Julie Graham · Rex Brennan *Editors*

Raspberry

Breeding, Challenges and Advances

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Editors
Julie Graham
Cell & Molecular Sciences
The James Hutton Institute
Dundee, UK

Rex Brennan
Cell & Molecular Sciences
The James Hutton Institute
Dundee, UK

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Preface

This book is intended as an update to *Raspberries and Blackberries: Their Breeding, Diseases and Growth* by D.L. Jennings, published in 1988 by Academic Press. All of the content of Jennings, 1988 is still relevant today; however, there have been significant advances and challenges in a number of areas including variety development and molecular breeding technologies, the impacts of climate change, lack of active compounds allowed for use on crops, the use of agroecology principles in plant defense, and new high-throughput plant phenotyping method developments. This book, which is focused on raspberry alone, aims to capture some of these advances as a companion to the 1988 text.

Dundee, UK

Julie Graham
Rex Brennan

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Contributors

Matt Aitkenhead Information and Computational Sciences, The James Hutton Institute, Dundee, UK

Graham Begg Ecological Sciences, The James Hutton Institute, Dundee, UK

A. Nicholas E. Birch Ecological Sciences, The James Hutton Institute, Dundee, UK

Rex Brennan Cell & Molecular Sciences, The James Hutton Institute, Dundee, UK

Alison Dolan The James Hutton Institute, Dundee, UK

Gina E. Fernandez North Carolina State University, Raleigh, USA

Julie Graham Cell & Molecular Sciences, The James Hutton Institute, Dundee, UK

Robert D. Hancock Cell & Molecular Sciences, The James Hutton Institute, Dundee, UK

Cathy Hawes Ecological Sciences, The James Hutton Institute, Dundee, UK

Pete Iannetta Ecological Sciences, The James Hutton Institute, Dundee, UK

Sophia Nikki Jennings James Hutton Limited, Dundee, UK

Hamlyn G. Jones Division of Plant Sciences, University of Dundee, Dundee, UK
University of Western Australia, Crawley, WA, Australia

Alison J. Karley Ecological Sciences, The James Hutton Institute, Dundee, UK

Stuart MacFarlane The James Hutton Institute, Dundee, UK

Susan McCallum Cell & Molecular Sciences, The James Hutton Institute, Dundee, UK

Gordon J. McDougall Environmental and Biochemical Sciences, The James Hutton Institute, Dundee, UK

Carolyn Mitchell Ecological Sciences, The James Hutton Institute, Dundee, UK

Ramon Molina-Bravo School of Agraian Sciences, National University of Costa Rica, Heredia, Costa Rica

Antonios Petridis Cell & Molecular Sciences, The James Hutton Institute, Dundee, UK

Craig Simpson Cell & Molecular Sciences, The James Hutton Institute, Dundee, UK

Fumiomi Takeda Appalachian Fruit Research Station, USDA-ARS, Kearneysville, WV, USA

Dominic Williams James Hutton Limited, Dundee, UK

Chapter 1
Introduction to the *Rubus* Genus

Julie Graham and Rex Brennan

1.1 Taxonomy, Botany and Growth

Raspberries, blackberries and the associated hybrid berries belong to the genus *Rubus* in the Rosaceae. The genus comprises of a highly diverse series of over 700 species with a chromosome number of x = 7 and ploidy levels ranging from diploid to dodecaploid (Jennings 1988; Meng and Finn 2002). The centre of diversity for *Rubus* is thought to be in China (Thompson 1997). Members of the genus can be difficult to classify into distinct species due to hybridization and apomixis, but molecular studies (Alice and Campbell 1999; Sochor et al. 2015; Wang et al. 2016) are now assisting the development of a robust phylogeny for *Rubus.* A brief history of the crop from ancient times can be found in Roach (1985) and Swanson et al. (2011) and Chap. 2 of this volume. However, the development of blackberry and raspberry as crops is much more recent, with cultivated forms of raspberry appearing in Europe in the mid-sixteenth century, although distinct cultivars were not reported until the late eighteenth century (Jennings 1995). Controlled breeding of raspberry began in the 1920s, and increased to the point where over 100 cultivars were released between 1981 and 2001 (Moore 2008). In the case of blackberry, cultivated forms appeared around 1830, and the development of the 'Loganberry' in 1890 is considered to be the first breeding effort in this crop (Swanson et al. 2011). The most widely grown blackberry cultivar is 'Marion', a trailing type, which is grown on over 2500 ha in Oregon alone (Finn 2008).

A comprehensive list of *Rubus* species, subgenera and sections is provided by Skirvin et al. (2005). The commercially important domesticated berries are contained within two subgenera, Idaeobatus and Eubatus. Idaeobatus contains the European red raspberry R. *idaeus* L. subsp. *idaeus,* the North American red raspberry *R. idaeus* subsp. *strigosus* Michx. and the black raspberry *R. occidentalis* L. Species

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J. Graham (✉) · R. Brennan
Cell & Molecular Sciences, The James Hutton Institute, Dundee, UK
e-mail: julie.graham@hutton.ac.uk

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within the Idaeobatus are distributed mainly in northern Asia but also East and South Africa, Europe, Australia and North America (Jennings 1988). Members of the Idaeobatus subgenus are distinguished by the ability of their mature fruits to separate from the receptacle. Almost all of the raspberry species are diploid (Thompson 1995), with only occasional variations as triploids or tetraploids.

In contrast, the Eubatus subgenus containing the blackberries (*R. fructicosus* and *R. caesius,* plus the North American *R. ursinus*) has a considerably more complex genetic makeup, and is mainly distributed in South America, Europe and North America (Jennings 1988). In terms of climate resilience, blackberries are more tolerant of drought, flooding and high temperatures than most raspberries, while the latter are more tolerant of cold winters. Plants exhibit vigorous vegetative reproduction by either tip layering or root suckering, permitting *Rubus* to cover large areas in some cases.

Raspberries and blackberries are woody perennial plants with a biennial cane habit, though primocane varieties (also referred to as fall-bearing) that fruit annually are increasingly important commercially, mainly because they provide an opportunity to extend the cropping season into late summer and beyond. Additionally, especially in regions with low chilling, primocane raspberry cultivars can be managed to produce a double crop in both summer and autumn (Pritts 2008).

Both types of raspberry form new shoots in the spring, followed by vegetative growth throughout the summer. In primocane varieties, flowers are initiated in mid-summer and develop to produce fruit in the autumn. In biennial fruiting varieties, floral initiation does not occur until the autumn, with fruiting occurring in the second year of development.

Changes in day length and temperature cause shoot elongation to cease, and the leaves form a terminal rosette at the shoot tip, after which dormancy sets in gradually. The environmental control of growth and flowering/fruiting in raspberry is reviewed by Moore and Caldwell (1985), Carew et al. (2001), Heide and Sønsteby (2011) and Graham and Simpson (2018).

Most blackberries and black or purple raspberries continue growth into the autumn and do not form a rosette, with growth stopped by rooting at the tips in contact with the ground or low temperatures. Age, genotype and environmental conditions play a role in timing and intensity of dormancy. Additionally, there appears to be some variation in the depth of dormancy achieved by some raspberry cultivars (Heide and Sønsteby 2011; N. Jennings pers. comm.).

The flowers have five sepals, five petals, a very short hypanthium, many stamens, and an apocarpous gynoecium of many carpels on a cone-like receptacle. Multiple ovaries each develop into a drupelet and the aggregate fruits are composed of the individual drupelets held together by almost invisible hairs.

Strik (1992) classified commercial blackberries into three groups based on plant habit, specifically trailing, semi-erect and erect. The trailing types such as 'Marion' form crowns with the primocanes trailing on the ground, where they are gathered and tied to a trellis. The semi-erect types grow on a trellis before arching over, and include 'Loch Ness' and 'Chester', while the erect types, such as 'Navaho' and

'Natchez', grow upright and do not form crowns directly, producing suckers below the soil instead.

In favourable conditions plantations can continue to fruit for more than 15 years and in some isolated parts of central Europe there are plantations aged 25 years or more. However, pest and disease pressures, together with changes in production methods, have ensured that plantation life can be seriously curtailed to uneconomic levels if the problems are not adequately addressed.

1.2 Economic Importance

Fruit of *Rubus* species represent a valuable horticultural commodity, providing both a source of income and also labour, as most fruit for the high-value fresh market is hand-harvested. After fresh fruit, individually quick frozen (IQF) berries are the next most valuable, and can either be hand- or machine-harvested (Moore 2008). The crops are also produced for processing markets, including high value berry juices for their flavour and perceived health benefits, and machine harvesting is usually normal for these markets.

Demand for raspberries, blackberries and other *Rubus* spp. is increasing rapidly in Europe and elsewhere (Clark et al. 2007; Strik et al. 2007, Swanson et al. 2011; Finn and Strik 2016), mainly due to improved shelf-life and fruit quality for the consumer through the use of refrigerated transport and storage, but also in part due to perceived health benefits. Across large parts of Europe especially, production of *Rubus* fruits for the fresh market is now largely conducted under polytunnels of varying design (Figs. 1.1 and 1.2), to give optimal growing conditions and the ability to extend the cropping season beyond the traditional short summer period. To meet the rising demand for berries on a year-round basis and to address the emerging

Fig. 1.1 High density production of *Rubus* under polytunnels

Fig. 1.2 New release Glen Carron from the commercially funded James Hutton Limited raspberry programme

challenges of environmental impacts and climate change, new cultivars are urgently required from plant breeders. In order to achieve this goal, breeders require several key resources; some are self-evident, eg. appropriate germplasm to enable the introgression of important traits into new cultivars, but contemporary breeding can also now utilise ‘omics’ technologies and phenotyping platforms to develop suitable cultivars in a more targeted manner than previously possible.

Rubus crops are of global significance in their production and value. For red raspberry, the biggest production is in Europe where the largest output is from Serbia, Russia and Poland, followed by the UK, Spain and Portugal, particularly for the fresh market. Figure 1.1 shows high density plantings under tunnel. Out-with Europe, the biggest producers of raspberry are the USA and Chile. Black raspberries are predominantly grown in North America for both the fresh and processing markets, with Oregon the leading production area, although there is also significant production in the eastern USA, and also in Korea. In recent years the demand for blackberries has increased sharply, particularly in Europe which now has the largest acreage in production. In North America, the main production of blackberries is also located in Oregon, and US production in 2014 was valued at over $50 m (National Agricultural Statistics Service, USDA, 2015). Mexican production of blackberry has increased significantly in recent years, mainly due to the opportunities for export and season extension to the USA, and raspberry is also exported in this way. Blackberries are also produced in Central America (Costa Rica and Guatemala) and in South America (predominantly from Ecuador and Chile). In Asia, China has seen a rapid expansion in production across several provinces. Production in

Oceania is mainly in New Zealand, particularly of the Boysenberry, and temperate parts of Australia, although the areas planted are relatively small. African production is in South Africa, Morocco, Algeria and Kenya.

There is localized interest in other *Rubus* species, notably in sub-arctic areas. Cloudberry (*Rubus chamaemorus*), in the subgenus Chamaemorus, is a highly valued berry in Scandinavia and northern Russia, with potential for domestication (Korpelainen et al. 1999). Similarly, the arctic bramble *Rubus arcticus* is grown for both fresh and processing purposes in Finland and northern latitudes in Scandinavia and North America (Ryynänen 1972), although production is often curtailed by various disease problems (Kokko et al. 1999; Lindqvist-Kreuze et al. 2003).

1.3 Climate Effects and Stress Tolerance

Abiotic and biotic stresses, both current and emerging, can limit crop productivity so resistant and resilient germplasm is required for cultivar development. Water stress is also forecast to increase at certain times of the year, with more erratic annual rainfall distribution described in many future climate models (IPCC 2014), intensified by soil physical conditions imposed by cultivation. Additionally, the cost of irrigation is likely to increase, so the selection of drought tolerant (DT) and water use efficient (WUE) genotypes will be crucial for some areas. For example, in the UK access to groundwater is now controlled through a government licensing programme, and abstraction licences are granted on the basis of availablity of water with no guarantee that applicants will be granted access to the volumes of water required to grow crops. In England, >50% of soft fruit holdings are in catchments defined as having no water available or defined as over-abstracted (HDC Project Report CP64, 2009). Conversely, cultivars tolerant to wetter flooded conditions may be required in other areas where climate change predictions suggest significant increases in rainfall.

Most *Rubus* plants require some level of chilling to develop normally from bud-break in the following season, and there is some variation between cultivars in this chill requirement. As the climatic trend is towards warmer winters, with less chilling hours, this may become an issue for plant breeders and the future sustainability of *Rubus* crops, particularly red raspberry. Most *Rubus* cultivars will not withstand winter temperatures below around – 30 °C (Moore 2008), although blackberries are more vulnerable to winter injury than raspberries. For a review of heat stress see Fernandez et al., Chap. 3.

High throughput phenotyping methods with the potential to accelerate both the assessment of crop performance under differing regimes and the identification of useful phenotypes for future production are under development, and several phenotyping platforms currently exist (eg. www.plant-phenomics.ac.uk/en/resources/lemnatec-system; http://www.plantphenomics.org.au/). However, these currently operate under artificially controlled conditions, and development of in-field phenotyping platforms is underway in berry crops including raspberry, which will enable improved selection regimes for research and plant breeding (see

Chap. 9, Williams et al.). In addition to advances in phenotyping, significant advances in genomics and metabolomics technologies are increasing our understanding of the links between genotype, phenotype and environment, with molecular tools emerging to assist and inform varietal selection strategies (see Chap. 8, McCallum et al.).

1.4 Cultivation and Challenges

The lifespan of most *Rubus* plantations is limited by a number of constraints predominantly linked to pest and pathogen pressures (see Chaps. 4 and 5) and crop biology (see Chap. 2). The plant's physical or architectural characteristics may therefore play a role in the viability of plantations. Plants with certain physical characteristics may be able to resist pests and diseases by exploiting morphological structures or biomechanical characteristics that interfere with pest/pathogen movement, host recognition, feeding or reproduction on or in the plant (Hanley et al. 2007), by making the plant less attractive visually, or presenting physical barriers to pests and diseases (Mitchell et al. 2013). Architectural traits in raspberry such as bush density and leaf hairs were shown to increase pest burden (Graham et al. 2014). Some plant traits however offered benefits, eg. cane hairs protect against fungal diseases (Graham et al. 2009a). Recently, Mitchell et al. (2016) reviewed the current understanding of the utility of herbivore resistance and tolerance traits as a strategy for improving the sustainability of crop protection, and the use of agro-ecological principles in resistance traits is discussed further in Chap. 5.

The planting of fully disease-free certified *Rubus* stocks into clean soils free from persistent viral, bacterial and fungal diseases and certain pests has a major bearing on the lifespan of plantations. These issues are underpinned by effective and robust quarantine arrangements and certification schemes to protect the propagation industry and downstream fruit production (Jones 1991; Smith 2003). Further information on plant certification highlighting the UK system as a case study is given in Chap. 6 of this volume.

Other crop management issues for *Rubus* fruits includes control of root spread across the inter-row space, requiring these young canes ('suckers' or 'spawn') be removed, mechanically or by contact herbicides, to prevent overgrowth in rows and avoid competition for light, water and nutrients (Knight and Keep 1960; Lawson and Wiseman 1983). Primocane numbers are controlled for the following seasons cropping by pruning in winter and early spring to reduce inter-cane competition. Fruit is harvested annually from each plant, although both non-fruiting vegetative canes (primocanes) and fruiting canes (fructocanes) are present. The crop is usually supported on a post-and-wire system designed to carry the weight of fruits and to protect canes from excessive damage due to wind, harvesting and cultivation. Old dead fruiting canes must also be removed by pruning after harvest. Such pruning operations remove sources of fungal inoculum from the plantation and are important for the long-term health of the crop.

The emergence of Spotted Wing Drosophila in many growing regions worldwide now poses a real threat to raspberry and blackberry crops, and in California alone it has been calculated that losses of ca. $39.4 million can be attributed to this pest between 2009 and 2014 (Farnsworth et al. 2017). Removal of all fruit (including dropped or damaged berries) is now an important part of crop health management programmes (Raffle and Fountain 2017)

1.5 Genetic Diversity

Modern cultivars of raspberry and blackberry remain only a few generations removed from their wild progenitor species, but domestication has resulted in a reduction of both morphological and genetic diversity (Haskell 1960; Jennings 1988), with modern cultivars relatively homozygous compared to wild accessions and genetically similar to each other (Dale et al. 1993; Graham and McNicol 1995). The lack of genetic diversity is a serious concern for future *Rubus* breeding, especially when seeking durable host resistance to pests and diseases. The genetic base can be increased by the introduction of unselected raspberry clones and species material (Knight 1986), thus protecting biodiversity for future *Rubus* breeding programs, and such work is essential to enable breeders respond to future environmental challenges, changing growing conditions and emerging pest and disease problems. However, the time required to produce finished cultivars from unselected wild material can be considerable, particularly if several generations of backcrossing are required to remove undesirable traits.

A number of studies have been carried out to characterize the levels of genetic variation in wild species and to examine the turnover of wild populations. In Scotland, Graham et al. (1997, 2003) examined the spatial genetic diversity in wild accessions of red raspberry, and barriers to gene flow across geographic locations were detected, partly explained by a separation of flowering period, with altitude proving to be particularly important in this context (Marshall et al. 2001; Graham et al. 2003). Further studies at the same sites 10 years later by Graham et al. (2009b) found widespread reductions in plant numbers which, since each population had unique alleles, also equates to a loss of alleles, a finding with potentially serious long-term consequences for diversity.

Similar studies using phenotypic characteristics were carried out on wild raspberry populations in Russia (Ryabova 2007) and Lithuania (Patamsyte et al. 2004) In this study, soil acidity rather than geographic distance significantly correlated with polymorphisms indicating an environmental effect on diversity within populations.

Research on natural populations of other *Rubus* species have shown varying results; in *Rubus arcticus* populations genetic diversity was estimated at levels near 50% for among and within population estimates (Lindqvist-Kreuze et al. 2003). Diversity in wild populations of *R. moluccanus* L. in the Philippines was examined by Busemeyer et al. 1997, and the results were similar to that of Graham et al.

(1997, 2003), with greater similarity present within populations at each location than between locations. Genetic diversity has been examined in natural populations of black raspberry (*R. coreanus*) (Hong et al. 2003) and populations have also been evaluated for traits of importance for use in red and black raspberry breeding (Finn et al. 2003). A study on 63 natural populations of *Rubus strigosus* across North America (Marking 2006) found the majority of the variation to be within populations (79.5%). Weber (2003) analyzed genetic diversity in cultivars of black raspberry (*R. occidentalis*) and red raspberry and found that black raspberry genotypes showed on average 81% genetic similarity. Five cultivars accounted for 58% of the observed variability in black raspberry, and none of the black raspberry cultivars were more than two generations from at least one wild ancestor. This compared well to the 70% similarity measured among red raspberry cultivars in Europe (Graham et al. 1995).

1.6 Genetic Resources

In the light of current climate change implications, collections of *Rubus* accessions from both wild and cultivated genepools represent a major resource for the development of new cultivars, better adapted to a changing natural environment. An example of this is the wild *Rubus* species collection held at the United States Department of Agriculture (USDA) Agricultural Research Service, National Clonal Germplasm Repository (Hummer and Finn 1999). Evaluation of this wild germplasm led to the identification of four sources of aphid resistance, two of which were introgressed into the elite breeding pool in two mapping populations (Bassil et al. 2014). A number of other major *Rubus* germplasm collections exist around the world, including at the Canadian Clonal Genebank (Luffman 1993), where over 140 accessions are maintained in a field collection and in protected culture, and in the UK where field collections of over 150 accessions exist at James Hutton Institute in Scotland and at East Malling Research in England (A. Dolan, pers. comm.). Collections from botanical surveys in Columbia consist of ten *Rubus* species recorded in open and/or disturbed habitats while plant material and seeds from exploration trips in Sakhalin territory are stored in gene banks including an orange *Rubus chamaemorus,* and a dark purple cloudberry (*R. pseudochamaemorus*) (Sabitov et al. 2007). In Europe, the ‘GENBERRY’ project (Denoyes-Rothan et al. 2008), was designed to ensure that agricultural biodiversity of small berries was preserved, characterized and used to improve varieties adapted to local European regions. The project focused on the construction of core collections, the development of a passport data list, the selection and definition of appropriate primary and secondary descriptors, characterization of genotypes using molecular markers, identification of health nutritional compounds and diseases evaluation for a large subset of the collections and the establishment of the European small berries database sustained by a continuous long term network (Denoyes-Rothan et al. 2008). In addition to the formally recognised collections, most breeders and their institutions retain a working germplasm collection that is accessed for parental material on a regular basis.

1.7 Breeding and Cultivar Development

In recent years the *Rubus* industry has relied on a small number of cultivars, often with limited pest and disease resistance, due to consumer and multiple retailer preferences, but at the same time the number of pesticides available for crop protection is decreasing. Additionally, consumers are increasingly concerned about the methods and environmental footprint of food production, and retailers are responding by requiring continued reduction in agricultural inputs and increased sustainability of local production. Conventional breeding has produced significantly enhanced cultivars (Finn and Hancock 2008), but progress in meeting the new challenges in today's marketplace can be slow in these highly heterozygous outbreeders. Selection of superior genotypes requires many years of assessment and evaluation, to ensure that new cultivars can deliver suitable quality in a sustainable way in commercial situations. The status of breeding progress in both Europe and North America are discussed in this volume in Chap. 2.

Breeding programmes in the various growing regions worldwide share common as well as specific goals, influenced by environmental conditions and challenges, end user requirements and available germplasm as well as financial and other resources. However, yield, fruit quality, abiotic and biotic stress tolerance, and ease of pick are key objectives for most programmes.

Fruit quality can be subdivided into physical quality, which includes berry size and shelf-life, and compositional quality. For physical quality, the size of berry is a key objective in many breeding programmes, as this trait can have a significant impact on the cost of harvest. Shelf-life and fruit softening also significantly impact costs in production due to losses on farm and also rejects from retailers. Fruit softening is an important agronomical trait that involves a complex interaction of plant cell processes. Recently QTLs were located primarily on linkage group (LG) 3 with other significant loci on LG 1 and LG 5 of the Latham x Glen Moy map (Simpson et al. 2017). The expression of key genes that underlie these QTLs, with roles in cell wall solubility, water uptake, polyamine synthesis, transcription and cell respiration were tested and gene expression patterns showed variable expression patterns across fruit development with highly significant positive and negative correlation between genes, supporting precise regulation of expression of different cell processes throughout raspberry fruit development.

Rubus berries have some of the highest levels of antioxidants and phytonutrients of any fruit crop, due primarily to their intense concentration of anthocyanins and phenolic compounds (Moyer et al. 2002). This has led to a number of investigations on antioxidant levels of raspberries (eg. Moore et al. 2008; Weber et al. 2008). A review of the chemical, sensory and health benefits of *Rubus* fruits is given by Hancock and McDougall in this volume.

Two of the main drivers of changing priorities and methodologies in *Rubus* breeding are the move towards primocane cultivars and the adoption of molecular breeding techniques (see Chaps. 2 and 8). In the case of primocane cultivars of raspberry, these offer the potential to extend the cropping season, reduce labour costs and achieve higher fruit quality (Pritts 2008). The first promocane-fruiting raspberry cultivar to achieve commercial success was ‘Heritage’ (Daubeny et al. 1992), still grown on significant areas in the USA and Chile. Initially, primocane raspberries were grown in warmer regions with limited chill hours (Pritts 2008; Graham and Jennings 2009), but they are now grown extensively as far north as Scotland and Norway (Sonsteby and Heide 2010). Further breeding of new and better-adapted cultivars is currently in progress in these areas.

In the case of blackberry breeding, a potentially important development was the initiation of a primocane-fruiting programme in the early 1990s at the University of Arkansas (Clark 2008). The primocane trait originated from a wild diploid plant from Virginia, and current breeding aims to improve the fruit quality of the primocane cultivars to commercially acceptable levels (Clark et al. 2012), together with the development of appropriate agronomic and management techniques. The potential impact of primocane blackberries is significant, with possible expansion of production regions and seasonal availability of fruit.

The development of linkage maps and molecular markers linked to key traits has led to significant increases in the efficiency of selection of desirable phenotypes in raspberry, and in particular this work has focused on pest and disease resistance traits such as aphid resistance and tolerance of raspberry root rot (Graham et al. 2006, 2011; Bushakra et al. 2015, Chap. 8 this volume). The genomics resources that have been assembled for *Rubus* are outlined below, but most of the emphasis in the downstream deployment of markers and gene information has been in diploid raspberry, rather than in polyploid blackberries.

Raspberry breeding at Washington State University is described by Moore and Hoashi-Erhardt (2016). Breeding raspberries in British Columbia (Dossett and Kempler 2016) describes breeding for aphid resistance as the major emphasis in raspberry breeding in British Columbia since the 1960s. Here, existing sources of resistance and the current status of knowledge is presented as well as instances of apparent genetic drift of aphid colonies in the greenhouse. New developments in raspberry breeding in Scotland are also described in the same volume (Jennings et al. 2016). Here the deployment of marker-assisted selection in raspberry is now a routine part of the selection process within the raspberry breeding programme at the James Hutton Institute. As a result, two productive genotypes combining resistance to root rot with high fruit quality are currently in commercial trials for further evaluation and potential release. In Poland the first private raspberry and blackberry breeding program conducted in Poland since 2012 is described (Orzel et al. 2016). High quality fruit, good yields, and suitability for fresh and processing market, machine harvesting ability, adaptation to Polish environment as well as improved pest and disease resistance are the key goals. Markers linked to the *Raspberry bushy dwarf virus* (RBDV) resistance locus are being developed in order to facilitate selection (MAS) of valuable clones. Here markers are also being used to assess genetic variability and relationships between cultivars.

1.8 Genomics Resources

Recent reports of marker-assisted breeding in *Rubus* breeding programmes highlights the availability of genomics technologies and their potential in breeding to assist in cultivar development. A number of marker techniques including isozymes, random amplified polymorphic DNA (RAPD), simple sequence repeats (SSR), amplified fragment length polymorphism (AFLP) and Single nucleotide polymorphisms (SNP) have previously been employed in genetic studies and in the construction of genetic linkage maps. Early work on linkage analysis utilised only morphological traits (Crane and Lawrence 1931; Lewis 1939), showing initial linkage between fruit colour (T) and pale green leaves (g or ch_1) in red raspberry. A further genetic linkage among 5 genes (waxy bloom *b*, apricot or yellow fruit *t*, pale green leaf *g*, red hypocotyl *x* and pollen tube inhibitor *w*), was identified producing the first genetic linkage group for *Rubus* (Lewis 1939, 1940). Sepaloid sx_3 was later added to the linkage group between *b* and *t* (Keep 1964). Crane and Lawrence (1931) and Lewis (1939, 1940) also postulated on a linkage between a semi-lethal allele with the unlinked *h* gene. Jennings (1967) added further evidence to this linkage, proposing the symbols *wt* for the locus linked to the fruit colour *t* locus and *wh* linked to the hairy locus (*h*) in place of *w* that Lewis (1939) used (Jennings 1967). Subsequent work in red raspberry has further elucidated the inheritance of hairiness and fruit colour as well as numerous other traits. Associations between the *H* allele for cane hairiness and resistance to spur blight, cane *Botrytis* and cane blight have been recognized (Jennings 1988; Keep 1989), and this association was later confirmed where Gene H and resistances to the diseases were both mapped on the first genetic linkage map for *Rubus* developed using molecular markers (Graham et al. 2004) in a reference mapping population of Latham x Glen Moy population (Graham et al. 2006).

Subsequent improvements in the Glen Latham x Glen Moy map have been generated (Graham et al. 2009a, 2011, 2014, 2015; Kassim et al. 2009; McCallum et al. 2010; Paterson et al. 2013; Simpson et al. 2017), culminating in the recent development of a high-resolution map developed through genotyping by sequencing (GbS) (Hackett et al., submitted). A range of linkage maps in other crosses have been generated: Pattison et al. (2004) used a population generated from NY00-34 (Titan x Latham) x Titan, while Sargent et al. (2007) used a Malling Jewel x Malling Orion population. Recently a map of tetraploid blackberry was generated (Castro et al. 2013) utilising a full-sib family segregating for thornlessness and primocane fruiting, from a cross between ‘APF-12’ and ‘Arapaho’. The development of linkage maps has allowed QTL mapping to proceed for a number of traits, and this is more comprehensively discussed in the Chap. 8 by McCallum et al.

The establishment of complete genome sequences is well-advanced in the Rosaceae (https://www.rosaceae.org/), and in *Rubus* a whole genome sequence for black raspberry *Rubus occidentalis* was reported by VanBuren et al. (2016). The development of genome sequences for Glen Moy and Latham have been completed at the James Hutton Institute in Scotland. These sequence scaffolds have been utilised in developing a GbS map of the Glen Moy x Latham population (Hackett et al. 2018). The completion of these sequence assembly will allow comparisons between species and the identification of key genes significant to important traits for raspberry production.

1.9 Future Challenges

The future expansion of *Rubus* crops globally is potentially constrained by factors including climate change, abiotic and biotic stress and the need for environmentally sustainable production techniques. The means of addressing these challenges lies in both the development of improved cultivation methods and in the breeding of better-adapted cultivars. The *Rubus* industry worldwide has previously demonstrated considerable foresight in adapting to changing market conditions, such as the development of protected cropping methods across Europe and elsewhere, and the increased range of tools available now can ensure that the continued expansion of *Rubus* crops can be maintained. Particular issues, around factors that limit production eg. *Phytophthora* root rot, need to be overcome, and there are hitherto unexplored sources of genes and alleles linked to aspects such as climate effects that can have real benefits for future production.

The rise in phenotyping technology is providing breeders and agronomists with the ability to assess exactly what is happening at the whole plant and subsoil levels, so that resources including water can be targeted more efficiently for maximum crop yields and enhanced quality. When coupled with the expansion of genomics and metabolomics technologies, the opportunities for the development of specific genotypes for prevailing climatic and indeed market conditions are considerable. In particular, the development of genomics resources, in terms of genome sequence and gene discovery, has advanced rapidly, and now the opportunity to fully exploit these resources, in conjunction with the available *Rubus* germplasm, needs to be taken as the next steps towards a fully integrated and sustainable cropping system.

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Chapter 2
Advances in Rubus Breeding

Sophia Nikki Jennings

2.1 Introduction

Demand for *Rubus* berries is significant and increasing at a time when the challenges in growing are also increasing. Climate change, pest and disease stresses, lack of crop protection compounds and consumer demand for sustainable, low-input, low food mile production, challenge breeders to develop resilient varieties, stable across a range of growing environments. Growers fear that within the next decade few if any plant protection chemicals will be available which on top of the climatic challenges bring abiotic and biotic stress back to the top of the breeding agenda, while still ensuring yield and quality expectations are met.

2.2 Biology

Raspberries and blackberries are separated botanically depending on whether the receptacle remains in the fruit when picked as is the case in blackberry or remains on the plant as in raspberry. Jennings (1988) and Swanson et al. (2015) described the history of *Rubus* in detail from the first artifacts of food remnants c. 8000 BCE to modern breeding efforts. By the 1500s, raspberries were cultivated throughout Europe and, with the introduction of North American raspberry *R. strigosus* into Europe in the early nineteenth century, many improvements were made and most cultivars dating from this period are hybrids of these two species (Dale et al. 1989, 1993; Daubeny 1983; Roach 1985; Jennings 1988). A comprehensive review of early domestication is given in Jennings (1988) updated in Swanson et al. (2015)

S. N. Jennings (✉)
James Hutton Limited, Dundee, UK
e-mail: nikki.jennings@huttonltd.com

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and recent individual efforts include Bradish et al. (2016), Clark (2016), Dossett and Kempler (2016), Finn and Strick (2016), Jennings et al. (2016), Orzel et al. (2016) and Salgado and Clark (2016). Briefly, five parent cultivars dominate the ancestry of currently cultivated red raspberries; ‘Lloyd George’ and ‘Pynes Royal’ entirely derived from R. *idaeus* and ‘Preussen’, ‘Cuthbert’ and ‘Newburgh’ derived from *R. idaeus* and *R. strigosus*. Controlled crossing began slightly earlier in the USA than the UK with the introduction of ‘Latham’ in 1914 (McNicol and Graham 1993). The first important black raspberry cultivar was ‘Ohio Everbearer’ introduced c. 1832 (Jennings 1988) and the most widely grown early cultivar ‘Cumberland’ released in 1896. Details of the domestication and early breeding of European and American blackberry cultivars is described in detail in Jennings (1988). Hybrid berries are also growing in importance from loganberry discovered in 1883 to modern hybrids.

2.3 Growth Habit

Details of the growth cycle have been described in detail by Jennings (1988). Briefly, raspberry plants produce biennial canes from a perennial root system. In the first year the canes are vegetative and termed primocanes which flower in year two becoming floricanes, producing the fruit crop, then dying off at the end of the fruiting season. Both non-fruiting vegetative canes (primocanes) and fruiting canes (fructocanes) are present on the plant after the first season thus fruit is produced from the plant annually. Primocane-fruiting red raspberry, black raspberry, and blackberry cultivars have been developed that flower and fruit on the first season cane.

Other than for primocane fruiters, flowers are initiated in the second year of planting after seasonal cues have been satisfied. The flowers of *Rubus* have five sepals, five petals, a very short hypanthium, 60–90 stamens, and an apocarpous gynoecium of many carpels on a cone-like receptacle. Sixty-80 ovaries are produced, each of which develops into a one seeded drupelet (a few contain two) generating an aggregate fruit held together by almost invisible hairs set together on a small conical core (Jennings 1988). Fruiting begins in the second year of planting and in favourable conditions, plantations can continue to fruit for >15 years. For a review of the developmental transitions to fruiting see Graham and Simpson (2018).

The main season summer-fruiting crop is usually supported on a post-and-wire system designed to carry the weight of fruits and to protect canes from excessive damage due to wind, harvesting and cultivation. Primocanes are produced in numbers excessive to requirements for cropping in the following season so many must be removed by pruning in winter and early spring to reduce inter-cane competition and create an open crop canopy for efficient light capture. Old dead fruiting canes must also be removed by pruning after harvest. Such pruning operations remove sources of fungal inoculum from the plantation and are important for the long-term health of the crop. Primocane-fruiting types also require support and modern systems have evolved into an adjustable horizontal system, e.g., using chrysanthemum netting, that can be raised to support the growth of the canes and subsequent flowers and fruit.

As primocanes and fruiting canes are in close proximity, the plant provides a spatial and temporal continuity for pests and pathogens (Willmer et al. 1996). The complex nature of the plant architecture also creates a barrier of foliage that impedes spray penetration of plant protectant chemicals requiring specialized chemical application equipment (Gordon and Williamson 1988). Quarantine arrangements and certification schemes are in place (Jones 1991; Smith 2003) as it is important that the soft fruit industry has access to planting material that is demonstrated to be free from diseases and genetic disorders. The most common approach is to establish mother plants of each cultivar that are not only tested by a wide range of methods to ensure that they remain pathogen-free but that they also conform to the phenotype of the cultivar, i.e., retain 'trueness to type'. Such tests involve visual assessments for virus symptoms on foliage and fruit, bioassays involving use of herbaceous virus-indicator plants or graft inoculation and enzyme-linked immunosorbent assay (ELISA). These three techniques are invaluable for the detection of a wide range of viruses and molecular methods are becoming more commonplace.

Red raspberries vegetatively produce spreading roots that form new canes so the plants can spread from the mother plant. The young canes ('suckers' or 'spawn') that develop from the root buds (Hudson 1959; Knight and Keep 1960) must be removed as a routine management practice either mechanically or by contact herbicides, to balance vegetative and reproductive growth and reduce competition of spawn for light, water and nutrients with the crop. In contrast, black raspberries are crown forming and do not spread underground, instead spreading vegetatively by tip layering like a blackberry.

Recent reports suggest that climate change is already impacting *Rubus* crops with reports of unpredicted phenotypic traits or variation in key transitional timings apparent in breeding and at the grower level. These include unpredictability in timing of key developmental processes altering scheduling; lack of evenness in expression of key developmental stages resulting in variety failure; spatial variations impacting at the plant, row and field level; modification of primocane and biennial behaviour; increased expression of fruit disorders particularly crumbly fruit (Jennings, personal communication; Graham et al. 2015). Understanding the genetic, environmental and epigenetic factors impacting flowering and fruiting and the development of breeding strategies to mitigate unwanted phenotypes are therefore crucial along with other key factors such as biotic stresses in order to ensure the continued future success of cane fruit.

2.4 Major Breeding Objectives

One of the obvious issues of breeding new cultivars is the time-consuming nature of collecting quality and yield data on early stage seedling populations (Stephens et al. 2009, 2012a). For fruit yield, visual scoring assessment methods are commonly used for seedling populations, but these may be poor predictors of yield. Consequently, visual scores for yield can result in less genetic improvement and thus can adversely affect successful cultivar development. Total yield measured by hand-harvesting is labor-intensive and does not assess machine-harvestability, but machine-harvesting yields are not practical to measure on individual plants.

2.4.1 Yield

Most plant breeding programmes focus on improving yield as a major priority, regardless of use, market or production system. Measuring accurate yield data in large, early stage seedling populations is difficult as hand-picking is costly and impractical. Attempts have been made to identify heritable traits of yield components in order to predict and identify productive genotypes before the plants reach maturity. Studies have found that marketable fruit yield had low narrow-sense heritability in both floricane cultivars for processing and primocane cultivars for the fresh market (Stephens et al. 2009, 2012b; Gonzalez 2016). Stephens et al. (2012a) identified berry weight and lateral length as two key components of yield that, when measured in the first two fruiting years from planting, were able to predict total yield in later years, enabling a breeder to concentrate on the most productive genotypes when selecting early stage material. Further studies on identifying productive machine harvesting floricane cultivars found that breeders should place selection emphasis on large berry weight and high numbers of laterals when identifying early-ripening floricanes, while for late season ripening, selection emphasis should be on long laterals and long cane length (Stephens et al. 2016). Gonzalez (2016) found that cane length and number of broken buds per cane were also found to correlate with yield in fresh-market primocane cultivars.

2.4.2 Fruit Quality

Fruit quality attributes are a high priority in breeding programmes. For the fresh market, colour, brightness, size and shape contribute to initial consumer acceptance; however, this must be followed up by satisfaction in terms of flavour to ensure repeat purchase. A consistent and large fruit size (berry weight) is attractive to both consumers and producers as it is more cost effective to harvest, and a prolonged shelf-life maintaining a uniformity and integrity in the punnet in storage for 7 days after harvest is preferred.

