out of 4:4 points for 4 correct. 2 for top 3 correct. 1 point for identifying the strongest.
Describe taste	BITTER - quinine		
Now rank the solutions in order from strongest to weakest			
Strongest – 1	951 = 0.08g/Litre (stock)		
2	693 = 0.03g/L (100mL stock plus 150mL water i.e., total of 250 mL) *(smaller quantities: 50mL plus 75mL for 125mL)*		
3	371 = 0.013 (100mL stock plus 400mL water, i.e., total of 500 mL) *(25mL plus 100mL for 125mL)*		
Weakest -4	174 = 0.005 (10mL stock plus 190mL water, i.e., total of 200 mL)		
Task Five – Please taste each of the drinks in the order given below. Two of the drinks are the same and one is different. Circle the code which you think is different and describe the difference			1 point
629	741	228	
Description of difference	629 & 741 = diet coke 228 = coke zero		

Task Six – Please sniff each of the tubes, one at a time, in the order given below, and describe what you can smell				
4	Eucalyptus			Out of 3
5	Orange oil			
6	Caramel			
Table 3				
Task seven – Colour blindness test. Please look at each 'plate' in the books provided and drew what you can see				
1	**12**	2	**74**	
3	**8**	4	**6**	
5	**29**	6	**45**	
7	**5**	8	**5**	
9	**3**	10	**7**	
11	**15**	12	**16**	
13	**73**	14	**Nothing**	1 point for all correct
15	**Nothing**	16	**Trace pattern**	
17	**Nothing**	18	**Trace pattern**	
19	**26**	20	**Trace pattern**	
21	**42**	22	**Trace pattern**	
23	**Trace pattern**	24	**Trace pattern**	
Task eight – Please sniff each of the tubes, one at a time, in the order given below, and describe what you can smell				
7	Mushroom			Out of 4
8	Thyme			
9	Onion			
10	Bouillon			
Task nine (PTC strips) – 1. Please take a strip, 2. Place the strip on your tongue for **1 sec**, 3. Describe **what you have tasted**.				5 points for the correct answer
Description	682 – PTC strip and 251 is the blind (coffee filters)			
Table 4				
Task ten – Complex taste solution recognition. Please taste each of the solutions in the order given below and describe the taste you recognise in each solution				
420	100mL stock sour + 100mL stock sugar			Out of 3
111	50mL stock bitter + 100mL sour stock + 100mL stock sugar			
938	100mL bitter stock + 100mL sugar stock			
Task eleven– Please taste each of the bread in the order given below. Two of the breads are the same and one is different. Circle the code which you think is different and describe the difference				1 point

429 = Hovis White	871 = Hovis White	333 = Asda white	
Description of difference			
Task twelve – Please sniff each of the tubes, one at a time, in the order given below, and describe what you can smell			Out of 3
11	Rose		
12	Basil		
13	Coffee		

Table 5		
Task thirteen - Please taste all the solutions and try to identify what it tastes. The taste will either be bitter, salty, sour, sweet or umami.		Out of 4:4 points for 4 correct. 2 for top 3 correct. 1 point for identifying the strongest.
Describe taste	UMAMI – monosodium glutamate	
Now rank the solutions in order from weakest to strongest		
Strongest -1	**495**=2.0 g/L (stock)	
2	**112**=0.7 g/L (350mL of stock plus 650mL of water**)** *(120mL plus 220mL to give 330mL)*	
3	**254**= 0.24 g/L (120mL of stock plus 880mL) *(60mL plus 440mL)*	
Weakest – 4	**819**= 0.08 g/L (40 mL of stock plus 960mL)	
Task fourteen - Please taste all the solutions and try to identify what it tastes. The taste will either be bitter, salty, sour, sweet or umami.		Out of 4:4 points for 4 correct. 2 for top 3 correct. 1 point for identifying the strongest.
Describe taste	SALTY –sodium chloride	
Now rank the solutions in order from strongest to weakest		
Strongest -1	**942**=1g/L stock	
2	**196**=0.69 g/L (690mL of 1g/L stock plus 310mL) *(345mL in 155mL)*	
3	**616**=0.5g/L (500mL of 1g/L stock plus 500mL of water**)** *(100mL plus 100mL)*	
Weakest – 4	**793**=0.24 g/L (240mL of 1g/L stock plus 760mL of water) *(120mL plus 380mL)*	
Task fifteen – Please taste all 6 custards and rank them in order of decreasing viscosity		
Rank order	Custard code	Out of 4:4 points for 6 correct. 2 for top 4 correct. 1 point for identifying the strongest.
Thickest – 1	**709 = 100mL custard Sainsbury's own**	
2	**184 = 100mL custard Sainsbury's own**	
3	**340 = 100mL custard Sainsbury's own + 16mL water**	
4	**992 = 100mL custard Sainsbury's own + 20mL water**	
5	**205 = 100mL custard Sainsbury's own + 25 mL water**	
Thinnest - 6	**674 = 100mL custard Sainsbury's own + 30mL water**	

Appendix 3. 1. Average total sugar, total acid, and total GSL concentration (mg. g $^{-1}$ DW) of a mapping population of 141 RILs of the F3 generation of *E. sativa* grown at two locations: Italy and the UK (n = 3) starting with the lowest to the highest concentration. Parent B (purple colour) and Parent C (orange colour) are included, with a red line showing similar total GSL content in both locations.

Italy (mg. g^{-1} DW)		**UK (**mg. g^{-1} DW)		**Italy (**mg. g^{-1} DW)		**UK (**mg. g^{-1} DW)		**Italy (**mg. g^{-1} DW)		**UK (**mg. g^{-1} DW)	
Trial no.	**Total sugar**	**Trial no.**	**Total sugar**	**Trial no.**	**Total acid**	**Trial no.**	**Total acid**	**Trial no.**	**Total GSL**	**Trial no.**	**Total GSL**
52x108_085	11.682	52x108_044	52.827	52x108_085	62.416	52x108_079	202.74	52x108_054	15.1011	52x108_058	14.7799
52x108_084	17.864	52x108_013	53.892	52x108_096	127.75	**Parent B**	204.83	52x108_013	15.2712	52x108_133	14.8829
52x108_096	18.441	52x108_091	54.382	52x108_080	159.33	52x108_095	205.41	52x108_041	15.321	52x108_090	14.9187
52x108_007	20.538	52x108_022	55.123	**Parent B**	159.4	52x108_103	212.05	52x108_031	15.3789	52x108_046	15.6772
52x108_066	20.997	52x108_101	55.141	52x108_131	184.92	52x108_068	216.13	52x108_037	15.4959	52x108_087	15.6821
52x108_106	21.115	52x108_113	55.148	52x108_001	187.91	52x108_094	218.05	52x108_122	15.5694	52x108_028	15.85
52x108_137	22.351	52x108_015	55.672	52x108_110	190.49	52x108_092	221.86	52x108_096	16.1132	52x108_013	15.8734
52x108_093	22.764	52x108_074	56.402	52x108_009	190.52	52x108_088	222.92	52x108_072	16.2301	52x108_077	16.2238
52x108_071	23.067	52x108_132	56.83	**Parent C**	192.21	52x108_100	224.31	52x108_051	16.2419	52x108_005	16.2667
52x108_122	23.434	52x108_031	57.301	52x108_027	195.23	52x108_040	224.53	52x108_127	16.6795	52x108_139	16.3024
52x108_127	23.634	52x108_082	59.491	52x108_025	195.79	52x108_007	224.63	52x108_065	16.7765	52x108_130	16.397
52x108_051	24.319	52x108_026	59.496	52x108_006	196.94	52x108_063	227.38	52x108_135	16.7997	52x108_023	16.9779
52x108_017	25.429	52x108_020	59.758	52x108_099	203.15	52x108_107	228.07	52x108_064	16.8795	52x108_120	16.9894
52x108_098	25.466	52x108_141	60.096	52x108_134	206	52x108_071	229.03	52x108_021	16.9247	52x108_132	17.0463
52x108_107	25.511	52x108_041	60.575	52x108_116	207.27	52x108_019	229.9	52x108_116	17.0136	52x108_122	17.3529
52x108_038	25.725	52x108_114	61.053	52x108_087	209.46	**Parent C**	230.24	52x108_084	17.1555	52x108_111	17.4165
52x108_052	25.737	52x108_095	61.593	52x108_109	211.73	52x108_134	230.48	52x108_066	17.5886	52x108_019	17.5892
52x108_040	26.207	52x108_097	62.293	52x108_089	211.8	52x108_033	231.56	52x108_106	17.6084	52x108_116	17.619
52x108_002	26.33	52x108_124	63.681	52x108_095	212.04	52x108_084	232.29	52x108_038	17.6184	52x108_138	17.6402
52x108_136	26.627	52x108_088	64.413	52x108_092	212.28	52x108_096	232.66	52x108_032	17.7337	52x108_105	17.6461
52x108_082	26.848	52x108_128	64.572	52x108_030	212.65	52x108_025	232.74	52x108_128	17.8022	52x108_022	17.7078
52x108_113	27.52	52x108_137	64.689	52x108_048	213.23	52x108_130	233.33	52x108_090	17.8131	52x108_106	17.7192
52x108_064	28.28	52x108_046	64.895	52x108_008	214.23	52x108_121	233.49	52x108_120	17.8549	52x108_103	17.7352
52x108_063	28.384	52x108_057	65.686	52x108_046	216.54	52x108_064	233.59	52x108_026	17.8791	52x108_031	17.7528
52x108_110	28.776	52x108_043	65.734	52x108_020	218.36	52x108_026	233.92	52x108_093	17.8947	52x108_052	17.7942
52x108_013	28.916	52x108_121	65.861	52x108_032	220	52x108_054	234.88	52x108_139	17.9155	52x108_041	17.9772
52x108_091	29.028	52x108_008	66.337	52x108_040	221.12	52x108_105	235.17	52x108_023	18.4877	52x108_135	18.0725

52x108_029	29.152	52x108_117	66.593	52x108_132	222.19	52x108_116	235.26	52x108_073	18.5263	52x108_007	18.0904
52x108_003	29.283	52x108_030	66.9	52x108_098	222.48	52x108_001	236.67	52x108_060	18.5768	52x108_037	18.1132
52x108_031	29.377	52x108_071	67.023	52x108_137	224.78	52x108_018	237.2	52x108_042	18.6047	52x108_009	18.1427
52x108_046	29.469	52x108_050	67.423	52x108_054	225.09	52x108_053	241.68	52x108_005	18.6376	52x108_020	18.2244
52x108_105	29.49	52x108_016	67.57	52x108_053	225.37	52x108_005	241.73	52x108_071	18.6958	52x108_085	18.3203
52x108_005	29.595	52x108_021	67.713	52x108_100	225.81	52x108_098	241.82	52x108_044	18.7172	52x108_008	18.3239
52x108_012	29.706	52x108_133	68.01	52x108_067	227.74	52x108_137	242.81	52x108_101	18.7276	52x108_057	18.3695
52x108_016	30.452	52x108_126	68.761	52x108_140	228.07	52x108_042	243.19	52x108_015	18.8058	52x108_024	18.5613
52x108_032	30.5	**Parent C**	68.787	52x108_103	228.98	52x108_070	243.63	**52x108_061**	**18.8193**	52x108_091	18.6077
52x108_077	31.052	52x108_032	68.905	52x108_055	229.51	52x108_020	243.81	52x108_012	18.8356	52x108_001	18.6209
52x108_083	31.069	52x108_052	68.971	52x108_097	230.23	52x108_123	243.84	52x108_007	18.9007	52x108_100	18.6941
52x108_128	31.146	52x108_038	69.17	52x108_013	230.45	52x108_044	244.06	52x108_077	19.0764	52x108_040	18.7341
52x108_117	31.393	52x108_131	69.37	52x108_141	230.55	52x108_050	244.68	52x108_033	19.0777	52x108_002	18.77
52x108_120	31.418	52x108_136	69.821	52x108_042	230.77	52x108_127	244.73	52x108_040	19.1118	52x108_088	18.7761
52x108_006	31.427	52x108_059	70.262	52x108_127	231.06	52x108_122	246.87	52x108_024	19.1169	**52x108_061**	**18.7875**
52x108_124	31.567	52x108_006	70.611	52x108_034	231.74	52x108_061	247.19	52x108_028	19.1953	52x108_051	18.8588
52x108_069	32.595	52x108_120	70.971	52x108_094	231.88	52x108_089	247.33	52x108_124	19.2326	52x108_101	18.9103
52x108_135	33.018	52x108_108	71.02	52x108_129	231.92	52x108_029	247.47	**Parent C**	19.4227	52x108_062	18.9311
52x108_070	33.702	52x108_073	71.158	52x108_138	232.1	52x108_075	248.11	52x108_027	19.5715	52x108_082	19.0039
Parent C	34.323	52x108_096	71.273	52x108_077	232.38	52x108_086	248.72	52x108_099	19.6288	52x108_113	19.0075
52x108_028	34.586	52x108_028	71.322	52x108_073	232.38	52x108_023	249.58	52x108_070	19.7067	52x108_072	19.0798
52x108_121	35.64	52x108_066	71.333	52x108_023	232.42	52x108_091	249.92	52x108_134	19.735	52x108_089	19.1046
52x108_010	36.192	52x108_009	71.611	52x108_130	233.3	52x108_048	250.27	52x108_138	19.8919	52x108_126	19.1878
52x108_101	36.23	52x108_119	72.543	52x108_123	233.48	52x108_027	251.14	52x108_141	20.0295	52x108_134	19.2007
52x108_090	36.445	52x108_045	72.564	52x108_093	233.69	52x108_032	252.6	52x108_014	20.1131	52x108_042	19.2731
52x108_130	36.446	52x108_018	73.086	52x108_126	233.71	52x108_017	252.88	52x108_022	20.1302	52x108_039	19.3152
52x108_099	36.536	52x108_093	73.148	52x108_038	234.61	52x108_132	252.95	52x108_117	20.1491	52x108_098	19.3544
52x108_059	36.678	52x108_011	73.651	52x108_018	235.08	52x108_090	253.54	52x108_058	20.1521	52x108_033	19.4023
52x108_065	36.686	52x108_129	73.68	52x108_064	235.1	52x108_002	254.38	52x108_115	20.2426	52x108_107	19.5028
52x108_072	36.971	52x108_086	74.294	52x108_017	235.98	52x108_036	254.4	52x108_008	20.257	52x108_128	19.5483
52x108_009	37.257	52x108_081	74.712	52x108_043	237.57	52x108_135	255.46	52x108_063	20.3062	52x108_016	19.5802
52x108_043	37.354	52x108_115	74.763	52x108_108	240.97	52x108_024	255.66	52x108_016	20.3208	52x108_038	19.5811