2.4.3 Pest and Disease Resistance

The commercial fruit industry relies on a decreasing number of chemicals, presenting serious challenges for future sustainable growth. No suitable high-quality varieties with resistant to pests and diseases are available. There is now a much greater emphasis towards integrated crop management (ICM). This is closely linked with climate change where unpredictability in phenotypic expression is increasingly evident at both small and large spatial scales.

The incorporation of novel resistance/tolerance to pests and diseases is regarded as essential for the development of cultivars suitable for growth under IPM systems. Sources of resistance in diverse *Rubus* spp. to many pests and diseases have been identified and exploited in conventional cross-breeding (Keep et al. 1977; Jones and McGavin 1998; Jennings 1988; Knight 1991; Williamson and Jennings 1992; Birch et al. 2002; Jones et al. 2002). Pest and diseases of raspberry in Europe have been extensively reviewed in Gordon and Williamson (2004), Gordon et al. (2006), Jennings and Dolan (2014) and Parikka et al. (2016). There has recently been greater interest in utilizing plant physical traits for tolerance breeding (Graham et al. 2014; Karley et al. 2016) as plants possess a range of anti-herbivore defences that could be exploited for crop protection. In the UK and EU, a transformation in cultivation practices has occurred from field plantations to protected cropping systems and trialling and selecting germplasm under protected cropping systems is now carried out routinely. There has also been a dramatic decline in field grown raspberries in northern Europe due to *Phytophthora* root rot with a move to less sustainable substrate production in pots or containers and breeding and selection for these conditions is now carried out alongside field selection. Advances in phenotyping however have not been realized to date. Recently a high throughput field phenotyping platform has been developed for cane and bush crops utilizing a range of imaging technologies (Anon 2016; Williams et al. 2017) with the aim of linking spectral signatures to desired phenotypes for use in breeding and research.

2.4.4 Machine-Harvesting

In areas where production focuses on processing, plantations may be harvested by specialist machines to reduce labour costs. This requires special attributes of plant habit and fruit quality to achieve this effectively. Cultivars suitable for machine-harvesting are required to produce high yields of good-quality fruit that is easily shaken from the receptacle during the harvest operations. Fruit destined for Individual Quick Frozen (IQF) production must maintain integrity without shattering into individual drupes on the freezing line. Upright, spinefree canes with well-presented fruit and robust laterals are necessary to endure repeated passes of the harvester for the duration of the fruit season with minimum damage to the following years' canes. Breeding programmes with this objective include data collection from machine-harvested trials to identify suitable genotypes.

2.5 Historic Breeding Efforts

Swanson et al. (2015) and Jennings (1988) have provided good reviews of red raspberry breeding and the following is a summary of these overviews. Dr. Brinkle of Philadelphia, Pennsylvania is recognized as the first successful raspberry breeder

(Darrow 1937). ‘Latham’ has been the most enduring cultivar from this early breeding period, introduced by the Minnesota Breeding Farm in 1914. It is still grown and has been used as a parent in widely utilized reference mapping population (Graham et al. 2004). ‘Pruessen’, ‘Cuthbert’ and ‘Newburgh’ were developed in Europe and are hybrids between the North American and European species, along with ‘Lloyd George’ and ‘Pyne’s Royal’, which are pure *R. idaeus*. These five cultivars dominate the ancestry of red raspberry with ‘Lloyd George’ in the direct ancestry of 32% of the North American and European cultivars in 1970 (Oydvin 1969) contributing traits including primocane fruiting, large fruit size and resistance to the American aphid (*Amphorophora agathonica*). Jennings (1988) speculated that the success of ‘Lloyd George’ hybrids “was possibly achieved because they combined the long-conical shape of ‘Lloyd George’ receptacle with the more rounded shape of the American raspberries”. ‘Willamette’ from a cross of ‘Newburgh’ x ‘Lloyd George’ is an example of a “‘Lloyd George’ hybrid” that dominated the industry in western North America for over a half century.

2.6 European Breeding Programmes

The East Malling Research (EMR) programme in the UK is responsible for the “Malling series” of raspberry. A number of selections were made prior to World War II and released in the 1950s: ‘Malling Promise’, ‘Malling Exploit’ and the very successful, ‘Malling Jewel’ (Jennings 1988). This programme continues to have a significant impact with the more recent release ‘Octavia’ (Knight and Fernandez 2008) which offers the industry late floricane fruit to extend the season and bridge the gap between summer and autumn fruit in the UK, particularly in Scotland. Releases since include ‘Malling Minerva’ and early season floricanes: ‘Malling Juno’ and ‘Malling Freya’. The programme has had a considerable emphasis on primocane types, releasing the early-season ‘Autumn Bliss’ in the 1980s, which has given a significant impetus to world development of primocane fruiting raspberries and these genetics have been used extensively for breeding around the world. Later releases have also laid a good germplasm base for primocane types, including ‘Autumn Treasure’ as a source of *Phytophthora* tolerance with good fruit quality (Jennings et al. 2016). Primocanes, ‘Malling Bella’ and ‘Malling Charm’ have since been released from this programme. The significant “Glen series” was developed at the Scottish Crop Research Institute (Now the James Hutton Institute) (Invergowrie, UK). Their first release was ‘Glen Clova’ in 1969 but the release of ‘Glen Moy’ and ‘Glen Prosen’ in 1981 offered great improvement in fruit size and flavour along with spinelessness. The lack of spines, together with an upright growth habit and the ease of pick from the receptacle gave the combination of traits suitable for harvesting by machine. The most successful of these cultivars thus far has been ‘Glen Ample’, which was released in 1996, and was a standard in the European wholesale raspberry market and suited both processing and fresh markets. More recent releases include ‘Glen Fyne’, well adapted for mechanical picking, ‘Glen Ericht’, which demonstrates good field tolerance to *Phytophthora,* ‘Glen Dee’ and ‘Glen Carron’, which offer large fruit and an extended shelf-life for the fresh market. More recently, this programme initiated breeding for primocane types and has concentrated on early autumn-fruiting and traits that contribute to increased picking efficiency to reduce labour costs.

In central Europe, Poland is one of the main producers of raspberry in the European Union. The Institute of Horticulture released important primocane cultivars ‘Polana’ and ‘Polka’, used widely as parental material in Western Europe. Since then the NIWA breeding programme has developed floricanes ‘Laszka’, ‘Radziejowa’, ‘Sokolica’ and ‘Przehyba’ with competitive quality traits (Orzel et al. 2016) and newer primocanes; ‘Polonez’, ‘Poemat’ and ‘Delniwa’.

2.7 Raspberry Breeding in North America

The United States is the world’s third largest producer of raspberries (FAOSTAT, 2016). Production occurs across much of the country but is concentrated around the Pacific Northwest in California, Washington and Oregon. The breeding programmes in the Pacific Northwest of North America at Washington State University (WSU; Puyallup, WA) and the USDA ARS in Oregon (USDA-ARS; Corvallis) have worked closely together and with several breeding programmes around the world, including Canada, New Zealand and the UK. Washington and Oregon mainly concentrate production on machine-harvested raspberries for processing. The cultivar ‘Meeker’, released in 1967 from WSU, is well suited to this area and to machine-harvesting, but it is susceptible to raspberry bushy dwarf virus (RBDV) and *Phytophtora* root rot. Despite the efforts of several breeding programs, ‘Meeker’ is still the predominant cultivar for commercial production in the PNW (Finn 2006; Stephens et al. 2012b). Several landmark cultivars from these programmes have included ‘Willamette’ and ‘Canby’ and, more recently, ‘Coho’ with its high yields of IQF fruit (Finn et al. 2001) and ‘Lewis’, adapted to the Pacific Northwest and to New Zealand’s Central and Southern districts for fresh market production. Primocane-fruiting types have become more important from this programme and ‘Summit’, ‘Amity’, ‘Vintage’ and ‘Kokanee’ have been released to extend the fresh market season. Newer releases ‘Cascade Delight’, ‘Cascade Bounty’ and ‘Cascade Harvest’ are root rot tolerant, and together with ‘Cascade Dawn’, which is immune to RBDV, are becoming widely planted (Moore 2004, 2006; Moore and Finn 2007; Moore et al. 2015).

Further north, the small fruit breeding programme at the Pacific Agri-Food Research Centre in Agassiz (PARC-Agassiz) in British Columbia has bred raspberries since the 1950s as part of Agriculture and Agri-Food Canada’s (AAFC) small fruit breeding programme, providing processing and fresh market varieties to growers in the Pacific Northwest and around the world. Initial releases such as ‘Haida’, ‘Chilcotin’, ‘Skeena’ and ‘Nootka’ have excellent fruit quality and high yields for a fresh market berry succeeded by ‘Chilliwack’, released in the mid-

1980s. ‘Tulameen’ released in 1989, is one of the world’s most popular cultivars, grown in a range of climates, and is regarded as the benchmark for fresh market fruit flavour. More recent releases ‘Esquimalt’, ‘Chemainus’, ‘Cowichan’, ‘Saanich’, ‘Nanoose’, ‘Ukee’ and ‘Rudi’ are being planted in North America and trialled in Europe (Kempler et al. 2005a, b, 2006, 2007).

In the eastern USA, the New York State Agricultural Experiment Station (Geneva) has the oldest raspberry breeding programme in North America, dating to the late 1800’s. Floricane cultivars such as ‘Taylor’ and ‘Hilton’ were early staples of the eastern industry. These have been replaced in recent decades with cultivars such as the large fruited ‘Titan’, released in 1985, early season ‘Prelude’ and very late season ‘Encore’, both of which were released in 1998. ‘Titan’ has proven to be an excellent parent, producing large fruited offspring but is susceptible to *Phytophthora* root rot. Primocane fruiting germplasm within the programme in combination with material such as ‘Durham’, developed in New Hampshire, was used to produce an excellent primocane fruiting germplasm pool that culminated with the release of ‘Heritage’ in 1969 and ‘Ruby’ (‘Watson’) in 1988 (Daubeny 1997).

In the southern USA, the North Carolina State University programme focuses breeding efforts on heat tolerance, selecting types adapted to high elevation production areas with warm, humid summers and warm, but fluctuating, winter temperatures (Ballington 2016). ‘Mandarin’ was the first cultivar derived from heat tolerant Asiatic species, *R. parvifolius*, which was adapted to the warm, humid conditions. More recently, ‘Nantahala’ is a late season primocane suited to production in the mountain regions of North Carolina and adjacent states (Fernandez et al. 2009).

2.8 New Practices

Primocane fruiting types have revolutionized raspberry production. They have become the standard in regions where cold winter temperatures caused considerable winter damage to canes of floricane fruiting raspberries, as well as in low chill areas where floricane cultivars do not receive adequate chilling to be productive. Private companies in California USA, such as Driscoll’s Strawberry Associates (Watsonville, CA), have developed cultivars and whole new production systems based around these primocane fruiting types in which plants are only in production for 18 months. ‘Driscoll Maravilla’ demonstrated a major change in fruit quality in terms of shipping, shelf-life and appearance. These cultivars and management systems have led to the rapid expansion of the California raspberry industry as well as industries in Central America and Southern America, southern Europe, Australia and South Africa. The University of Maryland has a coordinated breeding programme with Virginia Tech University, Rutgers University, and the University of Wisconsin – River Falls, and the primocane fruiting ‘Caroline’, ‘Anne’, and ‘Josephine’ developed in this programme have become standards throughout much of North America. In Europe, the increase and dominance of ‘Driscoll Maravilla’ has motivated many private breeding companies to adopt a similar business model, releasing proprietary cultivars with restricted licensing availability and exclusivity. Many of these breeding programmes were initiated by industry, i.e., propagators and marketing groups, and several new raspberry cultivars have been developed and released in a relatively short period of time. In the UK, Berryworld Plus Ltd. focus their breeding efforts on flavour and released 'T-Plus', 'Diamond Jubilee' and, in association with Five Aces Breeding LLC in Maryland USA, 'Sapphire', 'Pearl' and 'Jade'. In Holland, Advanced Berry Breeding released the Dutch primocane cultivars 'Imara', 'Kweli', 'Kwanza' and more recently, the early autumn cultivars, 'Mapema'and 'Rafiki'. Marionnet in France bred 'Paris' and the very large fruited 'Versaille'. Planasa in Spain produced 'Lupita' and 'Adelita', now grown successfully in Morocco for the European market during the winter months. In Italy the wholesale nursery, Vivai Molari, bred and developed 'Castion' and 'Enrosidira' and a grower cooperative, Sant'Orsola Societa Cooperativa Agricola, has had success with 'Vajolet' and 'Lagorai'.

2.9 Black Raspberry

Black raspberry (*R. occidentalis*) was not cultivated until the nineteenth century, probably because of its abundance in the wild and the public's preference for red raspberry. In the early twenty-first Century, black raspberry breeding efforts were renewed at the New York State Agriculture Experiment Station, the USDA ARS in Corvallis, OR and in Beltsville, MD, and with New Zealand HortResearch Inc. In Corvallis, Dossett et al. (2008) evaluated black raspberry genotypes from sibling families to assess variation and inheritance of vegetative, reproductive and fruit chemistry traits. Breeding progress has been hindered by a lack of variability in elite germplasm and a lack of disease resistance (Dossett et al. 2012). In New Zealand, 'Ebony', the first spineless black raspberry cultivar, was released for the home garden market (H.K. Hall, personal communication). The first primocane-fruiting black raspberry cultivar, 'Niwot', was bred by a private breeder in Colorado (Moore and Kempler 2014). Further populations segregating for primocane types are also among the germplasm in the Oregon programme.

2.10 Conclusion

With the year on year increasing consumer demand for fruits from the *Rubus* genera, the industry has shown it can adapt to some of the challenges faced through the use of protected cropping and primocane fruing as well as adopting markers into breeding programmes eg raspberry root rot. Future challenges particularly climate change will have a wealth of genetic/genomic and phenotyping resources to help support continued variety development and so cultivation.

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Chapter 3
What We Know About Heat Stress in *Rubus*

Gina E. Fernandez, Ramon Molina-Bravo, and Fumiomi Takeda

3.1 Introduction

With over 500 species spread across six continents, members of the genus *Rubus* can be found in a wide range of geographic and climatic regions (Graham and Woodhead 2011; Jennings 1988; Hall et al. 2009; Hummer 1996). Although there are sources of adaptation to climate extremes in wild germplasm, commercial raspberry cultivars have a limited range of production. Until recently, raspberries were produced primarily in the Western US, and where climates are considered temperate or Mediterranean (Kempler and Hall 2013). However, as the demand for year-round production increased, production expanded to regions including southern Europe, Chile and Mexico. In contrast, blackberry (*Rubus* spp.) cultivars are considered more widely adapted and perform well in both moderate Mediterranean climates and hot humid climates, however, the newer primocane-fruiting types struggle to produce flowers and fruit in warmer climates. With the advent of more frequent cold and warm temperature extremes that are a part of global climate change, both raspberries and blackberries will have heat stress as a major challenge for growth and expansion of these crops.

In plants, heat stress occurs when temperatures exceed an optimal value for a period of time and the result is irreversible damage to plant growth and development (De Souza et al. 2012). Heat stress can be short-term (transitory) and cause leaves to wilt during part of the day or damage floral development, often resulting in the

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G. E. Fernandez (✉)
North Carolina State University, Raleigh, USA
e-mail: Gina_Fernandez@ncsu.edu

R. Molina-Bravo
School of Agraian Sciences, National University of Costa Rica, Heredia, Costa Rica

F. Takeda
Appalachian Fruit Research Station, USDA-ARS, Kearneysville, WV, USA

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failure of the crop to produce fruit. Heat stress can also occur at night and can result in significant yield losses (Shah et al. 2011). In other situations, season-long periods of heat stress can inhibit photosynthesis, and as a result plants will have very limited growth and eventually die as is the case for raspberries in southern U.S. climates (Ballington and Fernandez 2008).

Heat stress is one of the most often cited factors that limit productivity in cultivated raspberry (*Rubus ideaus* and *R. strigosus*) (Stafne et al. 2000; Ballington and Fernandez 2008; Jennings 1988). Jennings (1988) stated that “red raspberries are not well adapted to southern parts of North America, because they tend to be short-lived and die before fruiting”. Various breeding programs have over time identified numerous species of *Rubus* are heat tolerant (Table 3.1). Since Jennings published his book in 1988, raspberry and blackberry production has spread to regions with warmer climates. However, compared to other crops, relatively little is known on the effects of heat in *Rubus*. The focus of this chapter is a compilation of recent discoveries in *Rubus* heat stress and tolerance with an emphasis on the impact of heat on physiological, biochemical, molecular and genetic/genomic mechanisms.

3.2 Physiological Effects of Heat

3.2.1 Photosynthesis

High temperatures can limit *Rubus* growth and development. One of the first studies that determined how heat impacts whole plant physiology assessed the impact of rising temperatures on photosynthetic assimilation (A), stomatal conductance (g), and evapotranspiration rate (ET; Fernandez and Pritts 1994). This study found that

Table 3.1 *Rubus* species reported to have high temperature adaptation

Species	Type	References
R. trivialis	Blackberry	Finn (2008)
R. cuneifolius	Blackberry	Finn (2008)
R. frondosus	Blackberry	Finn (2008)
R. parvifolius	Raspberry	Ballington and Fernandez (2008), Ballington (2016), Stafne et al. (2000, 2001)
R. kuntseanus	Raspberry	Ballington (2016)
R. hirsutus	Raspberry	Hall et al. (2009)
R. innominatus	Raspberry	Hall et al. (2009)
R. niveus	Raspberry	Hall et al. (2009)
R. occidentalis	Raspberry	Hall et al. (2009)
R. pileatus	Raspberry	Hall et al. (2009)
R. sumatranus	Raspberry	Hall et al. (2009)

the optimal rate of A in the floricane fruiting 'Titan' red raspberry was between 15 and 20°C, and as temperatures increased from 20 to 40°C, A values rapidly declined. In a later study, in the primocane-fruiting cultivar 'Heritage', A rates declined when temperatures were above 20°C (Percival et al. 1996). To determine heat stress in a wider range of germplasm, photosynthetic assimilation, ET and g of six red raspberry cultivars, 'Autumn Bliss', 'Dormanred', 'Heritage', 'Nova', 'Reveille', 'Southland' and one blackberry 'Arapaho' were assessed. As temperatures increased from 20 to 25°C (Stafne et al. 2001), the A rates of the raspberry cultivars declined once temperatures exceeded 25°C. However, A of the blackberry cultivar 'Arapaho' was almost always higher than all of the raspberry cultivars at all temperatures (20°, 25°, 30° a–nd 35°C), which suggests that blackberry may have a higher temperature optimum than raspberry. In the same study, all ET increased or remained the same with increasing temperatures, which suggests there was insufficient evaporative cooling. Stafne et al. (2001) concluded that 'Dormanred', a red raspberry, while having the lowest A throughout the trial, the rate of decline in g was lower compared to other cultivars thus, the ratio of A/g decline was not as great, indicating some sort of adaptation advantage of this cultivar. In another study, A was measured in 32 raspberry genotypes from a wide range of sources were grown at 35°C for 2–4 weeks (Stafne et al. 2000). A, g and ET varied widely among the genotypes and time exposed to the high temperatures. In general, genotypes from Oregon in the Pacific Northwestern US had lower A than those from southern US states and areas of Asia. They noted that most of the higher A were in genotypes that *R. parvifolius* in their background, including 'Dormanred'.

3.2.2 Chlorophyll Fluorescence (Fv/Fm)

Although photosynthesis is a useful tool to assess whole plant performance, measuring the physiological responses can be time consuming and cumbersome for screening germplasm in the field for breeding programs. Chlorophyll fluorescence has been used to quickly assess stress on numerous plant species and by breeders interested in assessing differences within germplasm accessions in response to stresses (Smillie and Hetherington 1983; Petkova et al. 2007; Kalaji and Guo 2008; De Souza et al. 2012). Numerous reviews detail the theory and practice of quantifying chlorophyll fluorescence (Krause and Weis 1991; Maxwell 2000). However, for the purposes of this review, put simply, light absorbed by the chlorophyll molecule in a leaf has three possible fates. It can be: 1. used for photosynthesis, 2. dissipated as heat or 3. re-emitted as light aka chlorophyll fluorescence. These three processes compete with one another and any rise in one will result in a decrease of one or more of the others. Modern measuring devices called 'chlorophyll fluorometers' have enabled *Rubus* (and many other) researchers to measure chlorophyll fluorescence parameters, such as the maximal quantum efficiency of photosystem II in dark adapted leaves (*Fv/Fm*), with relative ease.

The first report of the use of chlorophyll fluorescence in *Rubus* was in 2008 (Molina-Bravo et al. 2011). Breeders wanted to use chlorophyll fluorescence to assess a segregating population of red raspberry under heat stress. However, before they could use chlorophyll fluorescence equipment, they needed to develop a protocol. Over a series of several weeks and examination of a number of time scenarios of heat stress, they found that: optimal time to collect samples was in the morning, leaves could be detached and brought back to the lab to take measurements, and leaves needed to be heat shocked to a critical temperature of 45°C in order to detect extensive tolerance/susceptibility.

These chlorophyll fluorescence protocols were later used in two separate studies. A red raspberry population of (*R. parvifolius* × 'Tulameen') × 'Qualicum' (Molina-Bravo 2009) and two black raspberry population distributions (Bradish et al. 2016) were screened using chlorophyll fluorescence. Both the red and black raspberry populations segregated with similar near normal distributions at both ambient 25 °C and high temperatures 45 °C (Fig. 3.1). In both the red and black raspberry populations, there were individuals that had higher *Fv/Fm* than their heat tolerant parent/grandparents, suggesting that transgressive segregation occurred in the populations. In addition, although the experiments were not conducted at the same time, they were conducted at the same location and the black raspberry population had a higher mean *Fv/Fm* than red raspberry.

Although *Fv/Fm* is a useful tool, additional measureable traits that are related to plant growth needed to corroborate chlorophyll fluorescence data. Elevated temperatures from 20 to 27, 32 or 37°C impacted, chlorophyll fluorescence, chlorophyll pigment content, and floral development of raspberries (Gotame et al. 2013). Five cultivars 'Autumn Bliss', 'Autumn Treasure' 'Erika' 'Fall Gold' and 'Polka' were subjected to 1 week of the 3 elevated temperatures. Chlorophyll fluorescence decreased in all cultivars over the 7 day period, with 'Autumn Bliss' and 'Fall Gold' experiencing the largest decline in chlorophyll fluorescence. Chlorophyll content varied by cultivar after exposure to the heat stress. In two cultivars ('Autumn Treasure' and 'Erika') the levels of Chl a and Chl a/b, age of flowering laterals decreased in only 'Autumn Bliss' and flowering was delayed in 'Autumn Treasure' and 'Erika' under high temperature stress. They concluded that primocane (annual) fruiting cultivars vary in their ability to tolerate heat stress and knowing how a cultivar responds to heat stress could be used by to manipulate future cropping systems in warmer climates.

Vegetative and reproductive traits and *Fv/Fm* were measured in North Carolina, in two mapping populations (ORUS 4304 and ORUS 4305) of black raspberry (*R. occidentalis*) (Bradish 2016) over 3 years. Correlation analysis indicated that over time, although there was an overall loss of vigor in the plantings, primocane vigor, floricane vigor and winter hardiness were all positively correlated to high *Fv/Fm*. At the end of the trial, the combination of selection for high *Fv/Fm*, cane vigor and winter hardiness resulted in selection of approximately 1% of the population with improved vigor and heat tolerance (Bradish 2016). Although populations both share a common heat tolerant parent, NC 84-10-3, ORUS 4305 had significantly higher *Fv/Fm* than the other population ORUS 4304.

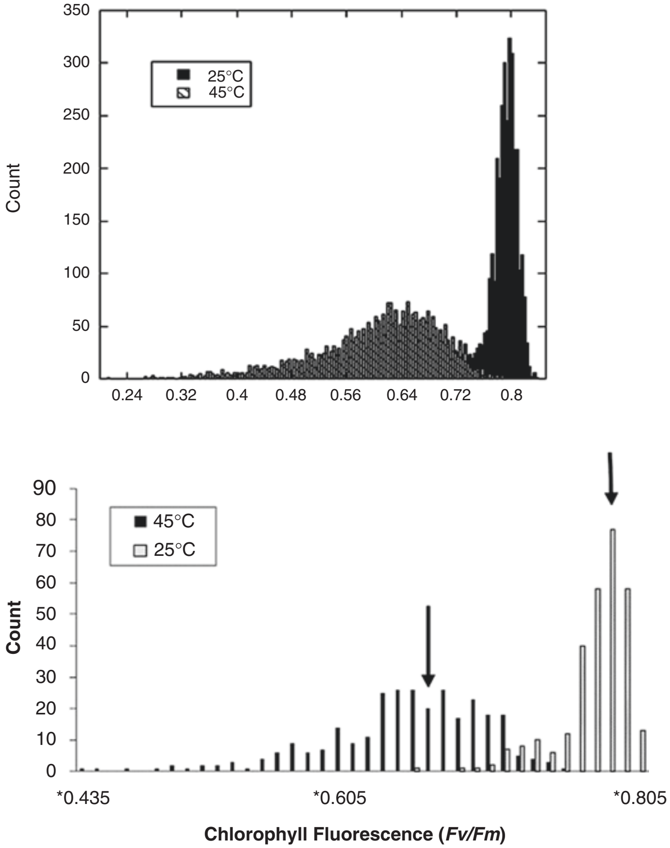

Fig. 3.1 Frequency distribution of maximal efficiency (*Fv/Fm)* for red raspberry (upper) and black raspberry (lower) segregating populations grown at the Sandhills Research Station in Jackson Springs, NC in 2008 and 2013 respectively. Detached leaves were subjected to ambient temperatures (25 °C) and high temperatures (45 °C). Arrows in lower figure represent average *Fv/Fm* for parents at ambient (25 °C) temperatures (Molina-Bravo 2009; Bradish et al. 2016)

3.2.3 Flower and Fruit Damage

High temperatures during summer months is attributable to increased levels of solar radiation, which is the major contributor to berry head load. Higher levels solar radiation account for as much as 0.15 MJ/m^2 of cumulative daily UV-A + UV-B radiation (Zibilske and Makus 2009). High temperatures have been shown to impact fruit set in a number of crops (Hatfield and Prueger 2015; Peet et al. 2003; Kadir et al. 2006; Shah et al. 2011). In *Rubus*, air temperatures impact floral initiation and development in peak summer months on primocane-fruiting raspberry (Privé et al. 1993; Lewers et al. 2008) and primocane-fruiting blackberry (Stanton et al. 2007). Although breeders were excited to develop primocane-fruiting blackberries in the 2000’s (Clark 2008), initial attempts to produce fruit in the southern U.S. in primocane fruiting types was limited. This was due to the inability of the plants to set flowers in the summer, although in Oregon's milder summer temperatures they would thrive and produce much more fruit on the primocanes in the late summer fall.

Researchers found that under high heat treatments of 35°/23.9°C day/night temperatures resulted in injury to both stamens and pistils of blackberry (Stanton et al. 2007). However, they suggested that the damage to the stamens was of major concern as the pollen from anthers is needed to stimulate the development of and the fertilization of ovaries. Conversely, low temperatures of −2.8°C have been implicated in the injury of the female part of the plant, the gynoecium (pistils), in blackberries during spring freezes (Takeda and Glenn 2016).

In red raspberries, heat stress, or fruit exposed to temperatures >42 °C, can result in sun scald, or more specifically photo bleaching of maturing fruit that have developed red pigmentation turning white (Fig. 3.2). Both high temperatures and UV light have been attributed to occurrence of white drupelet disorder in both raspberries and blackberries (Renquist et al. 1989).

Shading red raspberry plants for just a few days prior to fruit ripening was as effective as season-long shading for reducing white drupelet formation. Early studies with plastics and filters which absorb nearly all UV radiation was as effective as aluminum foil cover at preventing injury (Renquist et al. 1989).

In blackberry, red drupelet disorder, also called reversion, reddening or red cell disorder, occurs after fruit is harvested and previously black drupelets turn red. It is still unclear as to what exactly is the cause of this disorder. However, physical damage during harvest to the drupelets has been implicated, as have rapid changes

Fig. 3.2 White drupelet disorder in 'Apache' blackberry. (Photo Absalom Shank)

in temperature from the extreme hot field conditions to the sudden cold temperature of the refrigeration and nitrogen levels (Edgley pers. Comm.).

3.3 Mapping Studies

Many studies have looked for quantitative trait loci (QTLs) associated with heat stress response in other crops, especially in rice and wheat (Jha et al. 2014). Most of these studies used grain-related traits to measure heat tolerance to identify QTLs, such as grain-filling, seed weight, and seed set. However, other parameters have been used to find heat tolerance QTLs, such as canopy temperature, rate of senescence, photosynthetic rate and flowering stage (Cao et al. 2003; Paliwal et al. 2012; Pinto et al. 2010; Ye et al. 2010). In tomato, heat tolerance QTLs were identified by measuring fruit set at high temperatures (Grilli et al. 2007).

Using the chlorophyll fluorescence protocol described in Molina-Bravo et al. 2011, an interval mapping (IM) analysis identified three QTL regions that accounted for approximately 35% of the variation for heat tolerance in a segregating population on linkage groups 4, 5 and 7 (Fig. 3.2) as measured by *Fv/Fm* (Molina-Bravo 2009). A Kruskal-Wallis point analysis revealed markers significantly associated (at $p < 0.01$ or $p < 0.005$) with heat tolerance and colocalized within the same IM regions (Fig. 3.3). The QTL on LG5 explained most of the variation (13.1% LOD 4.69) and was strongly associated with microsatellite marker Rub35a ($p < 0.005$). In an 'ab × cd' allele model, the mean associated with the "c" allele for this QTL came from the 'Qualicum' parent and reduced the *Fv/Fm* value, and suggested an association with heat susceptibility. Each of the other two QTL accounted for

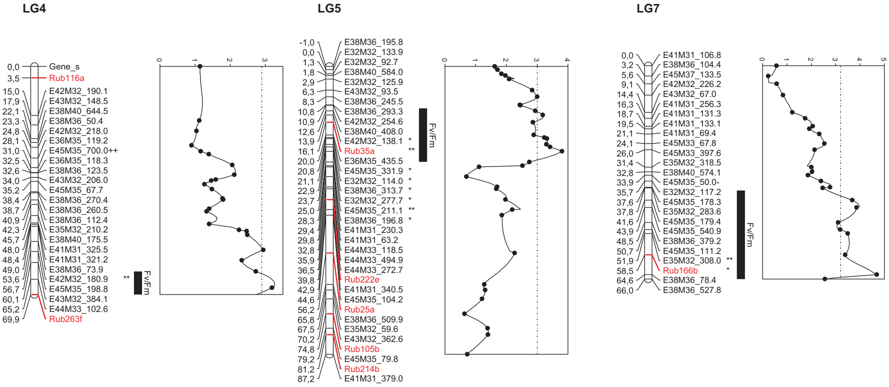

Fig. 3.3 QTL analysis using interval mapping (IM) and Kruskal-Wallis (KW) procedures for heat tolerance measured by maximal efficiency (*Fv/Fm*) in a red raspberry population ((*R. parvifolius* × 'Tulameen') × 'Qualicum') (Ballington and Fernandez 2008; Molina-Bravo et al. 2011, 2014). Significant LOD scores are shown by dashed lines. Markers were significant by KW analysis at 0.01(*) and 0.005(**). Linkage groups constructed using AFLP (black) and SSR (red) molecular markers. Group nomenclature as proposed by Bushakra et al. (2012)

approximately 10% of the variation and supported a model where both parents are heterozygous for heat tolerance, with "bc" alleles for heat tolerance, and "ad" alleles for susceptibility in both cases. Additionally, some individuals in the population surpassed the heat tolerant parent, i.e. there was transgressive segregation. These two observations would imply that, in spite of its poor heat tolerance, 'Qualicum' contributed alleles for heat tolerance.

Chlorophyll fluorescence is an indirect measure of the efficiency of the light harvesting complexes (Krause and Weis 1991). In a study in cotton, Crafts-Brander and Law (2000) demonstrated that heat stress causes irreversible damage to Rubisco and carbon assimilation through *Fv/Fm* measurements, and biochemical assays. Therefore, these QTL in *Rubus* could be associated with allele products that stabilize photosynthetic proteins such as Rubisco. Nevertheless, other parameters that measure heat tolerance should be explored in *Rubus*.

3.4 Transcriptome Studies

In a 2014 study (Gotame et al. 2014), a *Rubus* microarray was used to study the effects of two high temperatures (27 °C or 37 °C), applied for a short duration (24 h), on levels of total gene expression in four annual-fruiting raspberry cultivars ('Autumn Bliss', 'Autumn Treasure', 'Erika', and 'Polka'). 'Erika' and 'Autumn Treasure', considered to be heat tolerant cultivars, had 38 genes with elevated expression compared to 'Autumn Bliss' and 'Polka', considered to be not heat tolerant cultivars. Twelve of these genes were differentially expressed in two cultivars, 'Autumn Bliss' and 'Erika' when they were subjected to 37 °C heat stress. In addition, they found two genes (*P1P1* and *TIP2*) that were down regulated in 'Autumn Bliss' but upregulated in the other 3 cultivars at high temperatures.

Although the triggers in white drupelet disorder are likely environmental (UV and temperatures), differences in gene expression in white v. black drupelets are likely. In an attempt to identify genes responsible for white drupelet disorder (Fig. 3.2) in blackberry, researchers used RNAseq technology to develop transcriptome data of blackberry for white drupelet and reversion disorders (Fernandez et al. 2017). They found >12,000 genes that were differentially expressed between normal black drupelets and white drupelets, at FDR = 0.01 and 1.5 Log^2 fold change. They also found the amount of RNA in white drupelets was very low, suggesting the genes that are involved in nucleic acid biosynthesis were severely shut down, likely as a result of stress.

3.5 Other Omics

The genome of the black raspberry has been published (Van Buren et al. 2016). Red raspberry and blackberry are in progress. Fortunately for *Rubus* researchers, the genomes of other Rosaceous crops have been sequenced and can be used for comparing genomes and evaluating transcriptome response to abiotic stress. Gene discovery has helped to identify physiological traits involved with stress tolerance and to move tolerance genes between species. However, heat-stress tolerance is multigenic and manipulation will encompass transferring more than one gene at a time and incorporation into *Rubus* will take time.

Functional genomics, genetic engineering, transcriptome profiling, proteomics and metabolomics have been used to identify mechanisms of heat tolerance in other crops. Integrating data using various 'omic' approaches established other crops and within the Rosaceae will continue to help unravel heat stress in *Rubus*. *Rubus*, we believe will continue to serve as an excellent model plant for heat stress scientific discoveries.

3.6 Horticultural Mitigation of Heat Stress

Researchers have found that the rotatable cross-arm (RCA) trellis system provides some relief from heat stress (Takeda et al. 2013). The unique canopy configuration of the RCA trellis and cane training system has provided benefits on fruit quality. The RCA trellis technology allows fruit to be positioned on one side of the row. If the rows are oriented east-west, fruit can be positioned on the north side of the row and not exposed to direct sunlight in the morning or afternoon. If the rows are oriented north-south, the fruit would be exposed either to morning or afternoon sun depending on which side the fruit is positioned. A study by Takeda et al. (2013) showed that with 'Apache' blackberry the incidence of white drupe formation was similar whether fruit was on east or west side of the row. However, direct exposure to sunlight either in the morning or afternoon significantly increased the number and severity of white drupe formation compared to the fruit in the shade. The skin temperature was as much as 8 ° C higher in berries exposed to sun than those in the shade. In the Central Valley of California the RCA trellis and cane training system increased harvest efficiency 30% and, more significantly, fruit cull (berries with white drupes) was eliminated when the fruit was positioned on the north side of rows that were oriented east-west (Manuel Jimenez, personal communication).

Similar reduction in white drupelet disorder can be achieved by decreasing solar light transmission with a placement of a shade fabric over the plants. A large grower in the Central Valley of California has been able to grow 'Ouachita', 'Natchez', and 'Prime-Ark 45' blackberries without any signs of white drupe formation by growing them under high tunnels clad with a 50% shade cloth (personal observation). A study initiated at North Carolina State University's Piedmont Research Station to evaluate the fruit quality of 'Natchez', 'Ouachita', and 'Von' blackberries trained on the RCA trellis in rows oriented north-south and east-west with fruit positioned on east, north, west, or south side of plant canopy also confirmed production system that by changing row direction and positioning the fruit away from sunlight can be useful in reducing fruit quality loss and white drupe disorder in blackberry attributable to high light intensity (Takeda et al. 2013).

3.7 Looking Forward

According to the National Oceanic and Atmospheric Administration, the year 2016 marked the third straight year in a row that there were record warm temperatures on the globe (Dahlman 2017). Heat tolerance will be an increasingly important factor for all plant species, including *Rubus*. With increasing focus on local fruit production, raspberry and blackberry production can increase in areas located at high elevations (>1000 m) where high solar radiance, temperatures, and ultraviolet radiation during the growing season. With increasing episodes of extreme heat during the summer and longer duration of growing seasons, raspberry and blackberry will both be impacted. However, we suggest that through the use of emerging technologies both in the lab and in the field and the wide range of heat tolerance in the species, *Rubus* is an ideal crop to both study and develop heat tolerance.

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Chapter 4
Pathogens in Raspberry and Other Rubus spp.

Alison Dolan, Stuart MacFarlane, and Sophia Nikki Jennings

4.1 Introduction: Pathogens of Raspberry

Raspberry plants are susceptible to infection by a wide range of pathogens, however, whether any of these will cause significant disease in a plant at a specific location depends on many factors. Firstly, infection depends on the plant being exposed to the pathogen so that using only pathogen-tested plants to establish raspberry plantations will significantly reduce disease incidence. Some raspberry varieties carry natural resistance (or reduced susceptibility) to particular pathogens or their vectors. For example, resistance to aphid colonisation has been bred into many modern varieties. This reduces both damage due to aphid feeding and also reduces the transmission of certain viruses to the plants by the aphids. It should be noted that populations of aphids that overcome this resistance have emerged, and that the identification and deployment of sources of natural resistance needs to be an ongoing process. The application of chemical treatments to combat insects or fungi is common, though these treatments often have limited efficacy, may be overcome by development of resistance in the target organism, and are subject to environmental and consumer pressure to reduce their usage. Additionally, the introduction of new agronomic practices for the cultivation of raspberry plants can result in both an increase and a decline of infection by different pathogens. Raspberries are now widely grown under cover, in plastic tunnels, which increase the ambient temperature of the crop and extend the growing season. This can increase the multiplication rate and population size of insects in the crop, potentially increasing the prevalence of insect-transmitted viruses. Also, some raspberry crops are now planted in

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A. Dolan (✉) · S. MacFarlane
The James Hutton Institute, Dundee, UK
e-mail: alison.dolan@hutton.ac.uk

S. N. Jennings
James Hutton Limited, Dundee, UK

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artificial substrate rather than into field soil. This helps to prevent their exposure to soil-borne diseases such as root rot or some viruses that are vectored by soil-borne nematodes. There is a movement towards reducing the period over which individual plants are maintained in commercial production. This reduces the time over which plants are exposed to pathogens, preventing the development of disease in the crop and also reducing the potential for the crop to become a reservoir of disease that can be spread to other plants. Lastly, changes to the environment, such as increased temperature or rainfall, and introduction of plants from other geographies or as new varieties, can lead to the emergence of new (or at least previously unrecognised) diseases. An example of this is the recent rise to prominence of raspberry leaf blotch disease caused by a complex of raspberry leaf and bud mite and raspberry leaf blotch virus.