52x108_114	37.621	52x108_079	74.95	52x108_079	241.56	52x108_045	256.13	52x108_009	20.3638	52x108_034	19.5817
52x108_053	37.702	52x108_078	75.543	52x108_058	242.01	52x108_014	256.22	52x108_025	20.3804	52x108_071	19.5822
52x108_119	37.712	52x108_053	76.281	52x108_090	242.71	52x108_060	256.86	52x108_057	20.3945	52x108_079	19.6817
52x108_054	37.783	52x108_118	76.339	52x108_021	242.76	52x108_139	256.87	52x108_010	20.3987	52x108_124	19.721
52x108_057	38.313	Parent B	76.351	52x108_136	243.71	52x108_055	257.33	52x108_052	20.442	52x108_099	19.7489
52x108_014	38.491	52x108_094	77.452	52x108_115	244.32	52x108_129	257.55	52x108_088	20.4715	52x108_026	19.7996
52x108_061	38.536	52x108_025	77.62	52x108_078	245.08	52x108_067	257.57	52x108_020	20.5049	52x108_140	19.9732
52x108_087	38.557	52x108_122	77.9	52x108_124	245.37	52x108_087	258.19	52x108_091	20.5137	52x108_048	19.9742
52x108_078	38.696	52x108_109	77.956	52x108_031	247.5	52x108_010	258.2	52x108_048	20.5378	52x108_032	20.0071
52x108_023	38.777	52x108_092	78.128	52x108_026	248.64	52x108_118	258.38	52x108_108	20.6657	52x108_063	20.1273
52x108_116	38.96	52x108_039	78.269	52x108_007	248.8	52x108_136	258.93	52x108_119	20.6956	52x108_114	20.2083
52x108_080	39.09	52x108_135	78.897	52x108_015	248.88	52x108_119	258.97	52x108_110	20.7323	52x108_070	20.2217
52x108_079	39.125	52x108_062	79.651	52x108_047	249.08	52x108_128	259.34	52x108_079	20.7892	52x108_049	20.296
52x108_075	39.202	52x108_134	79.941	52x108_105	249.36	52x108_059	260.42	52x108_003	20.9154	52x108_108	20.2985
52x108_095	39.222	52x108_054	80.071	52x108_122	249.75	52x108_058	260.44	52x108_126	20.9544	52x108_059	20.307
52x108_089	39.224	52x108_100	80.104	52x108_010	250.02	52x108_062	261.97	52x108_082	20.9624	52x108_018	20.4347
52x108_109	39.394	52x108_099	80.17	52x108_012	251.93	52x108_099	262.34	52x108_059	21.0318	52x108_086	20.5312
52x108_076	39.669	52x108_007	80.313	52x108_118	254.14	52x108_093	262.52	52x108_132	21.1401	52x108_094	20.6306
52x108_068	39.904	52x108_040	80.389	52x108_057	254.77	52x108_038	263.73	52x108_129	21.1656	52x108_029	20.6821
52x108_112	39.928	52x108_017	80.576	52x108_082	255	52x108_035	263.87	52x108_019	21.2325	52x108_025	20.7251
52x108_048	40.047	52x108_019	81.058	52x108_139	255.17	52x108_138	264.16	52x108_137	21.2529	52x108_081	20.7276
52x108_027	40.2	52x108_103	81.891	52x108_084	255.82	52x108_008	264.7	52x108_133	21.2683	52x108_083	20.7554
52x108_008	40.845	52x108_061	82.797	52x108_003	256.8	52x108_022	264.89	52x108_067	21.331	52x108_076	20.8491
52x108_097	41.204	52x108_010	82.891	52x108_050	257.43	52x108_112	265.49	52x108_114	21.3673	52x108_097	20.8566
52x108_021	41.271	52x108_127	83.536	52x108_071	257.79	52x108_046	265.52	52x108_121	21.3886	52x108_056	20.8906
52x108_039	41.565	52x108_058	84.356	52x108_019	258.13	52x108_140	265.59	52x108_081	21.4662	52x108_050	20.9016
52x108_022	42.087	52x108_107	84.445	52x108_088	258.13	52x108_004	265.83	52x108_055	21.491	52x108_073	20.9019
52x108_126	42.35	52x108_069	84.525	52x108_014	258.19	52x108_030	266.04	52x108_140	21.5413	52x108_115	20.9579
52x108_108	42.644	52x108_105	84.96	52x108_120	258.2	52x108_015	266.48	52x108_018	21.5816	52x108_125	21.1147
52x108_015	42.839	52x108_068	85.783	52x108_063	259.14	52x108_081	266.89	52x108_006	21.6688	52x108_093	21.2755
52x108_058	43.574	52x108_084	85.934	52x108_005	259.3	52x108_003	267.07	52x108_083	21.6877	52x108_109	21.3014
52x108_067	43.863	52x108_065	86.043	52x108_076	259.77	52x108_074	268.27	52x108_075	21.705	52x108_084	21.3228

52x108_115	43.88	52x108_047	86.419	52x108_070	260.1	52x108_047	268.35	52x108_131	21.7971	52x108_078	21.3318
Parent B	44.098	52x108_089	86.754	52x108_117	261.69	52x108_115	268.75	52x108_107	21.9354	52x108_117	21.3376
52x108_018	44.416	52x108_001	86.858	52x108_004	261.77	52x108_065	268.84	52x108_056	22.0876	52x108_030	21.4375
52x108_044	44.445	52x108_087	86.872	52x108_113	261.98	52x108_110	268.88	52x108_097	22.1214	52x108_067	21.4836
52x108_094	44.809	52x108_080	86.97	52x108_107	262.59	52x108_114	269.33	52x108_109	22.2494	52x108_006	21.4869
52x108_055	44.991	52x108_112	87.025	52x108_041	262.99	52x108_085	270.05	52x108_136	22.2811	52x108_012	21.5522
52x108_141	45.535	52x108_063	87.213	52x108_069	263.56	52x108_041	271.29	52x108_076	22.3116	52x108_096	21.5679
52x108_060	45.677	52x108_085	87.553	52x108_133	263.75	52x108_031	271.4	52x108_078	22.4089	52x108_080	21.62
52x108_020	45.764	52x108_130	87.717	52x108_028	265.9	52x108_021	272.06	52x108_039	22.4925	52x108_047	21.7527
52x108_100	45.91	52x108_012	88.36	52x108_051	266.03	52x108_037	272.29	52x108_118	22.5253	52x108_092	21.756
52x108_132	46.062	52x108_090	88.841	52x108_044	266.05	52x108_077	272.73	52x108_011	22.5371	52x108_064	21.9008
52x108_073	46.077	52x108_076	89.039	52x108_091	267.97	52x108_124	272.83	52x108_017	22.5709	52x108_004	21.9349
52x108_092	46.18	52x108_037	89.081	52x108_106	268.13	52x108_066	275.4	52x108_113	22.6016	52x108_003	22.0592
52x108_049	46.32	52x108_060	89.421	52x108_111	268.25	52x108_101	275.56	52x108_045	22.7547	52x108_074	22.0645
52x108_074	46.331	52x108_051	90.159	52x108_033	269.02	52x108_052	276.05	52x108_036	22.9092	52x108_036	22.156
52x108_004	46.693	52x108_005	90.165	52x108_049	269.49	52x108_106	277.29	52x108_049	23.1002	52x108_112	22.2709
52x108_001	47.313	52x108_029	90.791	52x108_125	269.81	52x108_082	277.59	52x108_034	23.2802	52x108_129	22.2765
52x108_139	47.43	52x108_123	91.028	52x108_002	270	52x108_108	280.07	52x108_029	23.3896	52x108_123	22.29
52x108_056	47.455	52x108_056	91.4	52x108_059	270.32	52x108_113	280.37	52x108_046	23.4215	52x108_110	22.4375
52x108_140	47.925	52x108_077	91.81	52x108_052	270.63	52x108_057	280.48	52x108_130	23.4835	52x108_119	22.4854
52x108_088	48.033	52x108_110	92.359	52x108_066	271	52x108_012	280.68	52x108_098	23.5194	**Parent C**	22.5905
52x108_129	48.127	52x108_098	92.769	52x108_024	271.3	52x108_133	282.45	52x108_074	23.5721	52x108_065	22.5976
52x108_042	48.136	52x108_075	93.143	52x108_016	271.36	52x108_049	283.39	52x108_112	23.593	52x108_035	22.6599
52x108_133	48.581	52x108_042	93.688	52x108_083	272.53	52x108_013	284.43	52x108_062	23.714	52x108_027	22.7251
52x108_081	48.951	52x108_024	93.755	52x108_039	272.54	52x108_043	285.42	52x108_001	23.7143	52x108_118	22.8105
52x108_024	49.729	52x108_027	93.854	52x108_056	273.05	52x108_056	285.69	52x108_080	23.7441	52x108_069	22.8245
52x108_035	50.079	52x108_070	94.157	52x108_068	273.65	52x108_016	287.55	52x108_087	23.7876	52x108_017	22.9354
52x108_033	50.19	52x108_111	94.511	52x108_045	275.02	52x108_072	289.07	52x108_111	23.7906	52x108_141	22.9851
52x108_118	50.746	52x108_014	94.718	52x108_112	275.02	52x108_131	290.43	52x108_069	24.0371	52x108_066	23.0291
52x108_111	51.019	52x108_140	95.017	52x108_036	275.15	52x108_073	291.97	52x108_002	24.1216	52x108_136	23.0523
52x108_123	51.575	52x108_138	95.448	52x108_065	275.85	52x108_011	292.3	52x108_004	24.1779	52x108_121	23.1394
52x108_034	52.919	52x108_004	96.04	52x108_128	276.89	52x108_039	293.07	52x108_030	24.3215	52x108_055	23.1482

52x108_025	52.934	52x108_125	97.423	52x108_075	277.51	52x108_034	293.42	52x108_094	24.4803	52x108_010	23.158
52x108_103	54.401	52x108_106	98.082	52x108_114	277.71	52x108_141	293.81	52x108_125	24.4963	52x108_053	23.3097
52x108_125	55.283	52x108_048	100.68	52x108_022	280.86	52x108_009	294.1	52x108_095	24.5344	52x108_014	23.4697
52x108_138	56.246	52x108_055	101.12	52x108_061	282.79	52x108_076	294.26	52x108_103	24.545	52x108_011	23.5806
52x108_041	56.284	52x108_049	101.45	52x108_060	283.83	52x108_028	294.9	52x108_123	24.7045	52x108_054	23.597
52x108_037	56.575	52x108_023	101.59	52x108_135	285.16	52x108_006	295.49	**Parent B**	24.752	52x108_137	23.7205
52x108_030	57.473	52x108_003	102.99	52x108_119	288.64	52x108_109	297.02	52x108_035	24.8197	**Parent B**	23.7721
52x108_019	59.372	52x108_083	105.51	52x108_081	289.97	52x108_097	297.31	52x108_053	24.8464	52x108_015	23.818
52x108_045	59.394	52x108_064	107.41	52x108_029	289.97	52x108_051	299.2	52x108_105	24.8482	52x108_075	23.974
52x108_011	59.644	52x108_033	108.39	52x108_035	290.16	52x108_078	304.58	52x108_100	25.6066	52x108_021	24.4018
52x108_062	60.115	52x108_035	108.39	52x108_101	296.28	52x108_120	305.48	52x108_047	25.7942	52x108_127	24.5906
52x108_131	60.348	52x108_036	109.46	52x108_121	307.67	52x108_125	305.89	52x108_086	25.9202	52x108_044	24.9824
52x108_050	61.839	52x108_116	112.11	52x108_037	312.04	52x108_111	310.1	52x108_043	25.9227	52x108_045	25.1196
52x108_134	62.284	52x108_002	113.17	52x108_062	455.96	52x108_117	313.49	52x108_050	26.7039	52x108_060	25.4256
52x108_047	63.05	52x108_072	114.84	52x108_074	472.9	52x108_126	324.03	52x108_068	26.7444	52x108_068	25.5835
52x108_036	63.408	52x108_139	117.31	52x108_072	497.28	52x108_069	328.05	52x108_092	27.5073	52x108_043	25.8406
52x108_086	66.742	52x108_067	122.03	52x108_011	624.42	52x108_080	362.7	52x108_089	28.3161	52x108_131	25.9034
52x108_026	67.273	**52x108_034**	131.63	**52x108_086**	687.48	52x108_083	438.8	**52x108_085**	30.4475	52x108_095	27.7062

Appendix 3. 2. Average sucrose, glucose, galactose, and fructose concentration (mg. g $^{-1}$ DW) of a mapping population of 141 RILs of the F3 generation of *E. sativa* grown at two locations: Italy and the UK (n = 3) starting with the lowest to the highest concentration. Parent B (purple colour) and Parent C (orange colour) are included.

Trial no.	Sucrose-Italy (mg. g^{-1} DW)	Trial no.	Sucrose-UK (mg. g^{-1} DW)	Trial no.	Glucose-Italy (mg. g^{-1} DW)	Trial no.	Glucose-UK (mg. g^{-1} DW)	Trial no.	Galactose-Italy (mg. g^{-1} DW)	Trial no.	Galactose-UK (mg. g^{-1} DW)	Trial no.	Fructose-Italy (mg. g^{-1} DW)	Trial no.	Fructose-UK (mg. g^{-1} DW)
52x108_085	1.7	52x108_041	0.8	52x108_085	8.6	52x108_091	37.9	52x108_085	0.7	52x108_043	2.8	52x108_096	0.5	52x108_074	6.7
52x108_136	1.8	52x108_030	0.8	52x108_096	11.1	52x108_013	39.6	52x108_040	1.0	52x108_068	3.2	52x108_085	0.7	52x108_088	6.8
52x108_066	1.8	52x108_044	1.0	52x108_084	12.2	52x108_101	39.8	52x108_096	1.1	52x108_086	3.5	52x108_084	1.3	52x108_015	7.4
52x108_106	1.9	52x108_117	1.1	52x108_007	13.0	52x108_044	40.4	52x108_110	1.3	52x108_031	3.6	52x108_007	1.6	52x108_068	7.4
52x108_017	2.0	52x108_035	1.2	52x108_101	13.1	52x108_026	40.8	52x108_012	1.4	52x108_082	3.7	52x108_106	1.8	52x108_082	7.4
52x108_127	2.0	52x108_088	1.2	52x108_066	14.2	52x108_113	40.9	52x108_106	1.5	52x108_027	3.7	52x108_064	1.8	52x108_013	7.5
52x108_075	2.1	52x108_105	1.3	52x108_071	14.3	52x108_022	41.4	52x108_017	1.6	52x108_090	3.8	52x108_038	1.8	52x108_044	7.5
52x108_064	2.1	52x108_032	1.3	52x108_002	14.3	52x108_132	41.6	52x108_089	1.7	52x108_137	3.8	52x108_071	1.8	52x108_090	7.7
52x108_084	2.2	52x108_046	1.3	52x108_012	14.3	52x108_074	42.0	52x108_016	1.7	52x108_044	3.9	52x108_005	1.8	52x108_093	7.7
52x108_009	2.2	52x108_050	1.3	52x108_137	14.7	52x108_015	42.6	52x108_009	1.8	52x108_124	3.9	52x108_137	2.1	52x108_141	7.8
52x108_107	2.2	52x108_015	1.3	52x108_006	15.7	52x108_095	43.5	52x108_136	1.8	52x108_071	3.9	52x108_016	2.1	52x108_022	7.9
52x108_122	2.3	52x108_141	1.4	52x108_093	15.8	52x108_031	43.8	52x108_008	1.8	52x108_088	4.0	52x108_063	2.1	52x108_113	8.1
52x108_093	2.3	52x108_036	1.4	52x108_106	15.9	52x108_020	43.9	52x108_099	1.9	52x108_028	4.1	52x108_077	2.2	52x108_132	8.1
52x108_032	2.3	52x108_024	1.4	52x108_127	16.0	52x108_114	44.1	52x108_038	1.9	52x108_133	4.1	52x108_107	2.3	52x108_031	8.1
52x108_003	2.4	52x108_095	1.4	52x108_098	16.2	52x108_041	46.1	52x108_077	1.9	52x108_022	4.1	52x108_051	2.3	52x108_097	8.2
52x108_007	2.5	52x108_042	1.4	52x108_051	16.3	52x108_141	46.2	52x108_067	2.0	52x108_118	4.1	52x108_040	2.3	52x108_038	8.2
52x108_051	2.5	52x108_065	1.5	52x108_122	16.5	52x108_128	46.5	52x108_094	2.0	52x108_041	4.2	52x108_083	2.4	52x108_062	8.2
52x108_128	2.5	52x108_026	1.5	52x108_038	17.2	52x108_082	46.6	52x108_047	2.0	52x108_132	4.2	52x108_122	2.4	52x108_137	8.3
52x108_052	2.5	52x108_038	1.5	52x108_072	17.4	52x108_057	47.2	52x108_113	2.0	52x108_096	4.2	52x108_002	2.5	52x108_079	8.3
52x108_110	2.7	52x108_060	1.5	52x108_052	17.8	52x108_008	47.2	52x108_003	2.0	52x108_013	4.3	52x108_098	2.5	52x108_071	8.4
52x108_112	2.8	52x108_101	1.6	52x108_082	17.9	52x108_097	47.4	52x108_140	2.0	52x108_135	4.3	52x108_010	2.5	52x108_069	8.4
52x108_115	2.8	52x108_135	1.6	52x108_017	18.0	52x108_021	47.9	52x108_093	2.0	52x108_063	4.3	52x108_113	2.5	52x108_091	8.5
52x108_079	2.8	52x108_093	1.6	52x108_063	18.1	52x108_124	48.6	52x108_079	2.1	52x108_136	4.3	52x108_018	2.5	52x108_020	8.5
52x108_113	2.9	52x108_113	1.6	52x108_040	18.2	52x108_052	48.9	52x108_091	2.1	52x108_015	4.4	52x108_093	2.5	52x108_045	8.7
52x108_137	3.0	52x108_090	1.6	52x108_107	18.5	52x108_117	49.0	52x108_115	2.1	52x108_030	4.4	52x108_105	2.6	52x108_133	8.8
52x108_033	3.0	52x108_034	1.6	52x108_083	19.1	52x108_050	49.7	52x108_098	2.2	52x108_104	4.4	52x108_116	2.6	52x108_126	8.8
52x108_126	3.0	52x108_022	1.6	52x108_091	19.4	52x108_046	49.8	52x108_122	2.2	52x108_111	4.4	52x108_029	2.6	52x108_016	8.9
52x108_117	3.0	52x108_099	1.7	52x108_029	19.5	52x108_137	49.9	52x108_084	2.2	52x108_097	4.5	52x108_066	2.7	52x108_121	8.9
52x108_054	3.1	52x108_052	1.7	52x108_013	19.6	52x108_121	49.9	52x108_141	2.2	52x108_067	4.5	52x108_013	2.7	52x108_051	8.9
52x108_027	3.1	52x108_021	1.7	52x108_069	19.6	52x108_131	50.2	52x108_114	2.2	52x108_046	4.5	52x108_035	2.7	52x108_101	9.0
52x108_077	3.1	52x108_047	1.7	52x108_136	19.8	52x108_120	50.4	52x108_112	2.3	52x108_045	4.5	52x108_046	2.7	52x108_114	9.0
52x108_141	3.1	52x108_109	1.8	52x108_068	19.9	52x108_030	50.9	52x108_052	2.3	52x108_122	4.5	52x108_006	2.7	52x108_124	9.1
52x108_031	3.2	52x108_039	1.8	52x108_113	20.1	52x108_043	50.9	52x108_117	2.3	52x108_134	4.6	52x108_124	2.7	52x108_115	9.1
52x108_095	3.2	52x108_073	1.8	52x108_009	20.3	52x108_016	51.3	52x108_054	2.3	52x108_113	4.6	52x108_031	2.7	52x108_104	9.1
52x108_109	3.2	52x108_092	1.8	52x108_031	20.3	52x108_011	51.6	52x108_005	2.3	52x108_094	4.6	52x108_073	2.8	52x108_108	9.1
52x108_139	3.2	52x108_082	1.8	52x108_120	20.4	52x108_006	51.6	52x108_139	2.3	52x108_066	4.6	52x108_065	2.8	52x108_046	9.3
52x108_067	3.3	52x108_025	1.8	52x108_028	20.6	52x108_088	52.3	52x108_066	2.3	52x108_114	4.6	52x108_015	2.9	52x108_077	9.3
52x108_133	3.4	52x108_031	1.8	52x108_135	20.7	52x108_028	52.4	52x108_092	2.4	52x108_084	4.7	52x108_121	2.9	52x108_080	9.4
52x108_131	3.4	52x108_070	1.8	52x108_043	20.8	52x108_133	52.4	52x108_080	2.4	52x108_110	4.7	52x108_045	2.9	52x108_059	9.4
52x108_046	3.4	52x108_071	1.8	52x108_014	20.9	52x108_126	52.4	52x108_002	2.4	52x108_141	4.7	52x108_048	3.0	52x108_005	9.5
52x108_091	3.4	52x108_016	1.8	52x108_080	20.9	52x108_136	52.5	52x108_042	2.4	52x108_101	4.8	52x108_110	3.0	52x108_135	9.5
52x108_047	3.4	52x108_009	1.8	52x108_046	20.9	52x108_071	52.8	52x108_046	2.4	52x108_049	4.8	52x108_135	3.0	52x108_041	9.6
52x108_082	3.5	52x108_079	1.9	52x108_105	21.0	52x108_119	52.9	52x108_127	2.4	52x108_074	4.8	52x108_082	3.1	52x108_089	9.6
52x108_105	3.5	52x108_112	1.9	52x108_016	21.1	52x108_032	52.9	52x108_068	2.4	52x108_055	4.8	52x108_022	3.1	52x108_063	9.8

52x108_005	3.5	52x108_083	1.9	52x108_003	21.3	52x108_108	53.1	52x108_082	2.4	52x108_065	4.8	52x108_032	3.1	52x108_018	9.8
52x108_104	3.5	52x108_115	1.9	52x108_121	21.5	52x108_009	53.2	52x108_101	2.4	52x108_127	4.8	52x108_052	3.1	52x108_128	9.8
52x108_094	3.6	52x108_059	1.9	52x108_064	21.6	52x108_104	53.2	52x108_032	2.4	52x108_064	4.8	52x108_070	3.1	52x108_043	9.8
52x108_087	3.6	52x108_062	1.9	52x108_087	21.7	52x108_073	53.3	52x108_102	2.4	52x108_032	4.8	52x108_127	3.2	52x108_070	9.8
52x108_088	3.6	52x108_107	1.9	52x108_110	21.7	52x108_059	53.6	52x108_105	2.4	52x108_115	4.9	52x108_044	3.2	52x108_061	9.9
52x108_012	3.8	52x108_058	1.9	52x108_124	21.7	52x108_066	53.7	52x108_095	2.4	52x108_130	4.9	52x108_078	3.2	52x108_032	9.9
52x108_008	3.8	52x108_066	2.0	52x108_117	21.9	52x108_096	53.8	52x108_090	2.5	52x108_021	4.9	52x108_090	3.2	52x108_078	10.0
52x108_129	3.8	52x108_094	2.0	52x108_005	22.0	52x108_038	54.4	52x108_013	2.5	52x108_040	4.9	52x108_076	3.2	52x108_039	10.1
52x108_029	3.9	52x108_125	2.0	52x108_128	22.1	52x108_053	54.5	52x108_053	2.5	52x108_117	4.9	52x108_136	3.3	52x108_084	10.1
52x108_058	3.9	52x108_045	2.0	52x108_094	22.1	52x108_129	54.5	52x108_018	2.5	52x108_121	5.0	52x108_099	3.3	52x108_073	10.1
52x108_111	3.9	52x108_048	2.0	52x108_010	22.2	52x108_018	54.9	52x108_071	2.5	52x108_020	5.0	52x108_023	3.3	52x108_081	10.2
52x108_099	4.1	52x108_086	2.0	52x108_008	22.3	52x108_086	56.9	52x108_107	2.5	52x108_038	5.0	52x108_130	3.3	52x108_058	10.2
52x108_013	4.2	52x108_106	2.0	52x108_129	22.5	52x108_025	57.1	52x108_083	2.5	52x108_112	5.0	52x108_056	3.4	52x108_136	10.4
52x108_086	4.2	52x108_121	2.0	52x108_032	22.7	52x108_078	57.2	52x108_126	2.6	52x108_107	5.0	52x108_069	3.4	52x108_012	10.4
52x108_089	4.2	52x108_089	2.0	52x108_020	22.8	52x108_081	57.2	52x108_004	2.6	52x108_051	5.0	52x108_128	3.4	52x108_099	10.4
52x108_063	4.2	52x108_028	2.0	52x108_126	22.8	52x108_054	57.3	52x108_104	2.6	52x108_033	5.0	52x108_034	3.4	52x108_122	10.4
52x108_090	4.3	52x108_124	2.0	52x108_070	23.2	52x108_045	57.3	52x108_116	2.6	52x108_050	5.1	52x108_039	3.4	52x108_048	10.5
52x108_124	4.3	52x108_081	2.1	52x108_097	23.3	52x108_118	57.4	52x108_137	2.6	52x108_102	5.1	52x108_112	3.5	52x108_057	10.5
52x108_070	4.4	52x108_061	2.1	52x108_065	23.6	52x108_102	57.6	52x108_109	2.7	52x108_093	5.1	52x108_104	3.5	52x108_006	10.5
52x108_120	4.5	52x108_104	2.1	52x108_100	23.6	52x108_093	58.7	52x108_014	2.7	52x108_126	5.1	52x108_138	3.5	52x108_098	10.6
52x108_071	4.5	52x108_131	2.1	52x108_022	23.7	52x108_122	58.7	52x108_010	2.7	52x108_057	5.2	52x108_061	3.5	52x108_129	10.6
52x108_049	4.5	52x108_056	2.1	52x108_077	23.8	52x108_007	58.8	52x108_087	2.7	52x108_070	5.2	52x108_024	3.5	52x108_109	10.6
52x108_053	4.6	52x108_130	2.2	52x108_058	23.9	52x108_134	58.9	52x108_049	2.7	52x108_108	5.2	52x108_119	3.6	52x108_131	10.7
52x108_119	4.6	52x108_043	2.2	52x108_089	24.2	52x108_115	59.0	52x108_120	2.7	52x108_092	5.2	52x108_003	3.6	52x108_119	10.8
52x108_098	4.6	52x108_100	2.2	52x108_042	24.3	52x108_092	59.0	52x108_134	2.7	52x108_080	5.2	52x108_041	3.6	52x108_096	10.9
52x108_123	4.6	52x108_102	2.2	52x108_079	24.6	52x108_017	59.2	52x108_064	2.7	52x108_100	5.3	52x108_043	3.7	52x108_030	10.9
52x108_138	4.7	52x108_068	2.2	52x108_132	24.6	52x108_079	59.4	52x108_124	2.8	52x108_081	5.3	52x108_001	3.7	52x108_010	10.9
52x108_132	4.7	52x108_080	2.2	52x108_104	24.7	52x108_039	59.5	52x108_111	2.8	52x108_078	5.3	52x108_055	3.8	52x108_008	11.0
52x108_040	4.7	52x108_005	2.2	52x108_076	24.8	52x108_094	59.7	52x108_030	2.8	52x108_109	5.3	52x108_060	3.8	52x108_087	11.0
52x108_059	4.8	52x108_097	2.2	52x108_021	24.8	52x108_125	60.1	52x108_015	2.8	52x108_023	5.3	52x108_021	3.8	52x108_066	11.1
52x108_038	4.8	52x108_008	2.2	52x108_130	25.2	52x108_019	60.2	52x108_023	2.8	52x108_075	5.3	52x108_120	3.9	52x108_040	11.2
52x108_023	4.8	52x108_006	2.3	52x108_059	25.2	52x108_109	60.3	52x108_130	2.8	52x108_062	5.3	52x108_059	3.9	52x108_009	11.2
52x108_014	4.8	52x108_134	2.3	52x108_081	25.3	52x108_100	60.8	52x108_075	2.8	52x108_059	5.3	52x108_017	3.9	52x108_118	11.2
52x108_057	4.8	52x108_051	2.3	52x108_049	25.4	52x108_103	61.0	52x108_059	2.8	52x108_103	5.3	52x108_054	3.9	52x108_094	11.2
52x108_108	4.9	52x108_020	2.3	52x108_061	25.7	52x108_040	61.2	52x108_081	2.8	52x108_095	5.3	52x108_027	3.9	52x108_055	11.3
52x108_114	5.0	52x108_033	2.3	52x108_140	25.8	52x108_099	62.1	52x108_033	2.9	52x108_123	5.4	52x108_033	4.0	52x108_095	11.3
52x108_092	5.1	52x108_091	2.3	52x108_114	25.8	52x108_047	62.8	52x108_135	2.9	52x108_079	5.4	52x108_053	4.0	52x108_050	11.3
52x108_078	5.1	52x108_096	2.4	52x108_044	25.9	52x108_135	63.5	52x108_073	2.9	52x108_009	5.4	52x108_108	4.0	52x108_102	11.4
52x108_042	5.1	52x108_054	2.4	52x108_139	25.9	52x108_127	63.8	52x108_037	2.9	52x108_014	5.4	52x108_109	4.0	52x108_049	11.4
52x108_130	5.1	52x108_014	2.4	52x108_039	26.3	52x108_107	63.9	52x108_125	2.9	52x108_128	5.4	52x108_057	4.0	52x108_117	11.6
52x108_140	5.2	52x108_119	2.4	52x108_057	26.4	52x108_010	64.0	52x108_043	2.9	52x108_076	5.4	52x108_115	4.1	52x108_105	11.6
52x108_004	5.2	52x108_129	2.4	52x108_090	26.4	52x108_062	64.2	52x108_070	3.0	52x108_018	5.5	52x108_091	4.1	52x108_065	11.6
52x108_048	5.3	52x108_010	2.4	52x108_078	26.5	52x108_061	64.9	52x108_119	3.0	52x108_042	5.5	52x108_080	4.2	52x108_026	11.6
52x108_061	5.5	52x108_126	2.4	52x108_119	26.6	52x108_029	65.4	52x108_022	3.0	52x108_016	5.5	52x108_075	4.2	52x108_130	11.8
52x108_016	5.5	52x108_013	2.5	52x108_053	26.7	52x108_037	65.5	52x108_057	3.1	52x108_001	5.5	52x108_117	4.2	52x108_100	11.9