The following section describes some of the raspberry pathogens that have been noted in the past. As should be clear from the preceding section, the prevalence of these pathogens will vary from place to place and the emergence of new, locally-important diseases should be expected. It is also the case that the continued discovery of new raspberry pathogens, the production of new diagnostic tests for the detection of both new and old pathogens, and the in-depth investigation of the effect of these pathogens on raspberry production requires the involvement of researchers who are interested in and technically capable to do this work.

4.2 Raspberry Root Rot

One of the most widespread and economically important raspberry diseases is Root Rot caused by the oomycete *Phytophthora rubi* comb. nov. (Man In't Veld 2017). Although this organism is not a fungus, it does have some features similar to those of fungi (including production of spores and a hyphal mycelium) and can be controlled to a very limited effect by fungicide treatment (such as dimethomorph, developed to control potato diseases). The organism was previously known as *P. fragariae* var. *rubi* and produces underground spores that are distributed by wind and water splash and can persist in the soil for at least 20 years, so that fields that are contaminated with *Phytophthora* spores contain a ready-made inoculum to infect newly introduced plants (Wilcox et al. 1993; Heiberg 1999; Duncan and Cooke 2002). Root Rot disease is present in all temperate regions of the world, can cause complete crop loss in particular locations and is a major limitation to global raspberry production. Water-saturated soils are most prone to the disease. All parts of the plant at or below ground level can be infected, including raspberry roots, crowns and bases of young and old canes. Affected canes die in the first year of growth or their buds fail to emerge at the start of the second growing season. Lateral branches wilt and die at any time from emergence until late in the fruiting season. In the UK, root rot has caused losses of up to 60% in soil grown crops, with a substantial cost to growers and the industry and it has resulted in many growers ceasing raspberry production altogether (Thomson, pers. comm.). Growers have been encouraged to adopt integrated control methods in an attempt to limit the spread of the disease in the soil including; sourcing plants derived from high health, disease-tested, stock; planting on raised beds to improved drainage (McGregor and Franz 2002); using plastic mulch with trickle irrigation to control soil moisture during the growing season (Heiberg 1999). Many European growers have shifted production out of the soil in an attempt to avoid the disease. This involves growing raspberries in pots or containers in substrate, usually coir (cocopeat), with precision irrigation monitoring tools which requires high outlay costs. However, root rot remains to be a problem in this production system due to the use of infected propagation material and poor farm hygiene practises (Allen, pers. comm.). European pesticide regulations have reduced the number of available chemicals and limited the permissible number of applications (Garthwaite et al. 2016). Without varietal resistance to manage the disease the growth of the industry will be restricted by this pathogen.

Screening and selection of cultivars of red and other raspberries and wild *Rubus* species began soon after it became apparent that root rot was a major problem (Hall et al. 2009). Several raspberry breeding programmes are actively trying to incorporate natural resistance (or at least reduced susceptibility) to *P. rubi* into new varieties. In a conventional breeding programme, identification of new resistant or tolerant germplasm involves lengthy screening in infested soil. Field screening has the advantage of mimicking the conditions of commercial production, however, this screening method can add several years to an already lengthy breeding timescale and field infestation can become patchy and variability in disease development can occur. This has led to glasshouse screening in pots or in hydroponics culture (Kennedy and Duncan 1991; Pattison et al. 2004). Sources of strong genetic resistance include cultivars ‘Latham’, ‘Asker’, ‘Newburgh’, ‘Durham’, ‘Chief’, ‘Autumn Bliss’ and ‘Autumn Treasure’ and species *R. coreanus, R. illecebrosus, R. parviflorus, R. parvifolius, R. phoenicolasius, R. pileatus, R. spectabilis* and *R. sumatranus* (Barritt et al. 1979, 1981; Daubeny 1996; Heiberg 1999; Finn et al. 2002; Hall et al. 2009). There is a need for new resistant cultivars with superior agronomic traits more suited to the modern industry.The breeding programme at Washington State University has established field plots with very high root rot pressure and has released the varieties, ‘Cascade Delight’, ‘Cascade Bounty, ‘Cascade Dawn’ and ‘Cascade Harvest’, all with very high field tolerance (Moore 2004, 2006; Moore and Finn 2007; Moore et al. 2015). The Norwegian programme released ‘Hitra’, ‘Stiora’ and ‘Tambar’, each showing field resistance (Heiberg 1995; Roen et al. 2002). Canadian cultivars, ‘Chemainus’ and ‘Cowichan’, also show good field tolerance from the PARC-BC programme (Kempler et al. 2005, 2006). At the James Hutton Institute in Scotland, selecting types that are resistant or tolerant to root rot has been a major objective in the breeding programme. It is now possible to make use of genetic marker assisted selection within the programme as a result of the underpinning work developed at the James Hutton Institute (Jennings et al. 2016). A mapping population segregating for root rot resistance (‘Glen Moy’ x ‘Latham’) was screened under both field and glasshouse conditions over a number of seasons. Two quantitative trait loci (QTLs) associated with resistance were identified, as were overlapping QTLs for increased root vigour. Markers significantly associated with the traits were used to identify bacterial artificial chromosome (BAC) clones, which were subsequently sequenced to examine gene content. A number of genes were identified, including those associated with cell proliferation and elongation in the root zone, control of meristematic activity and organisation, cell signalling, stress response, sugar sensing and control of gene expression as well as a range of transcription factors including those known to be associated with defence. For marker assisted breeding the simple sequence repeat (SSR) marker Rub118b, a 110 bp allele from Latham, was found in root rot resistant germplasm but was not found in any of the susceptible germplasm (Graham et al. 2011). This marker is now used routinely in the breeding programme to identify resistant types early in the programme. Similar work has been carried out at Cornell University, New York, where marker assisted selection to identify resistant genotypes is also in development (Pattison et al. 2007).

4.3 Fungal Diseases

A large number of different fungi have been associated with disease in raspberry and other *Rubus* species. These fungi can infect various parts of the plant, including root, stem, leaves and fruit. The severity of the disease and the impact it has on the plant is dependent on various factors such as; variety, production system, environmental conditions, geographical location and disease pressure. The method of infection can be through a wound or direct invasive contact with the disease. The overall effect on fruit production can range from minimal damage to the crop to removal of plants and loss of revenue.

The principal approach to reduce impact of fungal diseases on plant and fruit production is to plant pathogen-free material and implement good crop hygiene and management practises.

4.4 Fungal Diseases Affecting Leaves and Cane of Raspberry Plants

4.4.1 Verticillium Wilt

Verticillium wilt is a disease of red raspberries in Europe caused by *Verticillium albo-atrum* (Reinke and Berth.) and *Verticillium dahlia* (Kleb.). It is also locally severe on black raspberries and some blackberries and hybridberries (Keep 1989; Ellis et al. 1991; Hancock 2008). Symptoms first appear on new canes in late summer, the lower leaves of canes turn yellow and drop prematurely or they may develop tiger striping through interveinal chlorosis. The entire plant is stunted and bluish lesions of infected tissue extend up the canes from the ground, matched by a brown discoloured sector in the wood beneath. Canes often die over winter. Fruiting laterals on diseased canes usually develop poorly and may die before the fruit ripens (Keep 1989). The fungus enters the roots and hyphae invade and block the xylem vessels. Conidia also move through the plant in the transpiration stream. The fungus returns to the soil in plant debris and can persist for many years in the absence of a known host. There is no effective chemical control but a few raspberry cultivars e.g. purple raspberry cv. 'Glen Coe' have field resistance to the disease (Fiola and Swartz 1994).

4.4.2 Armillaria Root Rot

Armillaria root rot is not common in *Rubus* but can cause serious losses when present. Symptoms occur as cane dieback and wilting. Infected roots often have a whitish to cream coloured mycelium just under the epidermis. Mycelia are fan shaped and have a characteristic mushroom odour (Ellis et al. 1991; Martin et al. 2017). The fungus can survive on dead roots years after an infected plant has been removed and this can be considered to be primary inoculum for further infection. Several species are attributed to the disease: *Armillaria tabescens* (Scop.) Dennis, Orton and Hora, *A. ostoyae* (Romagnesi) Herink, and *A. mellea* (Vahl:Fr.) P. Kumm. Removal and destruction of infected roots or stumps from the soil can slow the progression of the disease. The installation of barriers, such as ditches or buried plastic sheets may be used to prevent disease spread on an area that is adjacent to an infested one.

4.4.3 Spur Blight

Spur blight (*Didymella applanata* (Niessl) Sacc. (the anamorph is an unnamed *Phoma* sp.) can cause serious yield losses in red raspberry by reducing the number and vigour of fruiting laterals developing from infected nodes. Infections on mature leaves of young primocanes are initiated at the leaf margin and advance inward toward the midvein. This results in a brown v-shaped lesion with broad yellow margins. Infection spreads from the leaf through the petiole and into the node. Infected leaves are usually shed prematurely and a dark chestnut-brown spreading lesion develops on the cane below the node and around the axillary buds. During the winter, silver-grey lesions appear and tiny black pseudothecia, and later pycnidia develop on them. The buds are rarely killed because they are not invaded by the fungus and remain viable, but are retarded in growth compared to those at non-infected nodes. Yield losses result from reduction in number and vigour of fruiting laterals developing from infected nodes and, in some areas, from increased winter injury (Williamson and Hargreaves 1981; Ellis et al. 1991; Martin et al. 2017). Spur blight and cane *Botrytis* occupy the same ecological niche (Williamson and Jennings 1986; Gordon et al. 2006). Both fungi infect nodal areas of young canes after invading senescent leaves, neither pathogen can penetrate the mature polyderm and both have similar effects on the axillary buds and the emergence of fruiting laterals. The cultural practices and fungicide programmes for controlling cane botrytis also control spur blight. A major gene is responsible for resistance to both spur blight and cane *Botrytis*. Cane morphology has a considerable effect on resistance. The characteristic of cane pubescence or hairiness is determined by the presence of gene *H* (Jennings 1982; Williamson and Jennings 1986; Jennings and McGregor 1988; Graham et al. 2006), and cultivars with this gene and cane morphology, including 'Glen Carron', 'Glen Moy' and 'Glen Rosa', are resistant to both spur blight and cane botrytis.

4.4.4 Cane Botrytis

Cane *Botrytis* is caused by the fungus *Botryontinia fuckeliana* (de Bary) Whetzel. (anamorph *Botrytis cinerea*) that also causes grey mould on fruit. *B. cinerea* is a ubiquitous fungus which survives in dead and dying tissues of all plants and can therefore be imported from neighbouring plantations and crops. The fungus is unusual in that it can be dispersed by dry air, by splash dispersal and by insect dispersal. Cane *Botrytis* has many features in common with spur blight and the two diseases are often found in the same plantation or on the same canes and are often confused. Like spur blight, cane *Botrytis* can cause serious yield losses through bud failure, although cane *Botrytis* is considered to be the more damaging of the two diseases. Cane *Botrytis* infects mature or senescing leaves of primocanes in a similar way to spur blight, although the subsequent spread can be much more extensive. The fungus spreads through the petioles to the nodes and forms a tan coloured lesion which spreads rapidly around the cane. *Botrytis* lesions often show a characteristic banding pattern or watermark caused by changing growth rates during fluctuating environmental conditions. The lesions become white during the winter and develop black blister like sclerotia which release spores the following spring and are considered the principal initial sources of inoculum for infection of flowers and fruit (Ellis et al. 1991; Martin et al. 2017). Lesions of cane *Botrytis* can be distinguished from spur blight by their lighter tan colour and watermarks when they are present. Lesions of *B. cinerea* are generally longer and often inhibit growth of lateral shoots several nodes from the point of infection. Infected tissue causes a delay in bud development and the buds themselves are smaller at the end of the season than uninfected buds. Control of cane *Botrytis* in commercial plantations in Europe is effected by spray programs used for control of *Botrytis* fruit rots. Cultural practices are encouraged to control the disease: planting disease-free stock, opening the canopy by removing old fruiting canes immediately after harvest, adequate ventilation of protected cropping systems, good weed control and moderate nitrogen application.

4.4.5 Anthracnose

Anthracnose (*Elsinoe veneta)* (Burkholder) Jenkins (anamorph *Sphaceloma necator* (Ellis & Everh.) Jenk. and Shear) is commonly called cane spot or grey bark, occurs in several species of *Rubus*, including red raspberry, black raspberry, blackberries and hybrids. On young canes the disease appears in the spring as small, scattered, circular purple spots but later the lesions develop shrunken grey centres with red or purple margins. If the infestation becomes severe, lesions may coalesce to form large irregular areas which may extend right around the cane, resulting in defoliation, wilting of fruiting laterals, death of fruiting canes and damage to flowers, resulting in unmarketable fruit. In severe cases buds on infected canes fail to develop fruiting laterals the following season and yields are reduced. Fruit are also attacked, causing depressed spots. Future yields may also be reduced as, in severe cases, buds on infected canes fail to develop fruiting laterals the following season. Primary infection occurs in the spring after ascospores germinate and conidia are splash-dispersed from infected overwintered material by overhead irrigation or windblown rain. Only young green tissue is infected. Secondary infection occurs throughout the growing season. In problem areas, overhead irrigation should be avoided to prevent dispersal of the pathogen. Improving air circulation within a plantation by thinning excess young canes and removing weeds is recommended. Excessive nitrogen application should be avoided since it promotes excessive growth of very susceptible succulent plant tissue. Removal of overwintering inoculum is achieved by cutting-out old fruiting canes soon after harvest. The area surrounding the plantation should be kept free of wild *Rubus*. Broad spectrum fungicides can be used to control the disease early in the growing season. Liquid lime sulphur applied at the end of the dormant period may eliminate a portion of the overwintering inoculum if applied at the correct growth stage. Strong host resistance is present in several red raspberry cultivars, including ‘Autumn Bliss’, ‘Willamette’ and ‘Meeker’ (Hall et al. 2009).

4.4.6 Cane Blight

Cane blight (*Leptosphaeria coniothyrium)* (Fuckel) Sacc. (anamorph *Coniothyrium fuckelii* Sacc.) occurs in raspberry in close association with wounding on primocanes. These remain symptomless of the disease until late autumn when the epidermis of infected canes is scraped off to expose the vascular tissue and a brown stripe lesion can be seen spreading from the wound. By spring a lesion may extend across multiple internodes on one side of the cane, causing death of axillary buds or wilt of laterals as it spreads. Lesions can girdle vascular tissues during winter, causing cane death. When this occurs in late spring or summer the entire cane above the infected wound may wilt and die suddenly but, unlike *Phytophthora* root rot, healthy primocanes emerge from the base of infected plants (Ellis et al. 1991; Martin et al. 2017).

The fungus requires a wound or tissue damage in order to enter the vascular tissue of the plant. Old infected floricanes are the primary source of inoculum. Conidia are exuded from pycnidia and dispersed by splashing rain from early spring to late autumn. Disease outbreaks are considerably promoted by rainfall during the season. Since the fungus requires a wounding for this disease to develop, reducing damage to the canes is recommended. Wounds are made through the use of harvesting machines with spring loaded catching plates which cause considerable damage on primocanes. Protected cropping systems may promote wounding; when covers are removed after cropping, soft growing tissue is exposed to wind rock and abrasion on the support trellises, creating wounds and infection sites high on the cane. This disease is also associated with attacks by the raspberry cane midge. Control is also affected by keeping the bush open and chemical cane removal, to remove the basal leaves and laterals, as well as limit the new cane growth. When removing the fruiting cane, it is also important to prune as close to the ground as possible to prevent new canes rubbing against the vestigial old wood.

4.4.7 Midge Blight

Feeding damage caused by raspberry cane midge (*Resseliella theobaldi*) larvae predisposes raspberry canes to the disease known as midge blight which is responsible for major losses in raspberry in many parts of Europe (Gordon et al. 2006). Females lay eggs in splits and wounds in the bark at the base of the primocanes. The larvae hatch and feed in the outer cortical tissue protected by the covering of bark and, later, drop to the soil and pupate. Second and subsequent generations follow during the summer and early autumn. Affected canes continue to grow normally for the duration of the season without showing any visible symptoms. If patch lesions are extensive and girdling is common, a large proportion of the canes fail to produce flowering laterals the following spring, or they wilt and die before harvest, but unlike *Phytophthora* root rot symptoms, new primocane growth is unaffected. Midge blight is a disease complex involving cane damage created by the feeding of the larvae of the cane midge followed by infection by a range of fungi, including *Leptosphaeria coniothyrium, Didymella applanata, Phoma* and *Fusarium spp*. Effective control relies on reducing numbers of the cane midge.

A predictive model is used in Europe which uses the relationships between the historical dates of emergence of over-wintered cane midge and local meteorological data, particularly accumulated soil temperature, to give accurate forecasts of cane midge emergence in localized areas (Gordon et al. 1989). This allows growers to time chemical application based on local meteorological information. Cane midge can also be controlled by cultural methods to eliminate egg laying sites such as the use of herbicide to remove the first flush of primocanes of vigorous cultivars. This treatment stimulates the production of replacement canes that remain free of splits during the first generation oviposition period. If no alternative oviposition sites are present, the population declines rapidly. Research has shown that the control of midge blight may be achieved by the use of *Bacillus thuringiensis* (Bt) var. *israeliensis*, either on its own or in combination with other biological preparations. Cultivars with few natural cane splits can escape attack by the midge, such as 'Glen Prosen' and the hybrids 'Tayberry' and 'Loganberry'.

4.4.8 Raspberry Leaf Spot

Raspberry leaf spot (*Sphaerulina rubi)* Dem. and Wilc. is a serious disease of raspberries in the East and South of the USA, damaging canes and leaves and resulting in plant death in warmer humid locations, particularly at the southern limits of raspberry growing (Jennings 1988; Keep 1989). It is also found in warm, low-lying regions of South-Eastern Europe. Symptoms appear as inconspicuous lesions on canes and small, tan to brown, lesions on the lowest leaves which spread up the plant (Bost and Hale 2006). Infection of young expanding leaves causes greenish-black spots. As leaves mature lesions enlarge becoming grey or silver, these turn whitish and drop out, producing a shot-hole effect (Ellis et al. 1991; Martin et al. 2017). Heavily infected leaves turn yellow, become necrotic and fall from the plant and by late summer complete defoliation may result, making the plants more liable to winter injury. The fungus overwinters primarily on dead leaves. Reduction of the amount of overwintering inoculum is achieved by pruning out fruiting canes and dead or damaged canes soon after harvest. Improvement of air circulation within the plantation by thinning out primocanes and weeding to promote faster drying of foliage and canes after rain is recommended, These measures will lessen the time available for infection to occur. Most red raspberry cultivars are susceptible to this disease and it is the key limiting factor for their survival in the South East USA where leaves are shed in late summer and growth is markedly reduced. Breeding using the Asiatic species *R. parvifolius* in crosses with red raspberry in North Carolina produced cultivars with high resistance to leafspot, including 'Mandarin' and 'Southland' (Ballington 2016).

4.4.9 Cane Cankers

Cane cankers caused by *Sydowiella depressula* and *Gnomonia depressula* have been found in raspberry in temperate waterlogged soils in winter or in low lying areas susceptible to frost injury. Symptoms show silver coloration of the lower parts of overwintered canes, with numerous black spores giving the cane a warty like appearance. Water-soaked, black-brown vascular lesions circle the canes and can cause wilting before harvest. No control measures for *S. depressula* or *G. depressula* have been described.

4.4.10 Nectria Canker

Nectria canker (*Nectria mammoidea*) is a disease likely to be a secondary pathogen which affects raspberry. It is seen after periods of heavy rain and high winds where the cane bases and crowns are damaged. Bud break may be effected the following spring and result in reduction of cane productivity. Although no control measures have been described, good site choice and the use of windbreaks is advantageous (Ellis et al. 1991; Martin et al. 2017).

4.4.11 Powdery Mildew

Powdery mildew (*Sphaerotheca macularis*) is widespread on red, purple and black raspberries around the Northern Hemisphere but it is largely absent from the Southern Hemisphere. This disease thrives under warm, dry conditions; therefore crops produced under protected cropping systems can be particularly susceptible. Infected leaves develop light green blotches on the upper surface; the lower surface becomes covered by white mycelial growth (Ellis et al. 1991; Martin et al. 2017). Leaves remain small and tend to curl upwards at the margins. A white powdery growth appears on infected fruit. The pathogen survives the winter as mycelium in axillary buds. Cycles of infection of newly expanded leaves occur throughout the growing season as conidia are spread within the crop. Fungicides are available for control. Primocanes with symptoms should be removed, and air circulation within the plantation improved by weeding and thinning out canes.

4.4.12 Rosette

Rosette (*Cercosporella rubi*) also known as double blossom or witches broom, is a severe disease of erect blackberries in the USA. It rarely occurs on raspberry but affects hybridberries, particularly Boysenberry. Symptoms are striking and can result in a complete change in the physical appearance of the plant. Masses of short leafy shoots develop and form rosettes or witches brooms at the infected nodes. Infected flower buds are enlarged and malformed and have large leaf-like and often reddened sepals. As the petals unfold they are usually pink, wrinkled and twisted, giving the appearance of double flowers. No fruit develops. In some cultivars the fungus invades the stem and may enter the crown of a rooted stem. Axillary buds of primocanes are infected in early summer. The mycelial stage occurs within vegetative and floral buds of the host and conidia are formed in open blossoms. The first symptoms normally occur in the spring after infection occurred. Plants should be sourced from disease-free stocks and planted in an area isolated from wild blackberries and dewberries. Infected rosettes and flower clusters should be removed before they open to prevent dispersal of the fungus. Fruiting canes should be removed and destroyed immediately after harvest. Primocanes can also be cut to ground level and allowed to regrow, which is effective but may reduce yields.

4.4.13 Ascospora Dieback

Ascospora dieback (*Clethridium corticola*) has resulted in occasional outbreaks of dieback in North America and Europe on both raspberries and blackberries. The disease appears to infect only after low temperature injury. Ashen white lesions 7–20 cm long appear in late summer or early autumn on primocanes of red raspberries and on black raspberries they are bluish with a silvery bloom. In early spring or in autumn the lesions become dotted with reddish brown acervuli and after conidia have been discharged the bark surrounding the lesion becomes sooty black. Lesions develop mostly at nodes, suggesting that the infection develops through petioles or leaf scars (Ellis et al. 1991; Martin et al. 2017). No control measures for this disease have been described.

4.4.14 Downy Mildew

Downy mildew (*Peronospora sparsa*), although rare on raspberries, is a major problem for commercial plantations and nursery stock of blackberries, hybridberries, particularly boysenberries, and on arctic bramble (*R. articus*). Initial symptoms appear as a yellow discolouration to the upper leaf surface, changing to red then purple. Angular lesions may appear, bounded by leaf veins and midribs. In some cultivars, symptoms appear as purple blotches or lesions. On the lower leaf surface, pink or tan areas appear directly below the lesions which produce spores that are initially white, becoming grey with age. Infected fruits, termed ‘dryberry’, split at the receptacle due to dessication of the druplets. The pathogen is an obligate parasite and to reproduce, the fungus develops filamentous intercellular haustoria in the mesophyll tissues of leaves and in the outer layers of the cortex parenchyma of leafstalks and canes (Ellis et al. 1991; Martin et al. 2017). Clean planting stock should be used and areas with a history of the disease should be avoided. Alternate hosts, such as rose and wild blackberry in close proximity should be removed. If high temperatures and dry conditions are encountered, downy mildew is severely restricted.

4.4.15 Yellow Rust

Yellow rust (*Phragmidium rubi-idaei*) is a relatively minor disease of raspberry, except in warm humid climates where premature defoliation can occur if the infection is early and severe; the consequent reduction in winter hardiness can result in lost yield. Fruit can also be infected. The first symptoms are bright orange raised spots on the upper surface of young primocane leaves and laterals in early spring followed by orange-yellow uredinia on the lower leaf surface in summer which subsequently turn black in the autumn. These stick to canes and support trellises before releasing spores the following spring. There are five-stages of the life cycle of yellow rust. Bright orange aecia form on the upper surface of primocane leaves in early spring followed a month later by orange-yellow uredinia on the lower leaf surface. The latter pustules release numerous urediniospores that represent the main cyclic phase of infection in raspberries. Towards autumn, the yellow uredinia turn black as teliospores are produced, which is the winter survival stage of the rust. Improvement of air circulation within the planting by thinning out canes and effective weed control is recommended. Removal of the first flush of primocanes by hand or with herbicide is an effective control measure since it destroys the fungus at the initial stages of its life cycle. Cultivar resistance is present in ‘Malling Leo’, ‘Malling Jewel’ and ‘Tadmor’. In some germplasm, yellow rust resistance was considered to be correlated with resistance to cane spot, but Graham et al. (2006) found no association between the two pathogens.

4.4.16 Late Leaf Rust

Late leaf rust (*Pucciniastrum americanum*) is a disease of raspberry which causes premature defoliation, increasing susceptibility to winter injury and infects fruit, making it unfit for fresh market sales. On mature leaves small yellow spots develop and turn brown before leaf drop. Small uredinia are formed on the underside of infected leaves and these shed powdery yellow spores. Flowers, calyces, petioles, fruit and occasionally canes are attacked (Ellis et al. 1991; Martin et al. 2017). White spruce (*Picea glauca*) is a secondary host and severe outbreaks on raspberries often occur in association with a high level of infection on spruce. Growing resistant cultivars is the key to production in areas with high pressure for this disease. ‘Pocahontas’, ‘Ruby’, ‘Tola’ and ‘Trailblazer’ are highly resistant to late leaf rust in the field (Hall et al. 2009). Cultural practices to improve air circulation such as thinning out of canes and weeding within the plantation will help control the disease. Avoid establishment of new plantations near stands of white spruce.

4.4.17 Cane and Leaf Rust

Cane and leaf rust (*Kuehneola uredinis*) is a disease of blackberries that occasionally affects red and black raspberries during wet spring conditions. This disease is thought to be mainly confined to floricanes. The large yellow uredinia are found under the rind and on the underside of the leaves which, when severe, can cause early leaf drop. Infection of fruit also may occur but it is uncommon (Ellis et al. 1991; Martin et al. 2017). Control of this disease is through removal of fruiting canes after harvesting and applications of lime sulphur.

4.5 Fungal Diseases Affecting Raspberry Fruit

4.5.1 Botrytis Grey Mould

Botrytis grey mould (*Botrytis cinerea*) causes high losses in raspberry production worldwide and is the most important factor limiting the sale of fresh fruit to distant markets by rapidly reducing the shelf-life of harvested fruit. The fungus can infect fruit in the field before harvest (preharvest rot) particularly after persistent rain during flowering and in warmer climates when there are heavy dews, or rainfall at the time of fruit ripening but in many locations the main concern is the loss of picked fruits after harvest (postharvest rot). The fungus attacks open flowers and forms a symptomless infection. Conidia germinate in the stigmatic fluid in newly opened flowers, desend down the styles and enter the carpel. Mycelium grows in the developing druplet. The fungus persists in senescing styles and stamens, providing a further source of infection to infect the ripe fruit. The fungus also commonly affects drupelets in the fruit collar. Symptoms of the rot which develop are a greyish-brown dusty mass of hyphae and conidia. Raspberries grown in cool moist conditions are sprayed with broad spectrum fungicides routinely for control of grey mould from the flowering period onwards to reduce losses to rot during and after harvest, risking detectable fungicide residue in the fruit. Fruit rot from *Botrytis* infection has been significantly reduced by the use of protected cropping systems but growers are generally reluctant to reduce chemical control since rotting fruit would result in rejection of fruit by multiple retailers (Garthwaite et al. 2016; Bristow 1980). O'Neill et al. (2012) found that rapid post-harvest cooling and storage of fruit can effectively delay the onset of fungal rotting. Growers have been encouraged to use integrated control methods such as good weed control and thinning canes to maintain air flow in the plantation and reduce humidity. Reduction of nitrogen inputs and removal of fruiting canes immediately after harvest to reduce inoculum levels. Biological control agents, such as *Clonostachys rosea* and *Trichoderma harzianum* have potential for control but cool spring temperatures in temperate climates may be a limiting factor (Yu and Sutton 1998). Laminarin, derived from brown algae, has been successfully used as an elicitor to reduce grey mould in commercial plantations of raspberry in Poland (Krawiec et al. 2016).

4.5.2 Postharvest Soft Rot

Postharvest soft rot (*Rhizopus* spp. and *Mucor* spp.), also known as leak disease, infects only mature or damaged fruits of all *Rubus* species. Infected fruit may initially show water-soaking followed by the development of white mycelial growth on the fruit surface which becomes covered with black pin-head sporangia. The disease spreads rapidly, leading to maceration of the fruit which causes juice to leak into harvest containers. Improving air circulation through the plantation by thinning canes and weeding will help the surface drying of fruit. Ripe fruit should be picked regularly and cooled rapidly to reduce infection rate.

4.5.3 Minor Fruit Rots

Cladosporium rot is abundant in plantations and caused by *Cladosporium herbarum* (Pers.:Fr.) and *Cladosporium cladosporoides* (Fresen). The olive-green mould is usually found in the central cavity of overripe or damaged fruit (Ellis et al. 1991; Martin et al. 2017). In certain production systems in the UK, where fluctuating temperatures and inappropriate irrigation has caused guttation, resulting in increased moisture around the developing fruit, the disease has been problematic (Anon, pers. comm.).

Alternaria rot (*Alternaria* spp.) affects red and black raspberries. The disease produces a dark grey mycelium on fruit stored after picking (Ellis et al. 1991; Martin et al. 2017).

Penicillium rot (*Penicillium* spp.), also known as blue mould, affects all *Rubus*. Fungal growth can be seen on ripe and damaged fruit. The mould initially appears white and powdery, turning blue-green and causing the fruit to soften and leak juice (Ellis et al. 1991; Martin et al. 2017).

Colletotrichum rot (*Colletotrichum gloeosporioides*) can cause fruit rots in many crops, including *Rubus*. Sunken, water-soaked lesions with slimy fungal growth are characteristic of this rot on stored raspberry fruit (Ellis et al. 1991; Martin et al. 2017).

Control of these minor fruit rots is aided by good sanitation within the plantation and at harvest time by regular and careful harvesting followed by rapid chilling and storage below 4 °C.

4.6 Bacterial Diseases of Raspberry

4.6.1 Crown Gall

Crown gall (*Agrobacterium tumefaciens*) affects all *Rubus* species worldwide and can be a limiting factor in the nursery production of raspberry and blackberry. The plant produces galls near the crown in response to the disease, but is sometimes found on roots and canes. Severe infection can cause plants to lose vigour and become unproductive (Martin et al. 2017). Infection is usually seen after environmental stress such as waterlogging and frost damage. Effective control can be achieved using disease-free propagation material planted in soil free of crown gall.

4.6.2 Fire Blight

Fire blight (*Erwinia amylovora*) is not common on raspberry and blackberry in Europe but sporadic infection has been seen to cause damage and crop loss in North America. Flowers, leaves, fruiting laterals and green fruit can all show symptoms including water-soaked lesions, producing bacterial ooze, resulting in tissue becoming necrotic. Infected tissue turns brown and brittle but can remain attached to the plant with the tip of the primocane forming a shepherd's crook (Martin et al. 2017). No control measures are described but it is highly recommended that pathogen-free planting stock is used.

4.6.3 Pseudomonas Blight

Pseudomonas blight (*Pseudomonas syringae*) is a minor disease of raspberry. Symptoms appear in spring as brown, water-soaked spots on young growth. As the growing season progresses, the spots enlarge to produce darkened streaks which can infect and kill the cane (Ellis et al. 1991; Martin et al. 2017). This disease can sometimes be associated with buds already suppressed by spur blight. The most effective control can be achieved with cultivar resistance although some control is possible by application of copper sprays and avoiding the use of excessive nitrogen fertiliser.

4.7 Diseases Caused by Viruses

Although viruses have been known for many years to be the cause of several economically important raspberry diseases, previously most virus identification and characterisation relied on using biological tests (e.g. grafting between plants and mechanical transfer to herbaceous “indicator” plants) to try to differentiate between individual or groups of viruses that were suspected to be present in raspberry plants collected from the field. The last 20 years has seen the introduction of molecular (DNA- and RNA-based) techniques for virus cloning and sequencing, which has made it possible to precisely characterise individual viruses and to be able to detect them in plants even when they are present at very low levels (as raspberry viruses often are). Most recently, mass sequencing techniques (often referred to as Next Generation Sequencing or NGS) have been used to reveal that raspberry plants often contain mixtures of different viruses, and that many previously unknown viruses may be present in these plants. It is currently thought that, in many cases, visible disease symptoms are caused only when plants become infected with combinations of viruses, so that it may not necessarily be possible to say that a particular virus causes a specific disease. Actually it is technically very difficult to do controlled experiments where known combinations of viruses are introduced into individual raspberry plants, so for most viruses their contribution to disease symptoms, and yield loss particularly, is not known. Furthermore, disease symptom production is highly dependent on the cultivar of raspberry being used, so that in many cases infection of raspberry plants with particular viruses can cause no observable symptoms.

A recent review of viruses detected in *Rubus* species (Martin et al. 2013) listed 30 that had been reported to occur in red raspberry, black raspberry and blackberry, although this total has since been exceeded with new discoveries. Much of the recent blackberry and black raspberry virus research has been done in the United States, with the red raspberry research being done in the UK and Europe. There are currently some viruses that are detected only in the US (e.g. Blackberry virus Y) or Europe (e.g. Raspberry leaf blotch virus). This geographical separation may be real, caused by propagation of different plant stocks in the US and Europe, or may be artificial, caused by biases in the testing regimes employed in these continents. Similarly, whether particular red raspberry viruses are capable of infecting black raspberry or blackberry plants, and vice versa, is often not known. There may be limitations of, for example, blackberry-adapted insect vectors being attracted to or able to feed on red raspberry plants, or the specific vector for a particular virus may itself be geographically limited. An interesting example is Strawberry latent ringspot virus (SLRV) that is transmitted to red raspberry in Europe by the nematode *Xiphinema diversicaudatum*. This virus is not reported in *Rubus* in the US and the nematode vector is also not known to occur in the US. However, SLRV is present in strawberry and mint crops in the US, raising the possibility that there is a different vector transmitting the virus to these plants in the US.

Plant viruses are conveniently differentiated by their mode of spread between plants, which may involve different vector organisms or perhaps even no biological vector. Developing measures to protect against a particular vector type (e.g. aphids) can then prevent the spread of several viruses that share this transmission mechanism.

4.7.1 Aphid-Transmitted Viruses

Historically researchers identified four aphid-transmitted viruses as being components of Raspberry Mosaic Disease (RMD), causing leaf mottling and mosaic and being associated with severe decline in black raspberry but being less severe in blackberry and red raspberry. Recent research has shown that two of these viruses (Raspberry leaf mottle virus; RLMV and Raspberry leaf spot virus; RLSV), previously differentiated by causing slightly different symptoms in particular raspberry cultivars, are both isolates of the same virus. Similarly, a virus from black raspberry in the US was named as Raspberry mottle virus (RMoV) but is now accepted as another isolate of the same virus. All three viruses have now been accepted as different isolates of RLMV. The aphid vector of these viruses is the large raspberry aphid (*Amphorophora agathonica* in the US and *A. idaei* in Europe).

The two other RMD-associated viruses transmitted by this aphid are Black raspberry necrosis virus (BRNV) and Rubus yellow net virus (RYNV). In the Unites States a new virus transmitted by *A. agathonica*, Raspberry latent virus (RpLV), has been discovered recently and found to be widespread in raspberry plants also infected with RLMV. Control of the large raspberry aphid is by planting of varieties carrying aphid resistance genes, however, populations of aphids have emerged that can overcome A_1 and A_{10} resistance so that aphid-transmitted viruses are now commonly detected in raspberry crops. Some pesticides are licensed for use on raspberry, and biological control agents may have some utility.

4.7.2 Nematode-Transmitted Viruses

Tobacco ringspot virus (TRSV), Tomato ringspot virus (ToRSV), SLRV, Raspberry ringspot virus (RRV), Tomato black ring virus (TBRV), Arabis mosaic virus (ArMV), Cherry leaf roll virus (CLRV) and Cherry rasp leaf virus (CRLV) are all transmitted by longidorid nematodes of the genera *Xiphinema*, *Longidorus* or *Paralongidorus.* These viruses have wide host ranges that often include many wild plants, fruit trees, vegetable crops and *Rubus* species. As well as being transmitted by root-feeding nematodes they can also be spread by infected seed and infected pollen although the efficiency of these last two modes may be low and dependent on host plant species. Experimentally these viruses are very efficiently transmitted by mechanical inoculation to a wide variety of plant species and this property means that they are relatively easily detected during routine quarantine testing procedures. According to Martin et al. (2013), in the United States TRSV is one of the most important viruses infecting blackberries. Although it produces few if any symptoms when present by itself in these plants, co-infection of TRSV with one or more other viruses has the potential to cause very severe symptoms. In the UK and Europe, several of the nematode-transmitted viruses were previously found to cause locally important diseases in raspberry plantations, being apparent as isolated patches of disease that are geographically limited by the distribution of the nematode vector in the soil. Control of these viruses has been achieved primarily through fumigation of soil with nematicidal chemicals and provision of virus-tested planting stock. The trend towards planting raspberries in containers, free of contact with the field soil, will also significantly reduce the opportunity for nematodes to access the plant roots and transmit these viruses.