52x108_125	5.6	52x108_023	2.5	52x108_116	27.0	52x108_085	66.1	52x108_050	3.1	52x108_026	5.5	52x108_103	4.3	52x108_112	11.9
52x108_068	5.6	52x108_084	2.5	52x108_099	27.3	52x108_001	66.2	52x108_132	3.1	52x108_012	5.5	52x108_097	4.3	52x108_086	11.9
52x108_096	5.7	52x108_077	2.5	52x108_018	27.6	52x108_060	66.3	52x108_029	3.1	52x108_098	5.6	52x108_074	4.3	52x108_092	12.1
52x108_074	5.7	52x108_127	2.5	52x108_023	27.9	52x108_105	66.5	52x108_031	3.1	52x108_010	5.6	52x108_028	4.4	52x108_017	12.1
52x108_028	5.8	52x108_001	2.5	52x108_055	28.0	52x108_058	66.5	52x108_058	3.1	52x108_091	5.6	52x108_123	4.5	52x108_120	12.2
52x108_050	6.0	52x108_037	2.5	52x108_024	28.2	52x108_087	67.0	52x108_128	3.1	52x108_105	5.6	52x108_114	4.5	52x108_052	12.2
52x108_069	6.3	52x108_076	2.6	52x108_048	28.3	52x108_069	67.5	52x108_044	3.1	52x108_017	5.7	52x108_011	4.6	52x108_011	12.2
52x108_135	6.4	52x108_069	2.6	52x108_092	28.4	52x108_076	67.8	52x108_001	3.1	52x108_077	5.7	52x108_037	4.7	52x108_110	12.2
52x108_081	6.4	52x108_136	2.6	52x108_054	28.6	52x108_065	68.2	52x108_051	3.2	52x108_019	5.7	52x108_134	4.7	52x108_053	12.3
52x108_060	6.7	52x108_019	2.6	52x108_073	28.6	52x108_112	68.3	52x108_035	3.2	52x108_058	5.7	52x108_020	4.7	52x108_127	12.4
52x108_116	6.7	52x108_138	2.6	52x108_015	28.6	52x108_123	68.5	52x108_006	3.2	52x108_005	5.7	52x108_131	4.8	52x108_007	12.5
52x108_100	6.8	52x108_137	2.7	52x108_102	28.7	52x108_089	68.5	52x108_021	3.2	52x108_120	5.7	52x108_118	4.8	52x108_085	12.5
52x108_062	6.9	52x108_120	2.7	52x108_095	28.8	52x108_056	68.6	52x108_065	3.2	52x108_048	5.8	52x108_095	4.8	52x108_019	12.5
52x108_011	6.9	52x108_064	2.7	52x108_030	29.1	52x108_084	68.7	52x108_069	3.2	52x108_035	5.8	52x108_036	4.8	52x108_001	12.6
52x108_025	7.0	52x108_103	2.7	52x108_056	29.4	52x108_023	68.8	52x108_097	3.3	52x108_025	5.8	52x108_133	4.9	52x108_106	12.6
52x108_083	7.0	52x108_087	2.7	52x108_109	29.5	52x108_130	68.9	52x108_027	3.3	52x108_125	5.8	52x108_004	4.9	52x108_103	12.8
52x108_065	7.1	52x108_133	2.7	52x108_108	29.6	52x108_012	69.5	52x108_026	3.3	52x108_085	5.9	52x108_102	4.9	52x108_028	12.8
52x108_001	7.1	52x108_128	2.8	52x108_027	29.9	52x108_140	70.0	52x108_121	3.4	52x108_053	5.9	52x108_062	5.0	52x108_027	12.9
52x108_002	7.2	52x108_049	2.8	52x108_141	30.1	52x108_075	70.0	52x108_025	3.4	52x108_008	5.9	52x108_025	5.3	52x108_025	12.9
52x108_101	7.7	52x108_007	2.8	52x108_088	30.1	52x108_080	70.1	52x108_048	3.4	52x108_073	5.9	52x108_019	6.5	52x108_076	13.2
52x108_072	7.7	52x108_029	2.8	52x108_075	30.1	52x108_063	70.1	52x108_024	3.4	52x108_024	6.0	52x108_072	6.7	52x108_021	13.2
52x108_035	7.8	52x108_012	2.9	52x108_067	30.2	52x108_110	71.5	52x108_007	3.5	52x108_061	6.0	52x108_086	7.6	52x108_111	13.3
52x108_121	7.8	52x108_018	2.9	52x108_074	31.4	52x108_111	72.1	52x108_076	3.5	52x108_029	6.0	52x108_067	8.4	52x108_004	13.3
52x108_102	8.1	52x108_057	2.9	52x108_060	31.4	52x108_004	72.3	52x108_088	3.5	52x108_037	6.0	52x108_089	9.1	52x108_024	13.6
52x108_076	8.2	52x108_074	3.0	52x108_112	31.5	52x108_042	72.5	52x108_019	3.5	52x108_099	6.0	52x108_079	9.6	52x108_123	13.6
52x108_039	8.4	52x108_063	3.0	52x108_111	32.0	52x108_005	72.7	52x108_039	3.5	52x108_056	6.1	52x108_141	10.1	52x108_107	13.6
52x108_015	8.5	52x108_132	3.0	52x108_001	33.3	52x108_024	72.8	52x108_118	3.6	52x108_060	6.1	52x108_014	10.1	52x108_138	13.7
52x108_055	8.7	52x108_085	3.0	52x108_026	33.3	52x108_068	72.9	52x108_103	3.6	52x108_069	6.1	52x108_012	10.2	52x108_083	13.9
52x108_010	8.8	52x108_078	3.1	52x108_041	33.5	52x108_138	73.0	52x108_034	3.6	52x108_087	6.1	52x108_092	10.3	52x108_064	13.9
52x108_118	8.8	52x108_075	3.2	52x108_118	33.6	52x108_014	73.0	52x108_100	3.6	52x108_138	6.1	52x108_087	10.5	52x108_014	13.9
52x108_021	9.4	52x108_067	3.2	52x108_004	34.0	52x108_098	73.1	52x108_060	3.7	52x108_140	6.1	52x108_088	10.8	52x108_002	14.1
52x108_006	9.8	52x108_040	3.2	52x108_138	34.2	52x108_027	73.9	52x108_028	3.8	52x108_011	6.1	52x108_100	11.8	52x108_134	14.2
52x108_043	9.9	52x108_114	3.2	52x108_125	34.5	52x108_051	73.9	52x108_061	3.9	52x108_054	6.1	52x108_068	11.9	52x108_054	14.3
52x108_019	10.1	52x108_140	3.3	52x108_103	34.8	52x108_003	74.2	52x108_078	3.9	52x108_007	6.2	52x108_111	12.3	52x108_042	14.3
52x108_134	10.2	52x108_003	3.3	52x108_115	34.8	52x108_077	74.3	52x108_063	3.9	52x108_129	6.2	52x108_125	12.3	52x108_056	14.6
52x108_026	10.2	52x108_116	3.4	52x108_034	35.5	52x108_090	75.8	52x108_123	3.9	52x108_052	6.2	52x108_030	12.4	52x108_075	14.6
52x108_097	10.4	52x108_027	3.4	52x108_045	35.7	52x108_106	77.2	52x108_056	4.1	52x108_006	6.2	52x108_129	12.4	52x108_037	15.0
52x108_034	10.4	52x108_139	3.5	52x108_037	35.8	52x108_070	77.3	52x108_108	4.2	52x108_106	6.2	52x108_058	12.7	52x108_047	15.0
52x108_056	10.5	52x108_123	3.6	52x108_050	36.0	52x108_055	79.8	52x108_133	4.2	52x108_004	6.3	52x108_008	12.9	52x108_036	15.4
52x108_080	11.6	52x108_108	3.6	52x108_133	36.1	52x108_083	81.8	52x108_062	4.3	52x108_131	6.3	52x108_009	13.0	52x108_060	15.5
52x108_018	11.8	52x108_098	3.6	52x108_035	36.4	52x108_049	82.4	52x108_045	4.3	52x108_072	6.4	52x108_101	13.1	52x108_140	15.6
52x108_103	11.8	52x108_017	3.6	52x108_025	37.2	52x108_048	82.4	52x108_055	4.5	52x108_116	6.4	52x108_132	13.6	52x108_035	16.2
52x108_073	11.8	52x108_053	3.6	52x108_036	37.4	52x108_116	82.8	52x108_041	4.6	52x108_119	6.5	52x108_049	13.7	52x108_029	16.5
52x108_044	12.3	52x108_118	3.6	52x108_123	38.5	52x108_033	84.0	52x108_086	4.7	52x108_139	6.5	52x108_126	13.9	52x108_033	17.0
52x108_022	12.3	52x108_011	3.7	52x108_019	39.3	52x108_035	85.2	52x108_011	4.7	52x108_002	6.5	52x108_081	14.3	52x108_139	17.6
52x108_020	12.6	52x108_002	3.8	52x108_033	40.4	52x108_036	85.2	52x108_131	4.8	52x108_089	6.6	52x108_047	14.4	52x108_072	18.1
52x108_030	13.1	52x108_072	3.9	52x108_047	43.2	52x108_064	86.0	52x108_036	4.9	52x108_003	6.7	52x108_140	14.9	52x108_067	18.3
52x108_037	13.2	52x108_110	4.0	52x108_011	43.5	52x108_072	86.4	52x108_074	4.9	52x108_047	6.9	52x108_139	16.0	52x108_003	18.8
52x108_041	14.5	52x108_004	4.1	52x108_062	43.9	52x108_002	88.8	52x108_072	5.1	52x108_039	6.9	52x108_042	16.4	52x108_116	19.6
52x108_024	14.6	52x108_122	4.2	52x108_134	44.7	52x108_139	89.7	52x108_020	5.7	52x108_036	7.5	52x108_050	16.8	52x108_034	20.5
52x108_036	16.3	52x108_111	4.7	52x108_131	47.4	52x108_067	96.1	52x108_129	9.3	52x108_034	7.6	52x108_094	17.1	52x108_023	24.9
52x108_045	16.4	52x108_055	5.3	52x108_086	50.3	52x108_034	101.9	52x108_138	13.8	52x108_083	8.0	52x108_026	20.4	52x108_125	29.5

Appendix 3. 3. ANOVA pairwise comparisons (P≤0.05) of sugars, organic acids and glucosinolates for Parent B and Parent C between Italy and UK mapping population trials as representative averages (n = 3). Values are expressed as mg. g^{-1} dry weight. Letters within columns denote statistical significance; values with the same letters present are not statistically significant from one another.

mg. g^{-1} DW	Parent B*UK	Parent C*UK	Parent B*Italy	Parent C*Italy	P > F
Sugars					
Sucrose	2.205 a	2.095 a	8.057 a	3.490 a	0.061
Glucose	57.616 a	53.179 ab	28.720 bc	24.700 c	0.007
Galactose	5.117 a	4.410 a	2.435 a	2.635 a	0.039
Fructose	11.414 a	9.103 ab	4.885 bc	3.498 c	0.004
Total sugars	76.351 a	68.787 a	44.098 ab	34.323 b	0.015
Organic acids					
Citric	75.723 a	70.835 a	60.119 a	89.645 a	0.108
Malic	51.946 a	39.864 ab	31.407 ab	21.078 b	0.043
Succinic	77.159 a	119.541 a	67.872 a	81.489 a	0.090
Total acids	204.828 a	230.240 a	159.397 a	192.212 a	0.191
Glucosinolates					
Glucoraphanin	4.870 a	3.828 a	1.269 b	1.413 b	<0.0001
Progoitrin	0.228 a	0.214 a	0.124 a	0.316 a	0.511
Glucoalyssin	0.050 a	0.144 a	0.007 a	0.080 a	0.519
Diglucothiobeinin	0.367 a	0.370 a	0.059 a	0.122 a	0.030
Glucosativin	0.362 b	0.353 b	1.439 a	0.650 ab	0.022
4-hydroxyglucobrassicin	0.064 a	0.000 a	0.007 a	0.000 a	0.530
Glucoerucin	1.462 a	1.175 a	2.378 a	2.261 a	0.045
Dimeric-4-mercaptobutyl	15.866 a	15.844 a	17.480 a	12.375 a	0.119
4-methoxyglucobrassicin	0.048 b	0.264 b	1.455 a	1.324 a	0.000
Neoglucobrassicin	0.455 b	0.399 b	0.533 b	0.882 a	0.002
Total GSLs	23.772 a	22.590 a	24.752 a	19.423 a	0.174

Appendix 3. 4. Correlation matrix between various phytochemical components, with corresponding p-values.

Correlation matrix (Spearman (n)):

Variables	Sucrose	Glucose	Galactose	Fructose	Total sugars	Citric	Malic	Succinic	Total acids	Glucoraphanin	Progoitrin	Glucoalyssin	Diglucothiobeinin	Glucosativin	4-hydroxyglucobrassicin	Glucoerucin	Dimeric-4-mercaptobutyl	4-methoxyglucobrassicin	Neoglucobrassicin	Total GSLs
Sucrose	1	-0.456	-0.397	-0.318	-0.401	0.150	-0.466	0.113	-0.099	-0.590	-0.322	-0.451	-0.605	0.475	0.356	0.633	-0.065	0.521	0.389	0.138
Glucose	-0.456	1	0.800	0.689	0.981	-0.122	0.605	-0.088	0.166	0.780	0.405	0.504	0.594	-0.474	-0.406	-0.702	0.288	-0.784	-0.542	-0.019
Galactose	-0.397	0.800	1	0.584	0.827	-0.070	0.649	0.032	0.279	0.688	0.299	0.405	0.528	-0.497	-0.416	-0.663	0.217	-0.696	-0.493	-0.075
Fructose	-0.318	0.689	0.584	1	0.779	-0.216	0.505	-0.098	0.078	0.600	0.276	0.396	0.433	-0.249	-0.306	-0.462	0.278	-0.567	-0.391	0.078
Total sugars	-0.401	0.981	0.827	0.779	1	-0.141	0.620	-0.067	0.178	0.773	0.379	0.489	0.572	-0.454	-0.410	-0.680	0.304	-0.778	-0.534	-0.001
Citric	0.150	-0.122	-0.070	-0.216	-0.141	1	0.008	0.258	0.570	-0.225	-0.102	-0.121	-0.303	0.114	0.084	0.198	-0.071	0.111	0.312	-0.031
Malic	-0.466	0.605	0.649	0.505	0.620	0.008	1	0.235	0.602	0.580	0.290	0.357	0.534	-0.388	-0.334	-0.600	0.190	-0.545	-0.459	-0.047
Succinic	0.113	-0.088	0.032	-0.098	-0.067	0.258	0.235	1	0.750	-0.071	-0.059	-0.007	-0.057	-0.035	0.019	0.017	-0.050	0.086	0.035	-0.045
Total acids	-0.099	0.166	0.279	0.078	0.178	0.570	0.602	0.750	1	0.138	0.041	0.110	0.085	-0.170	-0.130	-0.197	0.040	-0.164	-0.054	-0.062
Glucoraphanin	-0.590	0.780	0.688	0.600	0.773	-0.225	0.580	-0.071	0.138	1	0.285	0.626	0.743	-0.520	-0.392	-0.672	0.378	-0.688	-0.631	0.154
Progoitrin	-0.322	0.405	0.299	0.276	0.379	-0.102	0.290	-0.059	0.041	0.285	1	0.291	0.455	0.103	-0.184	-0.351	0.216	-0.345	-0.234	0.105
Glucoalyssin	-0.451	0.504	0.405	0.396	0.489	-0.121	0.357	-0.007	0.110	0.626	0.291	1	0.514	-0.356	-0.321	-0.485	0.278	-0.407	-0.423	0.164
Diglucothiobeinin	-0.605	0.594	0.528	0.433	0.572	-0.303	0.534	-0.057	0.085	0.743	0.455	0.514	1	-0.389	-0.345	-0.680	0.248	-0.597	-0.583	0.029
Glucosativin	0.475	-0.474	-0.497	-0.249	-0.454	0.114	-0.388	-0.035	-0.170	-0.520	0.103	-0.356	-0.389	1	0.361	0.594	0.229	0.502	0.347	0.465
4-hydroxyglucobrassicin	0.356	-0.406	-0.416	-0.306	-0.410	0.084	-0.334	0.019	-0.130	-0.392	-0.184	-0.321	-0.345	0.361	1	0.556	-0.083	0.488	0.375	0.182
Glucoerucin	0.633	-0.702	-0.663	-0.462	-0.680	0.198	-0.600	0.017	-0.197	-0.672	-0.351	-0.485	-0.680	0.594	0.556	1	-0.048	0.730	0.629	0.327
Dimeric-4-mercaptobutyl	-0.065	0.288	0.217	0.278	0.304	-0.071	0.190	-0.050	0.040	0.378	0.216	0.278	0.248	0.229	-0.083	-0.048	1	-0.227	-0.165	0.855
4-methoxyglucobrassicin	0.521	-0.784	-0.696	-0.567	-0.778	0.111	-0.545	0.086	-0.164	-0.688	-0.345	-0.407	-0.597	0.502	0.488	0.730	-0.227	1	0.427	0.201
Neoglucobrassicin	0.389	-0.542	-0.493	-0.391	-0.534	0.312	-0.459	0.035	-0.054	-0.631	-0.234	-0.423	-0.583	0.347	0.375	0.629	-0.165	0.427	1	0.056
Total GSLs	0.138	-0.019	-0.075	0.078	-0.001	-0.031	-0.047	-0.045	-0.062	0.154	0.105	0.164	0.029	0.465	0.182	0.327	0.855	0.201	0.056	1

Values in bold are different from 0 with a significance level alpha=0.05

p-values (Spearman):

Variables	Sucrose	Glucose	Galactose	Fructose	Total sugars	Citric	Malic	Succinic	Total acids	Glucoraphanin	Progoitrin	Glucoalyssin	Diglucothiobeinin	Glucosativin	4-hydroxyglucobrassicin	Glucoerucin	Dimeric-4-mercaptobutyl	4-methoxyglucobrassicin	Neoglucobrassicin	Total GSLs
Sucrose	0	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.012	< 0.0001	0.059	0.098	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.275	< 0.0001	< 0.0001	0.021
Glucose	< 0.0001	0	< 0.0001	< 0.0001	< 0.0001	0.041	< 0.0001	0.142	0.005	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.745
Galactose	< 0.0001	< 0.0001	0	< 0.0001	< 0.0001	0.244	< 0.0001	0.589	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.000	< 0.0001	< 0.0001	0.211
Fructose	< 0.0001	< 0.0001	< 0.0001	0	< 0.0001	0.000	< 0.0001	0.100	0.190	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.193
Total sugars	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0	0.018	< 0.0001	0.259	0.003	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.983
Citric	0.012	0.041	0.244	0.000	0.018	0	0.899	< 0.0001	< 0.0001	0.000	0.086	0.043	< 0.0001	0.057	0.159	0.001	0.236	0.063	< 0.0001	0.602
Malic	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.899	0	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.001	< 0.0001	< 0.0001	0.430
Succinic	0.059	0.142	0.589	0.100	0.259	< 0.0001	< 0.0001	0	< 0.0001	0.237	0.327	0.910	0.340	0.554	0.753	0.782	0.401	0.152	0.558	0.449
Total acids	0.098	0.005	< 0.0001	0.190	0.003	< 0.0001	< 0.0001	< 0.0001	0	0.021	0.487	0.065	0.157	0.004	0.030	0.001	0.504	0.006	0.363	0.301
Glucoraphanin	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.000	< 0.0001	0.237	0.021	0	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.010
Progoitrin	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.086	< 0.0001	0.327	0.487	< 0.0001	0	< 0.0001	< 0.0001	0.085	0.002	< 0.0001	0.000	< 0.0001	< 0.0001	0.079
Glucoalyssin	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.043	< 0.0001	0.910	0.065	< 0.0001	< 0.0001	0	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.006
Diglucothiobeinin	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.340	0.157	< 0.0001	< 0.0001	< 0.0001	0	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.630
Glucosativin	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.057	< 0.0001	0.554	0.004	< 0.0001	0.085	< 0.0001	< 0.0001	0	< 0.0001	< 0.0001	0.000	< 0.0001	< 0.0001	< 0.0001
4-hydroxyglucobrassicin	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.159	< 0.0001	0.753	0.030	< 0.0001	0.002	< 0.0001	< 0.0001	< 0.0001	0	< 0.0001	0.166	< 0.0001	< 0.0001	0.002
Glucoerucin	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.001	< 0.0001	0.782	0.001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0	0.420	< 0.0001	< 0.0001	< 0.0001
Dimeric-4-mercaptobutyl	0.275	< 0.0001	0.000	< 0.0001	< 0.0001	0.236	0.001	0.401	0.504	< 0.0001	0.000	< 0.0001	< 0.0001	0.000	0.166	0.420	0	0.000	0.005	< 0.0001
4-methoxyglucobrassicin	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.063	< 0.0001	0.152	0.006	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.000	0	< 0.0001	0.001
Neoglucobrassicin	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.558	0.363	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.005	< 0.0001	0	0.351
Total GSLs	0.021	0.745	0.211	0.193	0.983	0.602	0.430	0.449	0.301	0.010	0.079	0.006	0.630	< 0.0001	0.002	< 0.0001	< 0.0001	0.001	0.351	0

Appendix 4. 1. Average values (with the letters as superscripts) for sugar, GSLs, the ratio for sugar and GSL, and sulphur content (mg. g^{-1} DW) of the six lines of *E. sativa* grown in Italy and the UK (n = 3).