4.7.3 Pollen-Transmitted Viruses

The most important pollen-transmitted virus is Raspberry bushy dwarf virus (RBDV) which affects raspberry, blackberry and Loganberry worldwide. This virus has been identified as a key component of bushy dwarf disease, where the plants are severely stunted and can have bright leaf yellowing, and also with crumbly fruit disease, where development of the fruit is affected so that each consists of only a few, large irregular drupelets instead of the usual many, small regular drupelets. Such malformed fruit cannot be harvested leading to significant (or even complete) loss of the crop. In both cases the appearance of severe disease symptoms requires the presence of at least one other, co-infecting virus. Work in the United States has shown that RLMV and RpLV are frequently involved with RBDV in crumbly fruit disease, and that the presence of RLMV leads to a large increase in the level of RBDV in these affected plants. In addition, the combination of RBDV, RLMV and RpLV leads to a very significant reduction in cane height very soon after initial planting. There have been several raspberry varieties identified as having resistance to RBDV, including Glen Clova, Malling Admiral, Malling Delight and Willamette. Resistance-breaking (RB) strains of the virus have been found in Europe, however, the basis for the resistance breaking activity is not known or whether different RB strains use different mechanisms to overcome the resistance.

4.7.4 Mite-Transmitted Virus

Yellow leaf blotching and fruit malformation of raspberry has been recognised since the 1920s and linked with infestation by leaf and bud mite (*Phyllocoptes gracilis*) since the 1940s. Since the early 2000s this disease has become widespread in the UK and Europe, leading to the discovery of a new virus, Raspberry leaf blotch virus (RLBV), as a component of the disease (McGavin et al. 2012). The precise roles of both the mite and the virus in causing the disease symptoms are not yet understood, although it has been found that using acaricides to reduce mite numbers does not prevent the disease occuring. Recent research suggests that some modern raspberry cultivars are particularly susceptible to the disease, which may explain the sudden increase in its incidence. However, preliminary work has found that resistance to leaf blotch disease does exist in some older raspberry varieties and may be usefully incorporated into new varieties during future raspberry breeding efforts.

4.8 Conclusion

Pests and pathogens are an unavoidable hazard for commercial (and amateur) producers of raspberries, blackberries and other *Rubus* fruit crops. Scientific research continues to provide faster and better ways to detect and diagnose these disease agents, although, 100% effective, cost-neutral, environmentally sensitive treatments and strategies are in short supply to the agricultural industry. Modern molecular genetic approaches are making it more feasible to analyse and then exploit naturally-occuring pest and pathogen resistances. These studies will without doubt provide many of the best, most durable solutions for growers to use in the future.

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Chapter 5
An Agroecological Approach for Weed, Pest and Disease Management in *Rubus* Plantations

Carolyn Mitchell, Cathy Hawes, Pete Iannetta, A. Nicholas E. Birch, Graham Begg, and Alison J. Karley

5.1 Introduction

Weeds, pests and diseases in *Rubus* plantations can have significant economic impact through their negative effects on yield and the costs of implementing control measures. Yield losses can be particularly high for certain pests and diseases, in some cases leading to total plantation losses. While the costs of weed control are rarely quantified, weeds can be a major detriment to profit in *Rubus* and other perennial fruit production systems. Although chemical control methods can be effective, growers are under increasing pressure to produce fruit sustainably with reduced chemical inputs to mitigate health risks and environmental damage (e.g. Łozowicka et al. 2012). In Europe, this has led to the requirement for each EU Member State to implement a National Action Plan with Integrated Pest Management (IPM; where the term 'pest' covers all injurious biotic stressors attacking crop plants) as a central crop protection strategy. Agroecological approaches offer an opportunity to develop sustainable weed, pest and disease management interventions by developing an understanding of the system processes and functions contributing to pest and disease regulation (Birch et al. 2011; Kremen et al. 2012). A framework for adopting an agroecological approach to promote integrated pest and disease management is provided by Birch et al. (2011) and includes (i) use of pest-resistant cultivars, (ii) optimising synthetic pesticide and biopesticide applications, (iii) use of bioactive substances (e.g. pheromones, semiochemicals), (iv) enhancing biocontrol using natural enemies, and (v) ecological engineering of habitats and landscapes to optimise pest and disease suppression. This chapter reports on recent progress and available options for these management measures in *Rubus* plantations.

C. Mitchell (✉) · C. Hawes · P. Iannetta · A. N. E. Birch · G. Begg · A. J. Karley
Ecological Sciences, The James Hutton Institute, Dundee, UK
e-mail: carolyn.mitchell@hutton.ac.uk

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5.2 Targets for Pest and Disease Control

A number of arthropod pests of raspberry and other *Rubus* species are considered economically important. The vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae), is a highly polyphagous pest whose host range includes *Rubus* species (Smith 1932). The larval stage, which feeds on plant roots, is considered the most economically damaging part of the lifecycle, as roots are exposed to feeding larvae for an extended period between late summer and the following spring. The adult insects feed on foliage during the summer and generally cause cosmetic damage only. Another beetle pest of raspberry and other *Rubus* species in Europe is the raspberry beetle, *Byturus tomentosus* (Coleoptera: Byturidae) (Gordon et al. 1997). Adult beetles over-winter in the soil, emerging in the spring to feed on the primocane tips and developing flower buds. Eggs are laid in the flowers and the hatching larvae feed on the developing fruit, rendering it unsaleable.

The European large raspberry aphid, *Amphorophora ideai* (Homoptera: Aphididae), is the most economically important aphid species attacking raspberry in Europe. It is a vector for at least four viral plant pathogens that cause significant yield losses (Jones 1976). This aphid species has an obligate holocyclic lifecycle with aphids present and feeding on plants between March and November (McMenemy et al. 2009). Although similar in appearance, the raspberry aphid in North America is differentiated taxonomically (*A. agathonica*) and is also a significant virus vector on red raspberry (Dossett and Kempler 2012). The small raspberry aphid, *Aphis idaei* (Homoptera: Aphididae), occurs on raspberry and other *Rubus* species, particularly in warmer regions. Feeding by *Aphis idaei* can cause leaves to curl, but more importantly this aphid species is a vector for the raspberry vein chlorosis virus (Alford 2007). The potato aphid, *Macrosiphum euphorbiae* (Homoptera: Aphididae), is a highly polyphagous pest with summer hosts that include raspberry and other *Rubus* species. High abundances of this aphid species can occur regularly in protected raspberry causing feeding damage that reduces leaf photosynthetic efficiency and leads to a build-up of sooty moulds on foliage and fruit contaminated with aphid honeydew. Similarly, damage caused by the large blackberry aphid, *Amphorophora rubi* (Homoptera: Aphididae), is generally associated with foliage feeding rather than transmission of persistent viruses (Gordon et al. 1997).

Raspberry leaf and bud mite, *Phyllocoptes gracilis* (Acari: Eriophyidae), feeds on the leaves of raspberries and other *Rubus* species during late spring and summer and causes yellow blotching, twisting and distortion of the leaves (Gordon and Taylor 1976). These symptoms are associated with the presence of Raspberry Leaf Blotch Virus, which is thought to be transmitted by the mite (McGavin et al. 2012). Blackberry mite, *Acalitus essigi* (Acari: Eriophyidae), occurs in Europe on wild and cultivated blackberry. Mites colonise the developing berries and inject toxic saliva into the fruit, which causes the fruit to remain red and hard and prevents further ripening (Alford 2007). Two- spotted spider mite, *Tetranychus urticae* (Acari: Tetranychidae), is a highly polyphagous pest whose host plants include raspberry and other *Rubus* species. Direct feeding damage causes reduced photosynthesis (Bounfour et al. 2002), which in turn reduces yield.

Spotted Wing Drosophila, *Drosophila suzukii* (Diptera: Drosophilidae), is a relatively new pest of *Rubus* species in Europe, where it has now become a major pest of soft and stone fruit. It causes significant economic damage, piercing the skin to lay eggs in the ripening fruit; the hatching larvae consume the fruit, causing it to collapse, and facilitate infection by microbial pathogens, ultimately reducing berry quality and yield (Lee et al. 2011). Raspberry cane midge, *Resseliella theobaldi* (Diptera: Cecidomyiidae), is a widespread pest in Europe. Midges lay eggs in splits that naturally appear in some canes as they grow; the emerging larvae feed on the cane, reducing yields by up to 50% (Hall et al. 2009). Cane midge damage is associated with Midge Blight and/or Cane Blight infection, *Leptosphaeria coniothyrium* (Woodhead et al. 2013). The Blackberry stem gall midge, *Lasioptera rubi* (Diptera: Cecidomyiidae), is a pest of blackberry and raspberry, laying eggs on the primocanes and the base of the flowering buds. The larvae bore into the cane and form galls (Milenkovic and and Tanasković 2008), which stunt plant growth and reduce both leaf and fruit production (Alford 2007).

While *Rubus* can succumb to a wide array of diseases, a number of these are considered particularly problematic in plantations and lead to impaired plant growth and reduced berry yield and quality. Raspberry Root Rot is a destructive disease caused by the oomycete *Phytophthora rubi* which infects the roots and basal parts of the canes (Harrison et al. 1998). Cane Blight, *Botrytis cinerea* (known as the teleomorph *Botryotinia fuckeliana* in the sexual stage of the life cycle), is an airborne pathogen of raspberry and other *Rubus* species and produces a grey mould on the fruit (Williamson et al. 2007). Verticillium wilt results from infection by a soil borne fungus (*Verticillium* spp.) which reduces yield through plant wilting, stunted growth and eventual death of the infected cane. Plants infected with the fungal pathogen causing Spur Blight (*Didymella applanata*) develop purple blotching on the canes and show reduced yield.

Weeds are potentially the largest cause of yield loss in many agricultural crops, estimated at more than 30% globally for annual arable crops, although effective weed management can reduce actual losses to less than 10% (Oerke and Dehne 2004; Oerke 2006). Weeds are particularly problematic in open field conditions (compared to container-grown plants) and for young establishing *Rubus* plants, due to competition for space, nutrients, light and water. Weed control also causes problems in mature plantations due to weed interference with harvesting operations (Atwood et al. 2012).

5.3 Plant Defensive Traits for Enhanced Pest and Disease Resistance

Genotypic variation in plant susceptibility and resistance to pests and diseases has long formed the basis of crop breeding and improvement. Breeding targets in *Rubus* crops can include resistance traits to prevent pest infestation or disease infection, and tolerance traits to limit the damage caused by infestation or infection (Mitchell et al. 2016).

An agroecological approach to pest and disease management using resistant or tolerant cultivars is more likely to be successful if the traits and mechanisms underlying resistance (or tolerance) are known so that their efficacy can be assessed in relation to other trophic groups in the agroecosystem (Mitchell et al. 2016).

In red raspberry, *Rubus idaeus*, significant genetic variation exists in susceptibility to a range of diseases for which candidate traits have been identified (Graham et al. 2014). Reduced susceptibility to *Phytophthora* root rot has been associated with enhanced root vigour, and QTL markers have been identified that co-locate with genes controlling a wide range of cell functions, including cell proliferation and meristematic activity (Graham et al. 2011). Although the precise mechanism is unclear, more vigorous root growth might allow raspberry root growth rate to exceed the rate of root rot infection and damage, thereby allowing plants to tolerate infection by 'growing out' of the damage. Such a mechanism might explain the observed inverse relation between root mass and root rot symptoms in a screen of 13 genotypes of *R. idaeus* (Fig. 5.1: Mitchell and Karley, unpublished data).

Genetic variation in *R. idaeus* susceptibility to *Botrytis* and spur blight appears to be linked to the presence of cane hairs (Graham et al. 2006), which are thought to reduce fungal spore contact with the cane surface both physically and by accelerating water run-off from canes (Jennings 1962; Jennings 1982). Genetic variation in cane splitting (Woodhead et al. 2013) could be exploited to breed genotypes with reduced propensity to split as a means to control cane blight infection associated with *Resseliella theobaldi* (Graham et al. 2014). Further work to identify the physicochemical basis of this trait indicated that cane height is a contributing factor (Graham et al. 2009; Woodhead et al. 2013), with cane splitting caused by rapid cane growth and radial expansion, which suggests that reduced cane height would be desirable for controlling cane blight disease.

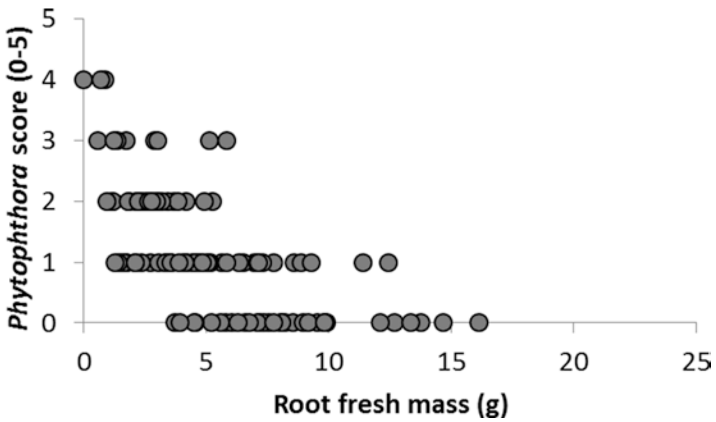

Fig. 5.1 Data for 13 genotypes of *Rubus idaeus* showing the relation between root fresh mass and severity of root rot symptoms on a scale of 0 (no symptoms) to 5 (severe root damage). Young plants were infected with *Phytophthora rubi* and grown in a polytunnel for 5 weeks prior to harvest ($n = 7$ plants per genotype)

Amongst arthropod pests of *Rubus*, a successful example of using plant traits for pest control has been the introduction of resistance genes that reduce susceptibility to the aphid genus *Amphorophora*, and thereby limit the damage caused by aphid-transmitted viruses (e.g. Birch et al. 2002; Sargent et al. 2007; Dossett and Kempler 2012). Emergence of resistance-breaking aphid biotypes has been a key factor driving IPM development and identification of new resistance genes in raspberry. Although the gene(s) contributing to aphid resistance have been identified, the traits and mechanisms underpinning resistance are not clear and might involve a combination of chemical deterrence at the leaf surface (e.g. Shepherd et al. 1999) and reduced phloem nutritional quality (Lightle et al. 2012). A recent review suggested that plant surface waxes and allelochemicals can be a useful trait for repelling or deterring several aphid species that colonise *Rubus* crops (Smith and Chuang 2013).

Varietal differences in fruit skin strength could provide much-needed resistance to egg-laying by the highly damaging pest *Drosophila suzukii* (Burrack et al. 2013). Other traits that impair pest oviposition include leaf trichomes, which form a dense woolly pubescence on the surface of raspberry leaves (Fig. 5.2). High leaf trichome densities can deter oviposition by *T. urticae* (Karley et al. 2016), and could be an important trait for limiting damage by other spider mite species. For example, abundance of the leaf and bud mite *P. gracilis* was inversely associated with trichome density (Mitchell and Fitzgerald 2010), although whether this leads to reduced incidence of mite-associated Raspberry Leaf Blotch Virus has yet to be tested. Leaf trichomes in strawberry have also been shown to deter foliage feeding by adult vine weevil (Doss et al. 1987), and might have similar effects in *R. idaeus*. By contrast, high leaf trichome densities of *R. idaeus* have been associated with enhanced abundance of *A. idaei* and *M. euphorbiae* aphids (Graham et al. 2014), although this might be driven by other trait(s) linked to leaf trichome expression, as there was little evidence for aphid preference for high leaf hair density in choice experiments (Karley et al. 2016).

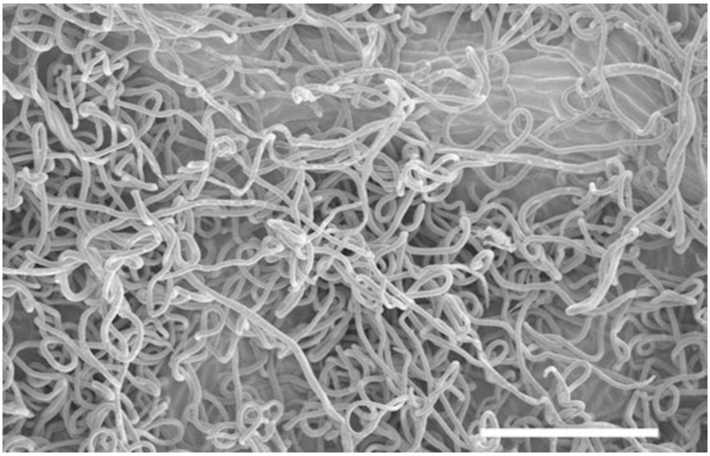

Fig. 5.2 Scanning electron microscope image of the abaxial surface of a *Rubus idaeus* leaf showing the form and structure of leaf trichomes. Scale bar represents 100 μm

Variation in plant phenology can be exploited to avoid pest damage by creating a mismatch between availability of suitable plant material and pest arrival (Mitchell et al. 2016). Differences between raspberry cultivars in damage incurred by infestation of *B. tomentosus* larvae arose from the variation in flowering time, with least amount of damage observed in late flowering cultivars (Arus et al. 2013). Using late-flowering cultivars, combined with the potential to introduce resistance traits from other *Rubus* species (Briggs et al. 1982), might contribute to better control of this pest.

Rubus genotypes with enhanced vigour might be able to tolerate infestations of root and shoot pests (e.g. Karley et al. 2016) with a smaller adverse impact on yield. Increased root vigour could provide a potential route to mitigate the damage caused by larvae of *O. sulcatus*; research indicates that varietal differences in susceptibility to root damage by vine weevil are linked to root mass and chemical composition (Clark et al. 2011; Johnson et al. 2011). Alongside crop breeding for increased vigour (Mitchell et al. 2016), conditioners and beneficial microbes could be incorporated into the soil to promote plant vigour (e.g. Orhan et al. 2006). Some of these types of products are already available commercially, and their efficacy should be tested as part of an IPM programme for *Rubus*. Enhanced root and shoot vigour might also provide a method to out-compete pernicious weeds in *Rubus* plantations.

The release of plant volatile compounds, either constitutively or following attack, could provide a trait of focus for future efforts to improve pest and disease resistance. Plant volatile compounds can act as a direct defence mechanism (e.g. release of deterrent volatile chemicals that repel pests or act as anti-microbial substances) or indirectly defend plants by increasing the recruitment of natural enemies (e.g. herbivore-induced plant volatiles that attract parasitoids or predators of arthropod pests: Stenberg et al. 2015). For example, raspberry aphids will respond to release by *R. idaeus* of the green leaf volatile (*Z*)-3-hexenyl acetate (McMenemy et al. 2012), suggesting that volatile composition could be exploited to deter aphid settling. This approach could also identify volatile compounds for use in lure traps (see Sect. 5.4).

5.4 Biocontrol of *Rubus* Pests and Diseases

A number of strategies are available to replace or reduce reliance on conventional fungicides and insecticides. These include application of biologically-derived biopesticides, release or augmentation of localised natural enemy populations, and use of attractants in lure traps used to monitor or kill the target pest. Some products are available commercially, although many are still at the stage of verifying efficacy for use in IPM programmes. A major challenge in deploying biopesticides and biocontrol agents effectively in *Rubus* plantations is the availability of clear guidelines for their use. Biocontrol products typically require several applications under suitable local conditions, and knowledge of their biology and/or mode of action can be critical for optimising efficacy.

5.4.1 Biopesticides

In Europe, biopesticides are products used to control weeds, pests and pathogens using naturally-derived biochemical products (e.g. nicotine extracted from plants) or microbial organisms (e.g. parasites or pathogens of the target organism). In the USA, the term biopesticides also includes plants engineered to produce pesticidal products (plant-incorporated protectants), although this category is not considered here. Recent research has shown that a plant-derived neem extract and a terpenoid blend suppressed aphid abundance in tunnel-grown raspberry crops infested with large raspberry aphid and potato aphid, particularly when applied with natural enemy control agents (O'Neill et al. 2014), and authorisations are currently in progress for these products. Essential oils extracted from Lamiaceae species show promise as contact and fumigant biopesticides of *D. suzukii* (Park et al. 2016), while 1-octen-3-ol incorporated into specialized pheromone and lure application technology (SPLAT) reduced *D. suzukii* oviposition significantly in raspberry fruit, and also increased flower visitation by bumble bees (Wallingford et al. 2016).

Microbial control of insect pests using entomopathogenic fungi can be highly effective under conditions that promote fungal development with long-lasting activity. Fungal spores germinate on the arthropod surface and grow into the body, causing death within 4–10 days. The fungus sporulates on the arthropod cadaver and thus generates new inoculum that can attack additional hosts. A number of entomopathogenic fungi, including *Beauveria bassiana*, *Metarhizium anisopliae*, *Hirsutella* spp. and *Verticillium lecanii* can be effective for control of arthropod pests such as two-spotted spider mite (e.g. Chandler et al. 2005), and vine weevil adults and larvae (e.g. Hirsch and Reineke 2014). Entomophagous nematodes of the genera *Heterorhabditis* and *Steinernema* are also available commercially and can be effective at reducing weevil survival independently or in combination with entomopathogenic fungi (Bruck 2007; Ansari et al. 2008), although temperature is an important consideration for deployment in field conditions due to the warmer thermal requirements of the fungus compared to nematodes (Bennison et al. 2014).

Microbial biopesticides are available, or have potential, for control of root and shoot diseases in *Rubus*. *Bacillus subtilis* is a soil bacterium with fungicidal effects that can be used to protect against *Botrytis* infection in cane fruit and could be effective against *Phytophthora* root rot (Wedgewood et al. 2014). *Trichoderma* spp. of fungi are opportunistic plant symbionts with multiple beneficial effects, including stimulation of plant growth and nutrient acquisition, induced resistance against root and foliar pathogens and direct biological control of pathogenic fungi (Harman 2006). *Trichoderma asperellum* is supplied commercially for control of root diseases and nematode damage in *Rubus*. The mycoparasite *Gliocladium* spp. can be effective for control of damping-off and root and wilt diseases by outcompeting or suppressing pathogenic fungi (e.g. Sutton et al. 1997).

There is increasing interest in 'priming' plant defence as a means of enhancing plant resistance to subsequent attack by pests or disease (e.g. Stenberg et al. 2015). Priming is caused by a stimulus that activates plant defence pathways without triggering a full plant defence response; this allows rapid induction of a strong and potentially more sustained response when the plant receives a later challenge, resulting in enhanced resistance to biotic or abiotic stress (Martinez-Medina et al. 2016). Plant priming can be achieved using synthetic 'elicitors' of plant defence pathways, including plant signal compounds such as jasmonates, and synthetic and plant-derived products (Aranega-Bou et al. 2014). While these have yet to be tested rigorously for their efficacy in enhancing the resistance of *Rubus* species to pest and disease attack, elicitors might offer an additional category of biopesticides in the future. There is some evidence that activation of defensive pathways by virus infection can alter *R. idaeus* suitability for subsequent aphid attack: aphids colonising plants infected with Black Raspberry Necrosis Virus and Raspberry Leaf Mottle Virus showed markedly slower development than aphids on uninfected plants, indicating reduced plant suitability for infesting aphids (McMenemy et al. 2012).

5.4.2 Bioactive Traps

Traps that provide optimal microclimate conditions and/or chemical attractants for arthropod pests in *Rubus* plantations can contribute to IPM as a means of pest monitoring and through lure-and-kill approaches. Semiochemical traps emitting floral volatiles attract adult *B. tomentosus* and are an effective tool for trapping and monitoring this pest in raspberry plantations (Mitchell et al. 2004). A sticky trap emitting the cane midge sex pheromone has been shown to provide reliable information about the degree of cane midge damage experienced in raspberry plantations across Europe, and could be used for monitoring pest thresholds for insecticide applications (Cross et al. 2008). Plant-derived volatile cues have also been shown to attract adult vine weevils (Karley et al. 2012) and traps containing a combination of the two plant volatile compounds, (*Z*)-2-pentenol and methyl eugenol, showed significantly increased catches of weevils compared to control traps (van Tol et al. 2012). Volatile compounds in wine and vinegar are important components of acid- and alcohol-baited traps that are effective for trapping *D. suzukii* (Cha et al. 2012).

5.4.3 Natural Enemies

Although efficacy of natural enemies on *Rubus* has not been widely tested, a number of natural enemies can be identified from studies of other crops that could be applied to aid control of invertebrate herbivore pests of *Rubus*. Predatory mites can be effective at tracking spider mite populations on raspberry (Bounfour and Tanigoshi 2002). Predatory mite species are available commercially that can be used for biocontrol of spider mite and leaf and bud mite (Mitchell and Fitzgerald 2010). Recent research indicates that several species of predatory mites have limited susceptibility to entomopathogenic fungi, suggesting that microbial biopesticides and predatory mites could be combined to optimise mite biocontrol (Wu et al. 2016). Parasitoid wasps are of particular interest for control of aphids on *Rubus* (e.g. Mitchell et al. 2010), and augmented release of multiple parasitoid species was compatible with the use of certain biopesticides for suppression of aphid infestations (O'Neill et al. 2014). Parasitoid wasps are also of potential interest as a biocontrol agent for *D. suzukii* (Miller et al. 2015; Gabarra et al. 2015). For soil pests such as vine weevil larvae, entomophagous nematodes can be highly effective at inducing mortality when applied in a range of plant growth media (Ansari and Butt 2011).

5.5 Vegetation Management to Optimise Ecosystem Services to *Rubus* Crops

Agricultural intensification has caused a decline in the diversity of farmland plants, particularly non-target species that are present at moderate or low abundance (Marshall et al. 2003). This loss of plant biodiversity has important implications for pest control as diverse plant vegetation in agricultural systems is more likely to support a diverse community of pest natural enemies (Taylor et al. 2006) and reduced abundance of crop pests (Letourneau et al. 2011). Two mechanisms have been proposed to explain this relation between plant and insect diversity (Root 1973): first, the 'resource concentration' hypothesis suggests that herbivores are less likely to find and remain on their hosts in mixed stands of vegetation, which reduces the potential for dominance by specialists; and second, the 'natural enemies' hypothesis argues that complex habitats provide a greater diversity of prey, refugia, alternative food sources and microhabitats, thus supporting more stable populations of both specialist and generalist natural enemies.

Agricultural habitats where plant diversity tends to be low are therefore likely to support a simplified arable food web with little functional redundancy and therefore low resilience to perturbation. A balance of different functional types of organisms in farmland foodwebs is necessary to maintain a stable and reliable provision of ecosystem services such as pollination, pest regulation, and carbon and nutrient turnover (Hawes 2017), which in turn depends on the presence of an adequate cover of plant types to support viable populations of beneficial invertebrates and other organisms. The challenge is to determine the optimal biomass and composition of the plant community with minimum impact in terms of competition with the crop.

Relaxed weed control to allow a low density of beneficial weeds as an understorey is the cheapest option to increase plant diversity, since savings can be made on the cost of the herbicide and manpower for hand weeding or mowing. In addition, environmental impact is reduced through a reduction in agrochemical inputs. However, management of the density and composition of the natural weed flora to achieve the optimal cover of beneficial species is hard to achieve. The grower risks a build-up of competitive weeds in the system that could become difficult to control and may incur a yield penalty over time. Using this option to increase background plant diversity will depend on the composition of the existing seedbank from which plants can be allowed to naturally regenerate. There is a need to test specific management options in perennial systems such as *Rubus* plantations to give the grower maximum control over the density and composition of the plant community. For example, conservation headlands arable systems have been optimised since they were first introduced by the Game Conservancy in the 1980s to increase the availability of insect food for partridges. The headland is created by switching off the outer boom section of the sprayer (usually 6–7 m) when broadleaved herbicides are being applied to the rest of the field, allowing weed regeneration along the field edge. Insecticides are not applied to this 6–7 m headland after 15th March, allowing an increase in both weed density and associated insect abundance (Sotherton 1991). However, to reduce competitiveness, particularly by nitrophilous weeds, and to increase species diversity of the weed flora, management has since been refined to exclude fertiliser applications to conservation headlands (Walker et al. 2007). Conservation headlands can also be rotated around the field each year to avoid excessive build-up of the weed seedbank. Similar approaches to those developed for arable systems could be explored in perennial crops to maintain a diversity of plants and the services they provide without detrimental impact to crop yield.

An alternative to natural regeneration is the use of sown understories, cover crops and margins which offer growers more control over non-crop plant densities and species composition. This provides an opportunity to tailor the resource supply for specific insect groups (e.g. pollinator mixes available from wildflower seed companies) or for particular functions (e.g. plants with complementary traits such as rooting depth, nitrogen-fixing capability and canopy structures that reduce the intensity of plant competition and enhance total capture of nutrient resources). The sown vegetative cover can suppress competitive weeds, and has potential to provide a wide range of additional functions to enhance the efficiency of crop production. This includes improved attraction of natural enemies: for example, undersowing raspberry plantations with a variety of flowering and aromatic herbs increased levels of parasitism of *B. tomentosus* larvae (Hanni and Luik 2006), and introduction of buckwheat to attract and retain hoverflies (Berndt et al. 2006) is currently being tested for the ability to suppress aphids on protected raspberry (Birch, unpublished). Legume species can provide high quality floral resources for insect pollinators and fix atmospheric nitrogen, thereby reducing the requirement for added mineral nitrogen. A 5 year study of *Rubus idaeus* plots sown with white clover understory demonstrated enhanced primocane growth (e.g. cane height, number of nodes, dry mass and nitrogen content) and leaf retention, and with no impact on fruit yield, compared to plots sown with perennial grass ground cover (Bowen and Freyman 1995). Inter-row cultivation of *Rubus* with white clover has been shown to improve soil wet aggregate stability, bulk density, organic carbon content, and total and mineralisable nitrogen content compared to perennial grass inter-row ground cover (Zebarth et al. 1993). The disadvantage of sown wildflower habitats is the cost of the seed and the reliability of plant establishment. Field margins and inter-rows tend to be nutrient-rich, creating a habitat unsuitable for many wildflower species and favouring dominance by nitrophilous and competitive types. Establishment under these conditions can be difficult and often requires repeated sowings.

Vegetation diversity can also be increased within the crop by growing two or more crop genotypes or species together. Polyculture systems including *Rubus* have not been tested widely, although intercropping with orchard trees and arable crops has been proposed (Waldo and Hartman 1947; Lawrence 1979). A study of blackberry-apple intercrops demonstrated poor *Rubus* growth and yield compared to monocultures (Rivera et al. 2004), suggesting that better knowledge of the optimal intercrop species, spatial design and management is needed to progress this approach. For floricane varieties, intercropping annual crops between rows is an option for the first year of growth, as long as the annual crop does not compete significantly with establishing *Rubus* plants for water and soil nutrients (Lawrence 1979). Intercropping in *Rubus* plantations could be achieved either by growing alternate genotypes within a plantation row or by alternating genotypes between rows or blocks or rows. While the former option presents practical challenges associated with harvesting operations and ensuring product uniformity when harvested from mixed genotype plantations, the latter option of growing alternate rows of different genotypes might be feasible. Although increasing diversity within the *Rubus* crop does not increase overall plant diversity to the same extent as using sown or natural regeneration of native plant communities, total productivity from the cultivated area can potentially be enhanced compared to the expected yield of crops in monoculture (Brooker et al. 2015). The phenomenon of 'over-yielding' in crop mixtures results from reduced competition between crop genotypes with differing physical characteristics and resource requirements, leading to improved resource capture, productivity and yield (Brooker et al. 2015). Intercropping might be an attractive option for improved pest and disease control in *Rubus* plantations by growing susceptible genotypes alongside one or more resistant genotypes. In other crop systems, the presence of resistant plants can mask the presence of susceptible genotypes and dilute the presence of infective or infesting colonies, limiting the overall pest and disease load (Tooker and Frank 2012). Such an approach might also slow the evolution of resistance-breaking pest and disease variants and thus enhance the durability of crop resistance (Tooker and Frank 2012).

5.6 Summary and Future Perspectives

Within the agroecological framework proposed for improved pest and disease management in *Rubus* (summarised in Table 5.1), certain components are already well developed, particularly the use of *Rubus* crop traits and/or resistance genes (Table 5.1). Continued investment in research to identify crop resistance traits and genetic markers and deploy them in breeding programmes is likely to remain a priority for future crop breeding programmes, aided by developments in high throughput crop genotyping and phenotyping (Mitchell et al. 2016), particularly for in-field crop characterisation to understand genotype-by-environment interactions. Imaging

Table 5.1 Summary of the main problems in *Rubus* plantations and existing or potential (in bold) agroecological tools for control of weeds, pests and diseases

	Common name	Latin name	Damage caused	Plant traits	Biopesticides/ bioactives	Biocontrol	Reference
Arthropod pests	Large raspberry aphid	*Amphorophora idaei*	Virus transmission Feeding damage	Leaf/phloem defensive chemistry Surface waxes and alleleochemicals **Defence priming**	Neem extract/ terpenoid blend	Parasitoid wasps	Shepherd et al. (1999), Lightle et al. (2012), Smith and Chuang (2013), O'Neill et al. (2014)
	Potato aphid	*Macrosiphum euphorbiae*	Feeding damage	Surface waxes and alleleochemicals	Neem extract/ terpenoid blend	Parasitoid wasps	
	Blackberry aphid	*Amphorophora rubi*	Feeding damage	**Plant surface physico-chemical traits**			
	Raspberry beetle	*Byturus tomentosus*	Flower damage	Late flowering	Lure-and-kill traps	Floral understory	Arus et al. (2013), Mitchell et al. (2004), Hanni and Luik (2006)
	Vine weevil	*Otiorhynchus sulcatus*	Root damage	**Root vigour**	Beneficial soil microbes lure traps	Entomopathogenic fungi Entomophagous nematodes	Orhan et al. (2006), van Tol et al. (2012), Hirsch and Reineke (2014), Bruck (2007), Ansari et al. (2008)
	Raspberry cane midge	*Resseliella theobaldi*	Cane blight	Cane height/growth rate	Pheromone trap		Woodhead et al. (2013), Cross et al. (2008)
	Two-spotted spider mite	*Tetranychus urticae*	Feeding, webbing	High trichome density	Entomopathogenic fungi	Predatory mites	Karley et al. (2016), Chandler et al. (2005), Bounfour and Tanigoshi (2002)
	Raspberry leaf and bud mite	*Phyllocoptes gracilis*	Virus transmission	High trichome density		**Predatory mites**	Mitchell and Fitzgerald (2010)
	Blackberry mite	*Acalitis essigi*	Uneven fruit ripening	**High trichome density**		**Predatory mites**	
	Spotted wing Drosophila	*Drosophila suzukii*	Berry collapse	Fruit skin strength	Plant-derived biopesticides Pheromone lure traps Baited lure traps	**Parasitoid wasps**	Burrack et al. (2013), Park et al. (2016), Wallingford et al. (2016), Cha et al. (2012)

Pathogens	Raspberry root rot	*Phytophthora rubi*	Root death	Root vigour	***Bacillus subtilis*** ***Trichoderma asperellum***		Graham et al. (2011)
	Cane blight	*Leptosphaeria coniothyrium*	Cane die back	Cane height/growth rate	Pheromone trap for cane midge		Woodhead et al. (2013), Cross et al. (2008)
	Botrytis fruit rot	*Botrytis cinerea*	Fruit rot	High cane hair density	*Bacillus subtilis* *Gliocladium* spp		Graham et al. (2006), Wedgewood et al. (2014), Sutton et al. (1997)
	Spur blight	*Didymella applanata*	Weakened canes	High cane hair density			Graham et al. (2006)
Weeds	Annual and perennial weeds		Reduced root/ shoot growth	**Root and shoot vigour**	**Soil conditioners to promote crop growth**	**Sown understory**	

platforms developed for high-throughput phenotyping could also provide tools for in-field detection of pest and disease problems and targeted control measures (Mitchell et al. 2016).

A mechanistic understanding of the effect of plant resistance or tolerance traits on pest and disease susceptibility is vital to ensure that multiple – and sometimes unanticipated – effects of plant traits are identified before being deployed in the field. For example, parasitoid efficiency was impaired on an aphid-resistant cultivar of raspberry, probably due to increased aphid restlessness on a poor quality host (Mitchell et al. 2010). High trichome densities, which can deter feeding and oviposition by mites and other herbivores, can also impair the searching efficiency and survival of predators (Riddick and Simmons 2014). Further, plant investment in herbivore-induced plant volatile release could influence the behaviour of beneficial organisms, for example by altering the quantity and composition of volatiles emitted by floral structures that attract pollinators and parasitoid wasps (Lucas-Barbosa et al. 2011).

The use of biopesticides and natural enemies for biocontrol is currently receiving significant investment and the number of available products is likely to increase significantly in the near future. There are opportunities for transfer of knowledge regarding well-characterised crop resistance traits and biocontrol products for particular pests and pathogens to address knowledge gaps in management of other pests and diseases (summarised in Table 5.1). Examples include testing the effects of leaf surface physico-chemical traits on blackberry aphid, and evaluating the efficacy of predatory mites for biocontrol of a wider range of phytophagous mite species. Microbial biopesticides such as *Trichoderma* and *Gliocladium* spp. could provide opportunities to tackle previously intractable root and shoot pathogens such as *Phytophthora* and fungal blights. The next step is to combine multiple agroecological tools for a number of targets (Table 5.1) to test their efficacy for integrated management of pests and diseases in *Rubus*. A good example of the type of approach that could be adopted is demonstrated in Bruce et al. (2016), which tested the efficacy of plant priming activators, plant-derived biopesticides and parasitic wasps for control of pest and pathogen targets attacking tomato plants.

Further research is needed to address gaps in the availability of specific tools for certain pest and disease targets (Table 5.1). For example, wider deployment of bioactive traps for suppressing pest populations (i.e. lure-and-kill), or simply for monitoring pest abundance to inform other control measures, could improve the management of mite and aphid infestations in *Rubus* plantations. The potential for vegetation and habitat management to contribute to pest and disease regulation also remains to be explored fully for *Rubus* crops. Enhanced vegetation diversity could be a useful strategy to address the lack of effective tools for weed control (highlighted by Table 5.1), one of several benefits demonstrated for vineyards undersown with endemic plant species in New Zealand (Shields et al. 2016). Implementing management approaches to conserve natural enemy populations also has the potential to be effective in perennial crop systems as disturbance levels tend to be lower than in annual crop systems, facilitating natural enemy persistence due to greater availability of refugia and overwintering sites (Landis et al. 2000). However, management measures to promote biocontrol by natural enemies at local scales are not always predictable in their outcomes: creation of unharvested refuges in short rotation coppice willow stands did not lead to expected effects on herbivorous leaf beetles and their predators (Liman et al. 2016). In addition, the impact of vegetation management might be minor at local scales due to the effects on arthropod populations of habitat composition at the landscape scale, which is increasingly recognised to play an important role in conservation biological control. Pest abundance tends to be reduced in landscapes with a high proportion of semi-natural habitat, and biological control by natural enemies tends to be more effective in these landscapes (Veres et al. 2013). This could explain why presence of a sown ground cover in olive groves had limited effect, compared to bare ground, on four common insect pests, whose abundances varied most significantly in relation to factors at landscape and regional scales (Paredes et al. 2015). These studies emphasise the need to develop empirical and theoretical approaches as potentially important tools for ecological engineering of landscapes to suppress pest and disease outbreaks (Birch et al. 2011; Begg et al. 2017) in *Rubus* and other production systems.

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Chapter 6
Pathogen Testing Requirements for Raspberry Material Entering the EU Certification Scheme

Alison Dolan

6.1 Introduction

Soft fruit production represents a valuable sector within the agricultural/horticultural landscape, and raspberries play a major part in this industry. As a perennial crop, raspberries require considerable initial and ongoing investment, and establishing a plantation with pest and pathogen free plants is the first step to a productive and profitable crop. Over successive seasons field-grown raspberry plants become increasingly infected with a variety of pests and pathogens, and this reduces their productivity and lifespan. It is therefore essential that growers re-stock their plantations and stock new plantations with certified, pathogen-free planting material, to maximise yields and keep crop losses due to infection as low as possible. To enable the raspberry industry to thrive, the availability of pathogen-free planting material, regulated through the Plant Health Certification Scheme, has been recognised as a significant contributor to the success of the industry. The scheme was based on a legislative framework that started with the introduction of inspections and certification of raspberry plants in the UK in the 1940s. The development of the scheme provided growers with raspberry material, and subsequently blackberry, loganberry and other *Rubus* hybrid berries, descended from plants proven in terms of varietal identity, health status and vigour.

The certification scheme in the UK was voluntary but was used widely due to its many benefits in meeting industry demands. The health of raspberry stocks was maintained by the regular introduction of pathogen-tested plant material into the propagation system, with an unbroken history of certification and time restrictions for eligibility in each grade within the certification scheme. Each EU member state had its own national certification scheme but from 2017, a new EU statutory scheme for fruit plant reproductive material was introduced. This new scheme operates in all

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A. Dolan (✉)
The James Hutton Institute, Dundee, UK
e-mail: alison.dolan@hutton.ac.uk

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EU member states and creates a single harmonised market. It covers registration of suppliers and varieties, defined labelling for each grade and certification and rules in relation to official inspections. The list of genera and species in the scheme has increased to include for example cherries and blueberries. The Nuclear stock grade is now referred to as Pre-basic, Super Elite as Basic 1, Elite as Basic 2, Standard as Certified and Non Certified material as *Conformitas* Agraria *Communitatis* (CAC) – Plant Passported.

The James Hutton Institute is the sole UK provider of buds and root entering the scheme from pest- and pathogen-tested *Rubus* Pre-basic 'mother' plants. These plants are tested and maintained to fulfil the requirements set in Scotland by the Scottish Government, in England and Wales by the Department for Environment, Food and Rural Affairs (Defra) and in Europe by the European and Mediterranean Plant Protection Organisation (EPPO) hort.marketing@scotland.gsi.gov.uk; https://www.gov.uk/guidance/fruit-propagation-certification-scheme; http://www.eppo.org/STANDARDS/standards.htm). Similar schemes exist in non-European countries such as in the USA where many, although not all, raspberry breeders go through a clean plant centre for testing and clean up, if necessary. Certification in the U.S. is voluntary for all crops except citrus. Most nursery stock producers and breeding companies do not have their raspberries in a certification scheme but they are grown under conditions that would meet most of the plant health requirements of such a scheme. To meet export requirements comprehensive testing is done on the top tier plants (G1), and these plants can also feed into domestic nursery production. Most raspberry and blackberry growers establish their plantations with material produced from either a certification scheme or from plants which have been pathogen tested at certified laboratories. In the U.S. there is also a National Clean Plant Network http://nationalcleanplantnetwork.org/ which focuses on the production of top tier plants. In Chile there are only a few plant nurseries that are involved with a certification scheme and in New Zealand there is currently no certification scheme for *Rubus*.

6.2 Production of Candidate Mother Plants for Pre-Basic Status

Established raspberry varieties and new varieties from commercial breeding programmes enter the scheme after the candidate mother plant of each variety is free after testing from the range of diseases listed in the UK and EPPO guidelines (https://www.gov.uk/guidance/fruit-propagation-certification-scheme; http://www.eppo.org/STANDARDS/standards.htm).

These plants are maintained in sterile compost with individual irrigation and UV sterilised water, in an insect-proof glasshouse, and are visually inspected to prevent infestation by pests such as aphids. When plants have attained Pre-basic stock status i.e. negative for all tests, the plant is moved to a designated aphid-screened area with restricted access. The plants are retested regularly and are inspected annually under a statutory regime by Government Plant Health Inspectors. Plants giving a positive result for any test are removed from the facility and destroyed.

Plant material provided for propagation and entry into the certification scheme is in the form of either root or buds. Since the introduction of *in vitro* bud propagation, root production has been overtaken as the main propagation method for Pre-basic stock plants in the production of material to be sold as certified stock (Dolan 2013, 2017). *In vitro* micro propagation is a non-destructive method, using only the buds from the top section of the floricane. The root and remaining cane can then remain in the facility to undergo further testing and provide the following year's mother plant.

The material leaving the Pre-basic stock facility, whether root or buds, is accompanied by a Plant Passport and a Plant Health Declaration, which details the pathogens and tests applied to the 'mother' plant from which the material is derived. At the James Hutton Institute a Scottish Government assigned 'Responsible Person' issues the Plant Passports and is the sole authorised signatory for the Plant Health Declaration. After propagation and multiplication from the root or buds, the resultant plants are either sold or remain in the certification scheme. To provide a robust, up to date scheme where the health status of the plants is paramount, it is essential that knowledge of existing and emerging pests and of new and more sensitive detection methods is adopted into the scheme at the earliest opportunity.

6.3 Pathogen Testing

All candidate mother plants prior to entering the scheme as Pre-basic stock are tested according to the UK and EPPO guidelines for viruses, virus-like pathogens and oomycetes. After the tests have been completed and the mother plant is found free from these known pathogens, they are vegetatively propagated to produce daughters i.e. the following year's mother plants. This refreshment each year and continuous testing provides the soft fruit industry with high health, vigorous new buds and root for release into the certification scheme for propagation.

Viruses and virus-like pathogens can be detected by a number of means, including visual assessment of the mother plant, graft inoculation to the virus indicator *Rubus occidentalis*, mechanical inoculation to herbaceous virus indicator test plants and enzyme-linked immunosorbent assay (ELISA). Polymerase chain reaction (PCR) detection is a recommended test for *Rubus* stunt phytoplasma, in addition to or instead of visual symptoms. As the genomes of uncharacterised agents become known, more molecular detection methods may be included in future testing schemes if these tests are scientifically proven to be as good as or better than existing tests. Biological assays remain valid as they are highly sensitive, efficient in detecting many viruses simultaneously and because not all viruses have yet been characterised meaning that molecular detection methods have not been developed.

The soil-borne disease raspberry root rot is associated with several species of the genus *Phytophthora*, the most destructive of which is *P. rubi* and to a lesser extent *P. idaei*, and it infects roots, crowns and the base of young canes. The pathogen is readily spread in water and has had a serious commercial impact on raspberry growing in Europe. For testing for this pathogen, two samples of root are collected and individually PCR tested from each candidate mother plant before entry into the Pre-basic stock facility, and a single sample is tested each year thereafter. To prevent the pathogen entering the system by other means, UV sterilised water and individual irrigation is applied, and protective clothing is worn in the Pre-basic stock facility.

Limiting the introduction of soil-borne pathogens, when plantations are established on ground not previously used for growing raspberries, is essential for the growth of the industry. If the planting material is infected, then the level of disease can reach proportions where field grow cropping will become commercially unsustainable.

6.4 Trueness to Type and Crumbly Fruit

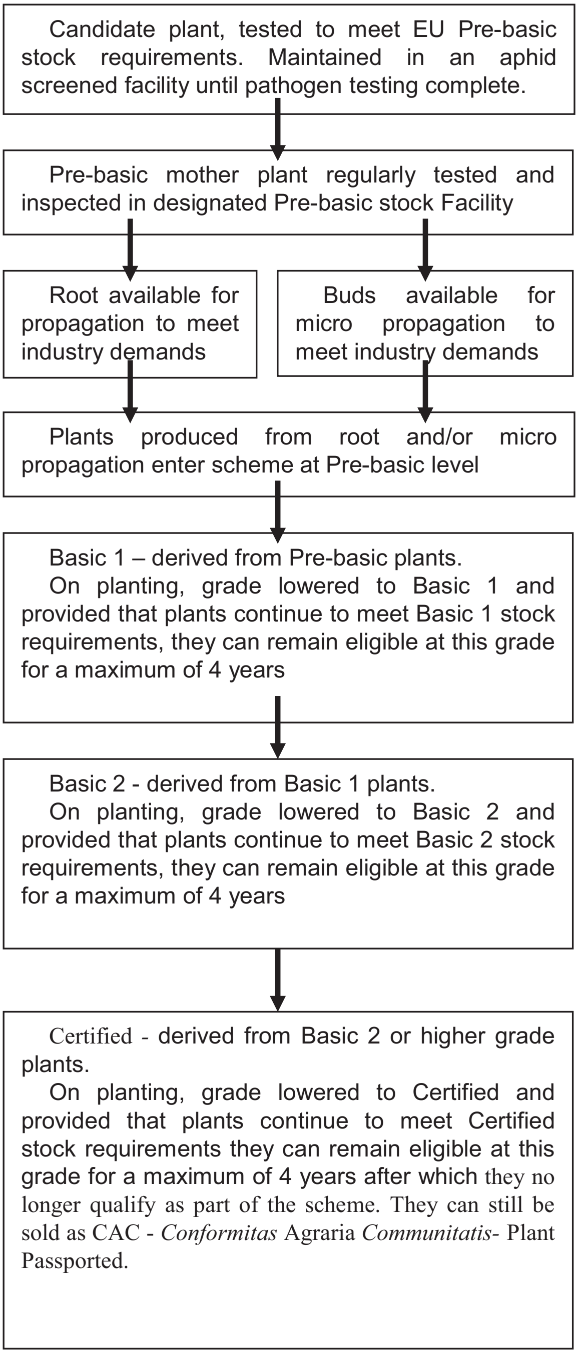
To ensure that the Pre-basic stock mother plants are distinct, uniform and stable and that their fruiting characteristics are not altered, canes of these stocks are allowed to flower and fruit each year in a separate facility from the Pre-basic stock facility. The plants are visually inspected for their phenotypic traits and the Crumbly Fruit Condition is assessed as part of the scheme to prevent fruit that readily crumbles, making it unmarketable when picked, being released to industry. It is unclear what the triggers for the condition are; crumbly fruit has been linked with pollen abortion and embryo sac degeneration, where drupelets may be greatly enlarged if their number is greatly reduced or, in the case of small reductions, cohere imperfectly so fruit readily crumbles when picked (Jennings 1988). Pollination is achieved with the use of commercial bee hives and visual assessments are made of the fruit development. No further propagation is permitted from mother plants that display atypical phenotypic characteristics or produce abnormal fruits.

6.5 The Certification Scheme

The UK *Rubus* Pre-basic stock facility is inspected and regulated by the Scottish Government. Growing season inspections are made by the Horticulture and Marketing Unit, who inspect plant material and issue Phytosanitary Certificates if required by an importing country.

Pre-basic stock plants are the foundation of the certification scheme and plants derived from these mother plants are used to establish the initial spawn beds for propagation at the beginning of the scheme. Government officials administer the strict regulations applied to provide certified stock.

Fig. 6.1 Diagram of certification scheme



Candidate plant, tested to meet EU Pre-basic stock requirements. Maintained in an aphid screened facility until pathogen testing complete.

Pre-basic mother plant regularly tested and inspected in designated Pre-basic stock Facility

Root available for propagation to meet industry demands

Buds available for micro propagation to meet industry demands

Plants produced from root and/or micro propagation enter scheme at Pre-basic level

Basic 1 – derived from Pre-basic plants.

On planting, grade lowered to Basic 1 and provided that plants continue to meet Basic 1 stock requirements, they can remain eligible at this grade for a maximum of 4 years

Basic 2 - derived from Basic 1 plants.

On planting, grade lowered to Basic 2 and provided that plants continue to meet Basic 2 stock requirements, they can remain eligible at this grade for a maximum of 4 years

Certified - derived from Basic 2 or higher grade plants.

On planting, grade lowered to Certified and provided that plants continue to meet Certified stock requirements they can remain eligible at this grade for a maximum of 4 years after which they no longer qualify as part of the scheme. They can still be sold as CAC - *Conformitas* Agraria *Communitatis*- Plant Passported.

Once the Pre-basic material has left the facility then propagation stock eligibility is limited to a 4 year generation between grades Basic 1 to Certified. Each time the plants are downgraded, fewer restrictions apply for entry into lower grades within the scheme. There are soil sampling requirements for Basic 1 and Basic 2 propagation for soil-living virus vector nematodes and the isolation and separation distances between grades and commercial production are grade dependent. Official inspections of plants (mainly visual), production records and labelling are essential for the traceability and eligibility of the plants within the scheme.

After completion of the final 4 years in the scheme the plants can still be sold but no guarantee of health status will be given.

The benefits of a certification scheme (Fig. 6.1) are to provide fruit producers and propagators with planting material of a known health standard, vigour and purity. It provides a means of preventing the spread of harmful pests and diseases by the regular introduction of pathogen tested material with an unbroken history of certification and limiting the time that stock can remain eligible for certification.

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Footnote: EU certification Scheme, raspberry, Pre-basic, Basic 1, Basic 2, and Certified

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Chapter 7
Raspberry Fruit Chemistry in Relation to Fruit Quality and Human Nutrition

Robert D. Hancock, Antonios Petridis, and Gordon J. McDougall

7.1 Introduction

In recent years raspberry fruit breeding has shifted its focus from traits associated with agronomic performance towards those associated with fruit sensory quality (Jennings et al. 2016) and potential health benefits (Mazzoni et al. 2016). Simultaneously, significant advancements have been made in raspberry genetics and genomics as well as analytical chemistry in soft fruit. These new tools are generating knowledge that has the capacity to significantly accelerate the development of new varieties that meet consumer expectations in terms of sensory experience and health benefits of fruit consumption. Significant research in recent years has identified the environmental, biochemical and genetic controls underlying the accumulation of specific compounds in raspberry fruit. Furthermore, increasing information is becoming available regarding the mechanisms of action of specific phytochemicals in relation to human health outcomes. This information is now providing the underpinning science for the development of new cultivars. In this chapter, we outline current understanding of the biosynthetic pathways associated with the accumulation of significant fruit phytochemicals and describe what is presently known regarding the influence of crop genetics and the growing environment on the accumulation of specific phytochemicals. Finally we outline the latest knowledge regarding how fruit phytochemicals modulate human health outcomes. It is anticipated that the work outlined here will guide molecular breeding targets for future crop improvement.

R. D. Hancock (✉) · A. Petridis
Cell & Molecular Sciences, The James Hutton Institute, Dundee, UK
e-mail: rob.hancock@hutton.ac.uk

G. J. McDougall
Environmental and Biochemical Sciences, The James Hutton Institute, Dundee, UK

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7.2 Primary Metabolites as Drivers of Fruit Flavour

Sugar-acid balance is considered a key driver of flavour in a range of fruits (Klee 2010) and achieving an appropriate sugar-acid balance is therefore essential for the sensory quality of the fruit. Few studies have been undertaken specifically in raspberry, however in a study of sensory preference in five cultivars soluble solids were positively correlated with fruitiness, sweetness and overall impression, while titratable acidity was positively correlated with sourness and astringency (Shamaila et al. 1993). Furthermore, a detailed sensory and preference analysis of several cultivars grown in the North-Western United States revealed three groups of consumers with different preferences. "Likers" showed a clear preference for raspberry aroma and flavour that was not shared with "nonlikers" and "nondistinguishers". However, what united all three groups was the frequency with which preference was associated with high scores for sweetness, while high scores for sourness and bitterness were associated with a disliking (Villamor et al. 2013). These data indicate the importance of sugars and acids in driving consumer preference in red raspberry.

The main sugars found in raspberry fruit are the monosaccharides glucose and fructose, and the disaccharide sucrose (Famiani et al. 2005; Lee 2015; Mazur et al. 2014a; Stavang et al. 2015). Smaller amounts of xylose, trehalose, myo-inositol (Dincheva et al. 2013a), sorbitol and mannitol (Lee 2015) have also been reported. As different sugars have different perceived levels of sweetness (Moskowitz 1970), the ratios of the major sugars in ripe fruit could influence the perception of sweetness in the fruit. Sugar content of fruit rises throughout development and at commercial harvest fructose and glucose are present at similar concentrations of around 15–30 mg g^{-1} FW, while sucrose has been reported at lower concentrations of approximately 10 mg g^{-1} FW (Mazur et al. 2014a; Stavang et al. 2015; Wang et al. 2009; Famiani et al. 2005).

Sugar accumulation in raspberry fruit is a complex process that proceeds *via* remobilization of starch reserves from root and canes as well as *via* direct translocation of sugars synthesized in leaves (Alvarado-Raya et al. 2007; de Oliveira et al. 2007). Interestingly, immature raspberry fruit can continue to accumulate soluble solids and sugars following detachment from the mother plant. Given that raspberry fruit does not accumulate starch, it has been proposed that the source of such sugars is the solubilization of cell wall components (Wang et al. 2009).

Sugars lie at the heart of central metabolism and participate in a broad range of both anabolic and catabolic biochemical pathways. Like other primary metabolites, varietal differences in fruit sugar content are strongly influenced by environment. For example, Mazur et al. (2014a) observed a significant impact of growing location on sugar content of 'Glen Ample' raspberry that was correlated with growing temperature and precipitation. Similarly, day length had a significant impact on fruit sucrose with fruit grown under long days (22 h) having lower sucrose content and soluble solids to titratable acidity ratio than fruit grown under shorter (10 h) days (Mazur et al. 2014b). Furthermore, in a study on heritability of pomological and quality traits in progenies derived by Meeker's yellow clone, heritability traits of reducing sugars were low and large genotype × year interactions were observed (Fotirić Akšić et al. 2011), suggesting that agronomic rather than genetic intervention may be the preferred method for optimising sugar content in raspberry fruit.

The primary organic acids found in raspberry fruit are citrate (10–15 mg g^{-1} FW) and malate (1.0–1.5 mg g^{-1} FW) (Famiani et al. 2005; Mazur et al. 2014a; Wang et al. 2009). Lower amounts of succinate, maleate, fumarate and glucarate have also been identified in ripe raspberry fruit (Dincheva et al. 2013a). Both malate and citrate levels decline during ripening, associated with an increase in the abundance and activity of phosphoenolpyruvate carboxykinase (PEPCK). This suggests that citrate and malate are diminished in ripening raspberry fruits by the activity of PEPCK leading to the formation of phosphoenolpyruvate which could be subsequently metabolized *via* gluconeogenesis, ethanol fermentation or other biosynthetic pathways as illustrated in Fig. 7.1, (Famiani et al. 2005).

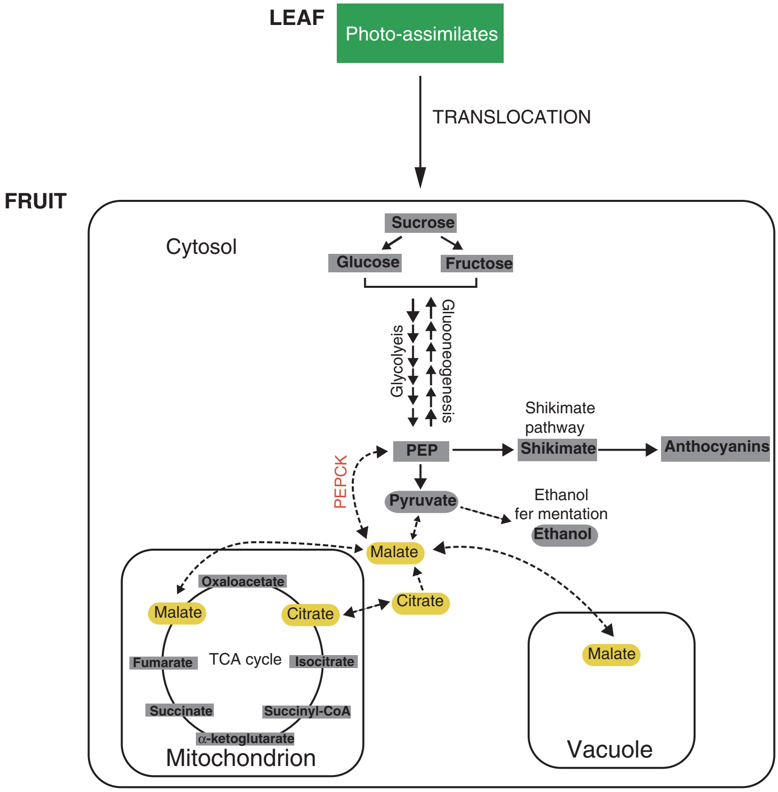


Fig. 7.1 Malate and citrate metabolism in developing raspberry fruit. At early stages of fruit development malate and citrate derive from the metabolism of sugars that translocate from the leaves to the developing fruit. During ripening, malate and citrate levels decline and several metabolic pathways are involved in their catabolism, including oxidation via Krebs cycle, gluconeogenesis, ethanol fermentation and biosynthesis of secondary metabolites such as anthocyanins. Malate and citrate can be found in different compartments within a cell. PEP phospoenolpyruvate, PEPCK phospoenolpyruvate carboxykinase

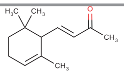
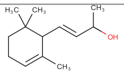
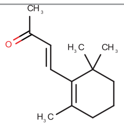
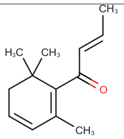
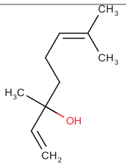
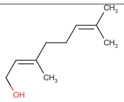
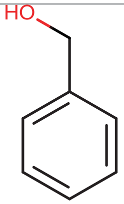
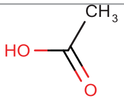
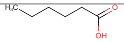
Similar to the sugars present in raspberry fruit, citrate and malate sit at the centre of primary metabolism being key components of the tricarboxylic acid (TCA) cycle. Besides its role in providing substrates (NADH, $FADH_2$) for ATP synthesis by mitochondrial oxidative phosphorylation, the TCA cycle provides substrates and intermediates for amino acid biosynthesis, secondary metabolite biosynthesis, ammonia assimilation, photorespiration, the glyoxylate cycle and purine nucleotide biosynthesis (Sweetlove et al. 2010). Like the sugars, total fruit acidity in raspberry fruits exhibited a low coefficient of heritability and a high phenotypic coefficient of variation indicating strong environmental effects on fruit acid content (Fotirić Akšić et al. 2011). This is confirmed by several studies that indicate that although there are significant differences in fruit acid content of different cultivars (Krüger et al. 2011; Mazur et al. 2014c), the growing environment also has a significant impact (Mazur et al. 2014a, c).

As sugar-acid balance is such a key component of raspberry fruit flavour, it is desirable for breeders and growers to have the capacity to predict and control the sugar-acid balance. However, these metabolites sit at the centre of fruit metabolism in highly branched and interconnected metabolic pathways. Whilst some minor QTL have been detected for sugars, these are not stable in different years and environments (J Graham, personal communication) highlighting the strong influence of the growing environment. It therefore appears that, in the short-term at least, management of the growing environment is the most promising opportunity to optimize fruit sugars and acids for quality.

7.3 Volatile Components of Flavour and Aroma Quality

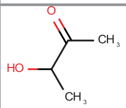
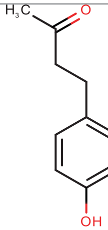
Almost 300 volatile compounds have been reported in raspberry fruit belonging to a range of chemical classes including acids, phenols, aldehydes, ketones, terpenoids, isoprenoids, esters, ethers and hydrocarbons (Aprea et al. 2015). However, only a fraction of these compounds have been demonstrated to be significant for fruit aroma and sensory properties. Larsen et al. (1991) identified ten compounds contributing to raspberry flavour by combining gas chromatographic analysis of solvent extracted aroma concentrates with sensory analysis of jams made from different cultivars. These comprised products of carotenoid cleavage, monoterpenes, fatty acid breakdown products and the key raspberry aroma compound *p*-hydroxyphenylbutan-2-one (raspberry ketone), which derives from the phenylpropanoid biosynthetic pathway (Table 7.1). Subsequent work expanded this list using a combination of simulated retronasal aroma generation in combination with gas chromatography-mass spectrometry (GC/MS) and GC-olfactometry (GCO). Using these techniques, a number of additional fatty acid derivatives, the phenylpropanoid vanillin, the amino acid derivative sotolon that imparts maple notes and the key strawberry aroma compound furaneol were identified as significant for raspberry aroma (Roberts and Acree 1996). This list of compounds was further expanded to 75 compounds following solvent extraction of fruit in combination with aroma

Table 7.1 Major raspberry volatiles and their sensory characteristics

Aroma compound	Chemical structure	Metabolic pathway	Sensory description
α-Ionone		Carotenoid	Woody, floral, balsamic and sweet tones, violet
α-Ionol		Carotenoid	Floral
β-Ionone		Carotenoid	Cedar wood, floral, fruity
β-Damascenone		Carotenoid	Floral, fruity, sweet
Linalool		Monoterpenes	Floral, spicy, woody
Geraniol		Monoterpenes	Sweet, fruity, floral
Benzyl alcohol		Fatty acids	Faint aromatic, sharp burning taste
Acetic acid		Fatty acids	Pungent, vinegar-like
Hexanoic acid		Fatty acids	Cheese, goat-like
(Z)-3-hexenol		Fatty acids	Intense grassy green

(continued)

Table 7.1 (continued)

Aroma compound	Chemical structure	Metabolic pathway	Sensory description
Acetoin		Fatty acids	Buttery, fatty creamy
Raspberry ketone		Phenylpropanoid	Raspberry, sweet fruity

Larsen and Poll (1990), Klesk et al. (2004), Paterson et al. (2013)

extract dilution analysis, a method for estimating the odour potency of volatile compounds (Klesk et al. 2004). It should be noted that while this greatly expanded list is useful for identifying components that may contribute to raspberry aroma, the extraction and concentration methods used might result in chemical transformations that do not normally occur during the ingestion of fresh fruit. Furthermore, some of the compounds identified in the concentrated extract might be below the limits of human detection in fresh fruit. Hence, Table 7.1 only lists the major volatile components.

Raspberry volatiles are synthesized from a range of metabolic pathways and here we describe only the metabolic pathways associated with some of the key volatile compounds. Raspberry ketone (*p*-hydroxyphenylbutan-2-one) is described as imparting the characteristic raspberry aroma and it exists both as the free ketone and as the glucoside (Borejsza-Wysocki and Hradzina 1994). The compound is synthesised in a two-step biosynthetic pathway from the precursors malonyl-CoA and *p*-coumaryl-CoA formed from acetyl-CoA and phenylalanine respectively (Fig. 7.2). The first step is a condensation reaction catalyzed by an atypical aromatic polyketide synthase (benzalacetone synthase, BAS) to form the intermediate *p*-hydroxybenzalacetone (Borejsza-Wysocki and Hrazdina 1996). In the second step, raspberry ketone/zingerone synthase catalyses the NADPH-dependent reduction of *p*-hydroxybenxalacetone to raspberry ketone (Koeduka et al. 2011). Genes encoding both enzymes have been cloned and kinetically characterized. BAS is encoded by *RiPKS4* and has both BAS and chalcone synthase activity – the latter activity producing precursors for flavonoid and anthocyanin biosynthesis. Raspberry ketone/zingerone synthase is encoded by *RZS1* and exhibits activity against hydroxybenzalacetone and to a lesser extent 3-methoxy-4-hydroxybenzalacetone. The reaction product of the latter compound with RZS1 is zingerone, which is not present in raspberry fruit. The abundance of both *RiPKS4* and *RZS1* transcripts increases during fruit ripening consistent with their function in raspberry ketone biosynthesis (Zheng and Hrazdina 2008).

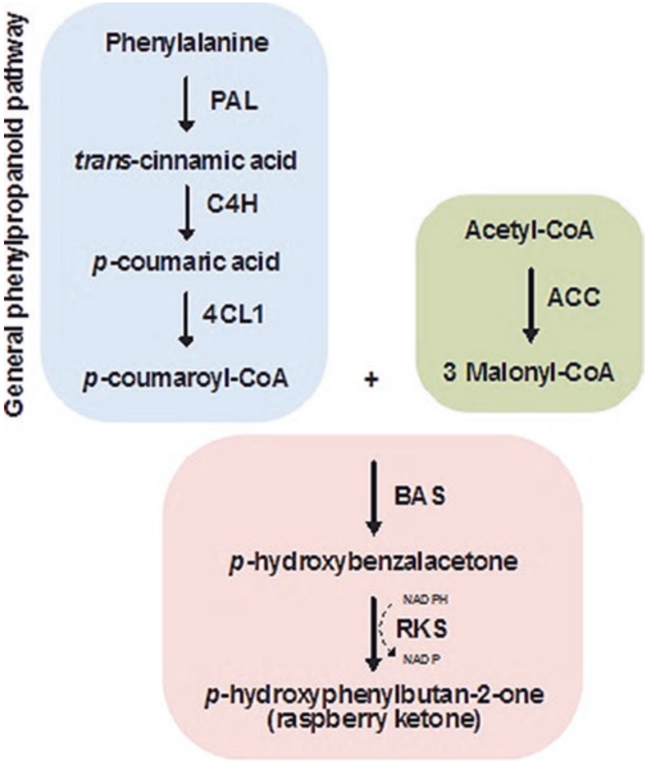


Fig. 7.2 Outline of the raspberry ketone biosynthetic pathway. Raspberry ketone is synthesised via a two-step reaction pathway using *p*-coumaroyl-CoA and malonyl-CoA as precursors. These compounds derive from the phenylpropanoid pathway and acetyl-CoA respectively. The first step of raspberry ketone synthesis is catalysed by benzalacetone synthase (BAS), leading to formation of *p*-hydroxybenzalacetone. The second step includes the NADPH-dependent reduction of *p*-hydroxybenzalacetone to raspberry ketone, which is catalysed by raspberry ketone/zingerone synthase (RKS). PAL phenylalanine ammonia lyase, C4H cinnamate-4-hydroxylase, 4CL1 4-coumarate:CoA ligase1, ACC acetyl-CoA carboxylase

In addition to its well established role in generating important raspberry aroma notes, raspberry ketone has also been shown to have anti-obesogenic properties. When supplied as a supplement to mice supplied with a high fat diet, raspberry ketone either alone (Morimoto et al. 2005) or in combination with ellagitannins (Luo et al. 2016) significantly inhibited weight gain and the accumulation of adipose tissue. Furthermore, raspberry ketone promoted lipolysis and fatty acid oxidation in adipocyte cell cultures that was associated with increased accumulation of adiponectin, an adipocytokine known to regulate lipid and glucose metabolism (Morimoto et al. 2005; Park 2010).

Alongside raspberry ketone, the norisoprenoids α- and β-ionone and the monoterpenes linalool and geraniol are considered to be significant aroma compounds in raspberry fruit (Larsen et al. 1991). Both groups of compounds share common precursors synthesized via the mevalonate (MVA) pathway in the cytosol or the methylerythritol phosphate (MEP) pathway in the plastid (Paterson et al. 2013). Both pathways produce the precursors isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which are condensed to geranyl pyrophosphate (C10, GPP), the precursor of the terpenoid aroma volatiles geraniol and linalool (Chen et al. 2011), or to geranylgeranyl pyrophosphate (C20, GGPP), the precursor of carotenoids (McQuinn et al. 2015) that are subsequently cleaved to α- or β-ionone (Fig. 7.3). However, labelling studies using deuterated pathway intermediates revealed selectivity concerning cytosolic or plastidial biosynthetic routes with linalool being synthesized solely *via* the cytosolic MVA pathway, while the ionones were labelled when fruit was provided with precursors from either pathway (Hampel et al. 2007). These data implied a cytosolic

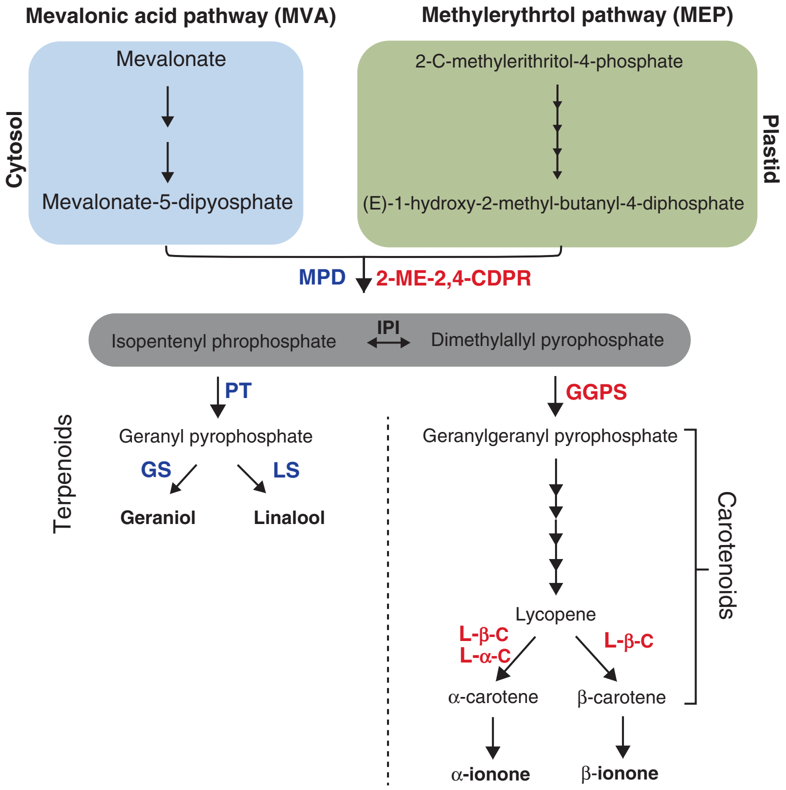


Fig. 7.3 Biosynthesis of major isoprenoids (geraniol, linalool, α-ionone and β-ionone) found in raspberry fruit. Precursors for the formation of these metabolites are isopentenyl pyrophosphate and dimethyallyl pyrophosphate that can be either formed through mevalonic acid (MVA) pathway in the cytosol or methylerythritol pathway (MEP) in the plastid. MPD mevalonate pyrophosphate decarboxylase, 2-ME-2,4—CDPR 2-C-methyl-D-erythritol-2,4-cyclodiphosphate reductase, IPI isopentenyl pyrophosphate isomerase, PT prenyltransferase, GGPS geranylgeranyl pyrophosphate synthase, GS geraniol synthase, LS linalool synthase, L-β-C lycopene-β-cyclase, L-ε-C lycopene-ε-cyclase

location for the monoterpene synthase required for linalool biosynthesis, contrary to the finding that the majority of known monoterpene synthases have been assigned as plastid localized enzymes (Sun et al. 2016). However, Aharoni et al. (2004) identified a dual function terpene synthase in strawberry fruit cytosol that was capable of synthesizing both linalool from geranyl disphosphate as well as nerolidol from C15 farnesyl diphosphate, indicating a cytosolic location for monoterpene synthases in the fruit of other rosaceous species. Carotenoid cleavage dioxygenases (CCDs), responsible for the synthesis of α- and β-ionone among other apocarotenoids, are found in both the plastid and cytosol (Rubio et al. 2008; Simkin et al. 2004), although it has been suggested that cytoplasmic enzymes are likely associated with the plastid membrane. In raspberry, a gene with 80% homology to Arabidopsis *CCD1* was cloned and expressed in carotenoid synthesizing *Escherichia coli* strains. The bacteria expressing the gene were found to produce α- and β-ionone from different substrates, while bacteria transformed with the empty vector did not. Furthermore, expression of the *CCD1* gene strongly correlated with the accumulation of ionones in developing raspberry fruit, suggesting that the encoded protein (RiCCD) catalyzed the synthesis of ionones from carotenoids in raspberry fruit (Beekwilder et al. 2008).

Raspberry volatile content depends both upon fruit genotype and growing environment. For example, several studies have shown variation between cultivars in raspberry ketone, α-ionone and β-ionone of up to fourfold, while the monterpenes geraniol and linalool typically vary tenfold and up to 100-fold, respectively (Larsen et al. 1991; Malowicki et al. 2008; Moore et al. 2002; Shamaila et al. 1993). Similarly, variation was observed when the same cultivar was grown at different sites in the same year or when the same cultivar was grown at the same site in different years, although environmental variation tended to be lower than between cultivars (Malowicki et al. 2008; Moore et al. 2002). This indicates the potential for developing molecular markers for fruit volatile content. Indeed, Paterson et al. (2013) were able to identify quantitative trait loci (QTL) for 11 of 12 volatiles mapped in a ‘Glen Moy’ × ‘Latham’ population. These loci exhibited a reasonable degree of consistency across years and cultivation sites, such as open field or polytunnels, indicating their potential utility. A number of candidate genes were identified underlying markers for fruit volatiles. One gene encoding for a deoxy-D-xylulose-5-phosphate reductase, a key enzyme of the MEP pathway, underlies a QTL for β-ionone, whereas genes encoding for terpene synthases underlie QTLs for linalool.

7.4 Lipophilic Components: Carotenoids, Tocopherols and Seed Oils

Raspberries exhibit an exceptional lipophilic antioxidant capacity having the highest value of 32 surveyed fruits, with the sole exception of avocado pears, and also scoring higher than a wide range of vegetables (Wu et al. 2004). Key lipophilic antioxidants in raspberries are the tocopherols and carotenoids.The latter group provides precursors for the key aroma compounds α- and β-ionone and play an important role in the colouration of yellow fruit. Tocopherols and carotenoids share a common biosynthetic precursor (geranylgeranyl pyrophosphate, GGPP), but despite their significance they have been little studied in raspberry fruit. Immature fruits are reported to contain relatively high contents of β-carotene and lutein that decline during fruit ripening (Beekwilder et al. 2008; Carvalho et al. 2013a). This was associated with a decline in chlorophyll-derived pheophytins (Carvalho et al. 2013a) and is consistent with dismantling of light harvesting complexes as green fruit starts to change colour. On a fresh weight (FW) basis tocopherols also declined during ripening (Beekwilder et al. 2008) although it was not clear whether this was due to degradation or dilution as the fruit size increased. In saponified extracts there was a decline in total lutein/FW during ripening (Beekwilder et al. 2008); however, a significant increase in lutein esters, primarily dilauroyl lutein, was observed during ripening. This was associated with an increase in the carotenoid precursor phytoene at the later stages of fruit development consistent with active lutein biosynthesis in ripening raspberry fruit (Carvalho et al. 2013a). Some variation in lutein and esterified lutein content was observed in yellow and red fruited raspberry cultivars, although there was no correlation with fruit colour. More variation was observed in the levels of different tocopherols indicating significant genetic diversity in this trait.