	ITALY						UK						p-value		
Compounds	21	25	68	72	112	130	21	25	68	72	112	130	lines	location	lines * location
Sucrose	5.012 c	7.730 abc	7.707 abc	3.891 c	4.620 c	5.446 bc	6.221 abc	12.803 a	11.142 ab	6.852 abc	12.097 a	9.400 abc	0.002	<0.0001	<0.0001
Glucose	7.029 d	19.665 d	54.520 bcd	13.220 d	28.233 cd	52.344 bcd	32.445 cd	68.486 abcd	127.703 a	85.106 abc	113.455 ab	114.875 ab	<0.0001	<0.0001	<0.0001
Galactose	2.795 d	3.155 d	4.854 cd	4.583 cd	4.077 cd	4.729 cd	5.339 bcd	6.111 abcd	7.068 abc	8.772 ab	8.812 ab	9.658 a	0.000	<0.0001	<0.0001
Fructose	3.345 c	6.799 c	7.402 c	6.235 c	8.887 c	7.680 c	14.129 bc	18.613 abc	20.415 abc	34.350 ab	36.855 a	33.918 ab	0.008	<0.0001	<0.0001
Total Sugars	18.182 c	37.350 c	74.483 bc	27.928 c	45.817 c	70.200 bc	58.133 bc	106.013 abc	166.328 a	135.080 ab	171.219 a	167.852 a	0.000	<0.0001	<0.0001
GIB	0.002 ef	0.002 def	0.004 c	0.001 f	0.004 c	0.003 cde	0.004 bc	0.006 ab	0.003 cde	0.003 cd	0.006 a	0.003 cde	<0.0001	<0.0001	<0.0001
GKR	0.538 cd	0.935 cd	2.343 ab	0.808 cd	1.442 bc	2.963 a	0.118 d	0.077 d	0.184 d	0.169 d	0.248 d	0.195 d	<0.0001	<0.0001	<0.0001
PRO	0.000 b	0.000 b	0.000 b	0.000 b	0.000 b	0.047 a	0.000 b	0.000 b	0.000 b	0.000 b	0.000 b	0.000 b	0.057	0.165	0.021
SIN	0.000 b	0.000 b	0.001 a	0.000 b	0.001 a	0.001 a	0.000 b	0.000 b	0.000 b	0.000 b	0.000 b	0.000 b	<0.0001	<0.0001	<0.0001
GRA	0.339 cd	0.392 cd	0.971 abc	0.226 d	0.465 bcd	1.046 ab	0.615 bcd	1.498 a	0.667 bcd	0.488 bcd	0.615 bcd	0.747 bcd	0.000	0.023	<0.0001
GRM	1.876 cd	1.902 cd	5.318 a	2.055 bc	2.364 bc	3.653 ab	0.383 cd	0.203 d	0.371 d	0.360 d	0.347 d	0.284 d	<0.0001	<0.0001	<0.0001
GAL	0.105 abc	0.050 cd	0.131 ab	0.046 cd	0.069 bcd	0.154 a	0.008 d	0.008 d	0.015 d	0.007 d	0.006 d	0.013 d	0.003	<0.0001	<0.0001
GPJ	0.014 b	0.025 ab	0.036 ab	0.038 ab	0.034 ab	0.078 a	0.000 b	0.000 b	0.000 b	0.000 b	0.000 b	0.002 b	0.216	<0.0001	0.000
GNP	0.000 c	0.000 c	0.001 bc	0.000 c	0.000 c	0.001 c	0.002 abc	0.001 abc	0.002 a	0.002 ab	0.002 a	0.003 a	0.019	<0.0001	<0.0001
DGTB	1.094 cd	1.783 bc	3.262 a	1.145 cd	1.967 bc	2.937 ab	0.639 cd	0.569 cd	0.960 cd	0.557 d	0.801 cd	0.803 cd	<0.0001	<0.0001	<0.0001
GBT	0.067 bc	0.144 ab	0.144 ab	0.051 bc	0.124 ab	0.182 a	0.003 c	0.000 c	0.009 c	0.003 c	0.003 c	0.007 c	0.017	<0.0001	<0.0001
4HGB	0.003 e	0.007 de	0.003 e	0.003 e	0.009 cde	0.005 e	0.011 bcde	0.030 a	0.016 bcd	0.019 abc	0.027 a	0.021 ab	<0.0001	<0.0001	<0.0001
GSV	1.038 ab	1.464 ab	3.193 ab	0.750 b	1.460 ab	3.706 a	1.512 ab	0.806 ab	3.285 ab	2.009 ab	3.343 ab	3.077 ab	0.000	0.265	0.001
DMB	22.666 c	25.080 bc	37.013 a	24.545 bc	31.576 ab	35.250 a	4.127 d	5.461 d	5.429 d	3.902 d	5.839 d	4.426 d	0.000	<0.0001	<0.0001
GTP	0.008 cdef	0.009 bcde	0.014 a	0.009 bcdef	0.011 abc	0.013 ab	0.003 g	0.004 fg	0.007 cdefg	0.005 efg	0.009 bcd	0.005 defg	<0.0001	<0.0001	<0.0001
GER	0.360 abc	0.301 bcd	0.466 a	0.211 cdef	0.402 ab	0.362 abc	0.125 ef	0.077 f	0.265 bcde	0.132 ef	0.175 def	0.150 def	<0.0001	<0.0001	<0.0001
GBC	0.002 c	0.005 bc	0.013 a	0.004 bc	0.010 ab	0.013 a	0.002 c	0.010 ab	0.008 abc	0.005 bc	0.010 ab	0.008 abc	<0.0001	0.380	<0.0001
4MGB	0.053 cd	0.079 bcd	0.182 ab	0.052 cd	0.149 abc	0.195 a	0.006 d	0.036 d	0.037 d	0.054 cd	0.045 d	0.035 d	0.002	<0.0001	<0.0001
GNT	0.000 b	0.000 b	0.000 ab	0.000 b	0.000 b	0.000 ab	0.000 ab	0.000 ab	0.000 a	0.000 ab	0.000 a	0.000 a	0.002	<0.0001	<0.0001
NGB	0.580 b	1.394 a	1.354 a	0.539 b	1.543 a	1.431 a	0.231 b	0.801 b	0.342 b	0.336 b	0.473 b	0.375 b	<0.0001	<0.0001	<0.0001
4MP	0.067 b	2.061 a	0.183 b	0.189 b	0.099 b	0.181 b	0.033 b	0.270 b	0.022 b	0.033 b	0.018 b	0.030 b	<0.0001	<0.0001	<0.0001
HEX	0.051 bc	0.000 c	0.116 ab	0.057 abc	0.050 bc	0.118 ab	0.023 bc	0.172 a	0.021 bc	0.044 bc	0.021 bc	0.014 c	0.215	0.224	<0.0001
BTL	0.188 ab	0.104 cd	0.122 abc	0.200 a	0.172 abc	0.113 bcd	0.000 e	0.012 de	0.000 e	0.000 e	0.000 e	0.003 e	0.072	<0.0001	<0.0001
Total GSL	29.051 d	35.740 cd	54.871 a	30.929 cd	41.951 bc	52.450 ab	7.846 e	10.042 e	11.646 e	8.129 e	11.989 e	10.201 e	<0.0001	<0.0001	<0.0001
Sugar:GSL	0.641 d	1.061 cd	1.338 cd	0.942 d	1.005 cd	1.420 cd	8.465 bc	10.988 ab	12.758 ab	16.968 a	14.284 ab	17.738 a	<0.0001	<0.0001	<0.0001
Sulphur	12.665 def	14.183 bcd	17.562 a	12.646 def	16.130 ab	15.460 abc	8.363 gh	8.918 gh	13.427 cde	7.950 h	10.334 fgh	10.938 efg	<0.0001	<0.0001	<0.0001

The table represents the result of 2-way ANOVA, pairwise comparisons (post-hoc Tukey's HSD test, $p \leq 0.05$). Different small letters (a,b,c,d,e,f,g,h) in each row indicate a sample significance from multiple comparisons (a = the highest level of significant difference). Abbreviations; DW: dry weight.

Appendix 4. 2. Average sugar, GSL, the ratio for sugar and GSL, and sulphur content (mg. g^{-1} DW) of the six lines of *E. sativa* grown in Italy and the UK for the 1st and the 2nd cut (n = 3).

	ITALY		UK		p-value		
Compounds	**1st cut**	**2nd cut**	**1st cut**	**2nd cut**	**cut**	**location**	**cut*location**
Sucrose	6.137 b	5.332 b	6.680 b	14.481 a	<0.0001	<0.0001	<0.0001
Glucose	19.502 c	38.835 bc	51.063 b	169.209 a	<0.0001	<0.0001	<0.0001
Galactose	4.040 c	4.025 c	6.160 b	10.778 a	<0.0001	<0.0001	<0.0001
Fructose	6.649 c	6.800 c	14.493 b	49.215 a	<0.0001	<0.0001	<0.0001
Total Sugars	36.328 c	54.992 bc	78.395 b	243.683 a	<0.0001	<0.0001	<0.0001
GIB	0.003 bc	0.003 c	0.004 a	0.004 ab	0.288	<0.0001	<0.0001
GKR	1.314 a	1.650 a	0.079 b	0.332 b	0.074	<0.0001	<0.0001
PRO	0.000	0.014	0.000	0.000	0.203	0.216	0.160
SIN	0.000 a	0.001 a	0.000 b	0.000 b	0.095	<0.0001	<0.0001
GRA	0.547	0.585	0.699	0.751	0.648	0.109	0.445
GRM	1.741 b	3.928 a	0.274 c	0.414 c	<0.0001	<0.0001	<0.0001
GAL	0.102 a	0.082 a	0.007 b	0.015 b	0.600	<0.0001	<0.0001
GPJ	0.042 a	0.032 a	0.000 b	0.001 b	0.549	<0.0001	<0.0001
GNP	0.000 c	0.000 c	0.001 b	0.003 a	<0.0001	<0.0001	<0.0001
DGTB	1.972 a	2.063 a	0.486 b	1.138 b	0.044	<0.0001	<0.0001
GBT	0.133 a	0.103 a	0.001 b	0.011 b	0.449	<0.0001	<0.0001
4HGB	0.005 c	0.005 c	0.016 b	0.029 a	<0.0001	<0.0001	<0.0001
GSV	1.565 bc	2.246 b	0.803 c	5.264 a	<0.0001	<0.0001	<0.0001
DMB	29.830 a	28.729 a	4.181 b	5.957 b	0.801	<0.0001	<0.0001
GTP	0.011 a	0.011 a	0.003 b	0.010 a	<0.0001	<0.0001	<0.0001
GER	0.385 a	0.317 ab	0.091 c	0.274 b	0.007	<0.0001	<0.0001
GNT	0.000 c	0.000 bc	0.000 b	0.001 a	<0.0001	<0.0001	<0.0001
NGB	1.025 a	1.244 a	0.432 b	0.378 b	0.286	<0.0001	<0.0001
4MP	0.542 a	0.395 ab	0.060 b	0.038 b	0.437	0.000	0.002
HEX	0.049	0.079	0.043	0.035	0.432	0.086	0.141
BTL	0.145 a	0.156 a	0.002 b	0.002 b	0.651	<0.0001	<0.0001
Total GSL	39.514 a	41.790 a	7.205 c	14.737 b	0.011	<0.0001	<0.0001
Sugar: GSL	0.885 c	1.236 c	12.325 b	17.244 a	0.001	<0.0001	<0.0001
Sulphur	14.007 b	15.542 a	9.000 d	12.146 c	<0.0001	<0.0001	<0.0001

The table represents the result of 2-way ANOVA, pairwise comparisons (post-hoc Tukey's HSD test, P <0.05). Different small letters (a,b,c) in each row indicate a sample significance from multiple comparisons. An absence of letters indicates no significant differences observed.

Appendix 4. 3. Average sugar, GSL, the ratio for sugar and GSL, and sulphur content (mg. g^{-1} DW) of the six lines of *E. sativa* grown in Italy and the UK for day 0 (intake) and day 5 (postharvest shelf life) (n = 3). Abbreviations: DW; dry weight.

	ITALY		**UK**		**p-value**		
Compounds	***Day 0***	***Day 5***	***Day 0***	***Day 5***	***Day***	***location***	***day*location***
Sucrose	6.392 bc	5.077 c	10.889 a	8.712 ab	0.032	< 0.0001	< 0.0001
Glucose	29.996 c	28.341 c	121.628 a	75.014 b	0.006	< 0.0001	< 0.0001
Galactose	3.659 c	4.406 c	9.065 a	6.949 b	0.112	< 0.0001	< 0.0001
Fructose	8.484 c	4.965 c	36.250 a	20.513 b	< 0.0001	< 0.0001	< 0.0001
Total Sugars	48.531 c	42.789 c	177.832 a	111.189 b	0.002	< 0.0001	< 0.0001
GIB	0.003 bc	0.002 c	0.004 a	0.004 ab	0.047	< 0.0001	< 0.0001
GKR	1.784 a	1.176 b	0.187 c	0.172 c	0.052	< 0.0001	< 0.0001
PRO	0.014	0.000	0.000	0.000	0.206	0.210	0.162
SIN	0.001 a	0.000 b	0.000 c	0.000 c	0.137	< 0.0001	< 0.0001
GRA	0.715	0.414	0.720	0.719	0.116	0.106	0.048
GRM	2.568 a	3.140 a	0.230 b	0.422 b	0.148	< 0.0001	< 0.0001
GAL	0.096 a	0.087 a	0.011 b	0.009 b	0.579	< 0.0001	< 0.0001
GPJ	0.031 a	0.043 a	0.001 b	0.000 b	0.491	< 0.0001	< 0.0001
GNP	0.000 b	0.000 b	0.002 a	0.002 a	0.060	< 0.0001	< 0.0001
DGTB	2.686 a	1.332 b	0.897 bc	0.603 c	< 0.0001	< 0.0001	< 0.0001
GBT	0.189 a	0.045 b	0.009 c	0.001 c	< 0.0001	< 0.0001	< 0.0001
4HGB	0.006 c	0.004 c	0.024 a	0.018 b	0.015	< 0.0001	< 0.0001
GSV	2.251	1.560	2.867	2.297	0.100	0.078	0.123
DMB	31.352 a	27.132 a	5.601 b	4.218 b	0.032	< 0.0001	< 0.0001
GTP	0.011 a	0.010 a	0.007 b	0.005 b	0.047	< 0.0001	< 0.0001
GER	0.335 a	0.366 a	0.171 b	0.156 b	0.731	< 0.0001	< 0.0001
GBC	0.010 a	0.005 c	0.009 ab	0.006 bc	< 0.0001	0.732	0.001
4MGB	0.146 a	0.088 b	0.041 b	0.036 b	0.039	< 0.0001	< 0.0001
GNT	0.000 b	0.000 b	0.000 a	0.000 a	0.282	< 0.0001	< 0.0001
NGB	1.089 a	1.184 a	0.326 b	0.490 b	0.094	< 0.0001	< 0.0001
4MP	0.577 a	0.355 ab	0.056 b	0.047 b	0.280	0.000	0.001
HEX	0.076 a	0.052 a	0.048 a	0.032 a	0.172	0.096	0.181
BTL	0.125 b	0.176 a	0.000 c	0.004 c	0.017	< 0.0001	< 0.0001
Total GSL	44.066 a	37.173 b	11.212 c	9.240 c	0.020	< 0.0001	< 0.0001
Sugar: GSL	1.042 b	1.021 b	14.960 a	13.637 a	0.446	< 0.0001	< 0.0001
Sulphur	14.546 a	15.003 a	10.063 b	10.453 b	0.351	< 0.0001	< 0.0001

The table represents the result of 2-way ANOVA, pairwise comparisons (post-hoc Tukey's HSD test, $p < 0.05$). Different small letters (a,b,c) in each row indicate a sample significance from multiple comparisons. An absence of letters indicates no significant differences observed. Abbreviations; DW: dry weight.

Appendix 4. 4. Correlation Table for phytochemical components, with the corresponding p-values.