Raspberry seed oils represent a potential value-added byproduct of fruit processing with dried raspberry seeds yielding in the region of 10–20% oil (Oh et al. 2007; Oomah et al. 2000). The oil primarily comprises neutral lipids (94% w/w) with small amounts of free fatty acids and phospholipids (Oomah et al. 2000). In terms of fatty acid composition, raspberry seed oils contain high levels of polyunsaturated fatty acids which exceed 80% of the total fatty acid content. The primary fatty acids are linoleic and α-linolenic acids that are present in a ratio of less than two to one. Oleic acid is the other significant fatty acid present at around 10–12% of total fatty acids, while smaller amounts of stearic (1–2%) and palmitic acids (2–3%) are also recorded alongside trace (<0.5%) amounts of longer chain (C20-C22) fatty acids (Bushman et al. 2004; Oh et al. 2007; Oomah et al. 2000; Parry et al. 2005; Radočaj et al. 2014; Yang et al. 2011). In addition to high levels of polyunsaturated and monounsaturated fatty acids, raspberry seed oils contain high levels of carotenoids (~25 mg/100 g) (Oomah et al. 2000). Similarly, raspberry seed oils contain high levels of tocopherols (up to 400 mg/100 g), higher than many other berry species (Bushman et al. 2004; Parry et al. 2005; Yang et al. 2011) and − sixfold higher than safflower and grape seed oils (Oomah et al. 2000). Very little information is available regarding environmental or varietal influences on raspberry seed oil composition. In one study fatty acid composition was similar between seed oils from yellow and red fleshed varieties, although seed oil yield was about 30% higher in the red variety (Oh et al. 2007).

7.5 Polyphenols, Important Components of Flavour, Appearance and Potential Health Benefits

Raspberry fruit contains a broad range of polyphenolic compounds that include phenolic acids, flavonols, anthocyanins, proanthocyanidins and ellagitannins (Dincheva et al. 2013b). These compounds have been subject to a great deal of research both in terms of their sensory properties, particularly in relation to wine, and for the potential health benefits associated with their consumption where they contribute the majority of the antioxidant capacity of raspberry fruit (Deighton et al. 2000).

Numerous studies have examined the influence of genotype and environment on the total polyphenolic and antioxidant content of raspberry fruit. According to Freeman et al. (2011), oxygen radical absorbance capacities (ORAC) ranged between 34 and 48 µmol trolox equivalents (TE) per g FW among raspberry cultivars, representing <1.5-fold variation between the lowest ('Caroline') and highest ('Autumn Bliss') cultivars. 'Autumn Bliss' was also shown to have a relatively high ORAC value compared against three other cultivars in a previous study, although again the variance in antioxidant capacity remained small with the highest cultivar being only 1.25 higher than the lowest cultivar (Wang and Lin 2000). Since differences in antioxidant capacity between cultivated varieties are low, the introduction of new germplasm into breeding programmes is required to boost antioxidant capacity in cultivated varieties further. However, in a study comparing wild varieties harvested in several Turkish provinces against the widely cultivated varieties 'Heritage' and 'Tulameen' both of the cultivated varieties scored favourably using the ferric reducing antioxidant potential (FRAP) and trolox equivalent antioxidant capacity (TEAC) methods. In particular, 'Heritage' scored higher than 12 out of 14 wild accessions in both measures, while 'Tulameen' also exhibited a greater antioxidant capacity than the majority of wild accessions (Çekiç and Özgen 2010), indicating that germplasm for breeding higher fruit antioxidant capacity might be difficult to obtain. Total polyphenol content is often closely correlated with total antioxidant capacity (Deighton et al. 2000; Dobson et al. 2012; Remberg et al. 2010) and like antioxidant capacity there is some variation in the total polyphenol content of different raspberry cultivars grown under similar environmental conditions. In the majority of reported studies, total polyphenol levels vary less than twofold between cultivars (Anttonen and Karjalainen 2005; Freeman et al. 2011; Krüger et al. 2011; Mazur et al. 2014c; Wang and Lin 2000) and only in a comparison of wild and cultivated genotypes did the variation exceed twofold (Çekiç and Özgen 2010). As with total antioxidant capacity, cultivated raspberry genotypes compared favourably with wild accessions and yellow fruited varieties did not have a conspicuously different phenolic content than red fruited varieties (Anttonen and Karjalainen 2005). In general, total phenolics were reported to be in the range of 200–400 mg/100 g FW, although there was some variation between studies. While environment does have a significant impact on fruit polyphenol content, studies have demonstrated a reasonable maintenance of hierarchy in fruit harvested in different years (Mazur et al. 2014c). Furthermore, several QTL for fruit phenolic content have been demonstrated to exhibit stability in multiple harvest years (Dobson et al. 2012).

Several studies have examined the influence of environment on fruit antioxidant content and phenolics. Mazur et al. (2014c) demonstrated significant variation in both FRAP activity and phenolics in fruit of 'Glen Ample' grown at three different sites in southern Norway that differed primarily in their altitude. Fruit grown at higher altitudes had significantly lower phenolic content and FRAP activity than fruit grown at lower altitudes and this was strongly correlated with the air temperature in the immediate 2 weeks prior to harvest with higher temperatures promoting greater polyphenol accumulation. These data were consistent with previous work in controlled environment chambers that showed a significant increase in FRAP and phenolic content as plant growth temperatures increased between 12 and 24 °C (Remberg et al. 2010). Regarding photoperiod, experiments conducted in controlled environments at 18 °C indicated that extended photoperiods had little impact on either polyphenols or antioxidant capacity (Mazur et al. 2014b).

Taking into consideration (1) the limited genetic diversity in terms of total phenol content and antioxidant activity, and (2) the stability of QTLs for these traits across different environments, there is limited potential for improvement through breeding. However, as outlined below, different polyphenolic compounds have specific impacts on fruit quality and therefore the polyphenolic composition of the fruit might be more important than the absolute amount of polyphenols in the fruit. Indeed, while the consumer may associate antioxidant capacity with potential health benefits, numerous studies indicate that this measurement does not provide meaningful information regarding the health benefits of consumption of specific fruits and any health benefits are likely to be imparted by specific polyphenolic components (Hancock et al. 2007) or their metabolites (Williamson and Clifford 2010).

7.5.1 Phenolic Acids

A number of simple phenolic acids have been detected in raspberry fruits including *p*-coumaric acid, caffeic acid and caffeoyl quinic acid (chlorogenic acid) (Kula et al. 2016; Maksimović et al. 2013). Sugar conjugates of *p*-coumarate and caffeate have also been reported (Dincheva et al. 2013b). Phenolic acids are found at low concentrations, usually less than 10 μg g^{-1} FW (Maksimović et al. 2013), which is at least an order of magnitude lower than concentrations of the more abundant ellagitanins (Gasperotti et al. 2010) and anthocyanins (Scalzo et al. 2008). As minor components they do not contribute significantly to the antioxidant capacity of raspberry fruit (Beekwilder et al. 2005; Mullen et al. 2002).

Phenolic acids represent the entry point into the general phenylpropanoid pathway and are synthesized by deamination of phenylalanine by phenylalanine ammonia lyase (PAL) to form cinnamic acid, which is further converted to *p*-coumaric acid by cinnamate-4-hydroxylase (C4H) (Yu and Jez 2008). *p*-Coumaric acid may serve as substrate for hydroxylation on the 3- position of the benzyl ring to form caffeic acid. The conversion of *p*-coumaric acid to caffeic acid is catalyzed by *p*-coumarate 3-hydroxylase (C3H). Less clear is the situation regarding caffeoyl quinate biosynthesis, where three possible routes have been proposed; in the most probable route (route 1), caffeoyl quinate is synthesised *via* caffeoyl CoA and quinate through the action of hydroxycinnamoyl CoA quinate hydroxycinnamoyl transferase (HQT). In route 2, hydroxycinnamoyl D-glucose/quinate hydroxycinnamoyl transferase converts caffeoyl D-glucose to caffeoyl quinate, while in route 3 cacaffeoyl quinate is formed through a two-step reaction involving hydroxycinnamoyl CoA shikimate/quinate hydroxycinnamoyl transferase (HCT) and C3H. However, the importance of the route 3 for plants has been questioned since *Arabidopsis thaliana* plants are unable to synthesise caffeoyl quinate, although HCT and C3H are normally expressed. (Niggeweg et al. 2004; Ferrer et al. 2008; Payyavula et al. 2015, Fig. 7.4).

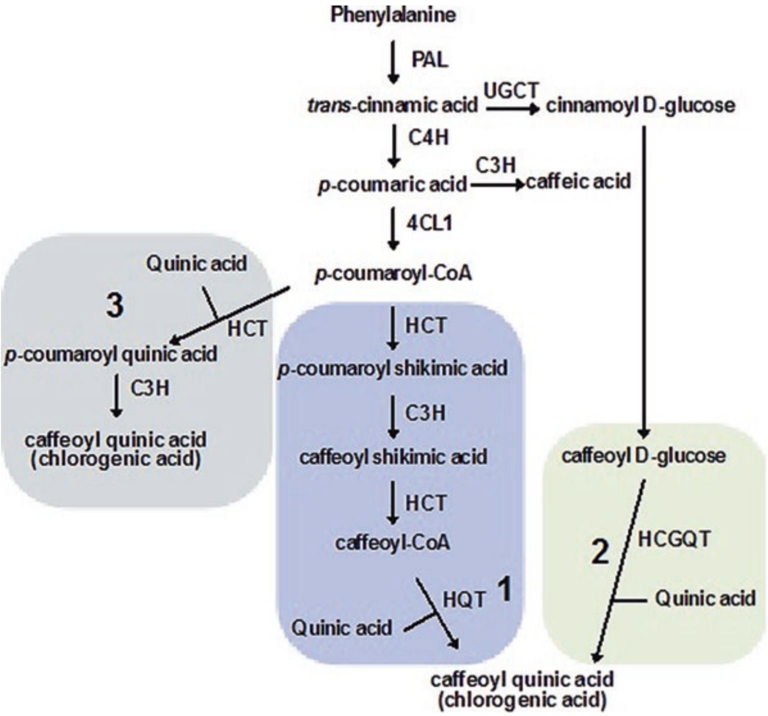


Fig. 7.4 Phenylpropanoid pathway and proposed routes leading to chlorogenic acid synthesis. The three possible routes are marked with different colours and numbered as 1, 2 and 3. PAL phenylalanine ammonia lyase, C4H cinnamate-4-hydroxylase, 4CL1 4-coumarate:CoA ligase, C3H *p*-coumarate 3-hydroxylase, UGCT UDP glucose:cinnamate glucosyl transferase, HCT hydroxycinnamoyl CoA shikimate/quinate hydroxycinnamoyl transferase, HQT hydroxycinnamoyl CoA quinate hydroxycinnamoyl transferase, HCQQT hydroxycinnamoyl CoA D-glucose:quinate hydroxycinnamoyl transferase

PAL is considered to be a key regulator of flux into the phenylpropanoid pathway. Two genes, designated *RiPAL1* and *RiPAL2*, encoding PAL proteins have been isolated from raspberry that exhibit 81% amino acid identity. Both genes were expressed to differing degrees in leaves, shoots, flowers, fruits and roots. In fruits, the pattern of expression depended on fruit development. *RiPAL1* transcripts were most abundant at early fruit stages, whilst *RiPAL2* transcripts were more abundant in fruits that were accumulating colour (Kumar and Ellis 2001). Thus, it was suggested that the two encoded enzymes may be involved in channeling of substrates into flavonoids and anthocyanins, respectively.

Activation of phenolic acids for subsequent synthesis of downstream phenylpropanoids occurs via acylation catalyzed by 4-coumarate:CoA ligase (4CL). Three genes encoding 4CL were cloned from different raspberry tissues with the predicted amino acid sequence for *Ri4CL1* and *Ri4CL2* showing greater similarity (approximately 72% amino acid identity) than the more divergent *Ri4CL3*, with approximately 60% amino acid identity to both *Ri4CL1* and *Ri4CL2*. Recombinant proteins of the three 4CLs exhibited different catalytic properties with hydroxycinnamic and benzoic acids as substrates. Ri4CL1 exhibited the greatest substrate specificity for *p*-coumaric acid, but also accepted caffeic, cinnamic and ferulic acids albeit at 10–30% of the activity exhibited against *p*-coumarate. Ri4CL2 was most active against cinnamic acid, but also accepted *p*-coumarate, ferulate and caffeate. Ri4CL3 exhibited high activity using *p*-coumarate and could also utilize caffeic acid, but not cinnamate or ferulate. In addition, the expression profiles of the three genes were different with *Ri4CL1* most highly expressed in leaves, whilst *Ri4CL2* transcripts most abundant in shoots and roots, suggesting a potential role in providing lignin precursors. *Ri4CL3* was highly expressed in early developing and ripening fruits and taken in conjunction with the high specificity of the enzyme for *p*-coumarate, it was suggested that this enzyme played a key role in the activation of substrates for flavonoid biosynthesis (Kumar and Ellis 2003).

7.5.2 Flavonoids

Anthocyanins play a critical role in raspberry fruit quality being the key compounds providing fruit colour in red-fruited varieties. Alongside the ellagitannins, anthocyanins are described as contributing significantly to the antioxidant capacity of ripe raspberry fruit (Beekwilder et al. 2005; Mullen et al. 2002). On the contrary, other flavonoids, such as flavonols, are present only in low amounts in raspberry fruit and hence their contribution to antioxidant capacity is negligible (Borges et al. 2010). However, as they share a common biosynthetic pathway with the anthocyanins (Fig. 7.5) they will be discussed here.

In red raspberry, the dominant flavonols are quercetin and kaempferol, derivatives of the latter generally being present at concentrations approximately tenfold lower than the former (Carvalho et al. 2013b). Although small amounts of free flavonols have been reported (Carvalho et al. 2013b), they are mainly found as

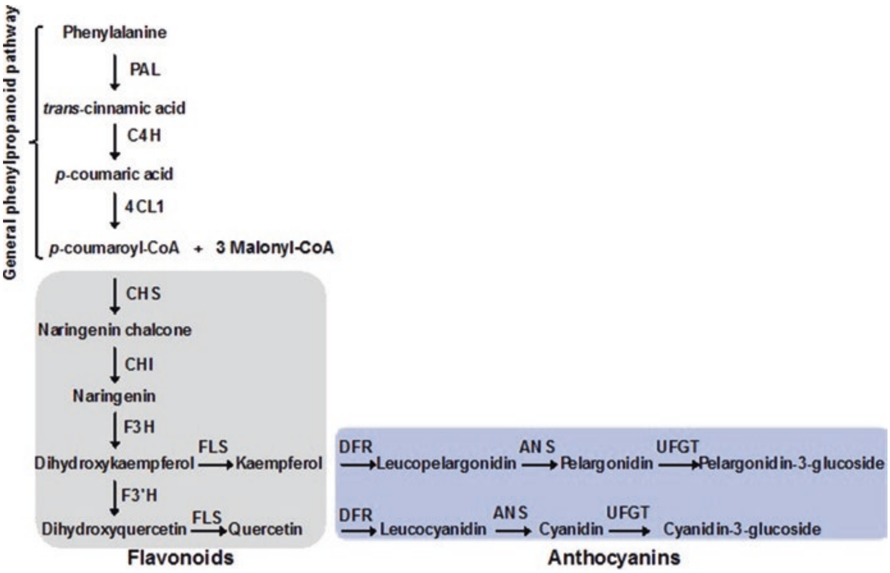


Fig. 7.5 Flavonoid and anthocyanin biosynthetic pathway. PAL phenylalanine ammonia lyase, C4H cinnamate-4-hydroxylase, 4CL1 4-coumarate:CoA ligase, CHS chalcone synthase, CHI chalcone isomerase, F3H flavanone 3-hydroxylase, F3'H flavonoid 3-hydroxylase, FLS flavonol synthase, DFR dihydroflavonol 4-reductase, ANS anthocyanidin synthase, UFGT UDP-glucose flavonoid 3-O-glucosyltransferase

conjugates of mono- and disaccharides, such as glucose, galactose, glucuronate, rutinose and sophorose (Borges et al. 2010; Carvalho et al. 2013b; Dincheva et al. 2013b; Kula et al. 2016). In total, flavonol content in raspberry fruit is generally lower than 0.1 mg g^{-1} FW (Carvalho et al. 2013b).

For anthocyanins, only cyanidin and pelargonidin derivatives have been described in red raspberry with cyanidin derivatives being dominant (Beekwilder et al. 2005; Borges et al. 2010; Chen et al. 2014; Kula et al. 2016; Scalzo et al. 2008). The anthocyanidin moieties are present as glucosides, sophorosides, glucosylrutinosides, rutinosides and xylosylrutinosides. Concentrations of cyanidin derivatives range from approximately 20 mg kg^{-1} fresh weight for the less abundant components, such as cyanidin-xylosylrutinoside, up to hundreds of mg kg^{-1} for the more abundant components, such as cyanidin-sophoroside and cyanidin-glucoside (Scalzo et al. 2008). Pelargonidin derivatives tend to represent less than a tenth of the total anthocyanins (Borges et al. 2010; Chen et al. 2014; Kula et al. 2016).

Flavonol and anthocyanin biosynthetic pathways are well established in many species, including raspberries. In the initial step in the pathway, the polyketide synthase, chalcone synthase, catalyses the conjugation of 4-coumaroyl-CoA with three molecules of malonyl-CoA to produce naringenin chalcone. Zheng et al. (2001) identified three polyketide synthase genes that were amplified by PCR-based cloning from cv. 'Royalty'. Heterologous expression in *E. coli* revealed that only one of the genes, *RiPKS1*, encoded a chalcone synthase, whilst the other two encoded a

4-coumaroyltriacetic acid lactone synthase and a non-functional protein, respectively. Subsequent work by the same group identified two further polyketide synthases in the 'Royalty' genome (Zheng and Hrazdina 2008). As discussed above, *RiPKS4* encoded an enzyme with both benzylacetone synthase activity, important for the production of a key aroma compound, and chalcone synthase activity. *RiPKS5* exclusively used 4-coumaryl-CoA as substrate synthesizing only naringenin chalcone. A recent RNA-seq transcript analysis of cv.'Nova' confirmed multiple sequences encoding polyketide synthases and 9 different unigenes encoding chalcone synthases were identified (Hyun et al. 2014). This confirmed a previous report in which 11 genes were cloned from cv. 'Meeker' (Kumar and Ellis 2003). Detailed expression analysis of 3 out of the 11 CS genes revealed that they were differentially expressed leaves, shoots, roots, flowers, and fruit. However one gene exhibited strong developmental control in ripening fruit that was consistent with a function in anthocyanin accumulation. All three of these genes encoded proteins exhibiting typical chalcone synthase activity.

Downstream of naringenin chalcone, chalcone isomerase is responsible for the formation of naringenin, which is further converted to dihydrokaempferol by the action of the P450 flavanone 3-hydroxylase. A second P450 flavonoid 3′-hydroxylase converts dihydrokaempferol to dihydroquercetin and these compounds can be reduced to the flavonols kaempferol and quercetin, respectively by the action of 2-oxoglutarate-dependent flavonol synthase. The observation that these compounds are minor components in raspberry fruit, with respect to anthocyanins, suggests that this enzyme is relatively inactive in this tissue. Many species additionally contain a flavonoid 3′,5′-hydroxylase that synthesises dihydromyricetin, the precursor of the flavonol myricetin and the anthocyanidin delphinidin. However the absence of these components suggests that raspberries lack an active enzyme, despite the identification of a gene potentially encoding this activity (Hyun et al. 2014). The anthocyanidins cyanidin and pelargonidin are synthesised from dihydroquercetin and dihydrokaempferol, respectively, by the combined actions of dihydroflavonol 4-reductase and leucoanthocyanbidin dioxygenase (anthocyanidin synthase). Subsequent glycosylation for the formation of mature anthocyanins is catalyzed by a series of glycosyltransferases.

Despite the detailed analysis of genes and proteins involved in the early parts of the biosynthetic pathway in raspberries, very little work has been reported concerning the later steps of biosynthesis. RNA sequencing indicates that all steps are encoded by multiple transcripts and differential expression analysis has indicated candidates significant for anthocyanin accumulation in fruit (Hyun et al. 2014). In a more targeted analysis in red and yellow fruited varieties, there was significantly higher expression of chalcone synthase, flavanone 3-hydroxylase, dihydroflavanol 4-reductase and glycosyltransferase in both varieties during fruit ripening than at earlier stages of development. However, anthocyanidin synthase expression remained low at both developmental stages in the yellow fruited variety. Furthermore, the yellow fruited variety had a 5 bp insertion in the coding region of anthocyanidin synthase resulting in an early stop codon. It was demonstrated by complementation of an Arabidopsis anthocyanin mutant that the inserted gene resulted in the synthesis of an inactive protein indicating that the mutation led to the yellow fruit colour, at least in the cultivar examined (Rafique et al. 2016).

The role of environment and genotype on fruit anthocyanin content has been examined in detail and at least two groups have identified quantitative trait loci associated with colour and anthocyanin content. Several studies have indicated a seasonal effect on the content of both total and individual anthocyanins (e.g. Mazur et al. 2014c) and specific environmental parameters, such as temperature (Bradish et al. 2012) and light (Wang et al. 2009) have been correlated with the content of specific anthocyanins. Although environment does have an influence on anthocyanin content, there is also a high potential for successful breeding of desired fruit anthocyanin content. Indeed, detailed work to investigate the heritability of total and individual anthocyanins in 42 families derived from 13 raspberry cultivars indicated high narrow sense heritability estimates both for individual and total anthocyanins, suggesting that breeding could be a powerful means towards increasing anthocyanin content in raspberry (Connor et al. 2005). Several studies have further enhanced the potential for breeding for bespoke anthocyanin content by developing molecular markers and identifying quantitative loci underlying the anthocyanin trait. Kassim et al. (2009) examined anthocyanin content of a raspberry population comprising a cross between the North American cultivar ‘Latham’ and the European cultivar ‘Glen Moy’. The parents of this population exhibited significant differences in total and individual anthocyanin content, with ‘Latham’ containing almost double the quantity of total anthocyanins. QTL analysis revealed two major chromosomal regions on linkage group 1 and 4 that were highly associated with the content of eight and seven individual anthocyanins, respectively. These QTL were consistent over two separate years and explained between 10% and 65% of the variation dependent on the specific anthocyanin, year of analysis and marker. Analysis of genes associated with the markers failed to identify any genes encoding biosynthetic enzymes; however, several genes encoding transcription factors were identified. In a separate study, a population comprising a cross between black (*R. occidentalis*) and red (*R. idaeus*) raspberry was phenotyped for anthocyanin content across three growing seasons (Bushakra et al. 2013). Linkage analysis in this population identified a total of 27 QTL of which 6 were stable in all 3 years of the analysis. This analysis not only confirmed some of the candidate transcription factors identified by Kassim et al. (2009),but additionally identified markers associated with several biosynthetic pathway genes.

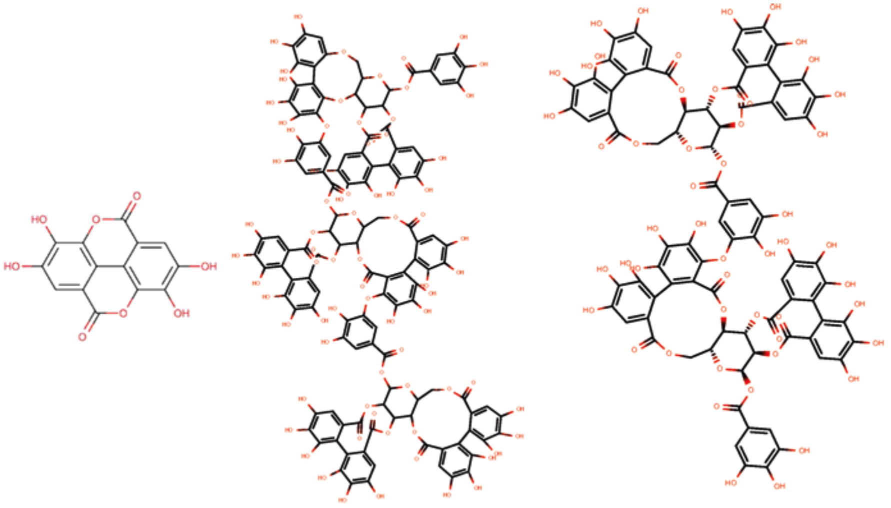
7.5.3 Tannins: Ellagitanins and Proanthocyanidins

A distinguishing feature of raspberries is their high level of ellagitannins in relation to other berry fruits. For example, the major ellagitannins, lambertianin C and sanguiin H-6, were reported at ~300–1000 nmol g^{-1} FW in raspberry fruits and represented >50% of the total fruit antioxidant capacity (Beekwilder et al. 2005; Borges et al. 2010). By comparison, anthocyanins totaled <900 nmol g^{-1} FW and represented less than 20% of antioxidant capacity. In addition to providing a significant proportion of fruit antioxidant capacity, the ellagitannins and proanthocyanidins impart important astringency, thereby having significant impact on the sensory properties of the fruit (He et al. 2015).

The levels of proanthocyanidins in raspberry fruit are considerably lower than the ellagitannins (Gu et al. 2003) and may be closely associated with seeds (Godevac et al. 2009; McDougall et al. 2014). Proanthocyanidins are polymers of flavan-3-ols synthesised from leucoanthocyanindins, in the case of raspberry leucocyanidin or leucopelargonidin, or anthocyanidins catalysed by leucoanthocyanidin or anthocyanidin reductase, respectively (He et al. 2015). Despite significant research effort, mechanisms of polymerization and the genes and enzymes required for these reactions remain largely unknown.

Ellagitannins are also polymeric polyphenolic compounds, in this case of ellagic acid. Raspberry fruits have been reported to contain free ellagic acid, ellagic acid glycoconjugates and a range of di- and tri-meric ellagitannins (Fig. 7.6). The most abundant compounds are the ellagitannins sanguiin H6 and lambertianin C, which were reported at concentrations in the ranges of 0.4–0.7 and 0.2–0.6 mg g^{-1} FW, respectively, in a number of raspberry cultivars harvested in northern Italy (Gasperotti et al. 2010). Other sanguiin and lambertianin derivatives were present at lower concentrations. Free ellagic acid and several ellagic pentosides were also abundant being reported at concentrations up to 0.16 mg gFW^{-1}.

Although the latter stages of ellagitannin biosynthesis remain obscure, progress has been made in the early parts of the biosynthetic pathways in recent years.



Ellagic acid Sanguiin H-6 Lambertianin C

Fig. 7.6 Structure of major ellagitannins found in raspberry fruit

Labelling experiments indicate that gallic acid is the precursor to ellagic acid in strawberries and raspberries (Schulenburg et al. 2016). Gallic acid has been demonstrated to be a product of the shikimate pathway, where atypical dehydroquinate dehydrogenases/shikimate dehydrogenases produce significant amounts of gallic acid both *in vivo* and in grapevine hairy root cultures (Bontpart et al. 2016). Further conversion of gallic acid to ellagic acid requires the glucosylation of gallic acid, with β-glucogallin as a key intermediate. Recent work identified five genes encoding putative gallic acid:UDP-glucose glucosyltranseferases in the genomes of *Fragraria vesca*, *Fragraria x ananassa* and *Rubus idaeus* based upon sequence homology to grapevine genes. Kinetic analysis of the five glycosyltransferases (GTs) revealed that FaGT2 had the highest catalytic efficiency (k_{cat}/k_m) against gallic acid (Schulenburg et al. 2016). Instead, RiGT2 showed higher affinity for cinnamic acid, coumaric acid derivatives and benzoic acid derivatives, similar to FvGT2. A better understanding of the genes involved in this key catalytic step would improve the prospects for breeding for high or low ellagitannin content in the future.

7.6 Potential Health Benefits Associated with Raspberry Polyphenol Consumption

Raspberries are dominated by high levels of polyphenols (total polyphenol contents of >200 mg/100 g $FW^{-1}$are common; Deighton et al. 2000) and these components have been a specific focus of attention with respect to understanding health benefits associated with raspberry consumption.

A concept has developed that the high antioxidant capacity of polyphenol components could contribute to health benefits by ameliorating the detrimental effects of reactive oxygen species (ROS) generated in the body through oxygen metabolism (Halliwell 2007). It was proposed that they could act as chain-breaking agents preventing the ROS from instigating free radical cascades that could damage cells, DNA and membranes and through accumulation cause diseases. However, this simple attractive precept is not generally tenable and high antioxidant capacity in the test-tube does not automatically translate into *in vivo* effectiveness. Although berry polyphenols may have high antioxidant capacity, their effectiveness is limited by their often low (μM – nM) uptake into the blood stream. Indeed, the effectiveness of even the small proportion taken up in to the serum is exacerbated by further metabolism in the liver and excretion through the bile or urine (Koli et al. 2010). In many cases, the original components are effectively absent and their circulating metabolites may differ greatly in structure and potential function (Williamson and Clifford 2010). In fact, different polyphenols have different stabilities, bioavailability and therefore potential effectiveness.

Berry polyphenols that are not taken up into the blood stream could still have beneficial functions in the gastrointestinal tract (GIT) as they pass through the digestive system such as preventing the formation of oxidation products from foods in the stomach and GIT (Gorelik et al. 2005) or by influencing food digestion, glucose levels and calorie usage (McDougall et al. 2008a). Berry polyphenols that pass through the upper digestive tract and reach the colon are subject to fermentation by bacteria and this produces a range of long-lasting phenolic metabolites that recirculate in the bloodstream, which could be the causative agents for some beneficial effects (Williamson and Clifford 2010).

The importance of understanding component bioavailability is neatly illustrated by the fate of ellagitannins, the major polyphenol components of raspberries. These large components remain in the GIT and break down to release ellagic acid (McDougall et al. 2014), which is subsequently metabolised by colonic bacteria to urolithin degradation products (Tomas-Barberan et al. 2017). These urolithins and their phase II metabolites reach significant levels in the serum (Ludwig et al. 2015) and these have been shown to have anti-cancer, cardiovascular and neuroprotective effects (Gonzalez-Sarrias et al. 2017a, b; Savi et al. 2017) at physiologically-relevant levels. Therefore, these microbiota-derived derivatives may deliver some of the health benefits associated with ellagitannin intake from raspberries.

7.6.1 Anti-Cancer Effects

There is considerable evidence that components from berries of *Rubus* species can influence the proliferation of human cancer cell models. Evidence has been obtained from a range of cancer lines originating from different organs and body locations from prostate to lung, breast to colon and blood to cervical cancers. Many studies have not adequately defined their extract composition so making assumptions about effective components is impossible and sometimes certain components are proposed to be causative without sufficient evidence. Within the polyphenols, many studies have highlighted the possible role of anthocyanins (Bowen-Forbes et al. 2010) but previous work suggested that ellagitannins from raspberry were more effective against cervical cancer cells than anthocyanins (Ross et al. 2007). Indeed, extracts from berry species rich in ellagitannins, such as the *Rubus* species, have been found to be particularly effective in cell line studies (McDougall et al. 2008b; Seeram et al. 2006) and individual ellagitannin components, such as Sanguiin H6, have potent effects in cancer cell models (e.g. Sakai et al. 2016; Lee et al. 2016; Lee and Lee 2005) or models of cellular inflammation (Sangiovanni et al. 2013), perhaps through degradation and release of ellagic acid *in situ* (Whitley et al. 2006).

Considering the issues of bioavailability outlined above, the use of cell lines derived from GIT cancers which could be in contact with active components in the digestive tract could be more physiologically-relevant models (Brown et al. 2012). For example, red raspberry extracts were found to inhibit the migration and invasion ability of oral cancer cells and altered metastasis by suppression of MMP-2 expression through the FAK/Scr/ERK signaling pathway (Hsieh et al. 2013). Modelling the effects of gastrointestinal digestion using *in vitro* digestion (IVD) systems provided a clearer picture of the physiological stability of red raspberry polyphenol components and confirmed that these gut-accessible components were protective in models of colon cancer (Coates et al. 2007). Other studies have suggested that anticancer effects of blackberry and wild *Rubus* extracts in colon cell models were enhanced by IVD treatment (e.g. Chen et al. 2016a, b) and again effectiveness was correlated with the survival of specific components. Furthermore, subsequent fermentation of IVD extracts using faecal bacteria provided a range of faecal metabolites and confirmed that anti-cancer effects survive bacterial degradation in the colon (Brown et al. 2014) although the original polyphenol components had been extensively metabolised to simpler phenolics.

7.6.2 Diabetes

The incidence of type 2 diabetes has reached near-epidemic proportions in the Western world. Following the outline of Hanhineva et al. (2010), there are four main areas where *Rubus* berries could beneficially influence glycemic control (i) protection of pancreatic β-cells from glucose-induced toxicity and oxidative stress, (ii) inhibition of starch digestion and absorption to control blood glucose levels, (iii) suppression of glucose release from the liver and (iv) improvement of glucose uptake in peripheral tissues such as muscles.

In the pre-diabetic state, the pancreatic β-cells (which secrete insulin) become dysfunctional and decline in number due to glucose-induced toxicity and oxidative stress (Hanhineva et al. 2010). Considerable evidence has accrued that polyphenols found in raspberries can maintain insulin secretion in β-cells grown in culture (Martineau et al. 2006), protect against oxidative damage induced by elevated glucose in rats (Rodrigo et al. 2011), and modulate insulin secretion and function in humans (Seymour et al. 2011; Stull et al. 2010). However, none of these studies used *Rubus* extracts.

Many dietary polyphenols are retained in the gastrointestinal tract and pass through to the colon without substantial absorption. These components can interact and modulate the digestion of glycaemic carbohydrates, namely starch and sucrose. Polyphenol-rich extracts from berries inhibit α-amylase and α-glucosidase *in vitro* (McDougall et al. 2005), the key enzymes involved in glucose production from starch. The degree of inhibition differed between the berries and was linked to their polyphenol composition. Fractionation studies suggested that tannins (ellagitannins and proanthocyanidins) were potent inhibitors of amylase (Grussu et al. 2011). Therefore, raspberry extracts were effective against amylase but less so against glucosidase. Indeed, a range of polyphenols may be capable of inhibition of glucosidase (Lo Piparo et al. 2008; Boath et al. 2012). Depending on their phytochemical composition, it is therefore possible that specific berries could inhibit both amylase and glucosidase and synergistically reduce the pool of glucose available for uptake into the blood. This raises the possibility of breeding to enhance the content of specific polyphenols that inhibit α-glucosidase further enhancing the capacity of raspberry fruit consumption for glycaemic control.

Purified polyphenols can additionally influence intestinal absorption of glucose through interaction with sodium-dependent glucose transporter (SGLT1) and the glucose transporter, GLUT2 (Hanhineva et al. 2010) in the GIT. In addition, strawberry polyphenols decreased glucose transport across gut epithelial cells through inhibition of both SGTL1 and GLUT2 (Manzano and Williamson 2010).

Purified polyphenols commonly found in *Rubus* spp. increased basal and insulin-stimulated glucose uptake in muscle cells (Claussnitzer et al. 2011) and basal glucose uptake was increased in muscle cells through activation of the adenosine monophosphate kinase system (Eid et al. 2010). The anthocyanin, cyanidin-3-glucoside, which is common in raspberries (and its metabolite protocatechuic acid) exerted insulin-like effects in a human adipocyte model (Scazzocchio et al. 2011).

Polyphenols may interact with the insulin-sensing pathway and modulate glucose release from the liver through reduction in glucose synthesis. Polyphenols appear to act on signal transduction pathways and influence the phosphorylation status of key transcription factors such as FOXO1a (Cheng and White 2011; Takikawa et al. 2010).

Taken together this research illustrates the capacity of raspberry polyphenols to modulate diabetic control at numerous levels. It is also clear that different components influence different aspects of glucose control opening up the possibility of breeding to enhance specific polyphenolic components to improve the potential benefits of raspberry fruit consumption even further.

7.6.3 Cardiovascular Disease (CVD)

Substantial evidence has been provided that berry components can influence parameters relevant to CVD in *in vitro* cell studies and animal models. For example, berry extracts including *Rubus spp* beneficially modulated function in endothelial cells *in vitro* (Tulio et al. 2012). Polyphenol-rich extracts of red raspberry, black raspberry and blackberry fruits attenuated angiotensin-II-induced senescence in vascular smooth muscle cells through reduction of intracellular reactive oxygen species (ROS). The different extracts operated by both NOx-1-dependent and independent mechanisms (Feresin et al. 2016).

Polyphenols may aid the muscle layer of blood vessels to relax (i.e. vasodilation). Endothelial cells, which make up the inner layer of blood vessels, produce nitric oxide which regulates blood pressure. Polyphenols found in berries can increase the activity of endothelial nitric oxide synthase (eNOS) to stimulate nitric oxide (NO) production and increase vasodilation (Galleano et al. 2010). Indeed, ethyl acetate extracts of red raspberries promoted antihypertensive effects in spontaneously hypertensive rats through enhanced NO production (Jia et al. 2011).

A review of human intervention studies with fruit polyphenols (Chong et al. 2010) found inconsistent but positive effects on CVD risk factors and suggested that the inconsistency was due to differences in experimental design and treatment groups. However, other analyses suggest that common berry components such as anthocyanins may beneficially influence clinical parameters associated with enhanced risk of CVD (Basu et al. 2010). Individuals with elevated cholesterol levels improved their endothelium-dependent vasodilation after berry anthocyanin intake along with improved serum lipid profiles and decreased markers of inflammation (Zhu et al. 2011).

7.6.4 Obesity

Obesity is often associated as an underlying risk factor in CVD, metabolic syndrome and diabetes. Berries have also been implicated in the prevention of obesity perhaps through interference with lipid digestion (McDougall et al. 2009) and/or modulation of lipid metabolism (Prior et al. 2011). Extracts from *Rubus* species were found to be particularly effective in inhibiting pancreatic lipase *in vitro* (McDougall et al. 2009), with ellagitannins particularly effective.

There is substantial evidence for effects of *Rubus* spp. in cell line studies. For example, water extracts of unripe *R. coreanus* caused anti-hyperlipdemic effects in liver cells through inhibition of cholesterol biosynthesis brought about by reduced apolipoprotein release and modulation of FOXO-1 signalling pathways (Bhandary et al. 2012; Jeong et al. 2013). Butanol extracts from unripe *R. coreanus* inhibited the differentiation of adipocytes (Oh et al. 2016) and five components (ellagic acid, erycibelline, 5-hydroxy-2-pyridinemethanol, *m*-hydroxyphenylglycine, and 4-hydroxycoumarin) were identified that could be responsible for this effect.

In an interesting study, a range of freeze dried berry powders (including black raspberry, red raspberry, and blackberry) did not prevent weight gain or hyperlipidemia in high-fat fed rats (Prior et al. 2009). However, purified anthocyanins from blackberries were effective, which suggests that these red pigments were important for lipid-lowering effects (Prior et al. 2010). Other components known to be present in *Rubus* fruits have also been implicated. Ellagic acid attenuated high fat/high sugar (HF/HS) diet effects on hyperlipidemia through antioxidant and anti-inflammatory effects (Panchal et al. 2013).

R. coreanus extracts reduced weight gain in mice whilst reducing white adipose tissue (Jeong et al. 2015) and other studies on brown pre-adipocytes also showed increases in thermogenic gene expression. Boysenberry (a *Rubus* hybrid) juice inhibited weight gain in high-fat fed rats and inhibited pancreatic lipase *in vitro* and triglyceride absorption from the gastrointestinal tract *in vivo*. Fractionation suggested that fractions enriched in ellagitannins and ellagic acid conjugates, and anthocyanins were most effective in inhibiting triglyceride uptake *in vivo* (Mineo et al. 2015). However, pure ellagic acid was not effective.

Raspberry juice and raspberry puree decreased weight gain in HF- fed mice with concomitant significant reductions in resistin levels (Luo et al. 2016). Extracts of *R. crataegifolius* significantly reduced weight gain in HF-fed rats with concomitant reductions in epididymal fat weight, serum cholesterol and triglycerides (Jung et al. 2016). Raspberry seed flour reduced HF/HS-diet-induced hyperlipidemia and biomarkers of inflammation in C57BL/6 male mice (Kang et al. 2016). The researchers suggested that ellagic acid derviatives may be important but raspberry seed flour would contain other phenolic components including proanthocyanidins (Godevac et al. 2009; McDougall et al. 2014) and substantial levels of dietary fibre.

7.6.5 Neuroprotective Effects

A body of evidence has developed that supports a role for berry polyphenol components in neuroprotection (Miller and Shukitt-Hale 2012). The berry components are proposed to protect against damage induced by ROS, which are known to be implicated in the development of neurological conditions such as Alzheimer's disease (Spencer 2010). The brain represents only 2.5% of the body's weight but it receives 15% of the cardiac output, and consumes 20% of total body oxygen and 25% of total body glucose utilization. Consequently this highly oxygenated environment means that the brain is particularly prone to damage induced by ROS and it has innately less-well developed antioxidant mechanisms. Berry components are proposed to mediate in cell signalling pathways that potentiate antioxidant mechanisms and influence inflammatory responses. A recent review has outlined neuroprotective effects of red raspberries (Burton-Freeman et al. 2016) and this section extends this to other *Rubus* species.

Polyphenol-rich *R. coreanus* extracts exhibited protective effects on neuronal PC-12 cells (Im et al. 2013). Extracts from wild, but not cultivated, blackberry varieties provided neuroprotective effects in cell models (Tavares et al. 2012, 2013) through reduction in intracellular reactive oxygen species, modulation of glutathione levels and caspase activation. These effects were potentiated in extracts that had undergone simulated gastrointestinal digestion. Red raspberry extracts protected both neuronal and microglial cells against H_2O_2-induced oxidative stress, lipopolysaccharide-induced inflammation and NO production (Garcia et al. 2012). The anti-inflammatory activity was retained by extracts that had undergone simulated intestinal digestion and appeared to act through inhibition of IBa1 expression and TNF-α release. *In vitro* studies also support the premise that pure polyphenols also found in *Rubus spp* can beneficially remodel amyloid-beta aggregation *in vitro* (Ladiwala et al. 2011), a process implicated in brain damage in Alzheimer's disease.

7.7 Conclusions

In recent years significant advances in our knowledge regarding the pathways of biosynthesis and mechanisms of control of accumulation of important raspberry fruit metabolites has greatly increased. Alongside this, knowledge about the phytochemistry underlying fruit sensory attributes continues to accumulate and mechanistic work to understand the role of specific fruit phytochemicals in modulating human disease status gathers apace. This has been accompanied by an increase in knowledge regarding raspberry genetics, and genes and loci underlying specific fruit phytochemical traits. Taken together, this raises the very real possibility of breeding new raspberry cultivars that deliver the consumer with the sensory experience they seek whilst delivering a fruit rich in phytonutrients that will protect and even reverse common degenerative diseases.

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Chapter 8
QTL Mapping and Marker Assisted Breeding in *Rubus* spp.

Susan McCallum, Craig Simpson, and Julie Graham

8.1 Background

Recent developments in genetics and genomics have advanced research in all crops including soft fruit species. Molecular markers which detect genome-wide variability in both protein coding and non-coding regions have enabled genetic mapping studies to move beyond linkages between simple morphological traits (Jennings 1967a, 1988; Ourecky 1975; Crane and Lawrence 1931; Keep 1968) to linkage maps containing numerous genetic markers which can be utilised in marker assisted breeding. Until recently, mapping in blackberry and other *Rubus* species has lagged behind that of red raspberry due to their more complex genetic make-up and lesser economic importance.

The marker techniques developed for linkage mapping include amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), simple sequence repeats (SSR), and Single nucleotide polymorphisms (SNPs) (Antonius-Klemola 1999; Hokanson 2001; Graham et al. 2002; Stafne et al. 2005; Woodhead et al. 2008, 2010; Castillo et al. 2010; Dossett et al. 2012; Bassil et al. 2014). Utilising a range of markers as they have been developed has led to the generation of linkage maps which provide a framework for increased efficiency of selection, where markers linked to the gene(s) or quantitative trait loci (QTL) underlying the trait(s) of interest can be developed further for use in breeding programmes, as well as a research tool for assisting in the understanding of the genetic control of desirable phenotypes.

The first marker-based genetic map of raspberry was developed by Graham et al. (2004) utilizing SSR and AFLP markers in a full sibling population of 'Latham' x 'Glen Moy'. SSR markers were developed from both genomic and cDNA libraries from the cultivar 'Glen Moy', and AFLP markers further saturated the map.

S. McCallum (✉) · C. Simpson · J. Graham
Cell & Molecular Sciences, The James Hutton Institute, Dundee, UK
e-mail: susan.mccallum@hutton.ac.uk

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The initial map consisted of nine linkage groups (LGs) with 273 markers covering 789 cM of map distance and has subsequently been enhanced in a number of QTL mapping studies (Graham et al. 2006, 2009, 2011, 2014; Woodhead et al. 2010, 2013; Kassim et al. 2009; McCallum et al. 2010; Paterson et al. 2013; Simpson et al. 2017). Aside from the 'Latham' x 'Glen Moy' population, a number of other linkage maps in red raspberry have been generated. A 'Latham' x 'Titan' population (Pattison et al. 2007) was used to construct a map based on AFLP, RAPD, and uncharacterized resistant gene analog polymorphism (RGAP) markers. Sargent et al. (2007) generated a map from a 'Malling Jewel' x 'Malling Orion' cross using AFLP and SSR markers. Ward et al. (2013) used Genotyping by Sequencing (GBS) to produce highly saturated maps for a *R. idaeus* pseudo-testcross progeny. GBS has also been applied to the 'Latham' x 'Glen Moy' population (Hackett et al. submitted.) to greatly enhance marker saturation. Castro et al. (2013) published the first genetic map of a primocane-fruiting and thornless tetraploid blackberry (*Rubus* subgenus *Rubus* Watson). Bushakra et al. (2015) constructed the first linkage map of black raspberry (*Rubus occidentalis*) using single-nucleotide polymorphism and simple sequence repeat markers representing seven linkage groups. This research group also created a new genetic mapping population from a cross between black and red raspberry. They performed comparative genomic mapping by using BLAST analysis of 131 markers from the black and red raspberry linkage map, with genomic sequence of strawberry, apple and peach. Over two-thirds of the markers showed a near perfect match with strawberry linkage groups and each of the seven *Rubus* LGs were aligned to each of the seven strawberry chromosomes supporting high synteny between *Rubus* and *Fragaria*. Synteny was reduced in apple and peach with *Rubus* LGs aligning with parts of the apple and peach chromosomes (Bushakra et al. 2012). Breeding and genetic studies are already greatly benefiting from these marker based linkage maps, which have allowed the linking of traits to chromosomal loci and in some cases, even the genes responsible for trait variation. In light of climate change and other legislative constraints on production, there is an increasing need for both conventionally valued traits like quality but also traits that will improve the sustainability and resilience of these crops by reducing the need for pesticides and other chemical inputs; managing water requirements and other climatic responses and responding to the desire by consumers for locally grown food. Many pests and pathogens can affect red raspberries including viruses, fungal diseases, aphids and beetles. Viruses are a particularly serious problem as an infected plant is unlikely to recover and future propagules will also be infected eg. RBDV (Ourecky 1975).

The development of new raspberry cultivars is a long and challenging process with breeders faced with increasing demands from consumers to produce high quality aromatic fruits. At the same time growers require pest and disease resistant varieties capable of utilising the extended growing season and with resilience traits adapted to changing climatic conditions. Breeding is discussed in Chap. 2 of this volume. In this chapter we discuss where QTL mapping has been applied towards the understanding and improvement of *Rubus* crops for a range of traits to meet the various challenges in production.

8.2 QTL Studies for Pest and Disease Resistance

Increasing pressure from governments, end-users and consumers to reduce chemical inputs has meant that the incorporation of resistance/tolerance traits into new cultivars is a key factor in ensuring future sustainability. The soft fruit industry faces particularly serious challenges, as it relies on a small number of cultivars which are generally bred for fruit quality. Together with a steady decline in the number of available chemicals for pest and disease control, there is a strong possibility that within 10–15 years the fruit industry will have to operate in an environment with few or no conventional pesticides and fungicides. In order to survive and retain viability, the soft fruit industry therefore needs a renewal of approaches that select either specific resistance genes, and/or physical or structural characteristics linked to resistance. An overall decline in plant defense in domesticated crops has been linked to changes in gross plant morphology, reduced variation in plant phenology and modification of secondary plant metabolites (Chen et al. 2015). Plant defensive traits could be exploited more widely as part of any crop protection strategy. Studies on the cost/benefits of fungicide/pesticides vs resistant varieties identified a x 4 return on investment in chemical controls but this increased to a 10–12 times return on resistance breeding with the added advantage of reducing environmental impacts and application costs (Pimentel et al. 1992, 1997; Morris and Heisey 2003).

Sources of resistance to many pests and diseases have been identified in diverse *Rubus* species and exploited in conventional cross-breeding (Keep et al. 1977; Jennings 1988; Knight 1991; Williamson and Jennings 1996). However, germplasm bearing single resistance genes when planted over extensive areas can in many cases, depending on the mode of action, eventually be overcome by the rapid evolution of new biotypes of pests or virulent races of pathogenic fungi. New types of host resistance are therefore required to sustain plant protection (Birch et al. 2002; Jones 2002). Although studies frequently focus on individual traits, plant defense is more likely to involve a suite of traits, and there is surprisingly little evidence for trade-offs in investment between multiple defenses (Koricheva et al. 2004). Widespread use of tunnels and covers in soft fruit growing systems in recent years enables more use of natural enemies against pests and development of resistant cultivars are a key factor in the success of such integrated systems (Birch et al. 2011). However, increasingly specific demands by multiple retailers and processors mean that any advances in the resistance status of new cultivars must maintain the harvested quality of the crop. Marker assisted breeding can therefore assist in the development of resistant varieties through identification and subsequent transfer of linked markers of resistance/tolerance traits into breeding programmes. A number of major pests and diseases affect *Rubus*, some sporadically while others have serious longer-term impacts on cultivation. In *Rubus* control of root rot, weevils, mites, aphids, cane diseases and viruses consistently require applications of pesticides. Pest and diseases of raspberry in Europe have been extensively reviewed in Gordon et al. (2006) and Jennings and Dolan (2014). Emerging pests such as spotted wing Drosophila (SWD) (*Drosophila suzukii*) with a wide host range including cane and bush crops will also require strategies for control, although so far there are few indications of robust sources of resistance.

8.2.1 Raspberry Root Rot

Root rot, caused by *Phytophthora rubi,* continues to be one of the most serious and destructive diseases of raspberry (See Seemüller et al. 1986; Duncan et al. 1987; Harrison et al. 1998) and poses a significant threat at present to the industry's survival in Northern Europe. There are no effective control strategies nor any commercially accepted resistant varieties, and uptake of new high-yielding but highly susceptible cultivars has been a major factor in the spread of the disease within Europe. Traditionally the lifetime of a raspberry plantation was >15 years, but raspberry root rot has greatly diminished this, with some growers now treating raspberry as a long cane annual crop whilst others assume a maximum lifespan of up to 6 years due to disease pressure. Large parts of the Northern European raspberry industry are now growing in pots with coir as a result of *P. rubi* infestation in available soils. Root vigour has been shown to have an impact on the ability of the plant to resist *Phytophthora* root rot infection (Graham et al. 2011). The demonstration of a highly significant correlation between the root sucker parameters and root rot resistance is of great value to breeders as it provides a simple visual screen for identifying germplasm with some level of resistance/tolerance to *Phytophthora* root rot (Graham et al. 2011). QTL for root vigour on LG3 and LG6 co-locate with resistance and can be used alongside markers identified.

In terms of QTL mapping, screening cultivars of red and other raspberries and wild *Rubus* species have identified a few potential sources of resistance including cvs. Latham and Winkler's Sämling and species material such as *R. strigosus, R. occidentalis* and *R. ursinus* (Barritt et al. 1979; Jennings 1988), though the basis of resistance remains unknown. 'Latham' is one of the few sources of root rot resistance that has remained durable, suggesting a complex basis of resistance, possibly several minor genes or more structural traits compared to R gene resistance, offering a feasible and effective long-term method of control. Recently, markers linked to a resistance QTL have been developed using a segregating population derived from a cross between a North American red raspberry 'Latham' and the European red raspberry 'Glen Moy' with good fruit quality characteristics but susceptibility to root rot (Graham et al. 2011). These have been utilised in the industry-funded UK raspberry breeding consortium, and resistant progeny are currently under selection for quality traits (Jennings, pers. com.) (Fig. 8.1). At present, the mechanism of resistance presented by 'Latham' is unknown but it may involve complex interactions between disease resistance processes and plant root development (Graham et al. 2011). Pattison et al. (2007) combined generational means analysis with molecular markers and QTL analysis to map resistance to *Phytophthora* root rot in a BC_1 population of NY00-34 ('Titan' x 'Latham') x 'Titan'. Separate genetic linkage maps of NY00-34 and 'Titan' were developed using RAPD, AFLP and resistance gene analog polymorphisms (RGAP) and analysed for QTL associated with various parameters of

Fig. 8.1 Selections with (left of post) and without (right of post) the root rot resistance marker on an infected site

root rot resistance assayed in a hydroponic system (Pattison et al. 2004). Regions on LG1, 5 and 7 were associated in multiple parameters of the resistance response. Bulked segregant analysis (BSA) corroborated this conclusion by identifying markers from these regions associated with bulked samples of resistant and susceptible genotypes. Generational means analysis suggests two major genes controlling resistance, possibly corresponding to the two regions on each parental linkage map associated with resistance. Genetic resistance through breeding offers a feasible method for control, although ensuring planting material is free from the disease is also very important as it is unlikely that the pathogen will be found in soil which raspberries have not been previously grown (Graham and Jennings 2009).

Given the parallel developments in genomic and transcriptomic technologies that are applicable to both raspberry and the *Phytophthora* pathogen, work is currently in progress to identify the genes that play a significant role in this interaction, determine the key resistance/susceptibility components and elucidate the mechanisms of resistance in 'Latham' (Graham et al., unpublished data). Comparison of the 'Glen Moy' and 'Latham' genome sequences is underway to identify any differences within QTL regions (Milne et al., unpublished data).

8.2.2 Raspberry Aphids

Aphids are a major problem on *Rubus* crops, with a number of aphid species reported, four of which (*Amphorophora idaei*, *A. agathonica*, *Aphis idaei* and *A. rubicola*) are known to cause economic damage mainly through their role as virus vectors, leading to virus build up over several years in long-lived soil based plantations. In the UK, the A_1 gene identified in the cultivar 'Baumforth A' by Knight et al. (1959), has been incorporated into many of the resistant cultivars released in Europe in the early 1970s but a virulent A_1-breaking biotype exists (Birch et al. 1994; Jones et al. 2000). Other resistance genes, designated A_2 to A_7, were identified by Knight et al. (1960) in the North American cultivar 'Chief', and further genes were identified in *Rubus idaeus* subsp. *strigosus*. Each of these genes was found to provide resistance against specific biotypes of *A. idaei* (Briggs 1965). As resistance-breaking biotypes against A_1 have emerged, a further resistance gene effective against *A. idaei*, designated A_{10}, identified in the black raspberry (*Rubus occidentalis* L.) cultivar 'Cumberland' (Keep and Knight 1967), has become widely used in breeding (Keep 1989; Birch et al. 2011). An A_{10} resistance-breaking biotype has emerged on 'Autumn Bliss' which carries A_{10} but not on 'Malling Leo' which carries both A_1 and A_{10} (Sargent et al. 2007). As a result, breeders in the UK are now combining A_{10} with other aphid resistance genes to increase durability, and it is hoped that developments in marker technology will facilitate this gene pyramiding strategy to bring aphid-resistant cultivars to the market in a shorter time scale. This is especially important as the use of aphid-resistant cultivars forms a significant part of Integrated Crop Management strategies in the UK (Mitchell et al. 2010; Birch et al. 2011). Sargent et al. (2007) mapped the A_1 locus conferring aphid resistance to LG3 in a population of 'Malling Jewel' x 'Malling Orion'. In black raspberry (Bushakra et al. 2015) a locus for aphid resistance, Ag_4, has also been mapped. In the Pacific Northwest the current standard commercial cultivar is highly susceptible to the aphid *Amphorophora agathonica* Hottes, a vector for the Raspberry mosaic virus complex. Sources of aphid resistance have been identified in wild germplasm and used to develop mapping populations to study the inheritance of these valuable traits (Dossett and Finn 2010). A mechanism located in the phloem for resistance to *A. agathonica* has been suggested by Lightle et al. (2012). The development of maps of progenies carrying resistance genes and the identification of molecular markers linked to these genes will thus provide a key tool in differentiating reported genes, identifying their presence in modern hybrid material and in managing strategies for pyramiding.

8.2.3 Raspberry Cane Diseases

Cane disease resistance is increasing in importance due to the impact on yield of lateral shoot loss due to spur blight (caused by *Didymella applanata*) and cane *Botrytis*. Field screening using either natural infection or a simple wound inoculation method (Jennings and Williamson 1982) remains the best method for analysis of resistance status, as glasshouse inoculations do not result in characteristic disease symptoms. However, breeders with limited resources can rarely include a primary screen for these diseases. These two pathogens occupy the same ecological niche on raspberry canes and it has been shown that a common resistance operates against them (Williamson and Jennings 1986), although the genetic control has yet to be determined. In attempts to control cane diseases, it has been known for some time that the presence of cane hairs in red raspberry (*Rubus idaeus* subsp. *vulgatus* Arrhen.) is associated with resistance to *Botrytis* and spur blight (Knight and Keep 1958; Jennings 1982; Jennings and Brydon 1989) and this was confirmed by linkage mapping (Graham et al. 2006). This effect may be due to linkage with major resistance genes or minor gene complexes that independently contribute to the resistance or susceptibilities of the six diseases affected or that the gene itself is responsible through pleiotrophic effects on each of the resistances (Williamson and Jennings 1992). Alternatively, it may be that cane hairiness affects the ability of fungi to adhere to and infect tissues (Jennings 1962). It may be that gene *H* acts early in development to affect several cell characteristics. For example, resistance to *Botrytis* and spur blight is highest in immature tissues, and it is possible that gene *H* increases resistance by delaying cell maturity. This hypothesis is supported by an effect of gene *H* on timing of fruit ripening (Graham et al. 2009). In a recent study (McKenzie et al. 2015), an attempt was made to explain the role of gene *H* by characterising gene content in this region. This study identified PDF2/GLABROUS2 as gene *H* and also provided an insight into the effects the region has on disease resistance through identification of a number of candidate genes that may suggest genetic resistance is a possibility. A mechanism for the delay to ripening was also hypothesized through the identification of a DIVIA like *Myb* transcription factor that could be controlling both trichomes and flowering time. *WEREWOLF*, a regulator of root hair pattern, has also been shown to be a post-transcriptional regulator of *FT*, a key floral regulator (Seo et al. 2011). There may therefore be more than one effect of gene *H*.

Cane spot or anthracnose (*Elsinoe veneta*) can develop in most raspberry tissues but it is most recognisable in the second year canes where it produces deep lesions that can lead to vascular damage, and therefore reduce yields. The resistance to this pathogen has been associated to the presence of hairy cane *H* in European red raspberry but not so in North-American cultivars (Graham et al. 2006). Genetic control of the trait has not yet been firmly established although Graham et al. (2006) identified two QTL in LG2 and LG4 of the 'Latham' x 'Glen Moy' progeny associated with response to the disease.

Yellow rust (*Phagmidium rubi-idaei*) increased its prevalence with cultivation under tunnels of susceptible cultivars e.g. 'Glen Ample' and 'Tulameen'. Recently, however, this has not been in evidence, though the reasons why are unclear (Jennings, pers. com.). A major resistance gene (*Yr*) from 'Latham' was identified by Anthony et al. (1986), and Graham et al. (2006) postulated this gene to be located on LG3 of the 'Latham' x 'Glen Moy' progeny. The inheritance of complete and incomplete resistance to rust in a half diallel cross including 'Boyne' was studied, which derives complete resistance from 'Latham' (Anthony et al. 1986). Here crosses of 'Boyne' to susceptible varieties all segregated for complete resistance and it was proposed that ‘Boyne’ was heterozygous for a single resistance gene, designated *Yr*, which was derived from ‘Latham’. Graham et al. (2006) proposed that ‘Latham’ is also heterozygous for *Yr*, and that this lies on LG3. Anthony et al. (1986) also found variation in the degree of susceptibility among offspring of ‘Boyne’ without complete resistance, and concluded ‘Boyne’ to also be a source of incomplete resistance. In the ‘Latham’ x ‘Glen Moy’ cross, there is some evidence, although not highly significant, for a gene on LG5, also from ‘Latham’, affecting the susceptibility of the offspring that do not carry the ‘resistant’ allele on LG3. This area on LG5 is also implicated in spur blight/botrytis resistance. There was no evidence, however, of gene *H* being related to incomplete resistance in this cross. None of the offspring in this cross were as susceptible to rust as the ‘Glen Moy’ parent suggesting another resistance gene, for which ‘Latham’ is homozygous.

8.2.4 Virus Resistance

Attempts to identify markers for viral resistance genes have been carried out for raspberry leaf spot and raspberry vein chlorosis. Field screening measured symptom production of these two viruses in two different environments. Significant linkages to mapped markers and resistance loci were found on LG2 and 7 of the ‘Latham’ x ‘Glen Moy’ map (Raluca et al. 2006).

8.2.5 Physical Resistance Traits

As an alternative to resistance gene based control, plants with certain physical and or structural characteristics may be able to resist attack by exploiting morphological structures or biomechanical characteristics that interfere with pest/pathogen movement, host recognition, feeding or reproduction on or in the plant (Hanley et al. 2007; Moles and Westoby 2000). These features could make the plant less attractive visually, or present formidable physical barriers to pests and diseases. Plant traits that may confer resistance/tolerance include structural traits (e.g. trichomes, spinescence, waxy cuticles, sclerophylly, and granular minerals), gross morphology (architecture and plant size), life history (flowering time, growth rate) and secondary metabolites that include a wide range of chemical classes. Allelochemicals such as glucosinolates, tannins and terpenoids can be effective in insect deterrence, as anti-feeding or toxic compounds, and as precursors to structural defense traits (Bennett and Wallsgrove 1994). Plant vigor has also been linked to tolerance in some plants (Price 1991). Breeding for physical resistance traits in crops has not been capitalized upon, despite the potential advantages of this approach compared to chemical resistance traits such as anti-feedants and toxins. Part of the reason for the under-exploitation of physical resistance traits in crops is that the genetic basis and heritability of these traits is poorly characterized and any associations of particular traits with pest and disease resistances have yet to be determined.

As described above for *Botrytis* and spur blight, the presence of cane hairs in red raspberry is associated with resistance. This may be through the hairs providing a physical barrier, or could be through a developmental response making the plant more or less susceptible. Regardless of the mechanism, cane hairs are easy to select for in crossing programmes (Graham et al. 2006) and provide a strategy for protection against these cane diseases.

Cane splitting in raspberry is another example of a physical trait that affects pest and disease burden. Cane splitting is a normal feature of raspberry growth and severe splitting can lead to plant infestation by cane midge (*Resseliella theobaldi* Barnes), followed by fungal infection by many pathogens leading to a disease complex called cane blight, with losses in yield of up to 50% if left untreated (Jennings 1988). Raspberry genotypes have been shown to differ considerably in the degree of cane splitting, and regions of the raspberry genome that are associated with cane splitting have been identified (Woodhead et al. 2013). A correlation between cane splitting and cane height has also been shown, with shorter genotypes exhibiting less cane splitting than longer ones. Loci accounting for 49% of height variation have been identified (Graham et al. 2009; Woodhead et al. 2013) thus indirectly allowing breeding for genotypes with reduced propensity to splitting.

Other plant physical traits with potential to affect pest resistance in *Rubus* (leaf trichomes, leaf density, cane density, bush density, overall density, lateral length and lateral numbers) have been investigated in raspberry to determine the heritability and therefore breeding potential of these traits. These traits had anecdotal evidence for an effect against insect pests (Jennings, pers. com.). This study showed the incidence of spider mites was positively associated with all the density traits assessed both in field and under polytunnel conditions. Thus, increases in plant mass supported a greater increase in observable mite damage. From other observations on field trials in raspberry, spider mites appear to prefer the middle of the plant, possibly to gain protection (Jennings, pers. com.). A number of factors might contribute here including microclimate effects, leaf quality differences and natural enemy evasion. For aphids, there was a positive association between aphid presence and leaf trichome density. This might be due to the hairs offering protection from predation. Analysis of heritability and QTLs for these physical traits identified candidate chromosome regions and associated markers that could be targeted for understanding the genetic control of these traits (Graham et al. 2014), leading to markers with utility for breeding.

8.3 QTL Studies for Quality Characteristics

Fruits from *Rubus* species are highly valued for their flavour and nutritive qualities, and this is the major factor behind the success of a variety. Flavour, appearance and shelf life are the main attributes of fresh market quality and are essential for repeat purchase of fruit by consumers. Flavour can be broken down into multiple descriptors for taste, texture and other sensory characteristics. Good, acceptable flavour in raspberry is fruity, sweet and floral with some acidity but no bitterness (Harrison et al. 1999). Colour, brightness, size and shape contribute to the appearance and are crucial to appeal to consumers. A dark colour can be perceived as overripe by fresh market retailers. Large fruit size is an attractive characteristic to both consumers and producers, in the latter case as it is more cost-effective to pick.

8.3.1 Flavour

To enable determination of what can be improved through both conventional and marker assisted breeding, an understanding of what drives flavour perception and how this is influenced by season and environment is required. In raspberries two main flavour attributes are sweetness and sourness (Harrison et al. 1999) and perception varies with season and environment, where flavour is considered 'good' or 'bad' depending on weather conditions (Jennings, pers. com.). Little work has been carried out in raspberry to correlate sensory evaluations with composition. A study by Zait (2010) aimed to understand the association between sugars and acids and sweetness and sourness perceptions. Here the work suggests that sugar levels are controlling flavor with acid level important when sugars are lower. Data from Paterson et al. (2013) on volatiles content, and from Kassim et al. (2009) on anthocyanin content from the 'Latham' x 'Glen Moy' population was utilized in a recent study to develop a preliminary flavour model. This model found that sweetness, sourness and flavor intensity traits were not adequately explained by singular contributions of either sugars or acids content, but through synergistic relationships between all flavour metabolites. Seasonal and environmental variability made it difficult to identify tight QTL, but this is not surprising as sugars and acids are central to metabolism. A number of overlapping QTL were identified on LG2, 3, 4 and 5. In peach, Etienne et al. (2002) identified several candidate genes involved in sucrose unloading in both the phloem and the cytosol: sucrose transporters (STP) and invertase (Inv) and to a lesser extent hexokinase (Hk). Tonoplast intrinsic proteins (TIPs) are members of the MIP family (major intrinsic proteins) which have been shown to act as water channels expressed predominantly within storage tissues. As glucose is accompanied by the transport of water, these genes are strong candidates for quantitative differences relating to the storage and transport of sugar molecules (Martinoia et al. 2000). Understanding the complexities of sugar uptake, accumulation and metabolism gives a greater insight into the potential candidate genes which control these, the nature of QTL which underlie the traits and the potential for associated molecular markers.

In the volatile study of Paterson et al. (2013), 12 raspberry character volatiles were quantified. Effects of season and environment were examined for their impact on the content of alpha-ionone, alpha-ionol, beta-ionone, beta-damascenone, linalool, geraniol, benzyl alcohol, (Z)-3-hexenol, acetoin, acetic and hexanoic acids, whilst raspberry ketone was measured in one season. A significant variation was observed in fruit volatiles in all progeny between seasons and method of cultivation. QTL were determined and mapped to six of the seven linkage groups, as were candidate genes in the volatiles pathways including phytoene synthase, CTR1, HMG CoA reductase and HMG CoA synthase, β-galactosidase, linalool synthase and terpene synthase.

8.3.2 Colour

Colour in raspberry is a complex trait with anthocyanin content (predominantly cyanidin and pelargonidin pigments) thought to be the major contributing factor (Jennings 1988; Wang et al. 2009). Jennings and Carmichael (1980) described the genes *R*, *So* and *Xy* necessary for synthesising the sugars rhamnose, sophorose and xylose respectively, which are required to give the array of different anthocyanin pigments observed in red raspberry, as well as a series of genes controlling pigment concentration (Jennings 1988). The final expression of fruit colour is influenced by both co-pigments and pH. Co-pigmentation allows the formation of complex interactions between pigments and colourless compounds which enhance colour intensity. Several compounds may act as co-pigments including flavonoids, alkaloids, amino acids, polysaccharides, metals, organic acids, nucleotides and other anthocyanins (Castañeda-Ovando et al. 2009). In most plants, the colour of fruit and flowers results from the accumulation of anthocyanins in cell vacuoles and as the absorption spectrum of anthocyanins depends on the pH of their environment, the observed tissue colour effectively reflects vacuolar pH (Yoshida et al. 2003).

Raspberry fruit colour was assessed in the ‘Latham’ x ‘Glen Moy’ mapping population by McCallum et al. (2010), and colour measurements were significantly associated with pigment content. Measures of individual anthocyanins mapped to the bHLH gene on LG1 and a bZIP gene on LG4 (Kassim et al. 2009; Bushakra et al. 2013) whereas colour and total anthocyanins mapped to overlapping QTL on LG2, LG3, LG4 and LG6 (McCallum et al. 2010). Major structural genes (F3’H, FLS, DFR, IFR, OMT, GST) and transcription factors (bZIP, bHLH, MYB) influencing flavonoid biosynthesis were shown to underlie the relevant QTL. Favourable alleles were identified for aspects of fruit colour and partitioning of individual pigments. Molina-Bravo et al. (2014) examined colour in a modified backcross between (*R. parvifolius* x ‘Tulameen’) and ‘Qualicum’. Unlike in the study of McCallum et al. (2010) where the pigments are mainly cyanidin based, fruit from this wider cross also contained pelargonidin pigments, presumably from the *R. parvifolius* parent. Here around a quarter of the population exhibited yellow fruits. Two QTL for berry colour were identified on LG1 and LG5 (relating to LG6 and LG5 of the map used in McCallum et al. (2010).

8.3.3 Health Traits

In terms of the value to health, epidemiological studies have suggested the efficacy of compounds found at high concentrations in berries for the prevention of a number of chronic diseases. Studies are now aimed at understanding the mechanisms of action of specific groups of phytochemicals (eg. Marinova and Ribarova 2007). For a review of the literature on the potential health benefits of berry fruits see McDougall and Stewart (2012).

Work towards understanding the genetic control of health-related compounds has been initiated in *Rubus* using a metabolomics approach to identify bioactive compounds in the 'Latham' x 'Glen Moy' under two different environments (Stewart et al. 2007). As a greater understanding of the relative importance and bioavailability of the different antioxidant compounds is achieved, it may become possible to develop and identify those raspberry genotypes with enhanced health-promoting properties from breeding programs (Beekwilder et al. 2005). Preliminary metabolic profiling showed that the fruit polyphenolic profiles divided into two gross groups segregating on the basis of relative levels of cyanidin-3-sophoroside and cyanidin-3-rutinoside, compounds implicated as conferring human health benefits. From the 'Latham' x 'Glen Moy' mapping population, data was collected on anthocyanin content across seasons and under different environments (Kassim et al. 2009). High performance liquid chromatography (HPLC) was used to quantify eight major anthocyanins, cyanidins, and pelargonidin glycosides: -3-sophoroside, -3-glucoside, -3-rutinoside and -3-glucosylrutinoside. All eight mapped to the same chromosome region on LG1 of the map of Graham et al. (2006), across both years and from fruits grown in the field and under protected cultivation. Seven antioxidants also mapped to a region on LG4 across years and for both field and protected sites. Candidate genes including *bHLH* (Espley et al. 2007), *NAM/CUC2* (Ooka et al. 2003) like protein and *bZIP* transcription factor (Holm et al. 2002; Mallappa et al. 2006) underlying the mapped anthocyanins were identified. In another study on red and black raspberry Bushakra et al. (2013) used ultra and high-performance liquid chromatography (UHPLC and HPLC) on two *Rubus* mapping populations to explore the presence of associations between concentrations of five anthocyanins in fruit and genotype. In total, 27 QTL were identified on the *Rubus* linkage maps, four of which were associated with molecular markers designed from transcription factors and three of which are associated with molecular markers designed from anthocyanin biosynthetic pathway candidate genes. Using the 'Latham' x 'Glen Moy' mapping population, total phenol content (TPC) and total anthocyanin content (TAC) in ripe fruit was examined over five seasons under two environments (Dobson et al. 2012) to examine variability. Corresponding measurements of antioxidant capacity (e.g. ferric reducing antioxidant capacity (FRAP) and trolox equivalent antioxidant capacity (TEAC)) were also carried out. TPC was highly correlated with TEAC and FRAP over the entire dataset. The subset of anthocyanin content was genotype-dependent and also correlated with TPC though the proportion of anthocyanin compounds contributing to total phenolic pool varied from progeny to progeny. QTL were identified on LG2, 3, 5 and 6. The QTL that influence TPC but not TAC are of particular interest to boost the antioxidant capacity of raspberry fruits, which is often related to their bio-activities.

8.3.4 Crumbly Fruit

In terms of fruit quality (as well as yield) the disorder known as ‘crumbly’ fruit has become a serious problem in the raspberry industry. In ‘crumbly’ fruit drupelets are generally reduced in number but greatly enlarged or, in the case of small reductions, cohere imperfectly so fruit readily crumbles when picked (Daubeny et al. 1967; Jennings 1988). There have been a number of causes suggested for the crumbly condition including infection with certain viruses (Jennings 1988; Murant et al. 1973; Daubeny et al. 1978). A genetic cause has been demonstrated where the crumbly phenotype arises from virus-tested mother plants (Jennings 1988). The cultivar ‘Latham’ can show a crumbly phenotype and this is thought to be due to mutation of the dominant allele at a heterozygous gene locus causing plants to become homozygous for a deleterious recessive gene (Jennings 1967b). From a ‘Latham’ self, Jennings (1967b) demonstrated that seedlings obtained could be classified into three groups: normal, crumbly and sterile. Studies have also shown that extensive tissue culturing of plants may increase the emergence of the condition (Jennings pers. com.). Additionally, environmental factors appear to play an important role with variations in the extent of crumbliness apparent from year to year (Dolan, pers. com.). A study on the ‘Latham’ x ‘Glen Moy’ population examined the occurrence of crumbly fruit over a 6 year period, in both open field and under polytunnel. This highlighted that seasonal, environmental and genetic factors all influence the condition. Two QTL that are important for the genetic control of the condition were located on LG1 and 3 (Graham et al. 2015). Contrary to the suggestion by Jennings (1967a) that crumbly fruit was related to the gene *H* region, no genetic association with this region on LG2 could be identified with the crumbly fruit syndrome. However there was an association with ripening, with the longer the fruit takes to fruit set and reach green fruit stage, the more likely it is to be crumbly. This may explain the association hypothesized by Jennings as the *Hh* genotype of gene *H* is associated with a slowing down of ripening across all stages from open flowers to the green/red stage compared to the *hh* genotype (Graham et al. 2009).

8.3.5 Fruit Size

Large size is an attractive characteristic to consumers and producers as it is both cost effective to pick and visually appealing. Uniformity of fruit size is also visually appealing to potential customers and when combined with regular shape can encourage sales (Graham and Jennings 2009). A definitive value of drupelet cohesion is one which can be assessed and measured within field trials, along with the overall size of fruits, considered individually or collectively as ten berry weights. Raspberry genotypes show a wide variation in fruit size. This range may be as a result of differences in cell number or cell volume. Genetic differences, season and environmental conditions as well as crop management practices have an effect on fruit size (Cheng and Breen 1992). Mutation of a major gene designated L_1 was identified in a large-fruited ‘Malling Jewel’ mutant which resulted in an increase in both drupelet number and size. However, the gene itself however proved to be unstable with the gene mutating back to its normal sized form (Jennings 1988). ‘Glen Moy’ was one of the early cultivars selected by breeders for its large fruit size which was attributed to both drupe size and number (Jennings 1988). Gene families like aquaporins may have a role in fruit size as these are water channel proteins capable of transporting water and small molecules across cellular membranes. Three main types of aquaporins are known in plants, membrane intrinsic proteins (MIPs), tonoplast intrinsic proteins (TIPs) and plasma membrane intrinsic proteins (PIPs) (Smart et al. 2001). Plant MIPs are reported to play an important role in cell division and expansion as well as water transportation in relation to environmental conditions (Oliviusson et al. 2001). Expansins are associated with cell growth, consequently with the later stages of fruit ripening in tomato and strawberries delaying or increasing fruit ripening (Cosgrove 2000).