Variables	Sucrose	Glucose	Galactose	Fructose	Total Sugars	Total GSL	sugar:GSL	Sulphur
Sucrose	**1**	**0.574**	**0.461**	**0.529**	**0.626**	-0.009	**0.396**	0.041
Glucose	**0.574**	**1**	**0.732**	**0.854**	**0.993**	-0.246	**0.852**	-0.105
Galactose	**0.461**	**0.732**	**1**	**0.718**	**0.754**	**-0.475**	**0.811**	**-0.424**
Fructose	**0.529**	**0.854**	**0.718**	**1**	**0.886**	**-0.420**	**0.809**	**-0.379**
Total Sugars	**0.626**	**0.993**	**0.754**	**0.886**	**1**	-0.260	**0.855**	-0.145
GIB	**0.468**	**0.587**	**0.316**	**0.574**	**0.604**	-0.005	**0.376**	-0.093
GKR	0.008	-0.212	**-0.419**	**-0.359**	-0.222	**0.947**	**-0.549**	**0.739**
PRO	0.176	0.218	0.104	0.138	0.202	0.154	0.035	0.181
SIN	-0.189	-0.273	**-0.380**	**-0.411**	-0.280	**0.825**	**-0.505**	**0.589**
GRA	**0.606**	**0.609**	**0.331**	**0.497**	**0.631**	0.163	**0.317**	0.188
GRM	-0.250	**-0.445**	**-0.566**	**-0.658**	**-0.476**	**0.830**	**-0.683**	**0.697**
GAL	-0.041	**-0.367**	**-0.528**	**-0.537**	**-0.376**	**0.922**	**-0.707**	**0.741**
GPJ	**-0.309**	**-0.387**	**-0.518**	**-0.547**	**-0.416**	**0.805**	**-0.617**	**0.643**
GNP	**0.591**	**0.862**	**0.661**	**0.877**	**0.876**	**-0.452**	**0.791**	**-0.337**
DGTB	0.230	-0.014	**-0.312**	-0.121	-0.009	**0.897**	**-0.432**	**0.692**
GBT	0.067	-0.285	**-0.474**	**-0.305**	-0.266	**0.834**	**-0.556**	**0.642**
4HGB	**0.499**	**0.718**	**0.632**	**0.851**	**0.748**	**-0.546**	**0.768**	**-0.440**
GSV	**0.570**	**0.489**	0.126	**0.320**	**0.487**	**0.487**	0.101	**0.468**
DMB	-0.063	**-0.298**	**-0.483**	**-0.442**	**-0.311**	**0.975**	**-0.682**	**0.719**
GTP	0.229	0.050	-0.224	-0.138	0.036	**0.877**	**-0.376**	**0.698**
GER	0.136	-0.265	**-0.404**	**-0.427**	-0.281	**0.772**	**-0.565**	**0.820**
GBC	**0.493**	**0.607**	0.220	**0.463**	**0.607**	**0.462**	0.234	**0.447**
4MGB	0.067	0.004	**-0.299**	-0.130	-0.005	**0.831**	**-0.366**	**0.665**
GNT	**0.474**	**0.875**	**0.621**	**0.819**	**0.877**	**-0.304**	**0.784**	-0.200
NGB	-0.098	-0.284	**-0.515**	**-0.475**	-0.298	**0.786**	**-0.559**	**0.620**
4MP	0.049	-0.287	**-0.466**	**-0.318**	-0.266	**0.734**	**-0.552**	**0.464**
HEX	-0.068	-0.009	-0.065	-0.087	-0.015	**0.417**	-0.204	0.219
BTL	**-0.352**	**-0.668**	**-0.578**	**-0.746**	**-0.684**	**0.710**	**-0.800**	**0.562**
Total GSL	-0.009	-0.246	**-0.475**	**-0.420**	-0.260	**1**	**-0.635**	**0.780**
sugar:GSL	**0.396**	**0.852**	**0.811**	**0.809**	**0.855**	**-0.635**	**1**	**-0.417**
Sulphur	0.041	-0.105	**-0.424**	**-0.379**	-0.145	**0.780**	**-0.417**	**1**

Variables	Sucrose	Glucose	Galactose	Fructose	Total Sugars	Total GSL	sugar:GSL	Sulphur
Sucrose	**0**	**<0.0001**	**0.002**	**0.000**	**<0.0001**	0.954	**0.008**	0.789
Glucose	**<0.0001**	**0**	**<0.0001**	**<0.0001**	**<0.0001**	0.107	**<0.0001**	0.497
Galactose	**0.002**	**<0.0001**	**0**	**<0.0001**	**<0.0001**	**0.001**	**<0.0001**	**0.004**
Fructose	**0.000**	**<0.0001**	**<0.0001**	**0**	**<0.0001**	**0.005**	**<0.0001**	**0.012**
Total Sugars	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**0**	0.088	**<0.0001**	0.346
GIB	**0.002**	**<0.0001**	**0.037**	**<0.0001**	**<0.0001**	0.976	**0.012**	0.549
GKR	0.961	0.166	**0.005**	**0.017**	0.148	**<0.0001**	**0.000**	**<0.0001**
PRO	0.253	0.154	0.500	0.369	0.188	0.316	0.821	0.239
SIN	0.218	0.073	**0.011**	**0.006**	0.066	**<0.0001**	**0.001**	**<0.0001**
GRA	**<0.0001**	**<0.0001**	**0.029**	**0.001**	**<0.0001**	0.291	**0.036**	0.222
GRM	0.102	**0.003**	**<0.0001**	**<0.0001**	**0.001**	**<0.0001**	**<0.0001**	**<0.0001**
GAL	0.790	**0.015**	**0.000**	**0.000**	**0.012**	**<0.0001**	**<0.0001**	**<0.0001**
GPJ	**0.042**	**0.010**	**0.000**	**0.000**	**0.005**	**<0.0001**	**<0.0001**	**<0.0001**
GNP	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**0.002**	**<0.0001**	**0.026**
DGTB	0.132	0.930	**0.040**	0.433	0.953	**<0.0001**	**0.004**	**<0.0001**
GBT	0.664	0.061	**0.001**	**0.044**	0.081	**<0.0001**	**0.000**	**<0.0001**
4HGB	**0.001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**0.000**	**<0.0001**	**0.003**
GSV	**<0.0001**	**0.001**	0.413	**0.035**	**0.001**	**0.001**	0.512	**0.002**
DMB	0.683	**0.050**	**0.001**	**0.003**	**0.040**	**<0.0001**	**<0.0001**	**<0.0001**
GTP	0.135	0.748	0.144	0.371	0.816	**<0.0001**	**0.012**	**<0.0001**
GER	0.378	0.082	**0.007**	**0.004**	0.065	**<0.0001**	**<0.0001**	**<0.0001**
GBC	**0.001**	**<0.0001**	0.151	**0.002**	**<0.0001**	**0.002**	0.126	**0.003**
4MGB	0.667	0.981	**0.049**	0.398	0.977	**<0.0001**	**0.015**	**<0.0001**
GNT	**0.001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**0.045**	**<0.0001**	0.193
NGB	0.524	0.062	**0.000**	**0.001**	0.050	**<0.0001**	**0.000**	**<0.0001**
4MP	0.752	0.059	**0.002**	**0.036**	0.081	**<0.0001**	**0.000**	**0.002**
HEX	0.660	0.952	0.675	0.575	0.922	**0.005**	0.184	0.153
BTL	**0.020**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
Total GSL	0.954	0.107	**0.001**	**0.005**	0.088	**0**	**<0.0001**	**<0.0001**
sugar:GSL	**0.008**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**0**	**0.005**
Sulphur	0.789	0.497	**0.004**	**0.012**	0.346	**<0.0001**	**0.005**	**0**

Appendix 4. 5. Correlation matrix between sensory components, with the corresponding p- values.

Correlation matrix (Spearman):		
Variables	**Bitter_T**	**Sweet_T**
Pun_A	**0.663**	**0.332**
Mus_A	**0.515**	**0.451**
Pep_A	**0.873**	-0.254
Gre_A	**0.657**	**-0.375**
Ear_A	**0.774**	**-0.483**
Cri_MF	0.173	**0.587**
Cru_MF	0.204	**0.538**
Fir_MF	0.096	**0.361**
Moi_MF	**0.343**	-0.152
War_MF	**0.728**	0.125
Num_MF	**0.386**	**0.559**
Bit_T	**1**	**-0.315**
Swe_T	**-0.315**	**1**
Uma_T	-0.238	**0.798**
Pep_F	**0.874**	-0.085
Gre_F	**0.559**	-0.178
Sop_F	**-0.434**	**0.755**
Mus_F	**0.590**	**0.340**
Bur_F	**0.679**	0.041
W_T0	**0.668**	0.117
W_T1	**0.632**	0.068
W_T2	**0.623**	0.015
W_T3	**0.575**	-0.075
T_T0	**0.654**	0.196
T_T1	**0.604**	0.111
T_T2	**0.628**	-0.045
T_T3	**0.567**	-0.067
G_T0	**0.723**	**-0.350**
G_T1	**0.684**	**-0.470**
G_T2	**0.631**	**-0.653**
G_T3	**0.526**	**-0.611**
D_T0	-0.004	**0.568**
D_T1	-0.041	**0.344**
D_T2	0.113	-0.023
D_T3	-0.017	-0.114
N_T0	**0.315**	**0.492**
N_T1	0.250	**0.544**
N_T2	0.234	**0.480**
N_T3	0.126	**0.520**
B_T0	**0.897**	-0.254
B_T1	**0.844**	**-0.359**
B_T2	**0.742**	**-0.456**
B_T3	**0.699**	**-0.585**

p-values (Spearman):		
Variables	**Bitter_T**	**Sweet_T**
Pun_A	**<0.0001**	**0.028**
Mus_A	**0.000**	**0.002**
Pep_A	**<0.0001**	0.096
Gre_A	**<0.0001**	**0.013**
Ear_A	**<0.0001**	**0.001**
Cri_MF	0.261	**<0.0001**
Cru_MF	0.183	**0.000**
Fir_MF	0.535	**0.017**
Moi_MF	**0.023**	0.325
War_MF	**<0.0001**	0.419
Num_MF	**0.010**	**0.000**
Bit_T	**0**	**0.038**
Swe_T	**0.038**	**0**
Uma_T	0.119	**<0.0001**
Pep_F	**<0.0001**	0.582
Gre_F	**0.000**	0.246
Sop_F	**0.004**	**<0.0001**
Mus_F	**<0.0001**	**0.024**
Bur_F	**<0.0001**	0.790
W_T0	**<0.0001**	0.447
W_T1	**<0.0001**	0.660
W_T2	**<0.0001**	0.923
W_T3	**<0.0001**	0.629
T_T0	**<0.0001**	0.201
T_T1	**<0.0001**	0.472
T_T2	**<0.0001**	0.770
T_T3	**<0.0001**	0.664
G_T0	**<0.0001**	**0.020**
G_T1	**<0.0001**	**0.001**
G_T2	**<0.0001**	**<0.0001**
G_T3	**0.000**	**<0.0001**
D_T0	0.979	**<0.0001**
D_T1	0.792	**0.023**
D_T2	0.464	0.884
D_T3	0.913	0.460
N_T0	**0.038**	**0.001**
N_T1	0.102	**0.000**
N_T2	0.126	**0.001**
N_T3	0.413	**0.000**
B_T0	**<0.0001**	0.096
B_T1	**<0.0001**	**0.017**
B_T2	**<0.0001**	**0.002**
B_T3	**<0.0001**	**<0.0001**

Appendix 4. 6. ANOVA pairwise comparisons (P<0.05) of sensory attributes of six lines of *E. sativa* between PAV/PAV and AVI/AVI diplotypes as representative averages.

Attributes	PAV/PAV	AVI/AVI	P> F	Significant
Aroma				
Pungent_A	33.9	36.2	0.082	No
Mustard_A	30.0 b	36.4 a	< 0.0001	Yes
Peppery_A	25.9 b	33.8 a	< 0.0001	Yes
Green_A	34.0 b	40.4 a	0.000	Yes
Earthy_A	16.3 b	31.6 a	< 0.0001	Yes
Mouthfeel				
Crisp_MF	38.2	38.0	0.891	No
Crunchy_MF	29.9 b	35.5 a	0.000	Yes
Firmness_MF	33.1	31.3	0.194	No
Moistness_MF	35.6 b	44.0 a	< 0.0001	Yes
Warming_MF	25.0	29.0	0.074	No
Numbing_MF	11.3 b	17.4 a	< 0.0001	Yes
Taste				
Bitter_T	40.9	41.2	0.846	No
Sweet_T	15.1 b	21.4 a	< 0.0001	Yes
Umami_T	16.0 b	24.5 a	< 0.0001	Yes
Flavour				
Peppery_F	30.4 b	36.2 a	0.004	Yes
Green_F	35.4 b	42.7 a	< 0.0001	Yes
Soapy_F	8.6 b	21.5 a	< 0.0001	Yes
Mustard_F	29.2 b	35.7 a	0.000	Yes
Burnt_F	5.2 b	18.0 a	< 0.0001	Yes
Aftereffect				
Warming_(T0)	17.3	16.6	0.648	No
Warming_(T1)	13.1 a	9.5 b	0.003	Yes
Warming_(T2)	10.4 a	6.0 b	< 0.0001	Yes
Warming_(T3)	8.6 a	4.3 b	< 0.0001	Yes
Tingling_(T0)	10.5	11.0	0.690	No
Tingling_(T1)	8.3	7.5	0.360	No
Tingling_(T2)	6.8 a	5.1 b	0.019	Yes
Tingling_(T3)	5.6 a	3.9 b	0.004	Yes
Green_(T0)	24.6	24.0	0.477	No
Green_(T1)	21.4 a	17.5 b	< 0.0001	Yes
Green_(T2)	18.7 a	13.5 b	< 0.0001	Yes
Green_(T3)	16.5 a	10.7 b	< 0.0001	Yes
Drying_(T0)	23.5 a	20.6 b	0.002	Yes
Drying_(T1)	21.6 a	17.0 b	< 0.0001	Yes
Drying_(T2)	19.8 a	13.9 b	< 0.0001	Yes
Drying_(T3)	18.1 a	11.9 b	< 0.0001	Yes
Numbing_(T0)	11.3	11.5	0.850	No

Numbing_(T1)	10.0	8.8	0.194	No
Numbing_(T2)	8.4 a	6.9 b	0.043	Yes
Numbing_(T3)	7.3 a	5.6 b	0.012	Yes
Bitter_(T0)	26.5 a	22.7 b	0.002	Yes
Bitter_(T1)	22.1 a	17.0 b	< 0.0001	Yes
Bitter_(T2)	18.6 a	13.0 b	< 0.0001	Yes
Bitter_(T3)	16.0 a	10.6 b	< 0.0001	Yes

Letters within columns denote statistical significances, with ‘a’ scoring higher values. Values with the different letters present statistically significant from one another. An absence of letters indicates no significant differences observed.

Appendix 4. 7. Correlation between TAS2R38 genotypes for sensory components, with p-values.