An increase in cell wall bound protein found in raspberry fruit has, in part, been attributed to expansin accumulation (Iannetta 1998). Several intrinsic processes involved in the transport of solutes across vacuolar membranes impact on fruit metabolite concentrations of fruit, making these ideal candidates for gene analysis as are other ripening associated genes such as auxins and ethylene regulators.

Recent work has been carried out in the ‘Latham’ x ‘Glen Moy’ population which segregates for fruit size (McCallum, pers. com.). Candidate genes for auxin, ethylene and specific size regulatory genes (fruit weight) and transcription factors were identified. Initial statistical analysis found one marker on LG3, which explained 23% trait variation in the mapping population, also contributed to 14.4% of variation in fruit size seen in two out of three unselected families assessed. A further SSR marker on LG1, contributing to 15% variation in the mapping population, contributed to 6.6% size variation across the same populations with an additive effect of 19.1% trait variation. Further work on these markers and putative candidate genes involved in fruit developmental processes are underway in a range of available germplasm and potential breeding parents. The transport of solutes across vacuolar membranes impacts on fruit metabolite concentrations, making the genes involved ideal candidates for gene analysis. In a *Rubus parvifolius* x ‘Tulameen’ cross one QTL was identified for fruit size (Molina-Bravo et al. 2014). A single major QTL for fruit weight (*fw* 2.1) was identified in tomato in close proximity to a cloned fruit weight gene *fw* 2.2 (Frary et al. 2000; Zygier et al. 2005).

8.4 Resilience to Environmental Change

Climate change is demonstrably impacting agricultural and horticultural production at local, national and global scales (e.g. Mackay et al. 2011; Huang et al. 2015; Innes et al. 2015) and these impacts are also apparent in soft fruit. Continuous and reliable production of high quality fruit is critical for the profitability and this is threatened by changing temperatures. There is now evidence that flowering time has shifted in response to changes in climate (Fitter and Fitter 2002; Amano et al. 2010). Many plant species are unable to respond to the florally inductive cues until they have reached a certain developmental stage, i.e. they have what is known as a juvenile phase. This is true for raspberry, which only responds to the prolonged period of cold (vernalization) after a certain stage of development. Crop resilience and adaptation is therefore essential for future sustainability of all crops and must be considered as a factor for breeding. Understanding the key genetic control points across development is a major challenge in *Rubus* breeding for both resilient variety development and season extension.

8.4.1 Dormancy

The time at which dormancy begins and the intensity it attains and subsequent transition to flowering are regulated by multiple environmental and physiological cues (Fornara et al. 2010; Pin and Nilson 2012; Song et al. 2012) and need to be fully understood in perennial crops. A major plant trait has to have the ability to tolerate fluctuating winter temperatures. Raspberry like other perennial crops has adapted by having high chilling requirements, however as winter temperatures increase, evidence of disruption to development is evident with irregular and unexpected timings of bud break (Jennings, pers. com.). Activity of CONSTANS (*CO*) a key component in leaves of the photoperiodic pathway accumulates in long day conditions and activates transcription of FLOWERING LOCUS T (*FT*) (Simon et al. 2015) which interacts with *bZIP* transcription factors (Abe et al. 2005; Cao et al. 2015) activating a cascade of downstream genes leading to flowering. In terms of temperature regulation, the MADS box FLOWERING LOCUS C (*FLC*) is central. *FLC* and short vegetative phase proteins (*SVP*) form a complex to represses flowering until the plant is exposed to the appropriate level of cold. In raspberry RiMADS_01 was identified as a potential candidate affecting vernalization through QTL mapping. This gene is similar to *SVP* modulating the timing of the developmental transition to flowering phase in response to temperature (Lee et al. 2007). In a colder season RiMADS_01 was associated with earlier flowering. SPL was also identified in raspberry on LG5 associated with a QTL for floral transition. Gene *H*, previously associated with cane morphology described above (Graham et al. 2006) was shown to be associated with a slowing down of ripening across all stages (Graham et al. 2009) and sequencing the gene *H* region (McKenzie et al. 2015) identified a DIVIA like Myb transcription factor (Werewolf) shown to be a post-transcriptional regulator of *FT* (Seo et al. 2011). Molina-Bravo et al. (2014) examined the progeny from a cross (*Rubus parvifolius* x 'Tulameen') x' Qualicum' for chilling requirement determined by measuring bud break in chilled cuttings. Four regions were associated with chilling requirement, and were mostly consistent across the 3 years of evaluation. This population is of interest to breeders in a time of climate change as a donor of higher chilling requirements (allowing germplasm to withstand fluctuations in winter temperature) and also as a donor of heat tolerance.

8.4.2 Heat Tolerance

In addition to a lack of winter chill affecting dormancy and bud break, high summer temperatures are also affecting raspberry cultivation. Gotame et al. (2014) carried out a study aimed at increasing our knowledge of temperature stress on raspberry cultivars with a view to mapping genes implicated in response to elevated temperatures. A range of cultivars were examined for the effects of high temperature stress on gene expression profiles at the flower initiation stage using a custom *Rubus* microarray (James Hutton Institute). An elevation of temperature (>10 °C) altered the expression of 40 genes (38 were down- and two up-regulated). Down-regulated genes included those encoding major latex-like protein (MLPs), plasma membrane proteins (PMPs), cysteine proteins and other stress-related proteins. A number of PMP candidate genes were located on the 'Latham' x 'Glen Moy' map.

8.5 Yield

High fruit yields have been shown to be associated with cane architecture traits, particularly lateral length, which alone accounted for 82% of the yield variation (Sønsteby et al. 2009). Stephens et al. (2012) reported a positive genetic correlation between cane diameter and total yield. In terms of QTL mapping, lateral length along with a range of other architectural traits (leaf trichomes, leaf density, cane density, bush density, overall density and lateral numbers) described above have been investigated in raspberry and candidate chromosome regions identified and associated markers that could be targeted for understanding the genetic control of these traits (Graham et al. 2014) leading to markers for breeding. Currently these are being investigated for any association with yield in a range of breeding populations at James Hutton Limited (Jennings unpublished data).

8.6 Further Development of Mapping Technology in *Rubus*

QTL mapping has proved an accurate methodology for identification of loci linked to traits of interest. Large scale sequencing has revolutionised our ability to sequence and assemble genomes of a wide range of crop species and gives the opportunity to develop novel markers and identify important genes. In *Rubus*, a whole genome assembly was established for black raspberry (*R. occidentalis)* consisting of 2226 scaffolds spanning an estimated 83% of the genome, which was further assembled into seven pseudo-chromosomes (VanBuren et al. 2016; Jibran et al. 2018). The black raspberry genome is largely collinear with the strawberry genome and has strong identity with red raspberry where 87% of the selected red raspberry genetic markers match the position of the black raspberry markers on the physical map (VanBuren et al. 2016). With the addition of an assembled red raspberry draft genome from 'Glen Moy' (Hackett et al. accepted), these genomes provide a physical genomic framework to compare between these related species, link the genetic maps to the physical genome and promote the development of high throughput, large scale mapping techniques. At its simplest, established markers are placed on corresponding genomic sequences of the *Rosaceae* and determine syntenic blocks of sequence (Bushakra et al. 2012; VanBuren et al. 2016). Candidate genes found in genomic regions identified by markers and linked to important traits can be selected by an understanding of the trait, variable expression of the genes in that region and comparing protein coding regions. For example, raspberry fruit softening is an important agronomical trait that involves a complex interaction of plant cell processes including cell wall solubility and water transport. QTL mapping followed by selection and expression analysis of genes that underlie these QTLs across different fruit stages identified candidate cell wall degrading and water movement genes that showed variability, in the timing of gene expression throughout fruit development (Simpson et al. 2017).

Recent efforts in the development of higher resolution Genotyping by Sequencing (GBS) maps in combination with genome sequencing have increased the utility and accuracy by which traits can be located and linked to underlying genes. A GBS map from a 'Heritage' x 'Tulameen' mapping population identified nearly 7000 SNP markers spanning all seven raspberry linkage groups. A second red raspberry GBS map of the 'Latham' x 'Glen Moy' population was aligned with the draft genome sequence of 'Glen Moy' and identified over 2000 high confidence SNPs. These confirmed previously established QTLs for fruit ripening and identified additional QTLs and underlying candidate genes (Hackett et al. submitted). The increase in marker saturation and availability of genomic sequences will help the development of genome-wide association study (GWAS) projects. These projects use SNP genotyping over a broad range of wild species, cultivars and breeding populations that are phenotyped for multiple selected traits. Raspberry has abundant natural and experimental populations that show adaptation to a range of habitats and variability to a range of traits that may be used to generate defined GWAS populations. GWAS may give an increase in allelic diversity and improve resolution, but it remains to be seen whether raspberry genetic heterogeneity and marker density is sufficient to identify loci or genes by association of markers with traits.

8.7 Other Omics Technologies

Gene expression and transcriptomic approaches provide large data sets that enable identification of important biochemical pathways and regulatory genes. Linkage analysis of quantitative genome-wide gene expression data from both microarray and large scale transcriptome sequencing and also from metabolic data has been combined in melon to identify quality trait genes (Galpaz et al. 2018; Giovannoni and Katzir, 2018). Expression QTL (eQTL) mapping has not been reported in *Rubus,* but the tools and some data are already available to utilise and develop expression patterns to identify genes that may be co-regulated. The first microarray experiment in *Rubus* was conducted to investigate bud dormancy phase transition (Mazzitelli et al. 2007). Over 220 clones exhibited up or down-regulation during the endodormancy – paradormancy transition. The results indicated that water and cell wall reorganization and sugar metabolism were key components of bud dormancy release. Transcription factors, including a *SVP*-type MADS box transcription factor, and hormone-induced genes were also identified, potentially indicating signalling molecules that may be required to release these buds from dormancy. Advancement in sequencing technology has also enabled large scale sequencing of transcriptomes that can be utilised in multiple ways. A red raspberry 'fruit transcriptome' comprising a database of 55,920 unigenes has been established and mapped to the genome scaffolds of 'Glen Moy' (Milne, personal communication). The unigene set is derived from various transcript sequences isolated from a range of raspberry tissues, developmental stages, including developing fruit and buds and also different conditions. Sequences originated from 454 and llumina transcript sequencing, Sanger Expressed Sequence Tags and BAC coding sequences. A total of 55,708 oligonucleotide probes were designed for generation of a custom Agilent microarray JHI_Ri_60k_v1 (Graham, pers.com.). Subsequent microarray experiments have investigated the effect of high temperature stress on total gene expression profiles in the annual-fruiting raspberry (*R. idaeus* L.) 'Autumn Bliss', 'Autumn Treasure', 'Erika', and 'Polka' using a customised *Rubus* microarray (Gotame et al. 2014) and examined gene expression in the development of crumbly and normal fruit to examine those within identified QTLs (Graham et al. 2015).

8.8 Conclusion

Significant developments have been achieved in *Rubus* in terms of QTL mapping and other associated developments such as genome scaffolds and microarrays. This is greatly assisting breeding practices and with the developments in high throughput phenotyping where image data can be utilized in QTL mapping, the ability to dissect more complex traits, particularly for environmental resilience should become a reality in the next few years.

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Chapter 9
Use of Imaging Technologies for High Throughput Phenotyping

Dominic Williams, Matt Aitkenhead, Alison J. Karley, Julie Graham, and Hamlyn G. Jones

9.1 Introduction

In this chapter we describe a high throughput phenotyping system that we have developed for raspberry and other soft fruit crops and its application to against individual (water stress regimes, vine weevil and *Phytophthora* root rot) and combined stresses. The term phenotype is used to describe the morphology, physiology, biochemistry and ontogeny of a plant, encompassing the diverse array of traits that contribute to the plant's functional form. Plant phenotype is expressed as a consequence of the interaction between the plant genetic background (i.e. genotype) and the biotic and abiotic conditions experienced by the plant in its growing environment. A key focus of raspberry and other crop breeding is to understand the genetic control of desirable plant traits and the influence of environmental conditions on trait expression, which relies on the ability to collect quantitative information on target traits across genetically-characterised populations of plants. The process of characterising plant traits in detail, referred to as plant phenotyping, is a major challenge when relating plant genetic information to traits for plants in realistic growing

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D. Williams (✉)
James Hutton Limited, Dundee, UK
e-mail: Dominic.williams@huttonltd.com

M. Aitkenhead
Information and Computational Sciences, The James Hutton Institute, Dundee, UK

A. J. Karley
Ecological Sciences, The James Hutton Institute, Dundee, UK

J. Graham
Cell & Molecular Sciences, The James Hutton Institute, Dundee, UK

H. G. Jones
Division of Plant Sciences, University of Dundee, Dundee, UK

University of Western Australia, Crawley, WA, Australia

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environments. Advances in genetics and genomics methods in raspberry including high throughput DNA sequencing which has provided a new GbS linkage map and genome scaffolds, developments in microarray and RNA seq data and data analysis tools have provided large amounts of detailed information on raspberry. There is, however, a lack of corresponding methodology for high throughput plant phenotyping, which limits the quantity of available trait information, and creates a bottleneck in raspberry and other crop breeding (Fahlgren et al. 2015; Pauli et al. 2016b). The key factor in understanding traits at a genetic level is an ability to accurately phenotype. The main limitation is the significant effort and complexity of measurements, depending on trait, required to capture phenotyping data from plants grown in relevant environments – i.e. field conditions.

Early developments in high throughput plant phenotyping focused on systems for automated data capture using robots and automated facilities that operate within controlled growing environments (glasshouse and growth chambers) and are not readily transferable to field conditions. Given that plant traits underpinning plant productivity and crop performance can be laborious to measure (e.g. photosynthetic activity, nutrient acquisition), field phenotyping has tended to focus on a small number of easy to measure traits for large scale plant phenotyping. More complex traits are often studied only in small scale field trials. Developing methods for high throughput phenotyping that are able to record more complex traits contributing to plant phenotype in field conditions has the potential to accelerate plant breeding and develop our understanding of how plant trait expression is regulated by genetic and environmental factors.

High throughput phenotyping using traditional methods of plant trait quantification poses a number of challenges due to the time, effort, cost and specialised equipment involved. For example, biochemical analysis of plant tissues (e.g. for nutrient composition) sampling of representative plant tissues, sample storage and transport to laboratories (with the risk of sample damage), sample preparation and analysis. Finally, there are costs, both time and financial, in collating data into a format that is suitable for analysis and interpretation. Some or all of these activities may seem spurious to include but the simple fact remains that the majority of time and money used in traditional phenotyping is spent doing manual, repetitive tasks. Developing an automated, integrated approach to characterise plant phenotype is a challenge that, if solved, would greatly reduce costs and accelerate progress in crop improvement.

Imaging technologies offer a potential solution to this challenge (Walter et al. 2015), allowing rapid non-destructive data capture from large numbers of plants across multiple time points and different environmental conditions. Imaging plants is far less labour intensive than other methods of plant characterisation and can be used in controlled environments, glasshouses and other protected growing environments, and in field based systems (Ghanem et al. 2015). Although working under controlled environmental conditions can facilitate accurate measurement of plant responses to specific conditions, controlled conditions cannot replicate the growing conditions experienced by plants in the field where multiple stresses are encountered and are also limited in scale, either by the number of plants, or the size and maturity of plants, that can be characterised indoors. Raspberry being a biennial crop requiring particular environmental cues for development further complicates this ability. Further, there is increasing recognition that future crops will need to cope with environmental uncertainty driven by a changing climate; field-based phenotyping could provide a better understanding of environmental regulation of plant phenotype and so accelerate the breeding process for crops that perform consistently in a fluctuating environment. These constraints and considerations have led to the development of techniques that are suited to field-based phenotyping of plant populations.

Imaging techniques for plant phenotyping are based on sensing how the plant interacts with the electromagnetic (EM) spectrum. This can involve quantifying the proportion of reflection, absorption and transmission of EM radiation in the visible and short wave infra-red regions or by the emission of thermal infrared radiation. The region of the EM spectrum most commonly used is between 400 and 2500 nm. This includes a number of different wavelength regions that correspond to light absorption and/or reflection by many different pigments within plant tissues, although the structure of a plant also has an effect on the reflectance spectrum. In the visible regions between 400 and 700 nm, absorption by photosynthetic pigments dominates. Most light from the sun reaches the surface of the earth in this region of the spectrum, so it is unsurprising both our eyes and plant leaves are adapted to interact with light in this region. In the near infra-red region (700–1400 nm), healthy plant tissue is highly reflective. In the short wave infra-red region (up to 2500 nm), water and some biomolecules contribute to reflectance characteristics. Water absorption is very strong in a number of regions of the spectrum, which has the effect of substantially reducing incoming solar radiation at the earth's surface in a number of bands due to absorption by water in the atmosphere. Figure 9.1 shows a typical plot of the reflectance of green vegetation in the visible and short wave infra-red region. Thermal or longwave infra-red radiation is the region between 8000 and 13,000 nm. Here the dominant source of radiation is thermal radiation emitted by all objects in proportion to their temperature, which can be exploited for remote measurements of canopy temperature (thermal imaging). Variation in plant phenotype can be related to variation in the plant response to radiation in these regions, detected using imaging cameras or sensors.

In summary therefore:

- High-throughput phenotyping is constrained by traditional techniques
- Plant characteristics related to phenotype can be linked to spectral properties of the plant tissues
- Spectral imaging offers an approach to capturing phenotype information that is also relatively automated and can be rapidly integrated into the data interpretation and phenotype characterisation process.

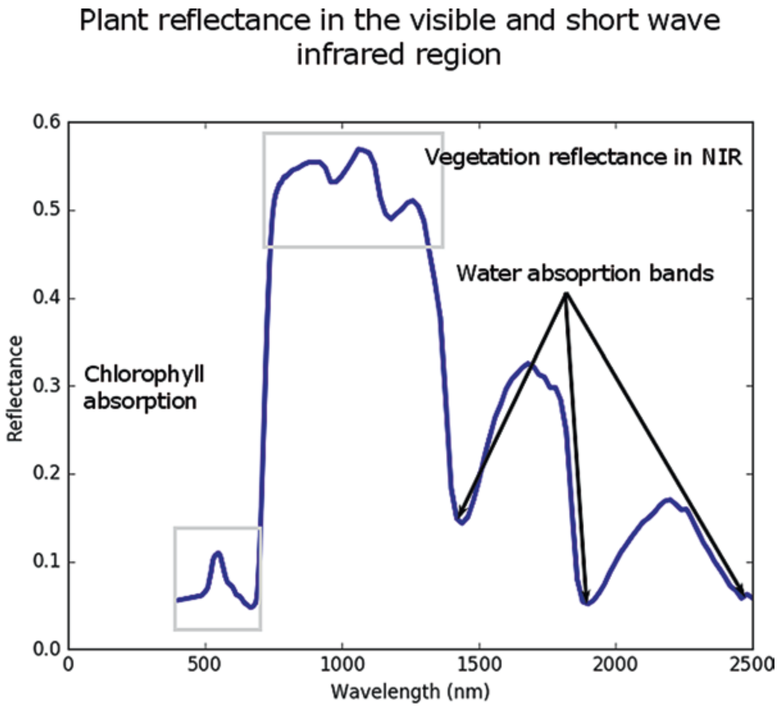

Fig. 9.1 Plot of reflectance of green vegetation at different wavelengths with key regions labelled. Data from (Baldridge et al. 2009)

9.2 Overview of Different Techniques/Technologies

Detailed information about the rationale and application of different sensors to phenotyping in both controlled environments and the field may be found in both environmental physiology and remote sensing texts (Jones and Vaughan 2010; Jones 2014). Here we consider the particular advantages and disadvantages of different sensors for application to *Rubus* breeding. The most widely used sensors are based on measurement of spectral reflectance of plant canopies at wavelengths extending from the visible through the near infrared (NIR) to the medium infrared up to about 2400 nm (short wavelength infrared or SWIR). These include both point sensors (such as spectroradiometers) and imaging sensors that give spatial information. Imaging sensors themselves may either be conventional frame cameras giving two-dimensional images, or line scanners where a single line of points is studied at one time and images are generated from movement of the sensor platform along the object to be imaged. The sensors themselves range from simple visible wavelength (red-green-blue or RGB) cameras through multispectral to hyperspectral imagers that can detect reflectance in several hundred individual wavelengths. Spectral reflectance gives important information about both the biochemical composition of plant tissues and canopy structure. For example, the position of the sharp transition in reflectance around 700 nm is a useful measure of chlorophyll content and leaf nitrogen status, while there are important water absorption bands at c.970, 1200 and 1450 nm that give information on leaf water content. The reflectance spectrum in the SWIR also gives useful information on biochemical components including sugars and proteins. In the past, hyperspectral sensors, especially those in the SWIR, have been large and expensive, but newer hand-held sensors are now becoming available (e.g. Tellspec) and these may well greatly expand the applicability of spectral sensing of canopy physiology.

While we have identified bottlenecks in traditional plant phenotyping (outlined above) that could be reduced or eliminated by the application of spectral imaging technology, there are a number of factors that need to be considered to ensure that the imaging does not suffer from its own, similar constraints. These can be considered in terms of the processing chain from the point of in-field sensing to the presentation of the results. Firstly, the spectral data capture process requires equipment design and operation that is intended to optimise the quality of the data and its subsequent use; this design can take significant effort and iterative trial and error engineering of the system. Secondly, experimental imaging systems can produce spectral data that is formatted, structured and stored in a manner more useful to the sensor manufacturer than the end-user of the data; this can mean time and effort in extracting and presenting the data in a useable manner. Finally, the raw spectral data from field sensing is likely to be noisier and less standardised than data from laboratory-based spectral systems. This means that the data must be prepared and transformed (pre-processed) effectively before it can be used for statistical modelling and plant characterisation.

Optical sensors can also be used in an active mode where the fluorescence emitted in response to an illumination beam gives information not only about pigment content and general biochemistry, but also about rates of photosynthesis. Unfortunately the high illumination intensities normally required for studies of chlorophyll fluorescence limit the use of these approaches in the field, but Laser Induced Fluorescence Transient (Kolber et al. 2005) and Solar Induced Fluorescence (Meroni et al. 2009) approaches may increase the potential for studies of photosynthetic responses in phenotyping.

Critical information relating to plant water relations can be obtained using thermal sensors that estimate canopy temperature through the detection of emitted long wave infrared (c. 9–12 m) radiation (Jones 2004). When combined with appropriate referencing techniques to allow for other factors that affect canopy temperature, temperature measurements can give useful information on plant transpiration and leaf stomatal opening in response to stress.

Other types of sensor that are particularly valuable for providing information on 3D-canopy structure are LiDAR and time-of-flight cameras, while even ultrasonic sensors provide useful structural information suitable for deployment on field buggies (Hosoi and Omasa 2009; Deery et al. 2014). Structural information can also be obtained from stereoscopic imaging using conventional RGB digital cameras.

Without proper interpretation, spectral data is just a long list of numbers. Classical statistical approaches are of very little use with hyperspectral data in particular, where individual wavelengths are often poorly correlated with characteristics of interest. A large number of techniques exist for pre-processing of spectral data and also for the statistical modelling of the data once it has been prepared. The selection of the best approach for either is very much a dark art, with literally hundreds of publications identifying specific pre-processing / modelling combinations for individual datasets. The issue is that each combination of instrumentation, lighting conditions and sensed material produces a different set of conditions under which the spectra are captured, and these conditions influence the character of the spectral data.

9.3 Imaging Under Controlled Lighting

High throughput phenotyping systems were first developed for use in glasshouses under controlled conditions. One of the first plants to be examined was *Arabidopsis thaliana*. In 1999, an automated analysis system was developed for colour images of *Arabidopsis* plants capable of measuring plant area (Leister et al. 1999). This method was able to produce a plant area estimation that showed a strong positive correlation with plant fresh weight for young plants. For older plants, leaf overlap created some problems in measuring plant mass from a planar image only.

Since then, phenotyping in controlled conditions has advanced rapidly. Automated systems where pots are moved on conveyor belts between weighing, watering, and imaging stations have been developed and used in a number of locations (European plant phenoyping network 2018). These systems allow images to be taken of the plant at multiple angles in addition to top down imaging. This permits detail on plant shape to be gathered for more accurate assessment of plant growth. In addition to colour imaging from multiple angles, different sensors have also been added to imaging systems to provide a greater range of information. In 2014 Chen et al. developed a framework for analysing high throughput phenotyping data (Chen et al. 2014). Barley plants grown in a glasshouse were subjected to water stress and imaging was carried out using both colour and infra-red cameras in a LemnaTec phenotyping platform (Klukas et al. 2014). These data were used to quantify barley plant growth and 52 phenotypic parameters associated with gene expression.

Another group in China (Yang et al. 2014) developed a glasshouse phenotyping platform for rice plants grown in controlled conditions that were then selected and removed to be imaged using a colour camera and rotating camera to produce a 3D view of the plants. X ray tomography was also carried out to image the roots of each plant. Using this approach, the authors were able to automatically detect 15 phenotypic traits and associate these with 141 loci on the rice genome, again showing the value of high throughput phenotyping in determining the genetic architecture of plants in relation to plant traits.

The examples discussed here are just a few of the uses that have been made of high throughput phenotyping in glasshouse conditions. They have shown great potential in developing useful phenotypic data that can be matched to genetic information. There are a number of disadvantages to these systems. The main one is the distance between controlled environments and actual field environments where plants are grown after breeding. Plant behaviour adapts in response to environmental conditions and one of the great strengths of imaging is the ability to detect this interaction. In order to do this most effectively, reducing the difference between conditions where phenotyping is carried out and where plants are grown commercially is necessary. Another disadvantage is the cost setoff setting up such systems. Substantial amounts of money are spent engineering glasshouses to install automated platforms to move pots into position. This requires investment that is often beyond the resources of plant breeders especially those involved in crops with a smaller market than mainstream grain crops. Finally, automated phenotyping systems are more suited for quick growing crops that will display differences rapidly. For longer lived perennial crops such as bush or tree fruiting crops, longer growing periods mean that phenotyping in field conditions is more appropriate. For these reasons there has been a push towards the development of field imaging systems.

The spectral signature of a particular plant trait or property of interest is often difficult to predict, and so the appropriate wavelength range cannot be identified prior to investigation. For this reason, it is important to carry out initial experimentation using a sensor or sensors that can cover a wide range of wavelengths. Additionally, the optimal combination of pre-processing and modelling for extracting as much information as possible about the desired trait is rarely known beforehand. It is important, therefore, to start with a general investigation across spectral processing techniques. This enables the researcher to identify appropriate methods to link with specific sample characterisation problems. Access to a generally applicable sensor or sensors, and the appropriate wide range of data interpretation skills, is an important consideration for plant spectral phenotyping.

9.4 Field Imaging

Any plant breeding programme requires rapid and effective methods for phenotyping. Although most effort has been invested in the development of phenotyping technologies applicable in controlled environments (Furbank and Tester 2011; Fiorani and Schurr 2013), an ability to perform phenotype assessment in the field is critical for progress in plant breeding. It is only very recently, however, that imaging technologies have started to become available for effective field phenotyping and that are beginning to provide capacity for data generation to match the potential of recent biotechnological advances in genomics and marker-assisted selection. Field phenotyping of complex traits associated with biomass development and yield and stress tolerance is now becoming increasing rapid and precise, reducing the time taken and the need for manpower, through the development of sophisticated imaging technologies mounted on mobile field platforms and Unmanned Aerial Vehicles (UAVs or drones) (Deery et al. 2016a; Hawkesford and Lorence 2017).

The approaches available for field phenotyping range from the use of simple hand-held sensors (such as thermal or hyperspectral cameras), through sensors mounted on fixed or mobile in-field platforms through to sensors mounted on UAVs or aircraft. The use of hand-held measurements can only rarely have adequate throughput for a plant breeding programme, while fixed systems where imagers are mounted over a fixed field array (e.g. the LemnaTech GmbH. Field Scanalyzer; http://www.lemnatec.com/product/scanalyzer-field) tend to be limited in the field area (and hence plot size and replication) that can be covered. Mobile platforms (buggies) are available that range from hand operated frames to larger motorised and autonomous vehicles carrying sophisticated sensor arrays through to sensors mounted on the standard agricultural machinery used for crop management (Deery et al. 2014).

Whilst field buggies are suitable for smaller field trials, the time taken to assess the whole trial can be a limitation, especially when studying rapidly changing physiological processes (e.g. stomatal conductance) which may be relevant for stress sensing. For larger experiments the time required may even be more than a single day with all the attendant problems. Therefore, there is much interest in the use of airborne sensing for plant phenotyping, whether from drone or from manned aircraft (Chapman et al. 2014; Sankaran et al. 2015; Deery et al. 2016a, b). Although most drones have only a limited payload for sensors and limited flight-times that restrict the area covered, their flexibility means that they can be particularly suited to phenotyping smaller area field trials. For larger areas, manned aircraft, which also can carry the heavier and more sophisticated cameras, come into their own (Deery et al. 2016b) and can provide imagery over large areas of crop in only a few minutes. Nevertheless, for typical plot sizes used for *Rubus* breeding programmes, mobile field platforms are the method of choice, offering the best combination of flexibility, ability to carry sophisticated sensors, and ease of operation.

The use of UAVs comes with its own set of challenges. Regulations for the use of UAVs can restrict their flight near buildings and power lines, and they require experienced users for successful application to crop sensing. Another issue is that of locating the position of individual pixels in the resulting imagery, which must be done with great precision. Modern UAVs are equipped with high-performance positioning systems and the accompanying software packages can assist in stitching together the images to produce usable hyperspectral imagery, but the costs can be significant. Additionally, light conditions can change significantly during a single flight, which means that the spectral data cannot be relied upon to be consistent across a single data capturing exercise. This introduces additional data processing requirements.

Here we describe the system that we have developed and its application to screening in raspberry and other soft fruit against individual (water stress regimes, vine weevil and *Phytophthora* root rot) and combined stresses. Work has been carried out at The James Hutton Institute, Dundee, UK, to develop an imaging platform for use in high throughput phenotyping of field trials (Williams et al. 2017). For this a ground based approach was used. There are two main advantages of using a ground based system. The first is that in Scotland most raspberry plantations are

Fig. 9.2 Picture of the James Hutton hyperspectral imaging platform in action. It is show here in polytunnel but has also been used on plant grown the open field

grown under cover in polytunnels. Most rotocopter UAV crop imaging protocols used in other species involve flying at heights of 50–100 m, while larger fixed wing systems fly at higher altitudes. The ability to fly and image effectively within a polytunnel is currently beyond the limits of available technology. The other advantage of ground based imaging is the larger payload of cameras that can be deployed. The cameras on the platform deployed at James Hutton weighed over 20 kg and were run using a generator for electricity supply. Figure 9.2 shows a photo of the platform in action in polytunnel.

The biggest challenge in taking hyperspectral imaging into the field is accounting for changing light levels. Hyperspectral imaging is based upon spectral reflectance from an object and entails measuring reflected light and light incident on that object. When imaging plants, the complicated 3D structure of plant leaves has a significant effect on the incident light received by the plant and that reflected from the plant to the camera. The signal received by the camera is dependent on lighting conditions, plant reflectance and angle of leaf relative to the camera. Measuring leaf angle is a challenging task for plants and the lighting conditions will vary dynamically with short term changes in the environment when imaging in the field (e.g. changes to cloud cover, sun moving across the sky). Images are often calibrated for changing light levels by use of reflectance standards. These are objects of a known reflectance that is constant across the range of wavelengths being imaged; Lambertian reflectance is a desirable characteristic of a reflectance standard that ensures reflectance is uniform across different angles of view (Fig. 9.3).

The group at James Hutton used the approach of having a surface of known reflectance held constantly in shot behind the plants being imaged. This allows for correction for changing light levels to be carried out. The use of a flat reference tile,

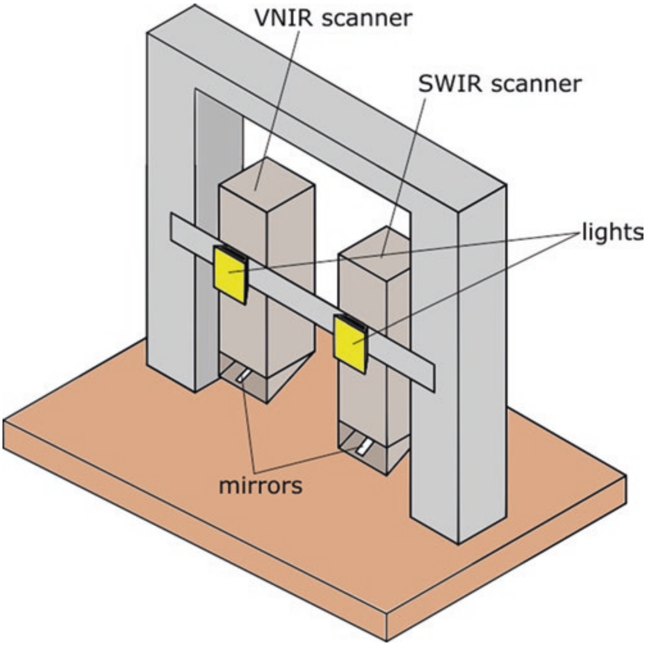

Fig. 9.3 Diagram of the imaging platform. Showing alignment of cameras and lights added to increase light levels when imaging in cloudy conditions

however, is an imperfect model for the 3D structure of plants. This means it is unable to remove completely the effect of changing light levels from the images and significant image variation was detected within the data. These image effects must be taken into account when designing imaging frameworks to allow for useful statistical analysis to be carried out on imaging data.

9.5 Non-destructive Data Capture Through Time

One of the major advantages of using imaging for phenotyping is the low cost of carrying out repeated and non-invasive measurements of plant traits. This allows studies to collect a greater volume of data to investigate temporal variation in plant phenotype and plant responses to environmental factors over time. This is a significant advantage over other phenotyping approaches as many traits are dynamic in nature, and it is beneficial to be able to capture trait information and map QTL expression as a function of time.

A key requirement is to demonstrate that plant traits and physiological status are linked to specific spectral signatures and that any variation in plant physiological status, either due to genetic control and/or in response to environmental conditions, is associated with a change in plant spectral signatures. These signatures might be generated via chlorophyll fluorescence or the specific biochemical composition and water status of plant tissues (e.g. Altangerel et al. 2017). High throughput phenotyping using spectral signatures that are diagnostic of desirable plant traits could facilitate selection of the best-performing genotypes and avoid undesirable plant phenotypes. For example, canopy temperature quantified with thermal imaging has been used to determine temporal changes in canopy water content in a number of studies. One study of cotton plants (Pauli et al. 2016a) used a high throughput phenotyping platform based on imaging to investigate plant responses to temperature and drought stress. The study detected QTLs for both imaging and more traditional traits, a number of these co-located to similar locations, across the growing season, with specific QTLs detected more frequently at certain time points, which was linked to plant development. Other studies have used chlorophyll fluorescence as a measure of photosynthetic health under different stress conditions (e.g. drought stress (Chen et al. 2014)). Changes in spectral signatures in response to stress conditions offer significant opportunities for high throughput phenotyping for pest and disease resistance traits (Goggin et al. 2015).

9.6 Future Challenges

More widespread adoption of high throughput phenotyping requires a number of challenges to be overcome. High throughput imaging technology is still in its infancy, and while a number of groups have developed platforms capable of imaging plants, each is designed for a specific application. As yet, there are no generic and commercially available solutions that would allow plant scientists to carry out high throughput phenotyping based on imaging. Instead, most work is based on interdisciplinary groups developing their own custom-made solutions.

As imaging technology develops, increasing amounts of data are created, which entails procedures and methods for data management and analysis. Simple low cost cameras can be used to produce large amounts of colour images quickly and cheaply; more expensive hyperspectral systems are slightly slower to cover ground but produce image data with high dimensionality. In order transform large numbers of images into usable plant data, computer vision techniques must be used. There have been some moves to develop and categorise image analysis methods for plants (Plant Image Analysis http://www.plant-image-analysis.org/) (Lobet et al. 2013), however these methods are typically suitable for specific image analysis problems and not generally suitable for developing new applications of imaging systems (Lobet 2017). For example, early imaging approaches have often used *Arabidopsis* as a model plant and have focussed on image capture from detached leaves (Gehan et al. 2017). This is because image analysis is much simpler when imaging plants from fixed angles and under controlled illumination. When carrying out phenotyping in field conditions, image analysis is more complex due to variation in illumination intensity and in the relative angle of the light source, plant and camera. This means, however, that imaging methods developed for use on *Arabidopsis* are not readily adapted for whole plant imaging.

To overcome this problem and generate interest from those within the computer science community, a number of well curated public image data sets have been created. While this has generated a good response, effort has mainly focussed on trying to extract specific features from images of plants. There are very few high throughput data sets publicly available but increasing the proliferation of high throughput phenotyping techniques may change this.

Another challenge faced in sharing data between different phenotyping trials is lack of standardisation in terminology assigned to target plant traits. Attempts are currently being made to set standards for measurement of phenotypic data and standardisation of metadata naming. Organisations such as the European Plant Phenotyping Network (EPPN) and International Plant Phenotyping Network (IPPN) aim to develop standards that will facilitate greater sharing and comparison of phenotypic data between researchers working on multiple species. Lack of alignment between different plant species in the focal characteristics measured has hindered efficient development of inter-species standard nomenclature.

For these future systems to work, specific skill sets are also needed: an understanding of the technology, and its use and limitations in research and applied environments; an understanding of the biology of the plants of interest and their response to different environmental conditions; the ability to manipulate spectral data from plant imaging to produce estimates of traits and recognise phenotypes. All of these skills need to exist within a research team or consortium that is able to communicate the different aspects of the work to one another, as the integration of the scientific and engineered components is a final step necessary to produce a phenotyping system and approach that can be applied successfully.

9.7 Conclusions

As advances are made in the technologies supporting plant breeding, high throughput phenotyping is increasingly becoming a bottleneck in the breeding process. A number of imaging technologies are being developed to address this gap. Imaging offers the potential for rapid large scale and non-destructive data collection on plant traits, which in raspberry include abiotic and biotic stress resilience and resilience to the rapidly changing and unpredictable climate. While there has been modest research effort invested in this area, the technology is relatively immature and fragmented, with the result that there are widely different phenotyping methods being used in different places. Ongoing research into this area will hopefully overcome these issues and set up widely adopted standards that can be applied across different crop species.

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