Correlation matrix (Spearman):				p-values (Spearman):		
Variables	**Burnt_F**	**Warming_(T1)**		**Variables**	**Burnt_F**	**Warming_(T1)**
Pungent_A	**0.505**	**0.535**		Pungent_A	**0.001**	**0.000**
Mustard_A	**0.626**	-0.025		Mustard_A	**<0.0001**	0.871
Peppery_A	**0.478**	-0.022		Peppery_A	**0.001**	0.888
Green_A	0.161	**-0.394**		Green_A	0.294	**0.009**
Earthy_A	**0.609**	-0.233		Earthy_A	**<0.0001**	0.128
Crisp_MF	0.233	**0.563**		Crisp_MF	0.128	**<0.0001**
Crunchy_MF	**0.537**	**0.384**		Crunchy_MF	**0.000**	**0.011**
Firmness_MF	0.077	**0.710**		Firmness_MF	0.618	**<0.0001**
Moistness_MF	**0.397**	**-0.585**		Moistness_MF	**0.008**	**<0.0001**
Warming_MF	**0.613**	**0.700**		Warming_MF	**<0.0001**	**<0.0001**
Numbing_MF	**0.714**	**0.442**		Numbing_MF	**<0.0001**	**0.003**
Bitter_T	0.202	0.031		Bitter_T	0.189	0.840
Sweet_T	**0.455**	0.018		Sweet_T	**0.002**	0.908
Umami_T	**0.603**	-0.273		Umami_T	**<0.0001**	0.074
Peppery_F	**0.433**	0.180		Peppery_F	**0.004**	0.242
Green_F	**0.381**	**-0.504**		Green_F	**0.011**	**0.001**
Soapy_F	**0.685**	-0.204		Soapy_F	**<0.0001**	0.184
Mustard_F	**0.609**	0.209		Mustard_F	**<0.0001**	0.173
Burnt_F	**1**	0.061		Burnt_F	**0**	0.695
Warming_(T0)	**0.362**	**0.911**		Warming_(T0)	**0.016**	**<0.0001**
Warming_(T1)	0.061	**1**		Warming_(T1)	0.695	**0**
Warming_(T2)	-0.119	**0.952**		Warming_(T2)	0.439	**<0.0001**
Warming_(T3)	-0.182	**0.918**		Warming_(T3)	0.237	**<0.0001**
Tingling_(T0)	**0.447**	**0.809**		Tingling_(T0)	**0.003**	**<0.0001**
Tingling_(T1)	0.274	**0.906**		Tingling_(T1)	0.072	**<0.0001**
Tingling_(T2)	0.097	**0.916**		Tingling_(T2)	0.530	**<0.0001**
Tingling_(T3)	0.032	**0.895**		Tingling_(T3)	0.836	**<0.0001**
Green_(T0)	0.155	0.192		Green_(T0)	0.314	0.212
Green_(T1)	-0.257	**0.489**		Green_(T1)	0.093	**0.001**
Green_(T2)	**-0.339**	**0.526**		Green_(T2)	**0.025**	**0.000**
Green_(T3)	**-0.467**	**0.600**		Green_(T3)	**0.002**	**<0.0001**
Drying_(T0)	0.050	**0.734**		Drying_(T0)	0.748	**<0.0001**
Drying_(T1)	-0.278	**0.811**		Drying_(T1)	0.068	**<0.0001**
Drying_(T2)	**-0.451**	**0.741**		Drying_(T2)	**0.002**	**<0.0001**
Drying_(T3)	**-0.552**	**0.634**		Drying_(T3)	**0.000**	**<0.0001**
Numbing_(T0)	**0.349**	**0.745**		Numbing_(T0)	**0.021**	**<0.0001**
Numbing_(T1)	0.219	**0.789**		Numbing_(T1)	0.152	**<0.0001**
Numbing_(T2)	0.086	**0.805**		Numbing_(T2)	0.576	**<0.0001**
Numbing_(T3)	0.076	**0.743**		Numbing_(T3)	0.623	**<0.0001**
Bitter_(T0)	0.055	**0.487**		Bitter_(T0)	0.724	**0.001**
Bitter_(T1)	-0.035	**0.696**		Bitter_(T1)	0.820	**<0.0001**
Bitter_(T2)	-0.167	**0.779**		Bitter_(T2)	0.277	**<0.0001**
Bitter_(T3)	-0.177	**0.765**		Bitter_(T3)	0.249	**<0.0001**

Appendix 4. 8. Correlation matrix between sensory attributes and phytochemical components, with the corresponding p-values.

Correlation matrix (Spearman):

Variables	GER	Total GSL	sugar:GSL	Numbing_MF	Bitter_T	Sweet_T
Sucrose	0.136	-0.009	**0.427**	**0.321**	0.137	**0.396**
Glucose	-0.265	-0.246	**0.873**	**0.664**	0.000	**0.648**
Galactose	**-0.404**	**-0.475**	**0.824**	**0.338**	**-0.344**	**0.580**
Fructose	**-0.427**	**-0.420**	**0.840**	**0.588**	-0.217	**0.817**
Total Sugars	-0.281	-0.260	**0.877**	**0.650**	-0.025	**0.685**
GIB	-0.131	-0.005	**0.402**	**0.530**	0.113	**0.487**
GKR	**0.715**	**0.947**	**-0.545**	0.087	**0.783**	**-0.493**
PRO	0.191	0.154	0.054	-0.015	0.134	-0.125
SIN	**0.518**	**0.825**	**-0.511**	-0.046	**0.498**	**-0.476**
GRA	0.118	0.163	**0.340**	**0.413**	0.266	**0.385**
GRM	**0.651**	**0.830**	**-0.694**	-0.229	**0.476**	**-0.776**
GAL	**0.778**	**0.922**	**-0.698**	-0.065	**0.742**	**-0.640**
GPJ	**0.521**	**0.805**	**-0.615**	-0.076	**0.614**	**-0.573**
GNP	**-0.352**	**-0.452**	**0.807**	**0.581**	-0.105	**0.782**
DGTB	**0.689**	**0.897**	**-0.415**	0.269	**0.825**	**-0.301**
GBT	**0.640**	**0.834**	**-0.549**	0.043	**0.720**	**-0.393**
4HGB	**-0.428**	**-0.546**	**0.783**	**0.397**	-0.294	**0.737**
GSV	**0.502**	**0.487**	0.122	**0.419**	**0.563**	0.076
DMB	**0.732**	**0.975**	**-0.665**	0.039	**0.762**	**-0.589**
GTP	**0.739**	**0.877**	**-0.356**	**0.303**	**0.793**	**-0.313**
GER	**1**	**0.772**	**-0.563**	-0.056	**0.660**	**-0.532**
GBC	**0.316**	**0.462**	0.254	**0.592**	**0.503**	0.270
4MGB	**0.627**	**0.831**	**-0.361**	0.208	**0.661**	**-0.306**
GNT	**-0.307**	**-0.304**	**0.794**	**0.635**	-0.032	**0.656**
NGB	**0.553**	**0.786**	**-0.560**	-0.144	**0.538**	**-0.561**
4MP	**0.434**	**0.734**	**-0.542**	-0.122	**0.511**	**-0.442**
HEX	0.193	**0.417**	-0.208	0.226	0.171	-0.046
BTL	**0.591**	**0.710**	**-0.801**	**-0.382**	**0.342**	**-0.777**
Total GSL	**0.772**	**1**	**-0.623**	0.042	**0.769**	**-0.590**
sugar:GSL	**-0.563**	**-0.623**	**1**	**0.458**	**-0.361**	**0.747**
Sulphur	**0.820**	**0.780**	**-0.424**	0.096	**0.691**	**-0.493**
Pun_A	0.239	**0.393**	0.127	**0.700**	**0.663**	**0.332**
Mus_A	0.072	0.251	0.263	**0.780**	**0.515**	**0.451**
Pep_A	**0.656**	**0.807**	**-0.376**	**0.346**	**0.873**	-0.254
Gre_A	**0.488**	**0.713**	**-0.532**	0.107	**0.657**	**-0.375**
Ear_A	**0.743**	**0.855**	**-0.544**	0.063	**0.774**	**-0.483**
Cri_MF	-0.012	-0.080	**0.638**	**0.556**	0.173	**0.587**
War_MF	**0.426**	**0.520**	0.074	**0.753**	**0.728**	0.125
Num_MF	-0.056	0.042	**0.458**	**1**	**0.386**	**0.559**
Bit_T	**0.660**	**0.769**	**-0.361**	**0.386**	**1**	**-0.315**
Swe_T	**-0.532**	**-0.590**	**0.747**	**0.559**	**-0.315**	**1**
Uma_T	**-0.543**	**-0.594**	**0.704**	**0.603**	-0.238	**0.798**
Pep_F	**0.632**	**0.687**	-0.189	**0.568**	**0.874**	-0.085
Gre_F	**0.354**	**0.564**	**-0.407**	0.110	**0.559**	-0.178
Sop_F	**-0.567**	**-0.705**	**0.720**	**0.303**	**-0.434**	**0.755**
Mus_F	0.193	**0.314**	0.252	**0.839**	**0.590**	**0.340**
G_T2	**0.748**	**0.809**	**-0.578**	-0.114	**0.631**	**-0.653**
G_T3	**0.640**	**0.749**	**-0.434**	-0.098	**0.526**	**-0.611**
D_T0	-0.232	**-0.373**	**0.724**	**0.624**	-0.004	**0.568**
B_T1	**0.691**	**0.808**	**-0.363**	0.293	**0.844**	**-0.359**
B_T2	**0.699**	**0.767**	**-0.397**	0.169	**0.742**	**-0.456**
B_T3	**0.686**	**0.794**	**-0.437**	0.011	**0.699**	**-0.585**

p-values (Spearman):

Variables	GER	Total GSL	sugar:GSL	Numbing_MF	Bitter_T	Sweet_T
Sucrose	0.378	0.954	**0.004**	**0.034**	0.375	**0.008**
Glucose	0.082	0.107	**<0.0001**	**<0.0001**	0.999	**<0.0001**
Galactose	**0.007**	**0.001**	**<0.0001**	**0.026**	**0.023**	**<0.0001**
Fructose	**0.004**	**0.005**	**<0.0001**	**<0.0001**	0.156	**<0.0001**
Total Sugars	0.065	0.088	**<0.0001**	**<0.0001**	0.872	**<0.0001**
GIB	0.394	0.976	**0.007**	**0.000**	0.463	**0.001**
GKR	**<0.0001**	**<0.0001**	**0.000**	0.575	**<0.0001**	**0.001**
PRO	0.213	0.316	0.727	0.924	0.385	0.419
SIN	**0.000**	**<0.0001**	**0.000**	0.765	**0.001**	**0.001**
GRA	0.445	0.291	**0.024**	**0.006**	0.081	**0.010**
GRM	**<0.0001**	**<0.0001**	**<0.0001**	0.134	**0.001**	**<0.0001**
GAL	**<0.0001**	**<0.0001**	**<0.0001**	0.674	**<0.0001**	**<0.0001**
GPJ	**0.000**	**<0.0001**	**<0.0001**	0.622	**<0.0001**	**<0.0001**
GNP	**0.020**	**0.002**	**<0.0001**	**<0.0001**	0.495	**<0.0001**
DGTB	**<0.0001**	**<0.0001**	**0.005**	0.077	**<0.0001**	**0.047**
GBT	**<0.0001**	**<0.0001**	**0.000**	0.781	**<0.0001**	**0.009**
4HGB	**0.004**	**0.000**	**<0.0001**	**0.008**	0.053	**<0.0001**
GSV	**0.001**	**0.001**	0.430	**0.005**	**<0.0001**	0.624
DMB	**<0.0001**	**<0.0001**	**<0.0001**	0.802	**<0.0001**	**<0.0001**
GTP	**<0.0001**	**<0.0001**	**0.018**	**0.046**	**<0.0001**	**0.039**
GER	**0**	**<0.0001**	**<0.0001**	0.716	**<0.0001**	**0.000**
GBC	**0.037**	**0.002**	0.096	**<0.0001**	**0.001**	0.076
4MGB	**<0.0001**	**<0.0001**	**0.017**	0.174	**<0.0001**	**0.044**
GNT	**0.043**	**0.045**	**<0.0001**	**<0.0001**	0.838	**<0.0001**
NGB	**0.000**	**<0.0001**	**0.000**	0.350	**0.000**	**<0.0001**
4MP	**0.004**	**<0.0001**	**0.000**	0.429	**0.000**	**0.003**
HEX	0.209	**0.005**	0.175	0.140	0.267	0.766
BTL	**<0.0001**	**<0.0001**	**<0.0001**	**0.011**	**0.024**	**<0.0001**
Total GSL	**<0.0001**	**0**	**<0.0001**	0.785	**<0.0001**	**<0.0001**
sugar:GSL	**<0.0001**	**<0.0001**	**0**	**0.002**	**0.016**	**<0.0001**
Sulphur	**<0.0001**	**<0.0001**	**0.004**	0.532	**<0.0001**	**0.001**
Pun_A	0.118	**0.009**	0.410	**<0.0001**	**<0.0001**	**0.028**
Mus_A	0.642	0.100	0.084	**<0.0001**	**0.000**	**0.002**
Pep_A	**<0.0001**	**<0.0001**	**0.012**	**0.022**	**<0.0001**	0.096
Gre_A	**0.001**	**<0.0001**	**0.000**	0.489	**<0.0001**	**0.013**
Ear_A	**<0.0001**	**<0.0001**	**0.000**	0.686	**<0.0001**	**0.001**
Cri_MF	0.941	0.603	**<0.0001**	**0.000**	0.261	**<0.0001**
War_MF	**0.004**	**0.000**	0.632	**<0.0001**	**<0.0001**	0.419
Num_MF	0.716	0.785	**0.002**	**0**	**0.010**	**0.000**
Bit_T	**<0.0001**	**<0.0001**	**0.016**	**0.010**	**0**	**0.038**
Swe_T	**0.000**	**<0.0001**	**<0.0001**	**0.000**	**0.038**	**0**
Uma_T	**0.000**	**<0.0001**	**<0.0001**	**<0.0001**	0.119	**<0.0001**
Pep_F	**<0.0001**	**<0.0001**	0.219	**<0.0001**	**<0.0001**	0.582
Gre_F	**0.019**	**<0.0001**	**0.006**	0.476	**0.000**	0.246
Sop_F	**<0.0001**	**<0.0001**	**<0.0001**	**0.046**	**0.004**	**<0.0001**
Mus_F	0.208	**0.039**	0.098	**<0.0001**	**<0.0001**	**0.024**
G_T2	**<0.0001**	**<0.0001**	**<0.0001**	0.459	**<0.0001**	**<0.0001**
G_T3	**<0.0001**	**<0.0001**	**0.003**	0.525	**0.000**	**<0.0001**
D_T0	0.129	**0.013**	**<0.0001**	**<0.0001**	0.979	**<0.0001**
B_T1	**<0.0001**	**<0.0001**	**0.016**	0.054	**<0.0001**	**0.017**
B_T2	**<0.0001**	**<0.0001**	**0.008**	0.272	**<0.0001**	**0.002**
B_T3	**<0.0001**	**<0.0001**	**0.003**	0.946	**<0.0001**	**<0.0001**